Radiotherapy Induces Intestinal Barrier Dysfunction by Inhibiting Autophagy

Wei Qu, Lijin Zhang, and Jinfang Ao *

ABSTRACT: Radiation enteritis is a common complication of abdominal irradiation (IR) therapy. However, the molecular mechanism of radiation enteritis accompanied by impaired intestinal barrier function is not clear. The aim of this study was to investigate the important role of autophagy in radiation-induced intestinal barrier function impairment. IR increased the abundance of autophagy-related genes in the colonic mucosa of mice. An autophagy activator (rapamycin) inhibited the oxidative stress (reactive oxygen species, reactive nitrogen species, malondialdehyde, and hydrogen peroxide) and inflammatory response (interleukin-1β, -6, -8, and tumor necrosis factor-α) in the colon samples. Antioxidant indices (superoxide dismutase, glutathione peroxidase, catalase, and total antioxidant capacity) in serum and colonic mucosa were significantly increased in the rapamycin group. Rapamycin can improve the activity of mitochondrial respiratory chain complexes I–V in colon mucosa. In addition, rapamycin reduced the gene expression and enzyme activity of caspase in the colonic mucosa. Levels of endotoxin, diamine peroxidase, D-lactic acid, and zonulin in serum and colonic mucosa were significantly reduced in the rapamycin group. Moreover, rapamycin significantly elevated the gene abundance of zonula occludens-1, occludin, claudin-1, and claudin-4. In contrast, completely opposite results were obtained for the autophagy inhibitor 3-methyladenine as compared to those of rapamycin. These results revealed that inhibition of autophagy is an important mechanism of intestinal barrier function damage caused by radiation. Collectively, these findings increase our understanding of the pathogenesis of radiation-induced intestinal barrier dysfunction.

INTRODUCTION

Radiation therapy encompasses treatment with ionizing radiation or radionuclides, and it is an important method currently used to treat malignant tumors. According to statistics, more than 50% of patients with malignant tumors require radiation therapy of which more than 50% are patients with pelvic and abdominal malignancies.1 Radiation therapy, as an important method for the treatment of abdominal tumors, often affects the intestinal organs and causes severe radiation enteritis. Approximately 50% of pelvic radiotherapy patients exhibit gastrointestinal symptoms that significantly affect their quality of life due to intestinal irradiation (IR) damage. In the acute stage of intestinal IR injury, prolonged diarrhea can easily cause malnutrition symptoms, anemia caused by repeated intestinal bleeding may result in decreased immunity, and fever and even endogenous infections may appear.

Intestinal epithelial cells are the main component of the intestinal barrier, and the zonula occludens/tight junction proteins (TJPs) between intestinal epithelial cells are the main determinant of intestinal barrier function.5,6 Intestinal epithelial cells cannot be effectively replenished within a short period of time after IR, and this results in the shortening and lodging of the intestinal villi and disappearance of intestinal crypts. Therefore, the damage to the intestinal epithelium barrier function caused by IR will destroy the body’s ability to absorb and metabolize nutrients. At present, the molecular mechanism of IR enteritis accompanied by an impaired intestinal barrier function requires further research.

Autophagy is the degradation of senescent organelles, long-lived proteins, and invading pathogens through lysosomes in eukaryotic cells under conditions of nutritional deficiencies, oxidative stress, ionizing radiation, and pathogen infection and the use of degradation products to maintain the pathophysiological processes required for their basic life activities.7,8 There are two processes that occur during autophagy to maintain the balance of the intracellular environment, and they consist of the selective elimination of invasive pathogens and the
removal of activated inflammasomes and reactive oxygen species.10

At present, many studies have shown that autophagy is closely related to diseases such as tumors, infections, cardiovascular diseases, and neurodegenerative diseases.11,12 With the deepening of the current knowledge on autophagy, there also has been intense research to determine how autophagy participates in intestinal barrier dysfunction. Inflammatory bowel disease (IBD) is also a gastrointestinal disease whose etiology and pathogenesis are not clear. It has been shown that changes in the intestinal barrier function are closely related to the occurrence of IBD, and autophagy plays a key role in maintaining the intestinal barrier function.13 There is evidence that autophagy has primarily protected the intestinal epithelial TJPs and the inflammatory response. However, it is unknown whether autophagy is involved in the pathological process of radiation-induced intestinal barrier impairment.

Therefore, we hypothesized that autophagy is an important molecular biological mechanism involved in radiation-induced intestinal barrier impairment. In this study, we investigated the important role of autophagy in radiation-induced intestinal barrier function impairment by constructing an abdominal IR model and an autophagy activator (rapamycin (RAPA))/inhibitor (3-methyladenine (3-MA)) intervention model in mice.

Figure 1. Effect of RAPA and 3-MA on colonic mucosal autophagy after IR. Colonic mucosal gene expression of (A) beclin-1, (B) ATG7, (C) ATG12, and (D) LC3. Data are represented as means ± SEM (n = 10). *P < 0.05 vs the CON group. #P < 0.05 vs the IR group.

Figure 2. Effect of RAPA and 3-MA on colonic mucosal oxidative status after IR. Serum levels of (A) ROS, (B) RNS, (C) MDA, and (D) H2O2. (E) Colonic mucosal ROS, (F) RNS, (G) MDA, and (H) H2O2 levels. Data are represented as means ± SEM (n = 10). *P < 0.05 vs the CON group. #P < 0.05 vs the IR group.
RESULTS

Effect of RAPA and 3-MA on Colonic Mucosal Autophagy after IR. In order to confirm the effect of RAPA and 3-MA on autophagy of colonic mucosa after IR, we measured the expression of beclin-1, ATG7, ATG12, and LC3 genes related to autophagy of colonic mucosa in mice. Compared with the control group, IR significantly reduced the gene expression of beclin-1, ATG7, ATG12, and LC3 in the colonic mucosa ($P<0.05$; Figure 1A–D). Meanwhile, RAPA significantly increased the expression of beclin-1, ATG7, ATG12, and LC3 compared with the IR group ($P<0.05$; Figure 1A–D). Additionally, the gene expression of beclin-1, ATG7, ATG12, and LC3 in the colonic mucosa of the IR + 3-MA group was significantly lower than that in the IR group ($P<0.05$; Figure 1A–D). These results suggested that IR may induce a series of deleterious biological effects by inhibiting autophagy.

Effect of RAPA and 3-MA on Colonic Mucosal Oxidative Stress after IR. In order to evaluate the effect of autophagy after IR on the oxidative stress of the colonic mucosa, we measured the levels of ROS, RNS, MDA, and H$_2$O$_2$ in the serum and colonic mucosa of mice. Compared with the control group, IR markedly increased the serum and colonic mucosal ROS, RNS, MDA, and H$_2$O$_2$ levels ($P<0.05$; Figure 2A–H). Meanwhile, RAPA significantly decreased the serum and colonic mucosa ROS, RNS, MDA, and H$_2$O$_2$ content compared with the IR group ($P<0.05$; Figure 2A–H). Besides, levels of ROS, RNS, MDA, and H$_2$O$_2$ in the serum and colonic mucosa of the IR + 3-MA group were significantly higher than those in the IR group ($P<0.05$; Figure 2A–H). These results indicated that IR-induced autophagy inhibition is a key step in mediating colonic mucosal oxidative stress.

Effect of RAPA and 3-MA on Colonic Mucosal Antioxidant Status after IR. In order to investigate the effect of autophagy on the antioxidant status of the colonic mucosa after IR, we measured the activity of the SOD, GPx, CAT, and T-AOC in the serum and colonic mucosa of mice. Compared with the control group, IR markedly reduced the serum and colonic mucosal SOD, GPx, CAT, and T-AOC activity ($P<0.05$; Figure 3A–H). Meanwhile, RAPA markedly increased the SOD, GPx, CAT, and T-AOC activity in the serum and colonic mucosa compared with those of the IR group ($P<0.05$; Figure 3A–H). Moreover, the SOD, GPx, CAT, and T-AOC activities in the IR + 3-MA group were significantly lower than those in the IR group ($P<0.05$; Figure 3A–H). These results demonstrated that IR-induced autophagy inhibition is a key step in mediating colonic mucosal oxidative stress.

Figure 3. Effect of RAPA and 3-MA on colonic mucosal antioxidative status after IR. (A) Serum SOD, (B) GPx, (C) CAT, and (D) T-AOC levels. (E) Colonic mucosal SOD, (F) GPx, (G) CAT, and (H) T-AOC levels. Data are represented as means ± SEM ($n=10$). *$P<0.05$ vs the CON group. # $P<0.05$ vs the IR group.

Figure 4. Effect of RAPA and 3-MA on the colonic mucosal inflammatory response after IR. (A) Serum IL-1β, (B) IL-6, (C) IL-8, and (D) TNF-α levels. (E) Colonic mucosal IL-1β, (F) IL-6, (G) IL-8, and (H) TNF-α levels. Data are represented as means ± SEM ($n=10$). *$P<0.05$ vs the CON group. # $P<0.05$ vs the IR group.
agy inhibition is a key step in mediating the colonic mucosal antioxidant system.

Effect of RAPA and 3-MA on the Colonic Mucosal Inflammatory Response after IR. To confirm the effect of autophagy on colonic mucosal inflammation after IR, we measured the pro-inflammatory cytokines content in the serum and colonic mucosa of mice. Compared with the control group, the serum and colonic mucosal interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor (TNF)-α levels were increased in the IR group (*P < 0.05; Figure 4A−H). Meanwhile, RAPA reduced the IL-1β, IL-6, IL-8, and TNF-α levels in the serum and colonic mucosa compared with those of the IR group (*P < 0.05; Figure 4A−H). Furthermore, levels of IL-1β, IL-6, IL-8, and TNF-α in the IR + 3-MA group were markedly higher than those in the IR group (*P < 0.05; Figure 4A−H). These data suggested that IR-induced colonic mucosal inflammation is closely related to autophagy.

Effect of RAPA and 3-MA on Colonic Mucosal Mitochondrial Respiratory Chain Complex Activity after IR. In order to study the effect of autophagy on the mitochondrial function of the colonic mucosa after IR, we examined the activity of the mitochondrial respiratory chain complex in the colonic mucosa of mice. The activity of mitochondrial respiratory chain complexes I−V was decreased in the IR group (*P < 0.05; Figure 5A−E). Meanwhile, compared with the IR group, RAPA increased the activity of mitochondrial respiratory chain complexes I−V in the colonic mucosa (*P < 0.05; Figure 5A−E). Furthermore, the activities of colonic mucosal mitochondrial respiratory chain complexes I−V of the IR + 3-MA group were significantly lower than those in the IR group (*P < 0.05; Figure 5A−E). These data indicated that IR-induced colonic mucosal mitochondrial dysfunction is closely related to autophagy.

Effect of RAPA and 3-MA on Colonic Mucosal Apoptosis after IR. In order to study the effect of autophagy on the apoptosis of the colonic mucosa after IR, we detected the gene expression and enzyme activity of caspases in the colonic mucosa of mice. Compared with the control group, the gene abundance and enzyme activity of caspases (caspase-3, -8, -9, and -10) in the colonic mucosa of the IR group were
significantly upregulated \((P < 0.05; \text{Figure 6A–H})\). Meanwhile, compared with the IR group, RAPA reduced the caspase gene expression and enzyme activity \((P < 0.05; \text{Figure 6A–H})\). Moreover, the caspase-3, -8, -9, and -10 gene expressions and enzymatic activities in the colonic mucosa of the IR + 3-MA group were significantly higher than those in the IR group \((P < 0.05; \text{Figure 6A–H})\). These data indicated that IR-induced colonic mucosal apoptosis is closely related to autophagy.

**Effect of RAPA and 3-MA on Colonic Permeability after IR.** To evaluate the effect of autophagy on colonic permeability after IR, we measured serum and colonic mucosal chemical markers (endotoxin, zonulin, diamine peroxidase, and \(\delta\)-lactate) in mice. Compared with the control group, IR markedly increased the levels of these markers in the serum and colonic mucosal \((P < 0.05; \text{Figure 7A–H})\). Meanwhile, RAPA decreased them compared with the IR group \((P < 0.05; \text{Figure 7A–H})\). Moreover, the amounts of endotoxin, zonulin, DAO, and \(\delta\)-lactate in the colonic mucosa of the IR + 3-MA group were significantly higher than those in the IR group \((P < 0.05; \text{Figure 7A–H})\). These findings implied that IR inhibits autophagy, thereby regulating REDOX balance, the inflammatory response, and apoptosis, ultimately increasing colonic permeability.
Effect of RAPA and 3-MA on Colonic Barrier Function after IR. In order to demonstrate the effect of autophagy on colonic barrier function after IR, we measured the gene expression of tight junction proteins (ZO-1, occludin, claudin-1, and claudin-4) in the mice colonic mucosa. Compared with the control group, IR significantly reduced the gene expression of ZO-1, occludin, claudin-1, and claudin-4 in the colonic mucosa ($P < 0.05$; Figure 8A–D). Meanwhile, RAPA increased them compared with the IR group ($P < 0.05$; Figure 8A–D). Additionally, the gene expression of ZO-1, occludin, claudin-1, and claudin-4 in the colonic mucosa of the IR + 3-MA group was significantly lower than that in the IR group ($P < 0.05$; Figure 8A–D). These findings implied that IR induces a series of negative biological effects by inhibiting autophagy, thereby disrupting colon barrier function.

**DISCUSSION**

IR therapy has been one of the main methods of treating various cancers in the past few decades. Although radiotherapy technology has been advancing, the effective use of IR therapy has been limited due to the damage caused by IR to normal tissues. A large number of studies have shown that abdominal IR therapy can lead to radioactive intestinal diseases, mainly manifested as acute and chronic intestinal injury. Radioactive intestinal disease usually results in anorexia, vomiting, diarrhea, dehydration, systemic infection, bloody shock, and death. IR-induced intestinal damage negatively affects patients’ treatment outcomes and reduce their quality of life. Therefore, it is important and urgent to study the underlying mechanism of intestinal IR injury. Autophagy is a process of degradation of proteins and organelles by lysosomes, and it assists in cellular adaptation to various undesirable stimuli and plays an important role in maintaining homeostasis and participating in self-renewal of the intracellular environment. In the current study, our results showed that the abundance of autophagy-related genes was downregulated in the colonic tissues of the irradiated mice.

Reactive oxygen species (ROS) and reactive nitrogen (RNS) produced under physiological conditions are important factors in the maintenance of cell life activities, but the overproduction of ROS and RNS is harmful to the human body. The toxic effects of these molecules include DNA/RNA damage, amino acid oxidation, and lipid peroxidation, resulting in intracellular nucleic acid damage, mutations, and protein and lipid damage. IR causes mitochondrial electron leakage and facilitates excessive ROS synthesis and RNS by nitric oxide synthase. Previous studies have shown that IR can cause oxidative damage to cells, leading to tissue and organ damage and loss of function. Consistently, our data suggested that IR-induced oxidative stress by increasing levels of ROS, RNS, MDA, and H$_2$O$_2$ in the serum and colonic mucosa of mice.

Organisms possess free-radical scavenging antioxidant defense systems, including enzymatic and non-enzymatic antioxidant defense mechanisms. In this study, the antioxidant system of IR group mice was damaged, mainly due to the loss of antioxidant enzyme activity. Autophagy can alleviate the damage caused by oxidative stress, thus protecting the survival of cells. Our data showed that an autophagy activator (rapamycin) significantly alleviated oxidative stress in the colon tissues of irradiated mice, while an autophagy inhibitor (3-methyladenine) further aggravated the degree of oxidative stress. These findings implied that radiation-induced autophagy inhibition is an important cause of the oxidation—antioxidant state imbalance.

Inflammation is one of the important clinical manifestations of IR intestinal injury. Previous studies have shown that IR induces high expression of pro-inflammatory cytokines such as IL-1$\beta$, IL-6, IL-8, and TNF-$\alpha$. TNF-$\alpha$ exerts an obvious destructive effect on the expression and distribution of tight junction proteins. Not only do IL-1$\beta$, IL-6, and IL-8 regulate the recombination of cytoskeletal proteins, but their action also directly leads to the rearrangement of tight junction proteins, thereby reducing barrier function. Consistently, our data suggested that IR induced the inflammatory response by increasing IL-1$\beta$, IL-6, IL-8, and TNF-$\alpha$ levels in the serum and colon of mice. Autophagy is closely related to inflammation. Cytokines can regulate the autophagy response, and autophagy can regulate the inflammatory response through multiple signaling pathways. Our data showed that an autophagy activator (rapamycin) significantly alleviated an inflammatory response in the colon tissues of irradiated mice, while an autophagy inhibitor (3-methyladenine) further aggravated the degree of inflammatory response. These findings suggest that inhibition of autophagy is an important factor in the inflammatory response to radiation-induced intestinal injury.

ROS is a byproduct of the energy production process of the mitochondrial respiratory chain. When the function of the cellular antioxidant system is impaired, excess ROS that cannot be eliminated can accumulate excessively in the mitochondria, resulting in oxidative stress damage and mitochondrial dysfunction. Emerging evidence showed that the mitochondria are the most sensitive organelles to radiation, and radiation can cause abnormal mitochondrial function. The primary respiratory chain and the secondary respiratory chain together constitute the mitochondrial respiratory chain in which complexes I, II, and III are composed of the primary respiratory chain, complexes IV, and V constitute the secondary respiratory chain, and complex V is ATP synthase. In the present study, our data showed that the activity of mitochondrial respiratory chain complexes I–V in the colon was significantly reduced after IR, suggesting the impairment of mitochondrial function.

Apoptosis refers to the spontaneous and orderly death of cells controlled by genes in order to maintain the stability of the internal environment. One of the classic mechanisms of apoptosis is the mitochondrial apoptosis pathway, which is characterized by the loss of mitochondrial integrity and transmembrane potential, leading to caspase-3 protein activation, DNA fragmentation, and cell death. Some scholars have shown that IR-induced intestinal damage was usually accompanied by intestinal epithelial cell apoptosis. Consistent with previous studies, we found that IR increased the gene expression and enzyme activity of caspases related to the mitochondrial apoptosis pathway in the colonic mucosa of mice. To investigate the role of autophagy in radiation-induced mitochondrial dysfunction and apoptosis, we also recruited mice with rapamycin and 3-methyladenine intervention. Our data showed that rapamycin significantly alleviated colonic mitochondrial dysfunction and apoptosis in irradiated mice, while 3-methyladenine further exacerbated these biological processes.

As mentioned above, IR can induce the production of excess ROS. Excessive ROS in intestinal tissue can disrupt the balance between damage and repair of intestinal epithelial cells, thereby...
increasing the risk of intestinal diseases. Researchers have conducted extensive research on the relationship between IR and intestinal injury. Among them, oxidative stress has caused widespread concern. IR-induced oxidative stress can inhibit the regeneration of intestinal stem cells, change the shape of intestinal villi, and increase the permeability of intestinal epithelial cells. Besides, when patients are exposed to high doses of IR, they will die of acute intestinal injury within 10 days. Fortunately, some nontoxic radioprotectors can protect intestinal health by adjusting the intestinal barrier function. In healthy individuals, the chemical indicators of intestinal damage such as endotoxin, DAO, β-lactic acid, and zonulin are relatively low or even undetectable, but when intestinal permeability increases, they will be present in the blood circulation in large amounts. In the present study, our data indicated that levels of chemical markers in serum and colon tissue of irradiated mice significantly increased. In addition, IR decreased the expression of the TJPs in the colonic epithelium of mice. Autophagy plays an important role in maintaining intestinal permeability and intestinal barrier function. Our data showed that RAPA significantly improved barrier function in the colon of mice, while 3-MA further impaired barrier function.

CONCLUSIONS

In summary, we report here that abdominal IR inhibited autophagy in intestinal epithelial cells and subsequently regulated biological processes such as oxidation-antioxidant balance, the inflammatory response, mitochondrial function, and apoptosis, ultimately increasing intestinal permeability and disrupting the intestinal barrier function in irradiated mice. Therefore, our findings provide a reference and theoretical basis for the screening of key targets for the prevention and treatment of radioactive intestinal injury.

MATERIALS AND METHODS

Animals. For this study, 40 male C57BL/6 mice aged 8–10 weeks (purchased from the Institute of Model Animals of Nanjing University) were selected. All mice are kept in an environment free of specific pathogens. The experimental environment guarantees constant temperature and humidity with a light/dark cycle of 12 h. All mice had free access to food and water and were fed adaptively at least 1 week before the experiment. The mice were maintained in accordance with the “Guidelines for the Protection and Application of Laboratory Animals” issued by the U.S. National Institutes of Health (NIH Publication no. 85-423, 1996 version) and the corresponding regulations of the Animal Management Committee of the Affiliated Jiangyin Hospital of the Southeast University Medical College (SU-20190324).

IR. C57BL/6 mice were anesthetized with 35 mg/kg 1% pentobarbital, then fixed on a cardboard, and subjected to local high-dose abdominal precision IR (a 225 Kv/17 mA Cs137 linear accelerator at 2 Gy/min for 5 min and a single dose of 10 Gy). The IR range was concentrated at the two-leg connection level to 2 cm above this area; the rest of the body was shielded with a 5 cm piece of lead.

Experimental Design. Four groups of mice (n = 10 each) were used in this study. Group 1 (CON group) consisted of mice that served as the untreated vehicle saline control group. Group 2 (IR group) received only IR every day. Group 3 (IR + RAPA group) received rapamycin (2 mg/kg/day) gavage every other day while receiving IR. Group 4 (IR + 3-MA group) was injected intraperitoneally with 3-methyladenine (24 mg/kg/day) every other day while receiving IR. The experimental period for all groups was 2 weeks.

Sample Collection. All mice were euthanized at the end of the experimental period. To reduce sample variability, intestinal segments were collected from the approximate middle position of the intestinal tract (colon). The colonic mucosa was separated from the muscular layers by blunt dissection and stored at −80 °C prior to further analysis. Blood samples were collected to obtain the serum. After blood coagulation and clot contraction, the samples were centrifuged at 3000 × g for 15 min to obtain the serum and stored at −80 °C for further analysis.

Measurement of Oxidative Stress Indicators. The amounts of reactive oxygen species (ROS), reactive nitrogen species (RNS), malondialdehyde (MDA), and hydrogen peroxide (H2O2) in serum and colonic samples were determined using enzyme-linked immunosorbent assay (ELISA) kits (Shanghai Enzyme-linked Biotechnology Co. Ltd., Shanghai, China) per the manufacturer’s instructions.

Measurement of Antioxidant Indicators. The activity of antioxidant indicators (SOD, GPx, CAT, and T-AOC) in serum and colonic samples were determined using commercial kits (Shanghai Enzyme-linked Biotechnology Co. Ltd., Shanghai, China). The detailed steps of the test operation refer to the manufacturer’s instructions.

Measurement of pro-Inflammatory Cytokines. The levels of IL-1β, IL-6, IL-8, and TNF-α in serum and colonic samples were determined using commercial kits (Shanghai Enzyme-linked Biotechnology Co. Ltd., Shanghai, China). The detailed steps of the test operation refer to the manufacturer’s instructions.

Measurement of Mitochondrial Respiratory Chain Complex Activity. The mitochondrial respiratory chain complex I–V activities in colonic samples were evaluated with mitochondrial respiratory chain complex assay kits (Suzhou Comin Biotechnology Ltd., China). The detailed steps of the test operation refer to the manufacturer’s instructions.

Measurement of Caspase Activity. Caspase-3, -8, -9, and -10 activities in colonic and intestinal segments were determined using commercial kits (Shanghai Enzyme-linked Biotechnology Co. Ltd., Shanghai, China). The detailed steps of the test operation refer to the manufacturer’s instructions.

Measurement of Colonic Permeability. The endotoxin, diaminoperoxidase (DAO), β-lactic acid, and zonulin content in serum and colonic samples were determined using commercial kits (Shanghai Enzyme-linked Biotechnology Co. Ltd., Shanghai, China). The detailed steps of the test operation refer to the manufacturer’s instructions.
RNA Isolation, cDNA Synthesis, and Real-Time Quantitative PCR. Total RNA from colonic samples was extracted using the TRizol reagent. The RNA concentration and quality in the extracted colonic samples were measured using a NanoDrop ND-1000 spectrophotometer (Thermo, USA). Next, 2 μg of total RNA was treated with RNase-Free DNase and reverse-transcribed per the manufacturer’s instructions. Diluted cDNA (2 μL; 1:20, vol/vol) was used for real-time PCR, which was performed using an Mx3000P real-time PCR system (Stratagene, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was unaffected by the experimental factors, was chosen as the housekeeping gene. All primers used in this study are listed in Table 1 and were synthesized by Generay Co. (Shanghai, China). The 2ΔΔCt method was used to analyze the real-time PCR results, and gene mRNA levels are expressed as the fold change relative to the mean value of the control group.

Table 1. Primer Sequences Used in This Study

| target genes | primer forward/ reverse | primer sequence (5′ → 3′) |
|--------------|-------------------------|--------------------------|
| GAPDH        | forward                 | GAAGACCTGGTGACCCGATG     |
|              | reverse                 | AAGGTGAGTGGTGTCAGAGG     |
| Beclin-1     | forward                 | GAGACGGCTGAAACCTGAGA     |
|              | reverse                 | GGTGGACAGAAAGAAAGC       |
| ATG7         | forward                 | GCTAAATTAGCGAACCAGAGG    |
|              | reverse                 | ACAAGTGGAGCCGAGGATG      |
| ATG12        | forward                 | ATATGACACGCGGCTGGTTG     |
|              | reverse                 | CCAACACTGCGATAGTAGAAGC   |
| LC3          | forward                 | AGGGCAAGATGGATGCCCCAAA   |
|              | reverse                 | AGCATTGTCCTCTGAAAGGGA    |
| caspase-3    | forward                 | AGTTGACTCCCTCTCTCAAT     |
|              | reverse                 | TGCTAGGCTGTCGAGTAGG      |
| caspase-8    | forward                 | GCTGGAGAGTGCACTGACAC     |
|              | reverse                 | GCTGCGAGGCGGGTAATAGT     |
| caspase-9    | forward                 | ATCGGAGGTGGAGAGGCTA      |
|              | reverse                 | GTTTGTGTTGCTGAGCGTGGGA   |
| caspase-10   | forward                 | GTAGCGCCTTGCAGGTTACCT    |
|              | reverse                 | CTAATGTCAGCTCTCAGGGGCT   |
| ZO-1         | forward                 | GCCCCACCTATCCTCTGCTAT    |
|              | reverse                 | CCTGTCCTCTATACAGGGGAC    |
| occludin     | forward                 | GGCAGCGACGGTGGTAAGCG     |
|              | reverse                 | ACTTGGCCGCGATGAGCAAGCA   |
| claudin-1    | forward                 | GTGTTGGAACCCCCGGAAGCA    |
|              | reverse                 | CTTCCCAAGATTTCTGCTGAGAT  |
| claudin-4    | forward                 | TCCCTCCAGAGATTTCTGAGATT  |
|              | reverse                 | CCACGTGCCTTCTGCGATAGG    |

<ref>synthesized by Generay Co. (Shanghai, China). The 2ΔΔCt method was used to analyze the real-time PCR results, and gene mRNA levels are expressed as the fold change relative to the mean value of the control group.</ref>

**STATISTICAL ANALYSIS**

Data are presented as means ± SEM. Statistical significance was assessed by the independent sample t test using SPSS (SPSS v. 20.0, SPSS Inc., Chicago, IL, USA) software packages. Data was considered statistically significant when P < 0.05. Numbers of replicates used for statistics are noted in the figures.

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**Author Contributions**

W.Q. designed the research. L.Z. conducted the research. J.A. analyzed the data. The manuscript was mainly written by W.Q., L.Z., and J.A. All the authors have read and approved the final manuscript.

**Funding**

This work was financially supported by the Wuxi Health Committee Youth Research Foundation, China (project no. Q201911), Li Jie-shou Gut Barrier Foundation, China (project no. LJS-201811C), and the Wuxi Science and Technology Bureau, China (project no. 2018B7).

**Notes**

The authors declare no competing financial interest.

**ABBREVIATIONS**

3-MA: 3-methyladenine; CAT: catalase; DAO: diamine oxidase; GPx: glutathione peroxidase; H2O2: hydrogen peroxide; IB: inflammatory bowel disease; IL: interleukin; IR: irradiation; MDA: malondialdehyde; RAPA: rapamycin; RNS: reactive nitrogen species; ROS: reactive oxygen species; SOD: superoxide dismutase; T-AOC: total antioxidant capacity; TNF-α: tumor necrosis factor-α.

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