Original Article
NADH protect against radiation enteritis by enhancing autophagy and inhibiting inflammation through PI3K/AKT pathway

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Abstract: Radiotherapy is an important method for cancer treatment but it has serious side-effects at high doses. One of the greatest challenges in radiotherapy is that radiation affects both healthy tissue and cancer tissues. For abdominal or pelvic lesions, the bowel is the most easily injured by irradiation. Radiation may cause radiation enteritis, intestinal inflammatory infiltration, or intestinal perforation. Coenzyme NADH involves in energy metabolism and transportation of nucleic acid, proteins and carbohydrates. In our study, NADH was used to protect the intestinal wall from irradiation injury in IEC-6 normal intestinal epithelial cells. By flow cytometry, we found that NADH can inhibit the cell death and the producing of reactive oxygen species (ROS). The immunofluorescence assay showed that cell autophagy was increased in the NADH group. Western blot data indicated that NADH promoted the microtubule associated protein 1A/1B-light chain 3(LC3)-I to LC3II and the expression of IL-1β and TNFα decreased in a dose dependent manner. Interestingly, a specific PI3K/AKT inhibitor (3MA) decreased the expression of inflammatory factors. In the animal experiment, after 12 Gy radiation, there were less TNFα and more LC3II in the RT+NADH group than that of RT group. Compared with the mock, there was no significant damage in the NADH group. Thus, our study provides the evidence that NADH may protect against radiation enteritis by suppressing inflammation and enhancing autophagy through PI3K/AKT pathway in normal intestinal cells.

Keywords: NADH, radiation enteritis, autograph, inflammation, PI3K/AKT pathway

Introduction

Cancer is a common disease, and the incidence is increasing year by year. More than half of cancers can be treated with radiotherapy (RT), but RT can also damage the surrounding normal tissues. This side-effect will diminish the quality of RT in cancer patients [1]. For example, after a few weeks of RT, the skin tends to develop painful, infection-prone severe epithelial barrier breakdown. 60-80% of patients experience temporary symptoms of bowel toxicity due to radiation therapy. Especially in abdominal or pelvic RT, 50% patients develop varying degree of chronic bowel dysfunction, one of the most common complications of long term cancer survivors [2]. At the early radiation enteropathy, because of epithelial barrier dys-function and mucosal inflammation, the main symptoms is nausea, vomiting, diarrhea or abdominal pain. The delayed early radiation enteropathy is mainly manifested as mucosal atrophy vascular sclerosis and intestinal wall fibrosis [2].

Radiation induced normal tissue injury is a complex process involving different mechanisms, such as DNA repair problem, inflammation, cell death and so on [3, 4]. Among them inflammation plays an important role in radioactive intestinal damage. Inflammation is an important part of immune system. Abnormal activation of inflammation can lead to severe tissue damage and even organ failure. Importantly, it is associated with a number of human disease, such as metabolic syndrome and

inflammatory bowel disease (IBD) [5, 6]. It is known that the activation of inflammatory lead to the increased production of reactive oxygen species (ROS) [7], which is important to ionizing radiation induced cell damage [8]. How to reduce the damage of normal intestines caused by radiation is an urgent problem to be solved.

Autophagy is a pathway of stressful lysosomal degradation. It maintains cytoplasmic homeostasis by eliminating intracellular misfolding or aggregation of proteins and damaged organelles. Autophagy is not only important to prevent inflammation-pron conditions by maintains cytoplasmic homeostasis but also directly by selective elimination of invading pathogens autophagy [9, 10] and the removal of activated inflammasomes and ROS [11, 12]. Mitochondria-selective autophagy, a type of autophagy, has become a participant in maintaining mitochondrial homeostasis and regulating inflammation activation. In addition, mitochondrial autophagy can inhibit the activation of inflammation by eliminating mitochondrial ROS [12]. Besides, an increasing number of evidence has proved that PI3K/AKT pathway is a key regulator of autophagy [13]. The pathway can inhibit the activation of autophagy.

NADH quinone oxidoreductase 1 (NQO1) is a ubiquitous cytosolic flavoenzyme that catalyzes two-electron donor. This NQO1 mediated reduction mechanism is responsible for the cellular defense against various damaging oxyradicals. Evidences have suggest that NADH play crucial roles in a variety of biological processes, including energy metabolism, mitochondrial function and gene expression [14]. In our study, we found that NADH can inhibit the inflammation after the IEC-6 normal intestinal epithelial cells treated with RT, and the autophgy increased. When we used an autophagy inhibitor to block this process, the inflammation was increased. So we hypothesized that NADH can reduce the inflammation by affecting autophgy in IEC-6 cells.

Method

Cell culture

IEC-6 normal intestinal epithelial cells were purchased from Cell bank of the Chinese Academic of Sciences (Shanghai, China) and cultured in RPMI1640 (Gibco BRL, USA) supplemented with 10% fetal bovine serum (FBS, Gibco BRL, USA), 1 mM L-glutamine, 100 U/mL penicillin and 100 ug/ml streptomycin in 5% CO_2 at 37°C. NADH was purchased from Roche (Roche, Switzerland).

Cell counting Kit-8 assay (CCK8)

Cell viability was evaluated by the Cell counting Kit-8 assay (CCK8) (DOJINDO, JAPAN). IEC-6 normal intestinal epithelial cells (1×10^4 cells/well) were seeded in the 96-well plate. When the cells stick to the wall later, different concentration of NADH was added in. Then the cells were incubated in 5% CO_2 at 37°C. After 48 h, the medium was removed, 100 ul of cell culture medium with 10% CCK8 was added to each well, then incubated for 1-4 h. The cell viability was measured at the absorbance of 450 nm with a microplate reader (Bio-rad, USA).

Annexin V-APC/7-AAD staining for apoptosis by flow cytometry assay

The IEC-6 cells were plated in the 6-well plates at a concentration of 2.5×10^5. After one night, cells were treated with 4 Gy X-ray irradiation. Then 0 ug/ml (NC group), 100 ug/ml NADH (100 ug/ml group), 200 ug/ml NADH (200 ug/ml group) were added to the wells respectively. 48 h later, the cells were collected, washed with PBS for three times, followed by staining with Annexin V-APC/7-AAD for 20 min at room temperature. A flow cytometer was used to analyze the percentage of apoptotic cells.

Measurement of ROS

The cells treated with RT and different does of NADH were incubated with 10 µM 2'7'-dichlorofluorescein diacetate (DCFDA) at 37°C for at least 30 min. Thereafter, the cells were washed with PBS for three times. Fluorescence was measured by flow cytometer and microscopy.

Animal experiment

All protocols in this study were approved by the National Institutes of Health Guide for the Care and Use of Laboratory animals (NIH Publications No. 8023, revised 1978). 6-week old male C57BL mice were purchased from Southern Medical University Animal Experimental Center (Guangzhou, China). The experiment was performed after 1 week of adaptation.
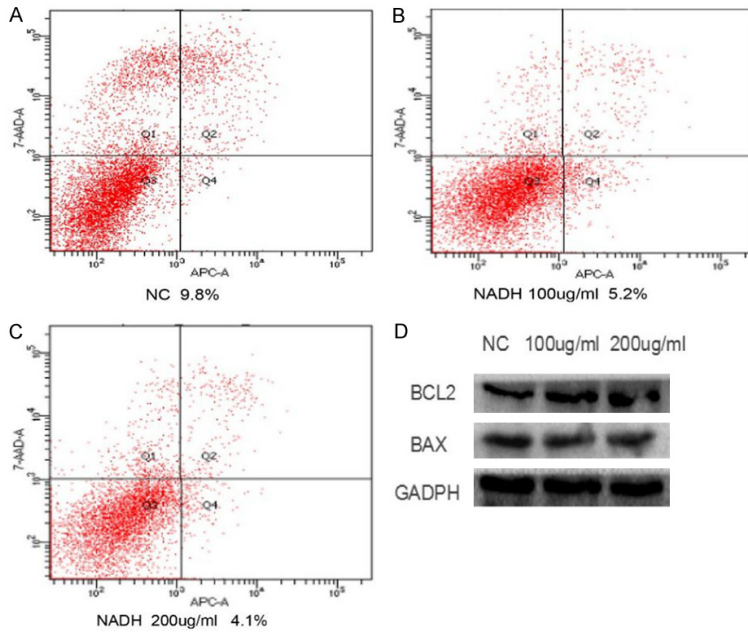
Figure 1. NADH inhibited the cells apoptosis. After RT (4 Gy), cells were treated with 0 ug/ml, 100 ug/ml, 200 ug/ml NADH for 48 h. Flow cytometry data showed that NADH had a protective effect on the IEC-6 cells in a dose-dependent manner (A-C). Western blot showed the anti-apoptosis protein BCL2 in the 100 and 200 ug/ml groups was more, the expression of pro-apoptotic gene, BAX, was less than no-treatment control group (D).

Mice were divided into 4 groups: mock, NADH, RT, RT+NADH (Four mice per group). 12 Gy of lethal dose were performed to the RT and RT+NADH groups. Then the NADH and RT+ NADH groups were given NADH by 10 mg/kg/day. After 7 days, the animals were sacrificed. Intestines were evaluated for immunohistochemistry and HE staining.

Immunohistochemistry and HE staining

Tissue sections (4 μm) from the intestines samples were deparaffinized, rehydrated and then heated in 10 mmol/L citrate buffer (PH = 6.0) for antigen retrieval. Then the sections were washed with PBS, blocked with 10% FBS for 30 min and incubated with primary antibody over night at 4°C. TNFα (Proteintech, Wuhan, China) was diluted by 1:300. LC3II (Cell Signal Technology, USA) was diluted by 1:500. The sections were subsequently incubated with the corresponding secondary antibody for 1 h and washed with PBS for three times. Images were obtained by a Leica DM4000B/M microscope (Leica Microsystems, USA). For HE staining, the intestines samples for all cases were fixed in 4% formaldehyde solution (PH 7.0) for periods not exceeding 24 h, then the tissues were

processed routinely as paraffin embedding, sectioned at 4 μm thick and placed on the glass slides. Tissue samples were stained with hematoxylin and eosin. Thereafter, sections were selected randomly to be observed under microscope (400 × and 200 × magnification, Leica Microsystems, USA).

Immunofluorescence (IF)

The Immunofluorescence assay was performed as previously did [15]. In brief, cells on the glass slide were fixed with 4% paraformaldehyde for 15-20 min. After washing in PBS with 0.2% Tween-20, blocked with 5% FBS for at least 1 h, the slides were incubated with primary antibody against LC3 (Cell Signal Technology, USA) at 37°C for 2 h. Thereafter, incubated with the anti-rabbit FITC-conjugated second antibody (Zhongshan, Jinqiao, Beijing) for 1 h. Photos were taken with a microscope (Olympus, BX53; Melville, NY, USA).

Western blot analysis

Total proteins were extracted by lysis buffer containing of 1% PMSF and phosphatase inhibitor (Beyotime, Shanghai, China). Equal amount of proteins were separated by SDS-PAGE gels, transferred to the PVDF membrane, blocked with 5% FBS at room temperature for 1 h on a shaker. The membranes were incubated with primary antibody over night at 4°C. IL-1β, TNFα, Gapdh and β-actin (Proteintech, Wuhan, China) were diluted by 1:1000. LC3II, P65, PI3K, AKT, p-AKT (Cell Signal Technology, USA) were diluted by 1:1500. The membranes were washed with TBST for three times, then incubated in an HRP-conjugated anti-rabbit Igg (1:5000) for 1 h at room temperature. Proteins were visualized by ECL Western blotting substrate, and the band intensity was assessed using Image J.

Statistics

All of the data were graphed and analyzed by Graphpad Prime 5.0 software. All the experi-
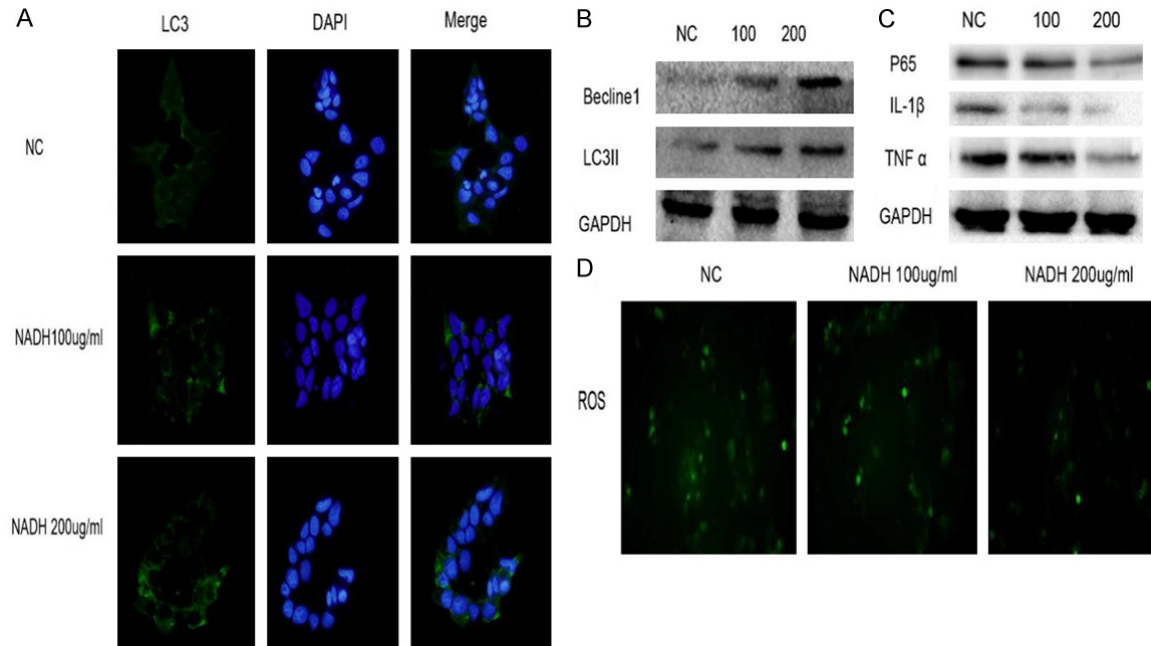
Figure 2. NADH can activate autophagy, suppress inflammation and ROS. Autophagy is closely related to apoptosis. After RT (4 Gy), cells were treated with 0 ug/ml, 100 ug/ml, 200 ug/ml NADH for 48 h. Immunofluorescence and western blot showed that after NADH treated, the marker of autophagy, LC3II (A and B) and Beclin1 (B), were increased. The expression of inflammation factor, IL-1β, P65 and TNFα, were decreased (C). The expression of ROS, an influencing factor of inflammation, also decreased compared to the NC group (0 ug/ml) (D).

ments were repeated at least three times. Differences between the groups were analyzed using Student's t-test. *P* values less than 0.05 were considered statistically significant.

Result

NADH did not result in significant toxicity on cell viability

To test whether NADH result in significant toxicity in IEC-6 cells, CCK8 assay was performed. The IEC-6 cells were incubated with NADH at 0, 100, 200, 300, 400, 500, 600, 700, 800 ug/ml concentrations for 48 h. The result showed that the cell viability was not affected at 1-500 ug/ml NADH (Supplementary Figure 1), so we chose 100 and 200 ug/ml as stimulate dose. All the experiments followed this dose.

NADH could inhibit apoptosis and reduce the inflammation in IEC-6 cells

IEC-6 cells treated with or without NADH after the 4 Gy RT were used to analyze the percentage of apotosis and inflammation. NADH could inhibit the IEC-6 cell from apoptosis (**Figure 1A**). Western blot data showed that compared

with the 0 ug/ml NADH cells, the anti-apoptosis protein BCL2 in the 100 and 200 ug/ml groups was more, the expression of pro-apoptotic gene, Bax, was less (**Figure 1B**). At the same time, the inflammation factors (IL-1β, P65 and TNFα) in the NADH treated groups also appeared a corresponding decline in expression (**Figure 2C**). All of them changed in a dose-dependent manner.

NADH promotes autophagy by suppressing the PI3K/AKT pathway

Western blot showed that NADH could inhibit the cells from apoptosis, and it is well known that autophagy is a self-degradation process, which is closely related to apoptosis. The result showed that in the group treated with NADH, the expression of autophagy protein LC3II showed a significant increase (**Figure 2A**). The immunofluorescence assay showed that in the cells treated with RT and NADH, the expression of LC3II, an important marker of autophagy, was significantly increased in a dose-dependent manner. Becline1 was also up-regulated (**Figure 2A** and **2B**). Moreover, we found that NADH could active autophagy through sup-
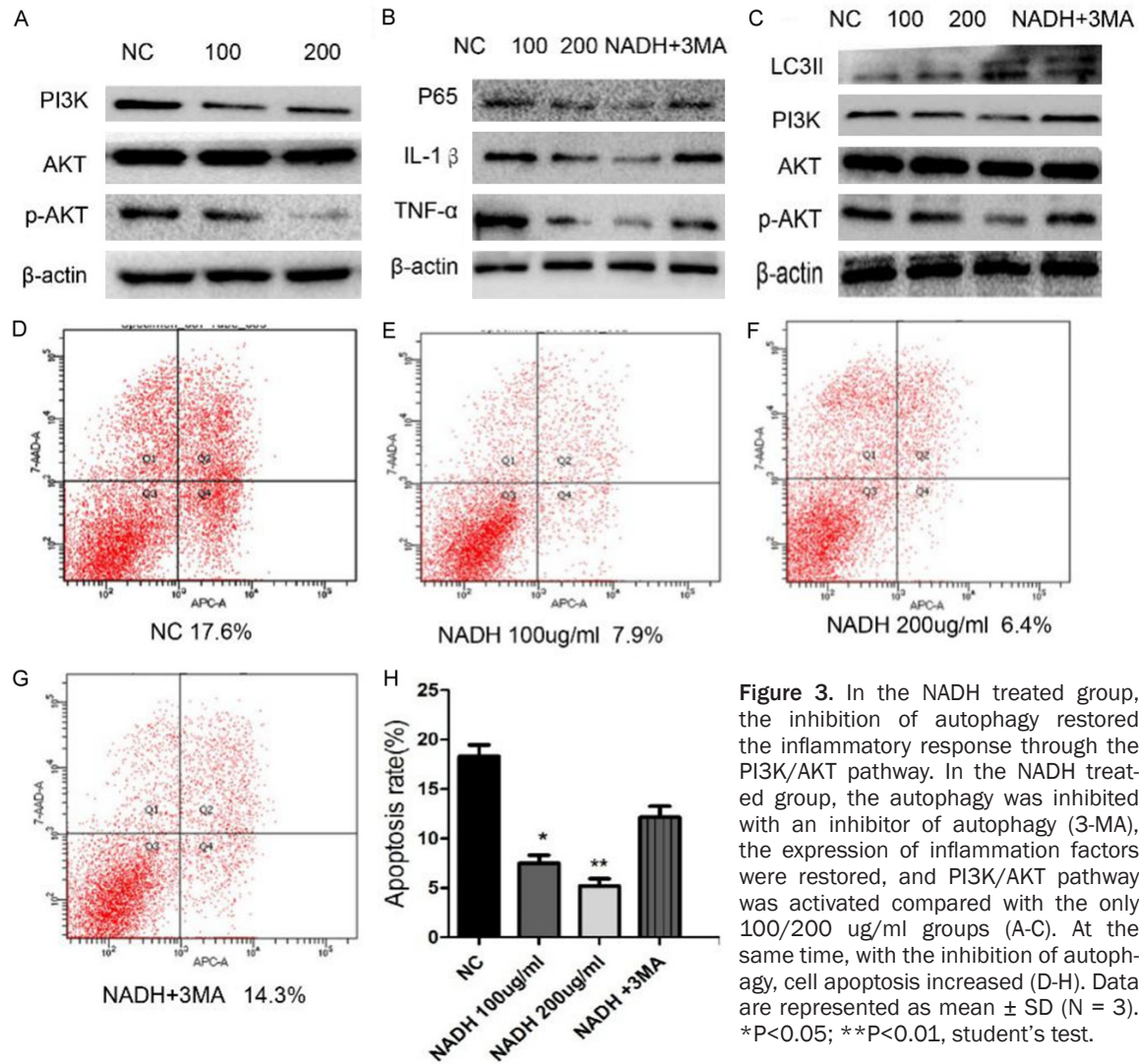
Figure 3. In the NADH treated group, the inhibition of autophagy restored the inflammatory response through the PI3K/AKT pathway. In the NADH treated group, the autophagy was inhibited with an inhibitor of autophagy (3-MA), the expression of inflammation factors were restored, and PI3K/AKT pathway was activated compared with the only 100/200 ug/ml groups (A-C). At the same time, with the inhibition of autophagy, cell apoptosis increased (D-H). Data are represented as mean ± SD (N = 3). *P<0.05; **P<0.01, student's test.

pressing the PI3K/AKT pathway. PI3K/AKT signaling pathway is a classical autophagic pathway (**Figure 3A**). The pathway can inhibit the activation of autophagy [13, 16]. To our surprise, when we use 3-Methyladenine (3-MA) (an inhibitor of autophagy) to stimulate the NADH treated group, the autophagy was inhibited, the cell apoptosis rate was also reduced, and at the same time, the expression of inflammation proteins, such as IL-1β, TNFα, P65, were also down-regulated (**Figure 3B-H**). Also the PI3K/AKT pathway was suppressed. Recently, it has been shown that autophagy has emerged as a central regulator for maintaining the cell function through eliminating damaged mitochondria, which leads to the inactivation of inflammation [17]. Previous study has suggested that ATP-induced mitochondria dysfunction lead to

the production of ROS, and ROS is an activator of inflammation [18]. In our study, we found that in the RT+NADH treated group, the production of ROS was less than the RT and RT+NADH+3MA group (Supplementary Figure 2). Taken together, all of these results showed that NADH could inhibit the inflammation activation through promoting the autophagy.

Animal assay showed that NADH could protect against the radiation-induced intestinal injury

The animals were divide into 4 groups: mock, NADH, RT, RT+NADH. After RT, NADH was orally administered to the NADH and NADH+RT groups. On the 7th day, we examined the intestinal injury by HE and immunohistochemistry, we found that NADH could significantly protect
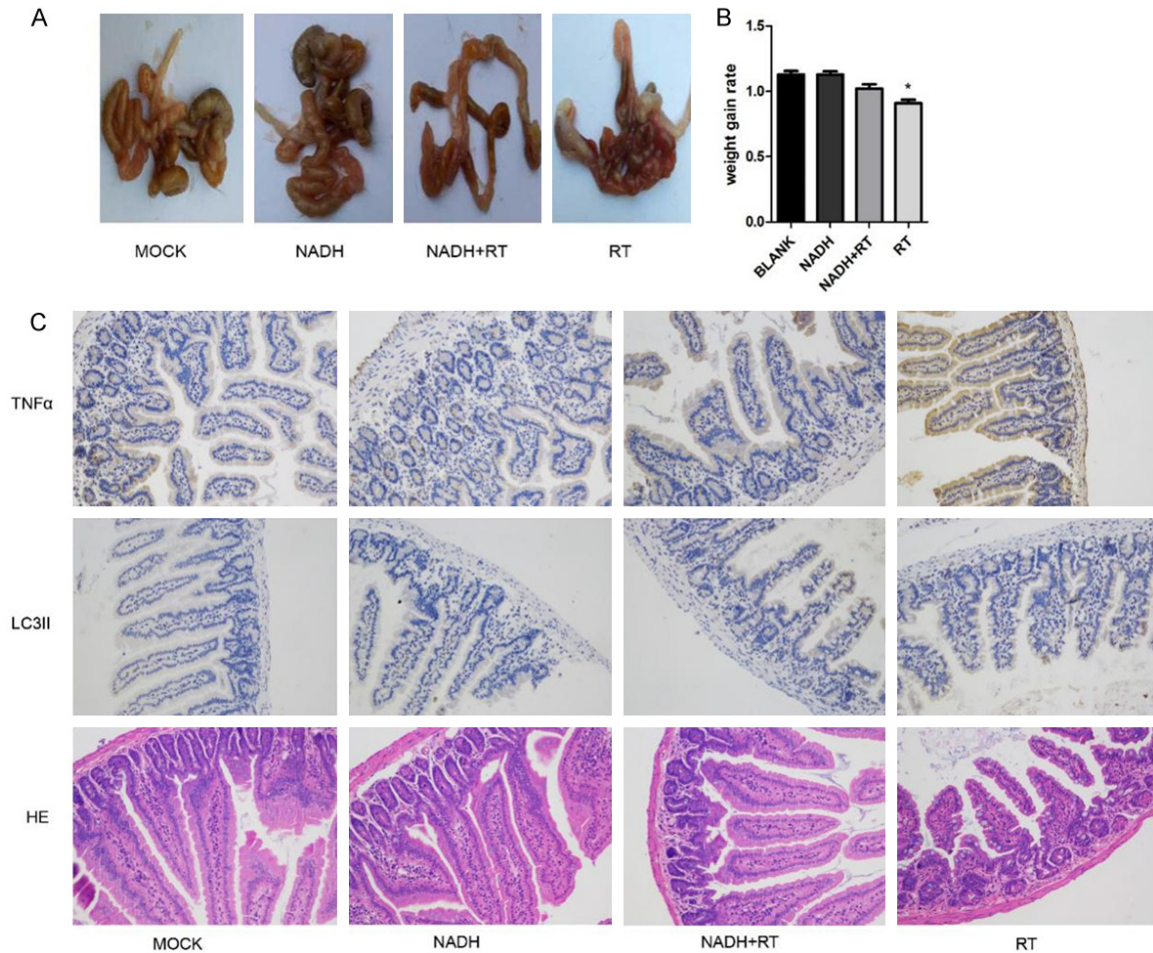
Figure 4. Animal assay showed that the expression of autophagy and inflammation were negatively correlated. Animals were divided into 4 groups, 7 days after RT. (A) representative images of intestines of mice. (B) quantification of weight gain rate of mice. We found that NADH could significantly suppress the intestinal congestion, edema and the loss of weight (A and B). IHC showed that in the RT group, the inflammation factors were over expressed, and autophagy were inhibited compared with the RT+NADH group (C). HE showed that NADH could protect the villi height. Compared with the RT group, intestinal mucosal damage was less in the RT+NADH group (C). Data are represented as mean ± SD (N = 3). *P<0.05, student's test.

the villi height and suppress the intestinal congestion and edema (**Figure 4A** and **4C**) in the RT treated cells. To confirm the radioprotective effect of NADH in vivo, the weight of all the animals were recorded. Among the 4 groups, animals in the RT group showed a serious reduction in weight, whereas, no obvious body weight lose was observed in the other 3 groups (**Figure 4B**). Compared with the mock group, NADH group had no significant systematic toxicity. In the RT and RT+NADH group, NADH led to a reduced expression of TNF-α, an increased expression of autophagy protein LC3II (**Figure 4C**). At the same time, in the RT group, there was a C57 mice died at the 5th day. These results suggested that NADH is a protective factor in radiation-induced injure.

Discussion

Approximately 50% cancer patients receive radiotherapy. Although advances in radiotherapy have been made with the advancement of science and technology, the radiation induced damage to normal tissues is still a big issue [19]. Early radiation-enteropathy occurs within 3 months of radiotherapy, which might influence not only the treatment outcomes, but also the living quality of patients. As a result, the RT treatment may be discontinued or has to be changed [2]. For long-term survival of cancer patients, delay the radiation toxicity of the intestine is very necessary. In our study, we found that NADH can inhibit the inflammation activation through promoting the autophagy,

which might be a big step forward for radiotherapy.

Inflammation is a major cause of tissue damage and cell death, and is closely linked to oxidative stress. Inflammation eliminates the body's pathogens by releasing free radicals, but excessive inflammation can impair normal cells. Inflammatory reaction plays an important role in radiation damage [20]. Inflammation underlies a wide range of physiological and pathological processes [21]. We found that, in the RT treated cells, NADH could inhibit the inflammation in a dose-dependent manner. The generation of ROS is involved in inflammation, proliferation and angiogenesis. It can induce apoptosis, senescence [22].

Autophagy is an important mechanism to maintain the homeostasis of cells. In recent years, it has attracted wide attention. Autophagy is a double-edged sword. Under physiological conditions, it can balance the recycling of cell components. But if it is over-activated, it will trigger non-apoptotic programmed cell death, which is called type II programmed cell death [23]. In our study, the apoptosis rate was down regulated determined as the expression of pro-apoptotic gene, BAX was down regulated and the anti-apoptosis protein BCL2 was up regulated. In our previous study, we found that NADH could inhibit the expression of P53, BAX in the LO2 cells undergoing X-ray irradiation. At the same time, level of intracellular ROS declined [24]. A study showed that X-11-5-27, a daidzein derivative, could inhibit the inflammasome activity via promoting autophagy to block the ROS generation. And the suppression of the PI3K/AKT signaling pathway was involved in this process [25]. This means that inflammation is closely related to autophagy and the activation of autophagy can suppresses the inflammation. In our study, after the RT, cells treated with NADH showed an increase expression of LC3, both in western blot and immunofluorescence, and the same to Becline1, which suggested that NADH enhanced the autophagy. moreover, in the NADH treated cells, the expression of inflammatory factor, IL-1β, TNF-α, p65, were down-regulated and when we used the autophagy inhibitor (for example, 3-MA) to block this process, the expression of inflammatory factors and PI3K/AKT signaling pathway were suppressed. These results might be explained by that the activation of autophagy

suppressed the reaction of inflammation through the PI3K/AKT signaling pathway.

In this study, we found that NADH has a significant protective effect on the intestines of irradiated mice. It is well known that more than 10 Gy of radiation can cause serious endothelial dysfunction and apoptosis, leading to acute or advanced organ failure [26, 27]. To confirm this effect, we evaluated the protective effect of NADH on the intestines treated by 12 Gy of radiation. In the four groups, there was no damage in the NADH alone treated intestines, obvious damage was observed in the RT group. However, when we combined RT with NADH, the damage had been reversed, demonstrating that NADH had a significant protective effect on the radiation-treated intestines. NADH can produce its protective effects by multiple mechanisms, including preventing mitochondrial alterations, enhance energy metabolism, preventing all forms of cell death, including apoptosis and necrosis, inhibiting inflammation [14].

In conclusion, our study has shown that NADH can effectively inhibit the generation of ROS and inflammation through the activation of autophagy in RT. Animal studies showed that the NADH+RT treated animals exhibited better conditions than the RT alone group. Intestinal inflammation of NADH+RT treated animals was lighter than the control group, while the autophagy was enhanced. At the same time, the suppression of PI3K/AKT signaling pathway was involved in NADH induced autophagy. Our studies support that NADH would be served as a promising radiotherapy protectant to improve the treatment effect and safety, and provide the preclinical rationale for future applications.

Disclosure of conflict of interest

None.

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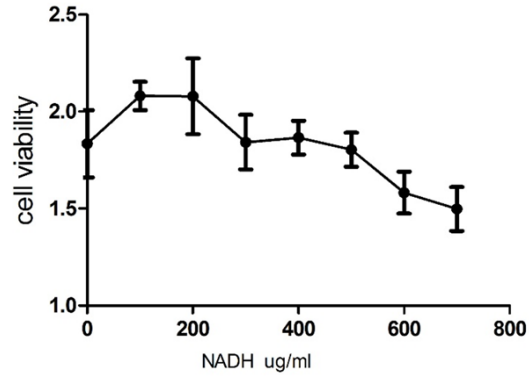
Supplementary Figure 1. NADH did not result in significant toxicity on cell viability. CCK8 assay showed that after 48 h NADH treated, 0-500 ug/ml doses did not affect the cell viability level. Above 500 ug/ml, there was slight inhibitory effect on the IEC-6 cells as the cell viability declined.
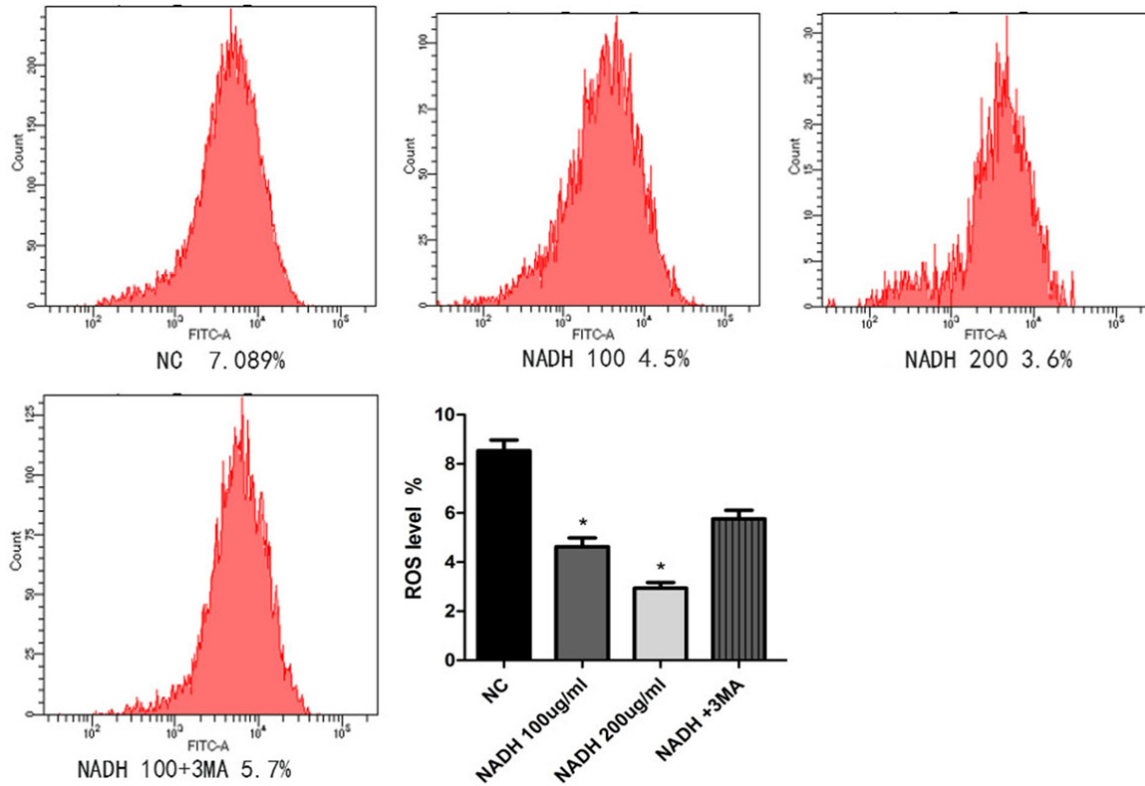
Supplementary Figure 2. Autophagy is important to prevent the activation of ROS. Inhibited the autophagy in the NADH group, the ROS increased. Data are represented as mean ± SD (N = 3). *P<0.05, student's test.