Baicalin Regulates Autophagy to Intervene with Intestinal Acute Graft-Versus-Host Disease via AMPK/Mtor Pathway and Abnormal Intestinal Flora

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Research Article

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Abstract

Acute graft versus host disease (aGVHD) is the main complication of allogeneic hematopoietic stem cell transplantation and the main cause of death. To verify if baicalin can protect the intestinal mucosal barrier by regulating abnormal autophagy and interfering with acute intestinal graft-versus-host disease, a mouse model of aGVHD was established. CB6F1 mice were transplanted with a mononuclear cell suspension from Balb/c donor mice bone marrow and spleen via vein injection after treatment with 60Co X rays. After treatment with different doses of baicalin for 15 days, survival time, survival time, the serum level of TNF- α and IL-10, the autophagy markers from intestinal and bacterial flora were detected. A intestinal barrier dysfunction cell model was also used to verify the effect of baicalin.

The results showed that baicalin could significantly prolong survival time, reduced aGVHD score compared to the model control group, significantly reduce TNF- α level, and increase IL-10 level. TEM examination showed that baicalin treatment increased the number of autophagic vacuoles and led to recovery of mitochondrial structure in the intestinal mucosal epithelial cells of mice and in Caco-2 cells. Western blot results showed that baicalin treatment enhanced autophagy *in vivo* by regulating the AMPK/mTOR autophagy pathway. Similar results were observed *in vitro* in Caco-2 cells. Furthermore, the effect of baicalin was reduced after combined treated with autophagy inhibitors 3-MA. The results of intestinal microbial sequencing also confirmed that there were significant changes in the type and quantity of bacterial flora before and after the application of baicalin. Baicalin can reduce the severity of intestinal acute graft-versus-host disease by regulating autophagy with influencing the unbalanced inflammatory cytokines and bacterial flora, which may be a new treatment for aGVHD.

Introduction

Acute graft versus host disease (aGVHD) is one of the most fatal complications in the early period after hematopoietic stem cell transplantation. Autophagy is the process of a cell phagocytosing its own cytoplasmic proteins or organelles, and encapsulating them into vesicles which fuse with lysosomes forming autophagic lysosomes. The autophagic lysosomes then degrade their contents which contributes to the metabolic needs of the cell and the renewal of certain organelles. Our work prior to this study suggests that autophagy protects the intestinal mucosa by degrading damaged organelles and other substances, and this slows aGVHD progression. Intervention with the mTOR pathway may protect against acute graft-versus-host disease after bone marrow transplantation^[1]. A study found that the intraperitoneal administration of metformin, which activates AMPK signaling, ameliorated the clinical severity of aGVHD and lethality^[2].

Multiple studies have shown that Huangqin decoction can regulate the Th17 / Treg balance and reduce intestinal mucosal barrier damage^[3]. This suggests that Huangqin decoction could help treat colitis via immunomodulation. Huangqin decoction contains a variety of ingredients, of which baicalin seems most promising. The baicalin molecular formula is C₂₁H₁₈O₁₁, and it is derived from the dried roots of the dicotyledon plant *Scutellaria baicalensis*. Baicalin has been demonstrated to significantly improve

cerebral ischemic injury via enhanced autophagy by up-regulating the Beclin-1 protein^[4]. Additionally, baicalin induces autophagy in PC12 cells by reducing p62 expression and increasing LC3-II expression which results in increased PC12 cell viability^[5]. Furthermore, baicalin can suppress neural apoptosis to protect the brain by ameliorating the mitochondrial apoptotic pathway^[6]. Despite its potential as a treatment, there have been few studies on baicalin's effects on intestinal aGVHD. Therefore, we performed experiments to explore the mechanism of baicalin regulated autophagy in the context of acute intestinal graft-versus-host disease to explore its potential as a novel clinical treatment of aGVHD.

Materials And Methods

Reagents and antibodies

Baicalin (98%), with batch number YS121121, was purchased from the Xi'an Yuansen Biotechnology Company. 3-MA, LC3-II, LC3-I, Beclin-1, P62, p-AMPK, AMPK, mTOR, and Tubulin monoclonal antibodies were purchased from Affinity Biosciences (Cincinnati, OH, USA). The CCK-8 kit, RIPA lysis buffer, polycarbonate Lucifer Yellow (dextran) and the reverse Transcription Kit were purchased from Sparkjade (Jinan, China). The Caco2 cell line was obtained from Chinese Academy of Sciences (Beijing, China). The Millicell system and fluorescence plate reader were provided by Sparkjade (Jinan, China).

Animals and experimental protocol

Mice were purchased from Beijing Weitong Lihua Experimental Animal Technology Co., Ltd. and the animal production license number was SCXK (Beijing) 2012-0001. 10 healthy SPF-grade Balb/c H-2d male mice were used as donors (weighing 18–22 g and aged 8–10 weeks); 56 SPF-grade CB6F1 female mice were used as transplant recipients (weighing 18–22 g and aged 8–10 weeks).

Inbred Balb/c H-2d mice were sacrificed by cervical dislocation, and the bone marrow and spleen mononuclear cells were obtained aseptically. Next, CB6F1 mice were irradiated with 60Co X rays, and then the transplant was completed by infusing the mononuclear cell suspension (1×10^7 bone marrow cells + 1×10^7 spleen cells), obtained from donor Balb/c mice (above), via tail vein^[7].

Mice were randomly divided into the model control, low dose, medium dose, and high dose groups. Model control group mice were fed with normal saline and treated group mice were fed with 15, 30 or 60 mg/(kg·d) baicalin for 15 days immediately after modeling.

In order to examine the influencing autophagy effect of baicalin, we also compared treated with 30mg baicalin alone group, to a group treated with 30mg baicalin combined with the autophagy inhibitor 3-MA^[8]. All animal procedures were performed with the approval of the Animal Ethics Committee of the Affiliated Hospital of Shandong University of Traditional Chinese Medicine. All experiments were performed in accordance with relevant guidelines and regulations.

Cell lines culture, tested by transepithelial electrical resistance

The human colon carcinoma cell line Caco-2 was grown in Dulbecco's modified Eagle's minimum essential medium (DMEM, pH 7.4) (Invitrogen) supplemented with 25 mM glucose, 10% inactivated fetal bovine serum (FBS) (Lonza), 1% penicillin streptomycin (PS) and 1% non-essential amino acid solution (Invitrogen). After the cells were stimulated by TNF- α (100 ng/ml) for 21 h at 37°C, transepithelial electrical resistance (TER) was tested to verified if the intestinal barrier dysfunction cell model was established. For the measurement of TER, 1×10^5 cells were seeded on polycarbonate transwell inserts with a diameter of 12 mm and a pore size of 0.4 μm . TERs were recorded using the Millicell system, and resistances obtained over a blank filter were subtracted from the obtained values. For assessment of Lucifer Yellow, or dextran, permeable transwell inserts were washed with HBSS followed by apical application of the tracer compounds. After 2 h of gentle agitation at 37°C, basal tracer concentration was measured using a fluorescence plate reader, and then used to determine relative sodium-to-chloride permeability according to the Goldmann-Hodgkin-Katz equation.

After cell model was established, cells were treated with 10, 20,40 $\mu\text{g/ml}$ baicalin, respectively. To confirm the autophagy effect 20 $\mu\text{g/ml}$ baicalin was chosen to be combined with 3-MA.

Clinical aGVHD classification criteria

Clinical score for aGVHD was determined by observing weight loss, posture, activity, hair, skin integrity, and diarrhea on the 15th day after transplantation (Table 1). The criteria for determining aGVHD was taken from work by Cooke et al^[9].

Table 1
aGVHD clinical score

criteria	Grade 0	Grade 1	Grade 2
Weight loss	< 10%	10–25%	> 25%
Posture	Normal	Hunching noted only at rest	Severe hunching impairs movement
Activity	Normal	Mile to moderately decreased	Stationary unless stimulated
Fur texture	Normal	Mile to moderate ruffling	Severe ruffling/poor grooming
Skin integrity	Normal	Scaling of paws/tail	Obvious areas of denuded skin
Diarrhea	≤ 1 time	2–3 times	≥ 4 times

Individual mice from coded cages received a score of 0 to 2 for each criteria (maximum score of 12), as described above.

Small intestinal mucosa histologic and pathological inflammation score analysis

Small intestinal tissue was collected from mice. The tissue was fixed in 4% paraformaldehyde for hematoxylin and eosin (H&E) staining following standard protocols. HE images were observed under a light microscope. Histological damage of the tissues was calculated using a pathological inflammation score (Table 2) which was determined by examining damage of the small intestine tissue, crypt destruction, and the degree of mucosal ulcer and the extent of cell infiltration (The scores for each criteria were added to get the pathological score of small intestine^[10]). Motic Images Advanced 3.0 software was used to measure intestinal villi height and crypt depth.

Table 2
Pathological inflammation score in small intestine

Small intestine	0	0.5	1	2	3	4
villous blunting crypt	normal	focal and rare	focal and mild	diffuse and mild	diffuse and moderate	diffuse and severe
crypt regeneration						
crypt loss						
luminal sloughing of cellular debris						
lamina propria inflammatory cell infiltrate						
mucosal ulceration						

Detection of TNF-α and IL-10 level

The expression of tumor necrosis factor (TNF)-α and interleukin (IL)-10 was measured using quantitative reverse transcription polymerase chain reaction (RT-PCR). Small intestine tissue was collected and quickly frozen in liquid nitrogen for subsequent mRNA extraction. Total RNA was extracted using the guanidine isothiocyanate-phenol-chloroform method. Extracted RNA was reverse-transcribed using the Sparkjade reverse transcription kit and the resulting complementary DNA was analyzed for the expression of the target molecules using the LightCycler 480 real-time PCR system. The primer sequences are provided in Table 3. The resulting gene expression levels of the target molecules were normalized based on GAPDH expression.

Table 3
PCR primer design

Gene	forward	reverse
GAPDH	5'-CAA CTT TGT CAA GCT CAT TTC C-3'	5'-GGT CCA GGG TTT CTT ACT CC-3'
TNF-α	5'-CAT GCA CCA CCA TCA AGG AC-3'	3'-GGC CTG AGA TCT TAT CCA GCC-3'
IL-10	5'-CTA TGC TGC CTG CTC TTA CTG-3'	5'-AGC AGT ATG TTG TCC AGC TG-3'

Western Blot Analysis

Cells were lysed using RIPA lysis buffer and total protein was collected. Next 20µg of each protein sample was loaded on to 10% SDS-PAGE gels, and the protein was concentrated at 80V for 20 min and separated at 120V for ~ 1h. After the electrophoresis was stopped, the samples were transferred to PVDF at 110V at 4°C based on the wet blotting method protocol. Each immunoblot was blocked in 5% nonfat milk TBST for 2h, then incubated with primary antibodies like LC3, P62, Beclin-1, AMPK, mTOR or β-tubulin at 4°C overnight. The membrane was washed 3 times, then incubated with diluted secondary antibodies for 2h. After the secondary antibody solution was fully washed away, the PTG ECL chemiluminescence detection kit was used to develop the blot. Then the blot was transferred to an imaging machine for exposure and analysis.

Immunohistochemistry (IHC) analysis

The tissues of small intestine (2–3 cm) were fixed with 10% formaldehyde, decolorized, cleared, paraffin embedded and sectioned. Antigen retrieval was performed for 20 min in a pressure cooker. The sections were cooled, rinsed with PBS, incubated in a 3% H₂O₂ solution for 10 min and rinsed with PBS again. The tissue sections were incubated in goat serum for 20 min to remove the serum, incubated with primary antibodies against AMPK and LC3 at 37°C for 1 h, rinsed with PBS, incubated with biotin-labeled secondary antibodies at room temperature for 30 min and again rinsed with PBS. Finally, the sections were incubated in DAB substrate solution for 5 min. After been washed thoroughly in tap water, counterstained in hematoxylin, dehydrated in absolute alcohol, cleared in xylene, and subjected to microscopy. The expression of proteins was quantified using an image analysis and measuring system (Image-Pro Plus 6.0). The mean area and mean integrated optical density (mean IOD) of the expression of these proteins were calculated.

Immunofluorescence staining of LC3 in vivo and in vitro

Tissue paraffin sections of the small intestine or Caco-2 cells were incubated with primary antibody at 4 °C for 24 h. Then, goat anti rabbit red fluorescent second antibody Cy3 was applied for 60 min at 37°C. Samples were fixed with mounting medium containing 4', 6-diamidino-2-phenylindole (DAPI) to stain the nucleus. Images were captured with fluorescence microscope immediately. Quantification of LC3 puncta was performed using the ImageJ software^[11].

DNA extraction and sequencing of mouse feces

Mice Feces were harvested with 2 ml perfectly clean eppendorf tube under sterile conditions 2 h before sacrifice and stored immediately in liquid nitrogen until processing for DNA isolation. The Genomic DNA extracted detection by 1% agarose gel electrophoresis after DNA isolation, PCR amplification were performed by ABI GeneAmp® 9700 model PCR instrumen. First, the purified DNA templates were amplified using the following primers of 16S rDNA V3-V4 regions: 5'- ACTCCTACGGGAGGCAGCAG- 3' and 5' - GGACTACHVGG GTWTCTAAT – 3'. AxyPrep DNA gel Recovery Kit (AXYGEN company) was used to recover PCR products by gel cutting. The PCR reaction were used TransStart Fastpfu DNA Polymerase, The reaction system consisted of PCR Mix 20 µL Reaction program: 95°C predenaturation for 3 min, 95°C denaturation for 30s, annealing-temperature of 55°C denaturation for 30s, 72°C extension for 45s, a total

of 27 cycles, 72°C final extension for 10 min. 10°C until halted by user. According to the preliminary results of electrophoresis, the PCR products were analyzed by quantifluor™-St blue fluorescence quantitative system (Promega company) to detection and quantification, sodium hydroxide denaturation were used to produce single stranded DNA fragments. Finally, miseq pe300 sequencer (Illumina company) were used for sequencing.

Statistical analysis

The experimental results were analyzed using SPSS 19.0 and GraphPad Prism 8.0. The results were expressed as mean ± standard deviation and analyzed by ANOVA test. $P < 0.05$ indicated that the difference was statistically significant.

Results

Effect of baicalin treatment on the recovery of intestinal aGVHD in mice

At first, the intestinal aGVHD model was verified by small intestinal mucosa histological analysis (Fig. 1. A) and aGVHD score (Fig. 1. D). The IL-10 and TNF- α expression level (Fig. 1.B) was also assessed to confirmed the intestinal aGVHD model established. Heavy infiltration of inflammatory cells was detected in the intestinal mucosa of the model group, which is consistent with the pathological characteristics of intestinal aGVHD (Fig. 1.A). After being treated with medium or high dose baicalin which structure showed in Fig. 1.C, the small intestinal mucosal epithelium structure recovered in multiple ways including decreased inflammatory cell infiltration, more regular arrangement of glandular cells, an increased number of goblet cells, less shedding of cellular debris, and less necrosis of mucosal epithelium. The pathological inflammation score of medium and high dose groups were significantly lower compared with to the model and low dose groups (Fig. 1.A). After using baicalin, the TNF- α expression was significantly reduced and IL-10 expression was significantly increased in the medium and high dose groups compared with the model group, ($P < 0.01$, Fig. 1.B).

The model group mice all died by d15 and the low dose group all died by d27, whereas, 50% of the medium dose and high dose group mice were alive beyond day 28 (Fig. 1.E). These data demonstrate that medium and high dose baicalin treatment effectively reduce morbidity and mortality by improving intestinal mucosal injury in aGVHD model mice.

Baicalin effects on intestinal aGVHD via influencing autophagy in mice

In order to find the mechanism of baicalin treatment, the mTOR signal mediated autophagy were tested. Medium dose of baicalin was chosen to be tested. Specifically, the AMPK autophagy pathway was activated, as indicated by increased levels of LC3BII/BI, Beclin1, and p-ampk with decreased level of P62 and mTOR ($P < 0.01$), (Fig. 2.A). After autophagy was inhibited with 3-MA, the baicalin induced increase

of autophagy related proteins was reversed to the same level as the model group. Additionally, there were more biphasic membrane autophagosomes with normal mitochondrial structure in baicalin group, compared to control and the 3-MA treatment group (Fig. 2.B). There was no significant difference between the baicalin + 3-MA group and the model group (Fig. 2.A and B). This indicates that autophagy mediates baicalin's effect on intestinal aGVHD.

As Fig. 3.A showed, the inhibiting autophagy with 3-MA reversed the beneficial effect that baicalin treatment had on aGVHD morbidity. The baicalin + 3-MA group had a far more damaged small intestinal mucosal epithelium structure, a higher level of inflammatory cell infiltration, an abnormal arrangement glandular cells, less goblet cells, and more mucosal epithelium shedding and necrosis when compared to the group given baicalin alone ($P < 0.01$) (Fig. 3.A). The level of LC3 was assessed by IHC (Fig. 3.C) and immunofluorescence staining (Fig. 3.D) in small intestine, results showed that the LC3 level was improved in tissues of baicalin group compared with the baicalin + 3-MA group and model group. Based on the proteins level of WB (Fig. 2.A) and IHC (Fig. 3.B), AMPK/mTOR induced autophagy should be the effective target of baicalin on intestinal aGVHD.

Baicalin can improve intestinal mucosal barrier by enhancing autophagy in vitro

After Caco-2 cells induced by TNF- α , the effect of baicalin was tested by using transepithelial electrical resistance (TER), western blot and immunofluorescence staining. We found a significant increase of level of LC3/ β -actin ratio (Fig. 4.A) and the number of LC3 positive dots (Fig. 4.D) in baicalin group with higher autophagic vesicle numbers (Fig. 4.C). The results were similar to what was found in vivo, which indicated that baicalin can promote autophagy by enhancing AMPK pathway activity and down-regulating mTOR activity in Caco2 cells. It was also found that the mitochondrial structure is rebuilt in the baicalin treated group (Fig. 4.C). If the autophagy inhibitor was added in baicalin treated group, the therapeutic effect of baicalin was decreased with lower autophagy level. Cause the intestinal barrier dysfunction can be alleviated by baicalin in TER test (Fig. 4.B), the mechanism of baicalin is by stimulating on autophagy was verified again in vitro experiments.

The diversity of intestinal flora in mice

The sequencing coverage of intestinal microflora in mice were about 99.614%, compared with the normal control group, 392 bacteria were same in baicalin group and model group, 176 bacteria were different between baicalin group and model group (Fig. 5.A). There were 18 bacteria in the structure of intestinal microflora at the phylum level, including 10 dominant phylum: Actinobacteria, Bacteria_unclassified, Bacteroidetes, Cyanobacteria, Deferribacteres, Firmicutes, Marinimicrobia_SAR406_clade, Patescibacteria, Proteobacteria, Tenericutes (Fig. 5B). The coverage of intestinal microbial sequencing in aGVHD model group were about 99.663%, which were higher than that in normal control group, and the expression of Bacteria_unclassified, Firmicutes, Proteobacteria were higher than that in normal control group. Actinobacteria, Bacteroidetes, Chloroflexi, Cyanobacteria, Deferribacteres, Marinimicrobia_SAR406_clade,

Patescibacteria, Tenericutes, Verrucomicrobia were lower than that in normal control group. Compared with the model group, the expression of Bacteroidetes, Proteobacteria, verrucomicrobia were increased and the expression of Firmicutes were decreased after baicalin treatment. The microbial community structure of the mouse intestinal flora is mainly composed of 227 genera at the genus level, of which 67 were dominant bacterial genera. Compared with the model group, the expressions of Akkermansia, Bacteroides, Clostridium_sensu_stricto_1, Eisenbergiella, Escherichia-Shigella, Lachnospiraceae_NK4A136_group, Prevotellaceae_NK3B31_group were increased after baicalin treatment, and the expressions of Alistipes, Lachnospiraceae_FCS020_group, Lactobacillus, Muribaculaceae_norank, Prevotellaceae_unclassified were decreased. And the relative abundance of bacteroidetes firmicutes, proteobacteria, verrucomicrobia, eferribacteres and the others was displayed in Fig. 5.C and D. The distribution abundance of intestinal flora changed significantly between baicalin group and model group(Fig. 5E).

The rarefaction curve tends to be flat, indicating that the selection of sequencing data is reasonable(Fig. 6. A, B). The range of the curve on the horizontal axis is large, the species abundance is high, and the curve on the vertical axis is gentle, indicating that the species are evenly distributed(Fig. 6.C). We use principal component analysis to extract the two coordinate axes that reflect the differences between samples to the greatest extent, so as to reflect the differences of multidimensional data on the two-dimensional coordinate diagram, and then calculate that the distance between baicalin group and control group is closer than that between model group and control group. This means that the composition of intestinal flora in baicalin group is more similar to that in normal group(Fig. 6.D).

Discussion

Intestinal aGVHD is one of the most common graft-versus-host reactions after transplantation. The intestinal symptoms of patients with acute graft-versus-host disease after transplantation are refractory diarrhea, serious colic, hematochezia and intestinal obstruction that will affect the implantation and endanger life. These symptoms are the same as those recorded in use of huangqin decoction, and that is why we tried to find an element in huangqin decoction to treat this disease. There are some studies that have shown that the occurrence of intestinal aGVHD is closely related to gastrointestinal flora^[12], inflammatory cell infiltration and other factors. One key cause recognized today is the pretreatment regimen which leads to cytokine storm-mediated inflammation and subsequent low immunity^[13]. In our study, the levels of TNF- α and IL-10 changed significantly after baicalin was used. Mucositis and infection^[14] can trigger the aGVHD via destruction of the intestinal mucosal barrier. So addressing how to protect the intestinal mucosal barrier is key in treating aGVHD.

Autophagy-dependent Treg cells are essential for immune regulation and reconstruction after transplantation^[15]. Autophagy-dependent control of Treg aGVHD activity was further supported by a study suggesting that increased autophagy increased the ability of thymus-derived Tregs to interfere with

CD8 + T cell mediated aGVHD ^[16,17]. Our experiment showed an increase in autophagy level changed after the application of baicalin suggesting that baicalin could help stimulate Treg control of aGVHD. Interestingly, if the C3aR/C5aR complex is inhibited, inducing mitochondrial autophagy in dendritic cells will likely reduce the symptoms of graft-versus-host disease on the basis of ensuring graft anti-leukemia effects^[18]. Our study showed that baicalin induced autophagy reduced the clinical aGVHD score of mice was decreased mirroring these results. In the initial treatment of standard risk aGVHD, mTOR inhibitor rapamycin had similar complete/partial remission rates as prednisone on day 28. Rapamycin can change the expression of autophagy related proteins, such as inhibit mTOR and promote autophagy^[19–20], this is similar to the effect of baicalin.

Baicalin is the main ingredient of Huangqin decoction which is often used to treat diarrhea symptoms. Baicalin itself can induce autophagy through PI3K/Akt/mTOR pathway which results in an anti-inflammatory effect^[21].

In our experiments, abnormal levels of IL-10 and TNF- α , inflammatory factors often dysregulated in aGVHD, were corrected by treatment with baicalin. This effect was most notable in the medium dose group. Additionally, after treatment with baicalin the viability of mice improved and the clinical aGVHD and pathological scores of small intestine decreased. This showed that baicalin had some ability to reduce the pathology of intestinal aGVHD. Next we postulated that mechanism by which baicalin regulated aGVHD was by regulating the unbalanced autophagy found in intestinal aGVHD, and we verified this by examining the AMPK/mTOR pathway after baicalin treatment. Baicalin can increase the expression of autophagy related proteins such as LC3-II/I and Beclin 1 so as to increase the production of autophagy vesicles and LC3 level to promote the autophagy of cellular debris and other substances, improve the function of mitochondria, and reduce the apoptosis and abscission of intestinal epithelial cells. There are some studies that suggest that activated autophagy may attenuate intestinal mucosal barrier dysfunction by preventing and reducing oxidative stress^[22–23]. Our experimental results show that low levels of autophagy make it difficult to improve the permeability of Caco-2 cells, and with the increased level of TNF in the small intestinal mucosa of acute graft-versus-host bearing mice, the barrier of intestinal mucosa will be destroyed.

To confirm this mechanism, 3-MA, an autophagy inhibitor, was used. The data showed that, after inhibition of autophagy with 3-MA, the effects of baicalin were reversed *in vivo*. Some studies have found that 3-MA can inhibit Caco-2 cell activity by inhibiting autophagy^[24]. For this reason we tested Caco-2 cell treated by 3-MA with or without baicalin. Medium and high dose of baicalin were found to improve intestinal barrier dysfunction by increasing the relative TER level of Caco-2 cells. Then 20 μ g/ml baicalin was combined with 3-MA for the further study. We found that the effect of baicalin was weakened after the application of 3-MA, providing further evidence that baicalin reduces aGVHD pathology by regulating autophagy.

Many studies have confirmed that intestinal flora is closely related to intestinal diseases^[25,26]. For example, the lachnospiraceae decreased with the Firmicutes increased^[27] in ulcerative colitis model^[28],

Bacteroidetes^[29], verrucomicrobia^[30] and lachnospiraceae^[31] would increase after treatment. These above bacteria have a certain inhibitory effect on inflammation, and play an important role in colitis and other intestinal diseases, which is consistent with our research results. Our data indicated that baicalin may reduce lachnospiraceae by reducing the diversity of flora, reducing the relative abundance of Firmicutes, increasing the relative abundance of Bacteroidetes, Proteobacteria and verrucomicrobia_FCS020. The relative abundance of lachnospiraceae was increased. The relative abundance of group bacteria can resist acute graft-versus-host disease.

This study provides strong evidence that baicalin can protect the intestinal mucosal barrier by promoting autophagy, reducing the death of intestinal mucosal epithelial cells, improve the abnormal intestinal flora and interfering with the progression of intestinal aGVHD. Our next experiments will focus on the effect of baicalin on Treg and Th17 cells in our aGVHD model, so as to further reveal baicalin's mechanism of protecting intestinal mucosal barrier by immunomodulation.

Conclusion

Based on the results above, the following conclusions can be drawn: baicalin can interfere with intestinal aGVHD by regulating autophagy-related protein level via the AMPK/mTOR pathway, baicalin increases autophagic vesicles and repairs mitochondrial morphology, and that baicalin intervention is weakened by inhibiting autophagy using 3-MA. Furthermore, baicalin can protect the intestinal mucosal barrier and interfere with the progression of acute graft-versus-host disease.

Declarations

Ethics approval and consent to participate

All experiments were conducted in compliance with the ARRIVE guidelines. We confirm that animal care and experimental procedures were carried out in accordance with the guidelines of the Animal Ethics Committee of the Affiliated Hospital of Shandong University of Traditional Chinese Medicine. The reference number is AWE-2019-037.

Consent for publication

Not applicable.

Availability of data and material

The datasets supporting the conclusions of this article are included within the article. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing financial interests to disclose.

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Authors' contributions

Dr. Xing Cui designed and performed the experiments, analyzed the data, and wrote the manuscript; Dr. Longjin Xu provided the experimental site and some equipment; Master Jie Xu and Wei Zheng obtained mouse tissue samples and tested; Master Xiaoqi Sun, Yanyu Zhang and Runjie Sun provided vital new reagents and performed experiments; Dr. Fumou Sun and Michael Pisano analyzed the data and wrote the manuscript. All authors have read and approved the manuscript.

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Figures

Figure 1

The survival rate and small intestine pathological analysis of mice A. Analysis of mouse small intestine pathological score. B: Inflammatory factor levels of IL-10 and TNF-α in experimental group. C: Baicalin's chemical structure: C₂₁H₁₈O₁₁. D: The clinical aGVHD score in each experimental group. E: Survival curve of experimental groups of mice.

A

B

Figure 2

Autophagy level analysis A: Detection of autophagy-related proteins LC3II/I, Beclin1, P62, p-AMPK, AMPK, and mTOR by Western Blot. B: Detection of autophagic vesicles by TEM. A: Representative intestinal histology of each experimental group. B: Analysis of Pathological Inflammation Score in Mice Small Intestine ** P<0.01.

Figure 3

Small intestine pathology, Immunohistochemical and LC3 level assessment A: Representative intestinal histology of each experimental group. A: Detection of autophagic vesicles by TEM and the number of autophagic vesicles. B: Immunohistochemical of AMPK analysis. C: Immunohistochemical of LC3 analysis. D: Detection of immunofluorescence staining of LC3.

Figure 4

Caco2 cell line test A: The levels of autophagy-related proteins LC3II/I, Beclin1, P62, p-AMPK, AMPK, and mTOR by Western Blot. B: Measurement of the permeability of Caco-2 cells. C: Detection of autophagic vesicles by TEM. D: Detection of immunofluorescence staining of LC3.

Figure 5

The effect of Baicalin on intestinal microflora of mice A: The similarity of intestinal microflora composition among normal group, model group and baicalin group in veen. B: species accumulation curves. C: The relative abundance of bacteroidetes firmicutes, proteobacteria, verrucomicrobia, eferribacteres and the others. D: Box chart of differential intestinal flora. E: Heatmap analysis of different intestinal microflora

Figure 6

Determination of sample size and similarity analysis of intestinal flora A: Rarefaction curve. B: Shannon-Wiener curve. C: Rank-Abundance curve. D principal component analysis diagram.