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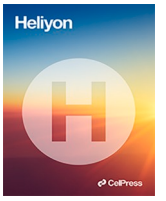

Research article

Oral administration of tea-derived exosome-like nanoparticles protects epithelial and immune barrier of intestine from psychological stress

Qianyuan Gong [*], Yueshan Sun, Lei Liu, Chunlan Pu, Yuanbiao Guo [**]

Medical Research Center, The Third People's Hospital of Chengdu, The Affiliated Hospital of Southwest Jiaotong University, Chengdu, 610031, Sichuan, China

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ABSTRACT

Psychological stress disrupts the integrity of the intestinal barrier and is strongly linked to emotional disorders, behavioral changes, and gastrointestinal dysfunction. However, there are limited treatment options available for repairing the damage to the intestinal barrier. As a natural plant-based health beverage, tea (*Camellia sinensis*) has been shown to have various potentially advantageous effects on the intestinal barrier and immune function. In this study, we extracted bioactive molecules from tea leaves, named exosome-like nanoparticles (ELNs), and then examined their potential protective properties for the intestinal barrier. Through in vitro experimentation, we investigated whether tea-derived ELNs (TELNs) could offer a protective effect against lipopolysaccharides-induced damage to the intestinal barrier. In an in vivo experiment, rats were exposed to water avoidance stress and subsequently administered TELNs orally. The administration of TELNs resulted in the enhancement of the epithelial barrier in the intestine, effectively preventing bacterial translocation to the submucosae. Additionally, TELNs were found to improve the function of the intestinal immune barrier through the mediation of interleukin-22 and the increased secretion of antimicrobial peptide Reg3g. Notably, miR-44 and miR-54 in TELNs exhibited similar protective effects on the intestinal barrier. These findings suggest that TELNs possess the ability to restore the integrity of the intestinal barrier in the context of psychological stress.

1. Introduction

Psychological stress is characterized by a non-specific defensive reaction triggered by the disruption of environmental homeostasis following exposure to intense psychological and physiological stimuli within a brief timeframe. Moderate levels of stress can facilitate the body's gradual adjustment to the environment, whereas excessive stress can disrupt the body's normal homeostasis, resulting in non-specific bodily harm and potentially leading to stress-related disorders, including functional gastrointestinal diseases such as irritable bowel syndrome (IBS). The intestinal tract is among the organs that exhibit early responsiveness to such stressors, mainly manifested with intestinal barrier damage and visceral hypersensitivity [1,2].

* Corresponding author.
** Corresponding author.
E-mail addresses: gqyscu@sina.cn (Q. Gong), guoyuanbiao@swjtu.edu.cn (Y. Guo).

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The intestinal barrier serves as a crucial defense mechanism against the infiltration of detrimental substances and pathogens into the intestinal tract, thereby preserving the stability of the body's internal milieu. It encompasses a physical barrier, a secretory barrier (comprising antimicrobial peptides, fluid, and mucus), and an immune barrier [3]. The physical barrier is established by the intercellular connections of intestinal mucosal epithelial cells, primarily consisting of tight junctions, adhesion junctions, and desmosomes [4]. Intestinal tight junctions are composed of transmembrane proteins, junction complex proteins, and cytoskeletal components. Some of these proteins, such as zonula occluden-1 (ZO-1) and occludin are used as markers of physical barrier [5]. The intestinal immune barrier comprises immune cells and immune molecules [6]. For example, type 3 innate lymphoid cells (ILC3) affect the secretion of antimicrobial peptides by epithelial cells through the release of cytokine interleukin-22 (IL-22) [7]. IL-22 plays a critical role in promoting intestinal immune and metabolic homeostasis [8]. Stresses significantly down-regulate the expression levels of ZO-1 and occludin, this impairs the intestinal physical barrier, leading to increased intestinal permeability, bacterial migration, and inflammatory responses [9,10].

Stress-induced intestinal barrier dysfunction is recognized as a significant contributor to the development of IBS. IBS is primarily characterized by changes in bowel patterns and persistent abdominal pain [11,12]. Recent research has indicated a correlation between IBS and compromised intestinal barrier function as well as immune activation. Individuals diagnosed with IBS exhibit elevated levels of intestinal permeability [13]. The underlying mechanisms responsible for the disruption of intestinal permeability in IBS remain uncertain, and the available treatments for this condition are limited.

Notably, tea (*Camellia sinensis*), as a naturally derived plant-based health beverage, exhibits intricate interactions with the intestinal barrier. The tea treatment for intestinal diseases was early recorded in The Song Dynasty's Extensive Annotation of Shennong Materia Medica and the Taiping Song Dynasty's Shengji General Record [14]. The oral consumption of theanine, derived from green tea, is absorbed by the mucosa of the small intestine, thereby modulating immune function [15]. Furthermore, the presence of polysaccharides in tea has been found to mitigate inflammation and subsequently reduce the risk of colon cancer [16]. L-theanine protects the integrity of intestinal physical barrier by inhibiting the expression level of inflammatory cytokines and reducing the degree of lipid peroxidation [17].

Exosomes are nanoparticles secreted by eukaryotic cells with a diameter of 40∼100 nm [18]. Plant exosomes can exert different biological effects on mammalian cells. The exosome-like nanoparticles (ELNs) derived from grapefruits can target intestinal cells and alleviate colitis in mice [19]. Plant ELNs deliver hydrophobic curcumin into colon tumors [20]. It has been found that natural tea-derived ELNs (TELNs) have the ability to prevent and treat inflammatory bowel disease [21]. Our previous studies have shown that TELNs can inhibit the activation of hepatic stellate cell and alleviate the progression of liver fibrosis in mouse [22].

Nevertheless, the therapeutic impact of TELNs on IBS and the precise mechanism by which TELNs safeguards the intestinal barrier remain uncertain. In this current investigation, with the hypothesis that oral TELNs has some protective effects on the intestinal barrier, we investigated whether TELNs could offer a protective effect against lipopolysaccharides-induced damage to the intestinal barrier in vitro, and then assessed the ability of oral TELNs to restore intestinal barrier integrity in an in vivo a rat model of intestinal barrier dysfunction.

2. Methods and materials

2.1. Isolation of TELNs

Fresh tea leaves were collected from Jiuru Ltd. (Chengdu, China) and preserved in liquid nitrogen and transferred to the laboratory for exosome (TELNs) extraction. We have selected three tea plants, including Fuding (FD), Meizhan (MZ) and old Chuancha (LCC) tea trees. Tea leaves (10 g) were placed into 50 mL phosphate buffer saline (PBS) and homogenized in a blender. The resulting juice was centrifuged at 1000×*g* for 10 min, 3000×*g* for 20 min and 10,000×*g* for 40 min to remove the large debris. The supernatant was filtered at 0.45 μm. TELNs were isolated by centrifuged at 10,0000×*g* for 4 h and purified on a sucrose gradient (8 %/15 %/30 %/45 %), using a bicinchoninic acid assay kit (Biosharp, Hefei, China) to determine the protein concentration after 1 mL PBS was used to suspend TELNs [23]. TELNs were stored at −80 °C. The concentration of TELNs isolated from 10 g of leaves was 0.145, 0.177 and 0.195 mg/g, respectively.

2.2. Transmission electron microscopy (TEM) and size characterization

For TEM analysis, samples were negatively stained with 2 % phosphotungstate solution for 2 min. Imaging was performed by a TEM (JEM-1400, JEOL, Japan).

The diameter of TELNs were measured by nanoparticle tracking analysis (NTA) (Particle Metrix, Meerbusch, Germany) using a 488 nm laser. The concentration of TELNs was measured by NTA software (1 cycle 9 positions) with three technical measurements.

2.3. Intestinal epithelial barrier function determination

Caco-2 cells were purchased from HonorGene Ltd. (Changsha, China), T84 cells were purchased from Jennio Ltd. (GuangZhou, China). Cells maintained in RPMI 1640 medium (Gibco, USA) with 10 % FBS (Biological Industries, Israel) at 37 °C in an atmosphere of 5 % CO_2. Cells seeded on transwell inserts (Corning, Kennebunk, USA) with polyester membrane (12-well, 0.4 μm pore size) at a density of 50,000 cells per well and monitored daily for 7 days starting on day 14. Transepithelial electrical resistance (TEER) was determined by EVOM^2 Epithelial Voltohmmeter system (World Precision Instruments LLC, Sarasota, USA). When cells reached

completely differentiated, 10 μg/mL TELNs were added for 24 h and then treated with or without 10 μg/mL LPS (Sigma-Aldrich, USASigma-AldrichSigma-AldrichSigma-AldrichSigma-Aldrich).

2.4. Cellular uptake assays

For internalization of TELNs into cells, TELNs were stained with lipophilic 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine perchlorate (DiI) (AAT Bioquest, USA) for 30 min at 37 °C. After treating Caco-2 cells (50,000 cells per well) with 1×10^{11} particles mL^{-1} DiI stained TELNs for 24 h, fixing the cells with 4 % paraformaldehyde (Biosharp, Hefei, China). The cell cytoskeleton was stained by Phalloidin-FITC (1:100, Abcam, UK) for 30 min at 25 °C, and then the cell nuclei was stained by 4′,6′-diamidino-2-phenylindole (DAPI) (Biosharp, Hefei, China) for 15 min at 25 °C. Finally, using a fluorescence microscope (Leica, Germany) to analyze the cellular uptake.

2.5. IL-22 in vitro blockade

For IL-22 blockade experiments, mast cells (HMC-1, 20,000 cells per well) were cultured on a 6-well plate with or without 10 μg/mL TELNs for 4 h, and then added with 10 μg/mL cyclosporine A (CsA) (Sigma-Aldrich, USA) for further 24 h or left untreated, the cells were collected and the expression of IL-22 was analyzed.https://www.sigmaaldrich.cn/CN/zh/life-science/sigma-aldrich.

2.6. Animal experiments

Male SD rats aged 8 weeks (150–200 g) were supplied by Dashuo Experimental Animal Ltd. (Chengdu, China) and housed under specific pathogen-free conditions. The rats were acclimated for 7 days with a temperature of 24 °C and a 12 h dark/12 h light cycle and ad libitum access to food and water. IBS in rats was induced by water avoidance stress (WAS) for 10 days according to the reference described [24]. Briefly, the rats were positioned on a platform measuring 6 × 6 cm in the middle of a water-filled tank (45 × 50 cm) with a temperature of 25 °C. The experiment was conducted between 9:00 and 10:00 a.m. each day to minimize the influence of circadian rhythms. For experiments, 40 rats were randomly divided into five groups: control, PBS, LCC, FD and MZ groups (n = 8 per group). Rats in the LCC, FD and MZ groups were orally administered with 100 μL TELNs (1 mg protein/kg per rat) for 4 days starting on day 7. The control group's platform had no surrounding water. Rats were sacrificed after being anesthetized on Day 10. After euthanasia, the intestinal tissues were collected and fixed in 4 % formaldehyde. For blood collection, the samples were collected from the tail vein prior to sacrifice under restraint.

2.7. Intestinal permeability assay

FITC dextran (mw. 4 KD, FD-4) was purchased from Sigma (Sigma-Aldrich, USA). 300 μl of 80 mg/mL FITC dextran was given to rats by gavage after a 16 h fast. Blood was collected 4 h after the FITC dextran gavage. 15 % v/v acid-citrate-dextrose solution was used as an anticoagulant. Plasma was prepared by centrifugation at 3000×*g* for 10 min. Fluorescence was determined at 530 nm with excitation at 485 nm using a spectrophotometer (VICTOR Nivo™). Permeability was expressed as relative fluorescence units between the groups being compared.

The serum levels of D-lactic acid, diamine oxidase and endotoxin were quantified according to the manufacturer's method (Zhongsheng Jinyu, Beijing, China).

2.8. Western blotting

Total proteins from cells or intestinal tissue were lysed with RIPA buffer (Beyotime, Shanghai, China). Protein concentration was measured by bicinchoninic acid assay. The proteins were separated via SDS-PAGE and then electrotransferred onto a PVDF membrane (Millipore, USA) at 85 V for 2 h. Membranes were incubated with corresponding antibody for 12 h at 4°C with mouse anti-β-ACTIN (1:5,000, proteintech, Wuhan, China), rabbit anti-ZO-1 and anti-occludin (1:2,000, proteintech, Wuhan, China), rabbit anti-IL-22 (1:1,000, Biorbyt, UK), then using IgG antibody conjugated to horseradish peroxidase as secondary antibody. Blots were incubated with secondary antibody for 1 h and chemiluminescence.

2.9. Image analysis: immunofluorescence

The fixed cells or tissues were washed with PBS and blocked in 10 % goat serum (Biosharp, Hefei, China) and incubated with corresponding antibody for 12 h at 4 °C with ZO-1 and occludin (1:200), IL-22 (1:50), Rorγt mouse monoclonal (1:200, BD bioscience, USA), IL-22RA1 rabbit polyclonal (1:100, Proteintech, Wuhan, China), Reg3g (1:100). Subsequently, the inserts incubated with secondary antibody (conjugated with AF488 or AF594) (Solarbio, Beijing, China) and photographed by microscope.

2.10. Microbial fluorescence in situ hybridization (FISH) analysis

The microbial abundance in the intestinal tissue was analyzed by EUB338 FISH Probe (Cy3 labeled) from Guangzhou exon biotechnology Co., Ltd. The tissues obtained were fixed in 4 % paraformaldehyde solution, embedded in paraffin, of which 5-mm-thick

sections were prepared and hybridized according to the manufacturer's instructions.

2.11. RT-qPCR assays

Total RNA was extracted with vazyme Trizol reagent (Nanjing, China) from epithelial cell or rat's intestinal tissue with indicated treatment. cDNA was prepared from total RNA by using vazyme RT SuperMix Kit (Nanjing, China). Real-time polymerase chain reaction (PCR) was performed by SYBR quantitative PCR (qPCR) Mix in qPCR machine (Roche, Switzerland). *β-ACTIN* was used as an internal control. Primers are listed in Supplementary Table 1.

2.12. The miRNA library preparation and sequencing

RNA extracted from TELNs, and the RNA quality inspection was measured using NanoPhotometer® (Implen, Munich, Germany). 10 ng of miRNA was used to library preparation with the miRNA Kit (bio scientific, USA). Adapters are ligated sequentially to the 3′ and 5′ ends of miRNAs in an unbiased reaction. The cDNAs were generated by reverse transcription of miRNAs with adaptor. The cDNA library building and library sequencing were completed by Huayin Ltd. (Guangzhou, China).

The raw reads were processed by removing the sequences without 3′end primer, low quality bases and undetermined bases. The clean reads were aligned to the genome using the Bowtie aligner (http://bowtie-bio.sourceforge. net/index.shtml). Mirdeep2 (https://www.mdc-berlin.de/content/mirdeep2-documentation) was performed to predict new miRNAs.

2.13. Quantification and statistical analysis

Unless otherwise indicated, one-way analysis of variance (ANOVA) with a post-hoc test (Least Significance Difference and Duncan) was used to determine whether means were statistically different (P value less than 0.05 was considered statistically significant).

3. Results

3.1. Characterization and cellular uptake of TELNs

To investigate the biological activities of TELNs to epithelial barriers, we extracted LCC, FD and MZ three types of TELNs from fresh tea leaves and analyzed the exosome-like structure and size distribution of TELNs by TEM and NTA (Fig. 1A-B). Studies have shown that plant ELNs can be absorbed by the intestine orally [20,25], we tested whether TELNs can be taken up by epithelial cells. We selected intestinal epithelial cells Caco-2 as in vitro model system to study functional epithelial barriers. To confirm the uptake of TELNs, DiI-labeled TELNs were added to the Caco-2 cell culture medium. Cell nuclei and cytoskeleton were stained with DAPI (blue) and Phalloidin-FITC (green), respectively. Microscopy analysis revealed that the DiI-labeled TELNs (red) from LCC, FD and MZ were widely distributed throughout the cell cytosol and surrounding the nucleus (Fig. 1C). These results strongly suggest that TELNs can indeed be effectively absorbed by epithelial cells, which is an essential step for their biological activity.

3.2. The TELNs protect epithelial barrier in epithelial cells

Caco-2 cells spontaneously differentiated into monolayer absorbent epithelial cells with mature structure. This maturation is completed about 2 weeks and the transepithelial electrical resistance (TEER) can assess the evolution of monolayer integrity [26,27]. TEER was increased along with the growth time of Caco-2 cells, while LPS remarkably decreased the TEER value ($P < 0.01$) compared with the control (Fig. 2A-B). When Caco-2 cells were pretreated with TELNs for 24 h before the stimulation of LPS, TEER values kept a higher level in the cells pretreated with TELNs than those pretreated with PBS ($P < 0.01$ and $P < 0.05$) (Fig. 2B).

LPS impaired tight junction through down-regulated *ZO-1* and *Occludin* mRNA expression levels in Caco-2 cells, in comparison with the control group ($P < 0.01$). Subsequently, TELNs pretreatment increased the mRNA expression levels of *ZO-1* and *Occludin* ($P < 0.01$) compared with the PBS group (Fig. 2C-D).

We also use immunofluorescent staining to observe the changes of the tight junction with marker protein ZO-1 in Caco-2 cells. In the control group, ZO-1 staining exhibited a continuous belt pattern around the cell membrane. As expected, in the LPS-treated cells, the staining intensity was reduced and the tight junction belt was discontinuous. In contrast, treatment with TELNs could markedly attenuated these rearrangements ($P < 0.01$) (Fig. 2E-F). Our results demonstrate that TELNs can effectively protect epithelial barrier in Caco-2 cells.

Moreover, we also examined the effect of TELNs on another human intestinal epithelial cells T84. As shown in Supplementary Fig. 1, the TEER value of T84 cells was significantly downregulated after LPS treatment. After TELNs were added, the TEER value was significantly upregulated, indicating that TELNs can also protect epithelial barrier in T84 cells ($P < 0.01$).

3.3. Retardation of WAS development by TELNs

We then examined the TELNs protective effects to intestinal barrier in WAS. Fig. 3A shows the scheme for the experimental investigation. During the entire experiment, the body weight in healthy control group showed a gradual increase, whereas the WAS control group was dramatically decreased in body weight ($P < 0.01$) (Fig. 3B). Interestingly, oral treatment with TELNs mitigated the

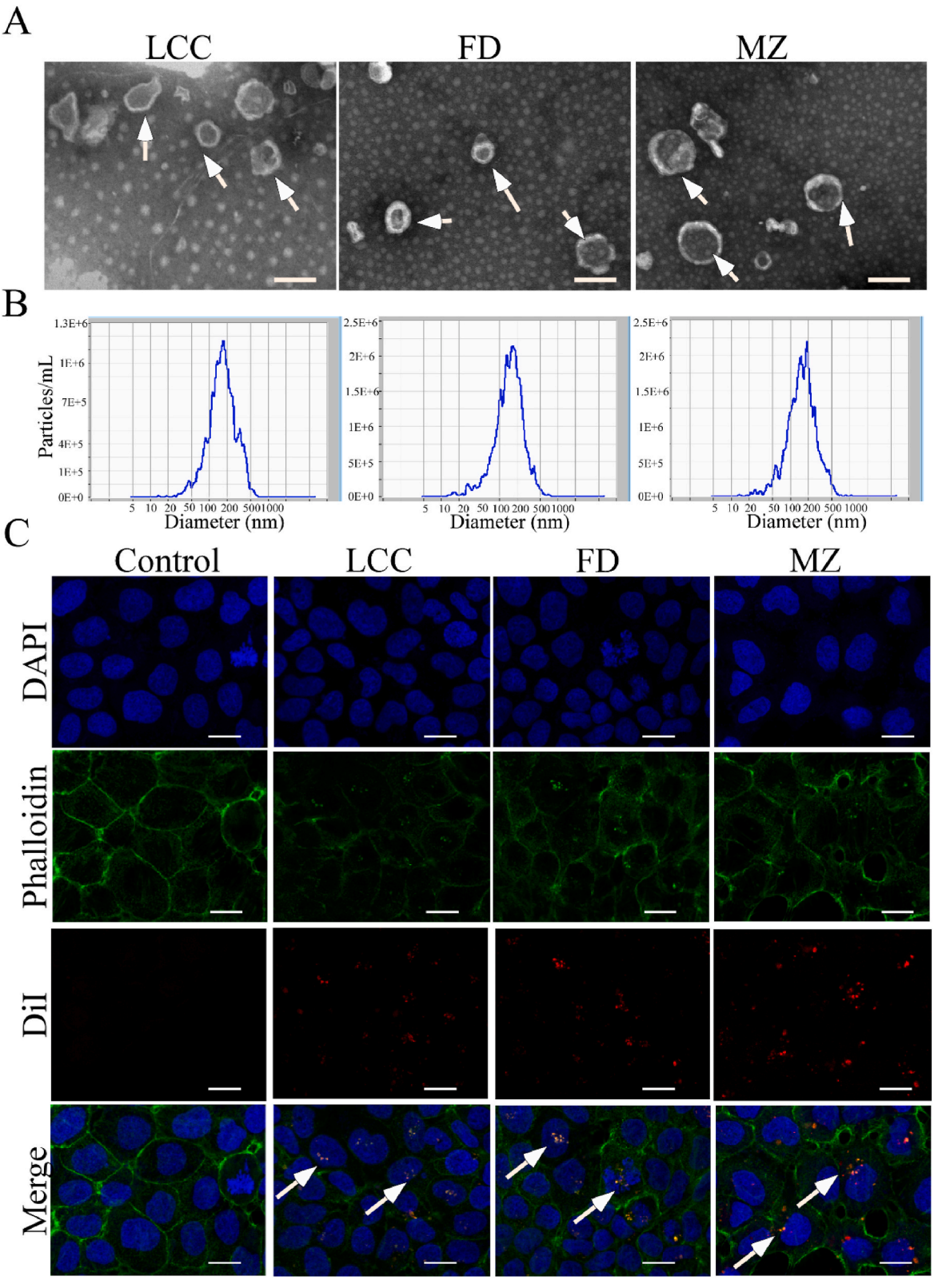

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Fig. 1. Characterization and cellular uptake of TELNs.
(A). TELNs were examined by transmission electron microscopy. Representative TELNs are indicated by white arrows. Magnification: 100000X. Scale bar = 200 nm. (B). The particle size of TELNs was analyzed by nanoparticle tracking analysis. (C). Cellular uptake of TELNs into Caco-2 cells. DiI staining indicates lipophilic staining that marks the TELNs. Phalloidin-FITC staining indicates actin filaments. DAPI staining indicates the cell nuclei. The merged images show the colocalization of the TELNs (red) with the cell cytoskeleton (green) and the cell nuclei (blue). Representative cellular uptake of TELNs are indicated by white arrows. Magnification: 200X. Scale bars = 20 μm.

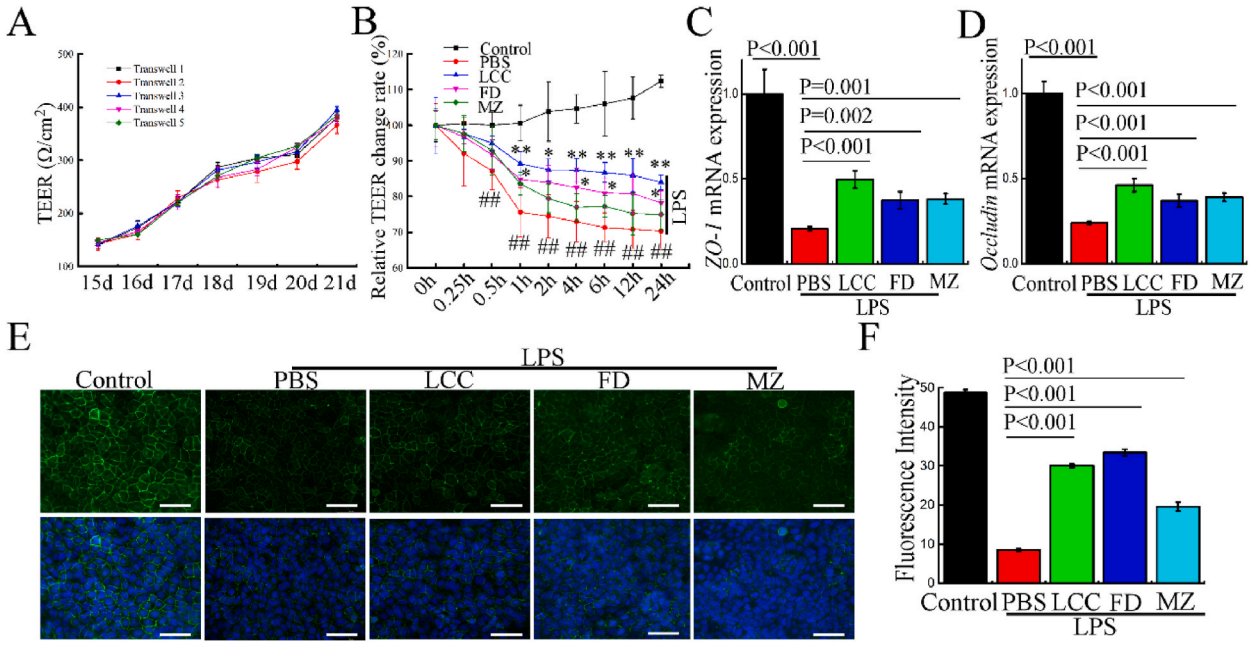

Fig. 2. The TELNs can protect epithelial barrier in Caco-2 cells.
(A). TEER was measured from day 15 to day 21 post-seeding. Data represent the mean ± SEM of three replicates.
(B). Relative TEER change of Caco-2 cells treated with various TELNs or PBS. Cells were co-cultured with TELNs for 24 h, and treated with LPS for different time. Relative TEER change is relative to the initial TEER value. Error bar represents SD ($n = 3$). *P < 0.05 and **P < 0.01, indicating significantly different from PBS group; ##P < 0.01, indicating significantly different from control group.
(C)-(D). Determination of the transcription level of *ZO-1* (C) and *Occludin* (D) in Caco-2 cells treated with various TELNs or PBS using RT-qPCR assays. Cells were co-cultured with TELNs for 24 h, and treated with LPS for 24 h. *β-ACTIN* was used as the internal control. Data were normalized by gene expression level in control. Error bar represents SD ($n = 5$).
(E). The immunofluorescence with antibody against ZO-1(upper panel) and merged with DAPI staining (lower panel) in Caco-2 cells treated with various TELNs or PBS. Cells were co-cultured with TELNs for 24 h, and treated with LPS for 24 h. Representative images are shown. Magnification: 200X. Scale bar = 50 μm.
(F). The relative fluorescence intensity in (E) was quantified using Image J software. Error bar represents SD ($n = 5$).

weight loss of WAS-treated rats. Particularly, in the LCC TELNs treated group, the body weights of rats remained stable during the whole experiment.

To investigate the permeability of intestine in vivo, rats was orally treated with FITC dextran, which would across the damaged intestinal barrier into bloodstream, so that blood FITC concentration could reflect the intensity of intestinal permeability. WAS caused an about twofold increase in FITC dextran absorption compared with controls. After treating with TELNs, the FITC dextran absorption could be significantly attenuated (P < 0.01) (Fig. 3C).

As the intestinal barrier is impaired, intestinal flora and its metabolites may be transferred from the intestinal tract to the systemic circulation, which may trigger endotoxemia [28–30]. This phenomenon known as intestinal microbiota translocation, which indicated by elevated levels of diamine oxidase, endotoxin and D-lactate [31,32]. We found that WAS can significantly increase the levels of these three indicators in the blood at day 7 (P < 0.01). After treating with TELNs, the D-lactic acid, diamine oxidase and endotoxin levels in the blood could be significantly attenuated (P < 0.01) (Fig. 3D-F). These results demonstrate that TELNs effectively protect the intestinal barrier, thereby attenuating bacterial translocation.

3.4. Tight junctions disfigured by WAS were reversed with TELNs treatment

Through electron microscope observation of rat intestinal mucosa epithelial cells, we found that compared to the control group, WAS group's tight junctions between the intestinal epithelial cells were cracked, paralleled with incomplete desmosomes, destructed

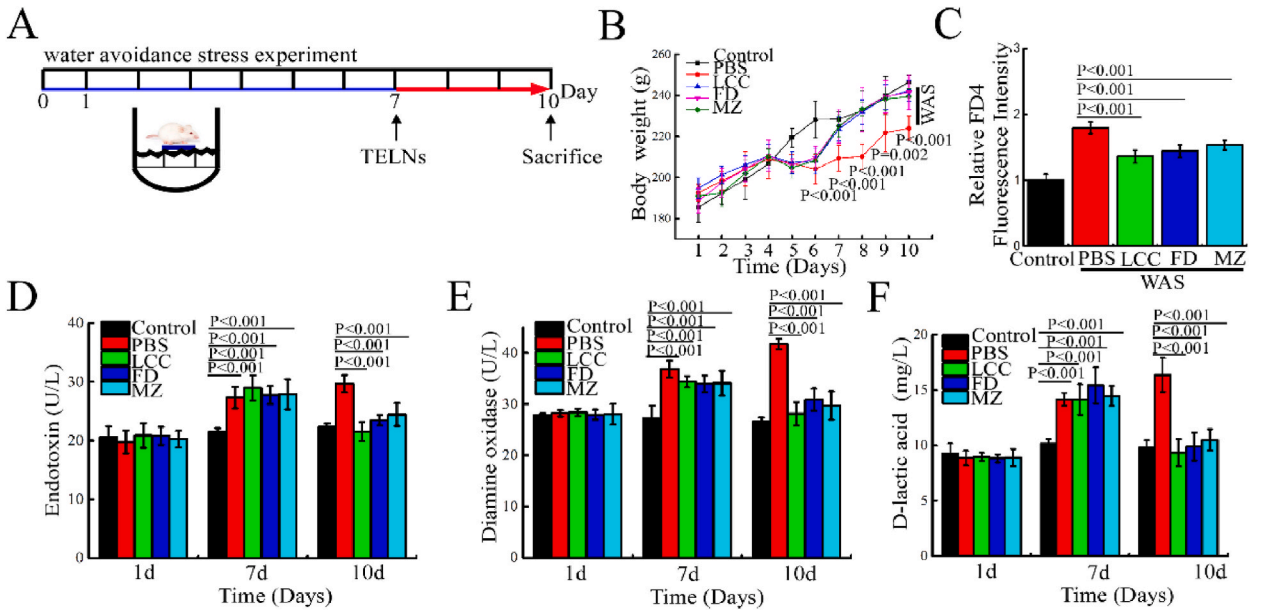

Fig. 3. Retardation of WAS development by TELNs.
(A). Flow diagram of experimental design employed in these studies. Rats were provided with WAS every day. On days 7–10, rats were orally administered with PBS or various TELNs (1 mg protein/kg rat).
(B). Variations of body weight over time in (A). $P < 0.05$, indicating significantly different from control group. Error bar represents SD ($n = 5$).
(C). In vivo detection of FD-4 in the blood stream 240 min after infusion into the lumen of the small intestine. Data were expressed as the mean ± SD. Error bar represents SD ($n = 5$).
(D)-(F). The indicators of intestine microbiota translocation, namely endotoxin (D), diamine oxidase (E) and D-lactic acid (F) levels in the blood. Error bar represents SD ($n = 5$).

microvilli structure, mild swelling of mitochondria (crest fracture and dissolved) and slightly rough endoplasmic reticulum expansion; whereas, in the TELNs intervention group, the tight junctions are complete, without obvious morphological damage, and desmosomes were relatively complete (Fig. 4A). These results suggest that TELNs can effectively fixed intestinal barrier damage caused by WAS.

As shown in Fig. 4B-D, ZO-1 and occludin protein and mRNA expression levels in the small intestine from the WAS rats were dramatically lower than those control group ($P < 0.01$). While treatment with TELNs strongly up-regulated the expression levels of ZO-1 and occludin ($P < 0.01$).

3.5. *Effect of TELNs on intestinal immune barrier protection mediated by IL-22*

IL-22 is an important cytokine to maintain immune barrier homeostasis during inflammatory diseases [7,8]. We further tested whether TELNs can activate intestinal immune protection through IL-22. As shown in Fig. 5, IL-22 expression levels were remarkably lower in the small intestine from the WAS rats than those control by western blots and immunostaining ($P < 0.01$). Interestingly, the WAS rats receiving TELNs treatment had significantly more IL-22 level than those PBS treatment group ($P < 0.01$) (Fig. 5). Further, interleukin-22 receptor (IL-22RA1) mediates the function of IL-22 and is expressed on epithelial cells in skin and gastrointestinal tract [33,34]. As expected, there were remarkable decreasing distribution of IL-22RA1 in the WAS group ($P < 0.01$), while TELNs treatment diminished this disrupted pattern ($P < 0.01$) (Supplementary Fig. 2).

In addition, CsA augmented the secretion of IL-22BPi1, which can bind and dissolves IL-22, thereby blocking IL-22 biological activity [35]. Therefore, human cultured mast cells (HMC-1) were cultured on a 6-well plate with TELNs, and IL-22 expression levels was noticeable increased compare to that control ($P < 0.01$). When the culture medium supplemented with CsA, the expression of IL-22 was same level whether or not treatment with TELNs (Fig. 5C, Supplementary Fig. 3). These findings indicate that IL-22 is essential for TELNs to protect the intestinal immune barrier. Interestingly, the marker protein from ILC3, Rorγt elicited an apparent distribution of IL-22 by immunostaining (Fig. 5D-E).

Taken together, our data demonstrate that oral administration of TELNs can effectively maintain the intestinal immune function though elevating IL-22 level.

3.6. *TELNs augment the production of intestinal antimicrobial peptide Reg3g*

IL-22 is produced by activated ILC3 cells and plays a crucial role in stimulating the production of antimicrobial peptides, such as Reg3g. This helps to maintain the integrity of the intestinal tissue by protecting it from the harmful effects of microorganisms [36,37].

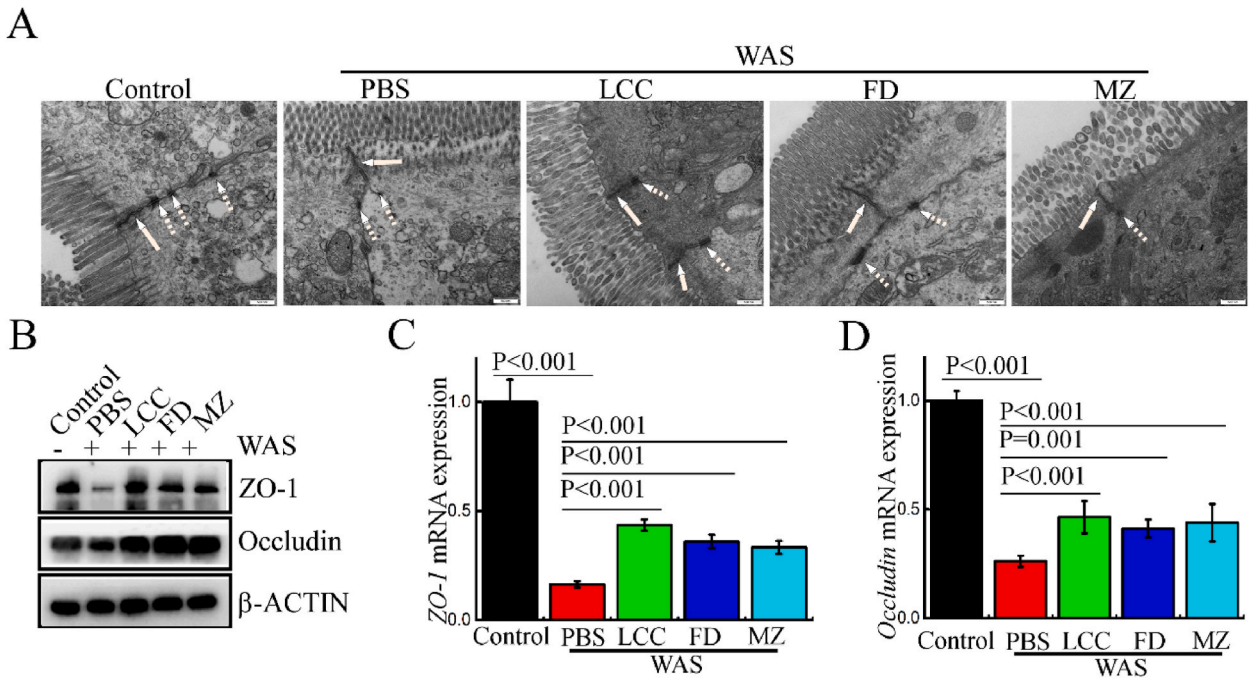

Fig. 4. WAS-induced TJ structural changes was reversed by TELNs treatment.
(A). The distribution of TJ in the different experimental groups. The solid lines pointed to tight junctions and dotted lines to desmosomes. Magnification: 30000X. Scale bar = 500 nm.
(B). Western blot of ZO-1 and occludin proteins in the different experimental groups.
(C)- (D). Determination of the transcription level of *ZO-1* (C) and *Occludin* (D) in the different experimental groups using RT-qPCR assays. Error bar represents SD ($n = 5$).

Oral administration of TELNs significantly increased the protein and mRNA levels of Reg3g in WAS rats ($P < 0.01$), compared to the sham treated WAS rats (Fig. 6A-C). Then we detected the submucosal bacteria in small intestine of rats with the FISH probe EUB338. As shown in Fig. 6D-E, more invasive bacteria appeared under intestinal mucosae in WAS rats than the control ($P < 0.01$), while TELNs treatment effectively abated the bacterial invasion ($P < 0.01$). Taken together, these data suggest that oral administration of TELNs could effectively defend the intestine from bacteria through antimicrobial peptides.

3.7. The miR-44 and miR-54 in TELNs were identified to protect intestinal barrier

Among the components contained in ELN, miRNAs have attracted much interest due to their functional and therapeutic importance. ELN miRNA derived from ginseng has a good effect on stimulating the differentiation of mesenchymal stem cells [38]. Herein, we identified the miRNAs contained in TELNs. With a sequencing depth of 22 million and 29 million clean reads, 65 and 63 novel miRNAs were identified as possible candidates with a length of 18–25 nt in LCC and MZ, respectively (Supplementary Table 2). In consideration of LCC displayed more therapeutic efficacy than MZ, among the most reads miRNAs, miR-54 and miR-44 in LCC were significantly more than MZ in sequencing abundance, while miR-6 and miR-56 showed more abundant in MZ than LCC, so these miRNAs were selected for the further research (Supplementary Table 3). The mimics of these miRNAs were transfected into Caco-2 cells, followed by LPS-induced barrier dysfunction. Interestingly, miR-44 and miR-54 could dramatically increase the ZO-1 expression levels ($P < 0.01$), while the expression of ZO-1 has no significant change in the miR-6 and miR-56 treatment group ($P > 0.05$) (Fig. 7A-B). Further research showed that the protective effect of miR-54 and miR-44 to intestinal barrier in a dose-dependent manner ($P < 0.01$) (Fig. 7C-F). Collectively, our data indicate that miR-44 and miR-54 in TELNs, but not miR-6 and miR-56, have the potential protective effects on intestinal barrier.

4. Discussion

Psychological stress is closely related to intestinal diseases. Stress can lead to a series of gastrointestinal diseases, such as IBS, inflammatory bowel diseases, gastrointestinal ulcer disease, etc. Intestinal diseases are usually the result of the dysfunction of intestinal barrier. There are several possible mechanisms about the mechanisms of altered intestinal barrier function under stress conditions, including hormone-mediated interactions between intestinal neurons, immune cells and epithelial cells, and vagal nerve activation [39,40].

Intestinal barrier dysfunction is characterized by an elevation in intestinal permeability, which enables the passage of enteric toxins

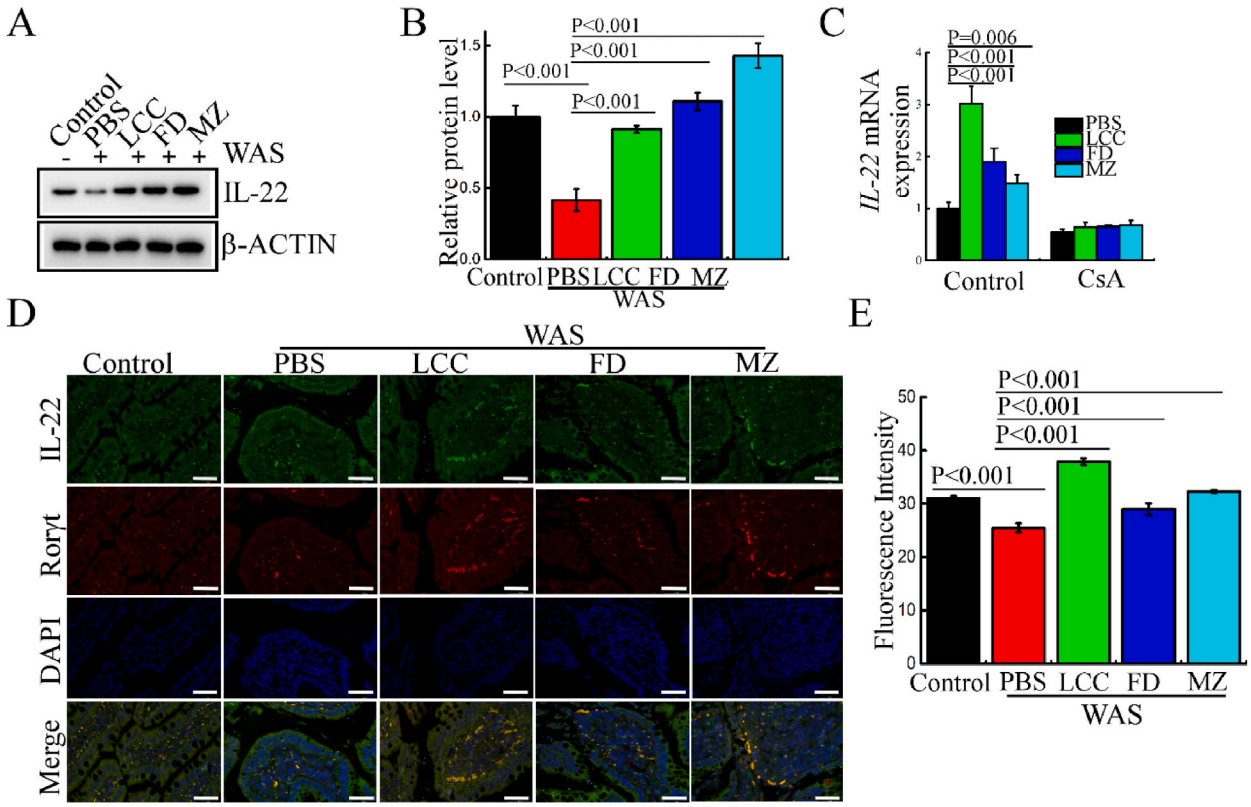

Fig. 5. Effect of TELNs on intestinal immune barrier protection mediated by IL-22.
(A). Expression levels of IL-22 in the different experimental groups.
(B). Quantification the protein levels of IL-22 in the different experimental treatment. Error bar represents SD ($n = 5$).
(C). Determination of the transcription level of *IL-22* in the control or Cyclosporine A (CsA) treated HMC-1 cells using RT-qPCR assays. Error bar represents SD ($n = 5$).
(D). Immunofluorescence detection Rorγt and IL-22 protein in the different experimental groups. Magnification: 400X. Scale bar = 50 μm.
(E). The relative fluorescence intensity in (D) was quantified using Image J software. Error bar represents SD ($n = 5$).

or pathogens [41,42]. When the intestinal barrier is compromised, there is a disruption in the expression of tight junction (TJ) proteins, leading to increased intestinal permeability. This allows harmful bacterial-derived endotoxins to enter the mucosa, initiating an uncontrolled inflammatory response [43]. TJ close the gaps between epithelial cell layers to regulate intestinal barrier permeability [44]. Our study found that these TJs of intestinal mucosa were seriously destroyed and the protein expression of ZO-1 and occludin were significantly reduced in WAS model rats. Oral TELNs significantly raised the TJ molecules at both protein and mRNA levels. Also, the intestinal permeability induced by stresses could be ameliorated by TELNs. Thus, we concluded that TELNs alleviate the phenotype of intestinal barrier dysfunction by facilitating the repair of TJs between epithelial cells.

Studies have shown that the destruction of the intestinal immune barrier is associated with the activation of intestinal immune cells. The cytokine IL-22 acts on epithelial cells to maintain barrier function and tissue repair [45,46]. ILC3s are considered a primary source of IL-22, which can protect intestinal immune system from inflammatory damage induced by bacterial infections [47–49]. Our study displayed the effect of WAS-induced IBS on IL-22 expression in rats. Arguably, TELNs activated type-3 ILC3 to produce IL-22, and then stimulate the production of antimicrobial peptides. TELNs are therefore believed to protect the intestinal immune barrier. The intestinal immune barrier is also regulated by intestinal neurons [50]. The neuropeptide vasoactive intestinal peptide can activate intestinal ILC3s through the G protein-coupled receptor and then increase the expression of IL-22 and enhance the barrier function of the small intestine [50,51]. The detailed mechanism of IL-22 activation by TELNs in the promoting of intestinal immunity should be further elucidated.

TELNs are double-layer membrane-wrapped exosome-like structure under electron microscope (Fig. 1A) [22], including lipids, proteins and microRNA (miRNA) molecules [21]. The major lipid components in TELNs were phosphatidic acid, phosphatidylglycerol, phosphatidylcholine, and phosphatidylinositol. These lipids in TELNs largely promote the formation of nanovesicles and maintain their spherical structure. The mainly proteins in TELNs related to metabolic and genetic information processing. Proteins in plant ELNs are transferred to recipient cells and induce various cellular functions. For example, lectin family proteins can mediate the internalization of ELNs into HepG2 cells through interaction with CD98 [52], and heat shock protein family A can activate aromatics receptor signaling pathways to exert anti-inflammatory effects [53]. The miRNA from plant ELNs can be ingested and absorbed by the

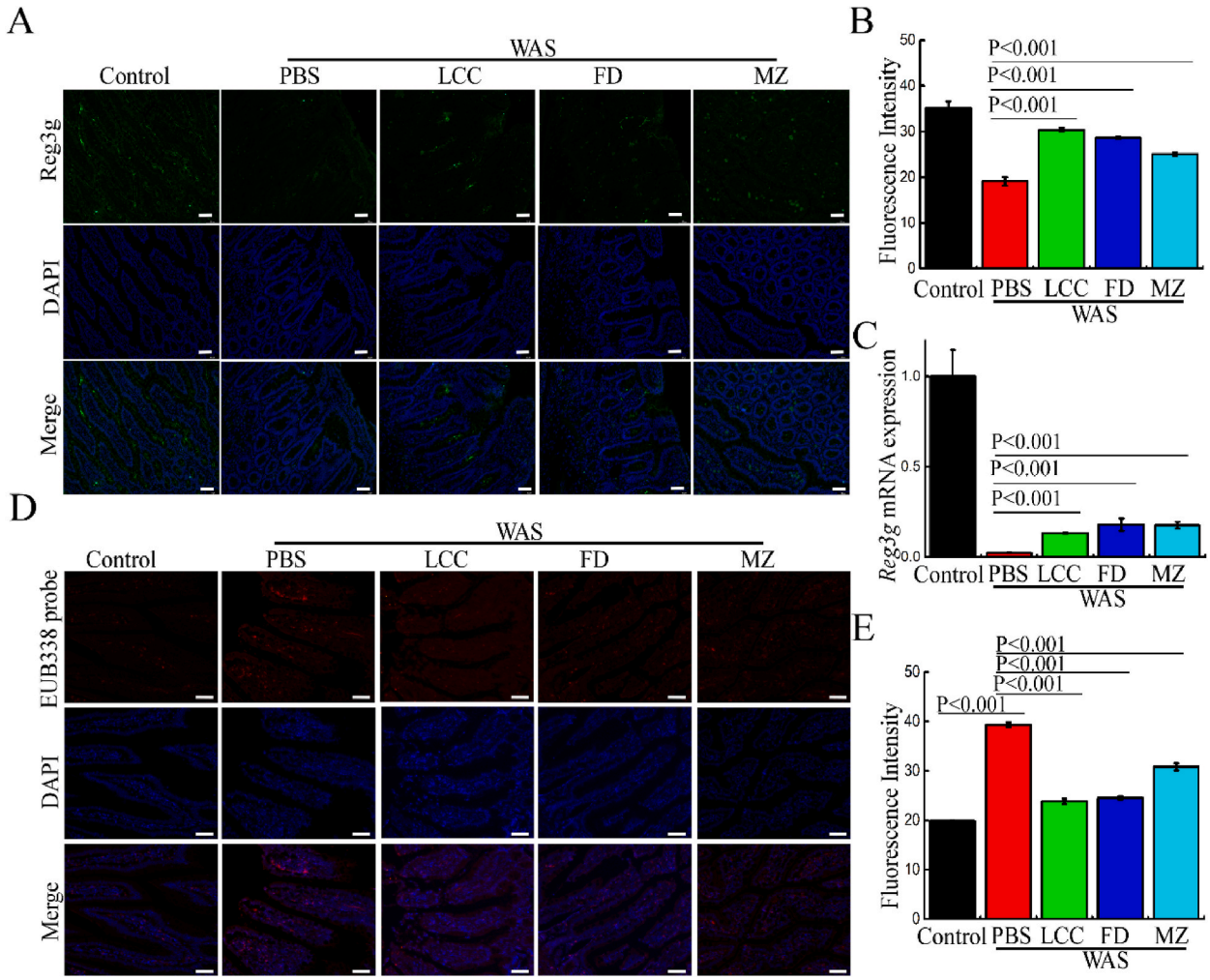

Fig. 6. WAS-induced Reg3g expression and invasive bacteria in small intestine was reversed by TELNs treatment.
(A). Immunofluorescent detection Reg3g protein in the different experimental groups. Magnification: 200X. Scale bar = 50 μm.
(B). The relative fluorescence intensity in (A) was quantified using Image J software. Error bar represents SD (n = 5).
(C). Determination of the transcription level of *Reg3g* in the different experimental groups using RT-qPCR assays. Error bar represents SD (n = 5).
(D). Representative images of FISH detecting enrichment of invasive bacteria in the different experimental groups. Magnification: 200X. Scale bar = 50 μm.
(E). The relative fluorescence intensity in (D) was quantified using Image J software. Error bar represents SD (n = 3).

intestine and subsequently transported to organs [54,55]. For example, miRNA-168a derived in rice was substantiated in mouse serum and liver with decreased low-density lipoprotein receptor levels [55]. The miRNA-7267-3p in ginger-derived ELNs could suppress the *ycnE* gene expression in gut microbiota *Lactobacillaceae* [56]. In our studies, miRNA-44 and miRNA-54 of TELNs could alleviated barrier dysfunction in experimentally LPS-induced barrier injury in Caco-2 cells. The miRNA can function by binding to their target genes. Therefore, we predicted the target genes of TELNs miRNA and found that miR-54 and miR-44 likely to regulate genes involved in cell division and proliferation, such as adhesion G protein-coupled receptor and cell division cycle protein [57,58], miR-54 may also affect the secretion of cytokines interleukin-21 and miR-44 may influence the synthesis of cell adhesion molecule 1 (Supplementary Table 4). IL-21 can induce the production of IL-22 by $CD4^+$ T cells to regulate colon inflammation [59]. Adhesion molecules are involved in the establishment and maintenance of intestinal barrier [60]. Future research needs to clarify whether miRNAs from TELNs can be absorbed by the intestine and exert any protective effect on the intestinal barrier function in consumers.

Our studies have shown that TELNs not only protect intestinal epithelial barrier in vitro, but also effectively protect intestinal barrier from psychological stress in vivo. TELNs might be promising candidate bioactive molecules derived from tea to treat intestinal barrier-related diseases in the future.

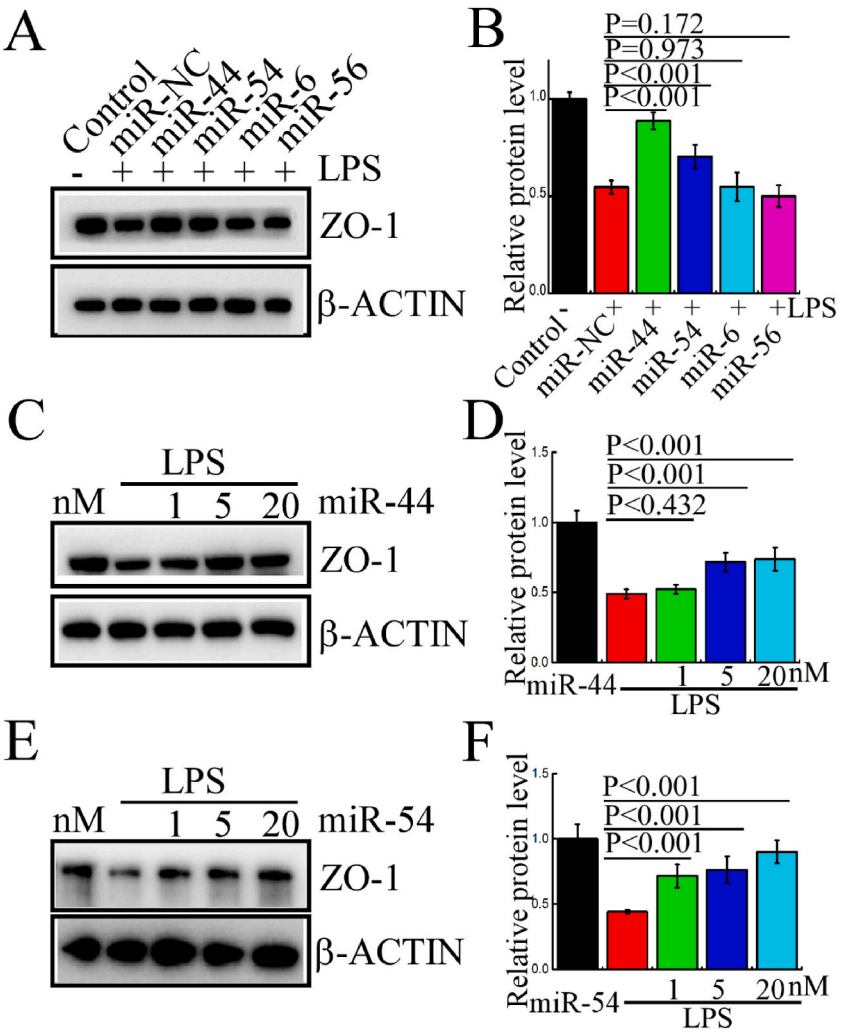

Fig. 7. The miR-44 and miR-54 in TELNs were identified to protect epithelial barrier in Caco-2 cells.
(A). The miR-44 and miR-54 protect epithelial barrier. 20 nM of miRNA (miR) mimic or miRNA negative control (NC) were transfected. The protein expression levels of ZO-1 were detected.
(B). Quantification the protein levels of ZO-1 in (A). Error bar represents SD ($n = 5$).
(C). The miR-44 dose-dependently increased ZO-1 protein expression. Different amounts of miRNA negative control were co-transfected with miR-44 to ensure the total transfected RNAs of 20 nM.
(D). Quantification the protein levels of ZO-1 in (C). Error bar represents SD ($n = 5$).
(E). The miR-54 dose-dependently increased ZO-1 protein expression. Different amounts of miRNA negative control were co-transfected with miR-54 to ensure the total transfected RNAs of 20 nM.
(F). Quantification the protein levels of ZO-1 in (E). Error bar represents SD ($n = 5$).

Ethics statement

This study was reviewed and approved by Ethics Committee of Southwest Jiaotong University with the approval number: SWJTU-2203-NSFC (026), dated March 14, 2022.

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Data availability statement

The miRNA raw sequence data reported in our study have been deposited in https://ngdc.cncb.ac.cn/gsa (repository name: Genome Sequence Archive in National Genomics Data Center, accession number: CRA007888).

CRediT authorship contribution statement

Qianyuan Gong: Writing – original draft, Data curation, Conceptualization. **Yueshan Sun:** Funding acquisition, Data curation. **Lei Liu:** Funding acquisition. **Chunlan Pu:** Funding acquisition. **Yuanbiao Guo:** Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e36812.

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