Polysaccharide From *Laminaria Japonica* Attenuates Radiation-Induced Xerostomia In Mice Via Activation of Nrf2 Signaling Pathway

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Abstract

Background: Xerostomia is one of the most common complications during radiotherapy for head and neck cancer patients that seriously affects their quality of life. However, optimal treatment for radiation-induced xerostomia is currently unavailable. Therefore, this study aimed to investigate the protective effects of *Laminaria Japonica* Polysaccharide (LJP) on radiation-induced xerostomia and decipher its underlying mechanism in mice.

Methods: Male eight-week-old mice were randomly divided into four groups: normal control group, LJP control group, irradiation group, and irradiation with LJP treatment group. LJP (40mg/kg) was intraperitoneally injected at 1 day before irradiation once daily for consecutive 5 days. The mice irradiated received a single radiation dose of 15Gy. Body weight, daily food and water intake were measured at 28 days post irradiation. Oxidative stress parameters (ROS and MDA) and antioxidant enzyme (MnSOD) in submandibular glands (SMG) were also assessed post irradiation. In addition, histopathology, function and weight of SMG were detected after irradiation. The expressions of Nrf2 pathway-related genes (Nrf2, HO-1 and NQO1) were measured using immunofluorescence or immunohistochemical staining and real-time PCR method.

Results: Compared with the control group, irradiated mice showed increases in the levels of oxidative stress parameters (ROS and MDA), water intake and expression levels of Nrf2 pathway-related genes (Nrf2, HO-1, and NQO1), and decreases in body weight, food intake, saliva flow. Nevertheless, LJP treatment significantly increased body weights, food intake, and saliva flow in irradiated mice, markedly downregulated oxidative stress levels, improved the morphology and structure of SMG, and further promote expressions of Nrf2 pathway-related genes.

Conclusion: LJP treatment alleviated radiation-induced SMG oxidative stress injury through activating Nrf2 signaling pathway, implying that LJP might be a novel agent for treating radioactive xerostomia in the future.

Background

Head and neck carcinoma (HNC) is the sixth most common cancer worldwide, and radiotherapy is a primary treatment method for head and neck tumors such as nasopharyngeal carcinoma[1, 2]. However, radiotherapy causes unavoidable damage to salivary glands (SGs) due to their anatomical location adjacent to the radiation treatment field[3, 4]. Hyposalivation occurs due to impaired SG function, which contributes to secondary effects of xerostomia, taste problems, oral mucositis and oral ulcers. This greatly affects patient's quality of survival and limits treatment progress[5–8]. Although researches are available to preserve or restore radiation-induced SG dysfunction, most therapies are limited to palliative cares[5]. As a result, identifying new radioprotective strategies and agents is a pivotal task for treating HNC patients.

Owing to poor efficacy and adverse effects of synthetic drugs, natural herbal medicine and plants have garnered global attention to treat various diseases[9–11]. The brown seaweed, *laminaria japonica*, is a popular and economically viable in several Asian countries, including China, Korea and Japan. For many centuries, *L. japonica* has been regarded as a beneficial drug in traditional Chinese medicine. Polysaccharide components extracted from *L. japonica* possess strong antioxidant capacity against oxidative injury caused by various free radical generating agents[12]. Pharmacological studies on *laminaria japonica* Polysaccharide (LJP) have demonstrated that it has multiplex medical properties, such as antioxidant[13], anti-radiation, anti-tumor[14, 15], anti-inflammation[16] and neuroprotective[17]. Nonetheless, the effect and underlying mechanism of LJP on radiation-induced SGs dysfunction remain unknown.

Radiation has been demonstrated to induce tissue injury through mediating reactive oxygen species (ROS) production and DNA damage[9, 18]. Nuclear factor erythroid 2-related factor 2 (Nrf2) works as a transcriptional activator to protect cells from oxidative stress damage by regulating its downstream target genes such as NADPH quinine oxidoreductase 1 (NQO1), heme oxygenase 1 (HO-1), and manganese superoxide dismutase (MnSOD). Nrf2-related pathway has been demonstrated to be a key signaling pathway in the resistance to oxidative stress[19]. It has been shown that Nrf2 promotes survival of irradiated cells and tissues via facilitating ROS detoxification, damaged DNA repair and potentially regulating inflammatory responses[20]. One previous study reported that when exposed to oxidative stress, such as radiation, Nrf2 existed in cytoplasm is translocated into nucleus and combines with antioxidant response element (ARE) to promote the expression of downstream reductases[21]. Moreover, a recent study has revealed that activating agents of Nrf2-ARE signaling pathway prevent radiation-induced damage[22]. Traver *et al.* have demonstrated that Nrf2 loss promotes pulmonary fibrosis following ionizing radiation[23]. Based on these observations, we hypothesized that Nrf2 pathway could play a vital role in mitigating radiation-induced SG oxidative stress damage.

To address these gaps, we herein investigated the impact of LJP on function and oxidative stress injury of SGs in mice following radiation. In addition, to better understand the mechanism and test the hypothesis, we examined the effect of LJP on Nrf2 pathway-related genes expression in mice post-radiation.

Methods

Animals

Male SPF mice weighing 28 ± 2g and aged eight weeks, of Kunming strain were obtained from Guangxi Medical University Laboratory Animal Center. The mice were housed in a controlled temperature (22 ± 2°C) lab with a light/dark period of 12 h/12 h. Before experiment, all mice were adaptively fed for one week with a standard food and water. This present experimental study was conducted in accordance with the ethical guidelines of Animal Care and Use and the Animal Research: Reporting of In Vivo Experiments

(ARRIVE) guidelines[24]. All animal procedures were approved by the Animal Ethics Committee of Guangxi Medical University (No. 201806196, Nanning, China).

Animal grouping and drug treatment

The mice were randomly divided into four groups: (I) normal control group (Con, n = 8); (II) LJP control group (LJP, n = 8); (III) irradiation group (IR, n = 8); (IV) i irradiation with LJP treatment group (IR + LJP, n = 8). The mice in the LJP and irradiation plus LJP group were administered intraperitoneally (i.p.) with LJP diluted in 0.9% NaCl (once daily, 40 mg/kg, Sigma-Aldrich, USA) starting 24h prior to radiation for consecutive five days, while mice in the first and third groups were injected with same volume of saline. LJP dose selection is based on our previous study[25].

Radiation procedure

The mice were anesthetized with sodium pentobarbital (50 mg/kg) through intraperitoneal (i.p.) injection and fixed in a custom-made thermoplastic jig. The radiation procedure was performed as describe previously[26, 27]. The mice in irradiation and irradiation + LJP groups received a single dose of 15Gy using Cobalt-60 teletherapy unit (Nanning, China, source-to-skin distance = 80 cm, dose rate = 0.49 Gy/min), whereas mice in the control and LJP groups received 0 Gy. All body parts except for head and neck regions were protected from radiation with lead shielding.

Saliva collection

Anesthetized mice were subcutaneously injected with 0.2% pilocarpine (0.2 mg/kg) at days 28 after radiation. As described previously[28], the volume of saliva secretion was collected using three to five small pre-weighed cotton balls placed into subungual region of oral cavity for 30 minutes immediately after pilocarpine stimulation. Following that, saliva flow was calculated and normalized by body weight.

Histopathological studies

At the end of the experiment, all animals were euthanized by decapitation under sodium pentobarbital (50 mg/kg, i.p.) anesthesia and then submandibular glands (SMG) were removed, weighted and fixed with 10% neutral buffered formalin. Tissue samples were embedded in paraffin and cut into 4 mm thick serial sections. The morphology and histopathological characteristics of SMG tissues were examined using hematoxylin and eosin (HE) staining.

Immunohistochemistry and immunofluorescence

Antigen retrieval was carried out following deparaffinization. The sections were placed into a pH 6.0 citrate buffer and heated at boiling temperature with a pressure cooker for 5 min. The 3% H_2O_2 was used for blocking endogenous peroxidase activity at room temperature for 10 min. The specimens were washed with 0.01 mol/L phosphate buffer saline (PBS, pH 7.4) (5 min×3 times) and incubated using primary antibodies diluted in 0.1 mol/L PBS with 0.25% Triton X-100 and 0.2 % gelatin (PBSGT, pH 7.4) at 4°C overnight, including rabbit anti-Nrf2 (1:250, Abcam, UK), rabbit anti-MnSOD (1:100, Abcam, UK), rabbit

anti-Heme Oxygenase 1(HO-1) (1:150, Abcam, UK) and mouse anti-NQO1 (1:100, Abcam, UK). After washing in PBSGT, sections were incubated with Alexa Fluor 488/594-conjugated (1:1000, Abcam, Cambridge, UK) or HRP-conjugated (1:800, Cambridge, UK) secondary antibodies at room temperature for 1 h. Images were captured respectively using a fluorescent or optical microscope (Olympus, Tokyo, Japan). According to published literatures, immunohistochemistry and immunofluorescence analysis were performed with computer image analysis system (ImageJ; National Institutes of Health)[29]. At least five representative fields per animal and three different mice per group were taken for comparative analysis.

Assessment of oxidative stress indicators

Levels of reactive oxygen species (ROS) (Nanjing Jian Cheng Institute of Biological Engineering, Nanjing, China) and malondialdehyde (MDA) (Suzhou Comin Biotechnology Co., Ltd., Suzhou, China) were detected according to these manufacturer's protocols.

Real-time polymerase chain reaction (RT-PCR) analysis

Total RNA extracted from submandibular glands was employed to synthesize complementary DNA (cDNA) using PrimeScrip RT Master Mix Kit (#RR036A, Takara, Dalian, China) according to the manufacturer's instruction. Real time PCR was conducted using a standard SYBR Green PCR kit (#RR820A, Takara, Dalian, China) on an ice box according to manufacturer's protocol. These sequences of mouse primer were designed by Takara, as listed Table 1. The target gene relative expression levels in each group were normalized by GAPDH mRNA levels and calculated based on $2^{-\Delta\Delta CT}$ method[30].

Table 1. Primers sequences used for real-time PCR amplification

Gene	Primer sequence (5'-3')	Product (bp)
Nrf2	Forward: TTGGCAGAGACATTCCCATTTG	172
	Reverse: AAACTTGCTCCATGTCCTGCTCTA	
SOD	Forward: TCCCAGACCTGCCTTACGA	115
	Reverse: TCGGTGGCGTTGAGATTG	
NQO1	Forward: TCAAGAGGAGCAGAAAAAGAACAAG	162
	Reverse: CTGAAAGCAAGCCAGGCAAAC	
HO-1	Forward: ATGAGGAACTTTCAGAAGGGTC	130
	Reverse: GTGGGGCATAGACTGGGTT	
GAPDH	Forward: TGTGTCCGTCGTGGATCTGA	150
	Reverse: TTGCTGTTGAAGTCGCAGGAG	

Statistical Analysis

Statistical Analysis was performed using GraphPad Prism 7.0 software (Inc. La Jolla, Calif.). Data were presented as mean ± standard errors of the mean (SEM). The data were compared by using ANOVA and Tukey post-hoc test. Statistical significance was considered at $p < 0.05$.

Results

Effect of LJP on general conditions in mice after radiation

To evaluate the effect of LJP on general conditions in mice post radiation, body weight, water intake and food consumption were measured at one week prior to the end of experiment. As displayed in Fig. 1, mice from the irradiation group showed a significant weight loss, food consumption reduction and water intake increase when compared with those of the control group. Conversely, LJP administration obviously reduced water intake, and increased body weight and food intake in mice after radiation. These results indicate that LJP contributed to recovery of irradiation-induced general condition abnormalities.

Effect of LJP on SGs' function in mice after radiation

To investigate the effect of LJP on SGs' function in mice post radiation, we detected saliva flow, histology and weight of submandibular gland (SMG). H&E staining in the irradiated mice showed disrupted acinar architecture, vacuolization and atrophy in acinus and ducts, and fibrosis in interstitium compared to the control group (Fig. 2a), with no obvious differences between the control and LJP groups. The above changes were mitigated following LJP administration. In addition, radiation significantly induced SMG weight loss and saliva secretion downregulation (Fig. 2b, c). However, LJP treatment significantly increased saliva flow and SMG weight in the irradiated mice. These data suggest that LJP treatment may be involved in recovery of salivary glands' dysfunctions in the irradiated mice.

Effect of LJP on oxidative stress in SGs after radiation

Tissue injury caused by radiation is commonly associated with oxidative stress[31]. To examine the effects of LJP treatment on radiation-induced oxidative stress damage in SMGs, we investigated levels of MnSOD, ROS and MDA in SGs. Expression of MnSOD (Fig. 3a), a frequent antioxidant enzyme marker, was obviously increased in the irradiation group, and levels of ROS and MDA, markers of oxidative stress, were also significantly increased (Fig. 3b, c) compared with the control group. However, LJP treatment further upregulated expression of MnSOD in mice following radiation. In contrast, LJP significantly downregulated expression of ROS and MDA. However, expression levels of ROS, MDA and MnSOD in the LJP group were not significantly different from the control group. These data suggest that LJP preserves SGs from radiation-induced saliva secretion reduction by inhibiting oxidative stress in SGs.

Effect of LJP on Nrf2, HO-1 and NQO1 protein expression in SGs after irradiation

It is well known that Nrf2 signaling pathway is the most important pathway against oxidative stress[32]. Hence, we investigated the effect of LJP on Nrf2 and its downstream antioxidases, heme oxygenase-1

(HO-1) and quinone oxidoreductase-1 (NQO1), post radiation using immunofluorescence or immunohistochemistry. Compared with the control group, expression levels of Nrf2, HO-1 and NQO1 protein were significantly were reduced in the irradiation group (Fig. 4). Moreover, the IR + LJP group exhibited a further increase in Nrf2, HO-1 and NQO1 protein expression when compared to the IR group (Fig. 4). No significant difference in Nrf2, HO-1 and NQO1 protein expression was observed between LJP and control groups.

Effect of LJP on Nrf2, HO-1 and NQO1 mRNA expression in SGs after irradiation

We further examined the effect of LJP on Nrf2, HO-1 and NQO1 mRNA expression in SGs after irradiation by using RT-PCR method. The mRNA expression levels of Nrf2, HO-1 and NQO1 in the SMGs of the irradiated group were higher than those in the control group (Fig. 5). Additionally, the mRNA expression levels of Nrf2, HO-1 and NQO1 in the IR + LJP group were higher than those in the IR group ($P < 0.05$). No obvious difference in Nrf2, HO-1 and NQO1 mRNA expression was observed between LJP and control groups.

Discussion

Although the anti-radiation effect of LJP, a natural polysaccharide compound extracted from *laminaria japonica*, is already known[33], its effect of LJP on radiation-induced submandibular gland damage and the underlying mechanism remain unclear. In this study, treatment with LJP not only attenuated weight loss and hyposalivation in mice after irradiation, but also inhibited morphological damage of the SMG. The above results indicated that LJP intervention had a protective effect on radiation-induced SMG injury. It is well known that ionizing radiation leads to oxidative stress and macromolecular damage in tissue [34, 35]. Antioxidant activity of sulfated polysaccharide and fucoidan extracted from *laminaria japonica* in vitro have been reported in some early studies[36, 37]. In addition, Liang, Z. *et al.* have found that fucoidan (FUC) from *L. japonica* improves oxidative stress of aortic smooth muscles in type 1 diabetic rats induced by instreptozotocin through enhancing glutathione and superoxide dismutase activity[38]. Similarly, recent studies in vivo have demonstrated that FUC from *L. japonica* attenuates oxidative stress injury in cardiac, hepatic, and renal tissues induced by diazinon[39] and microcystin-LR[40]. These studies illustrate that LJP is a potent natural antioxidant.

Tissue damage arises from accumulated ROS radicals in cells, followed by forming peroxidation products, particularly in malondialdehyde (MDA)[41]. MDA is regarded as a tumor promoter and carcinogenic agent due to its high cytotoxicity and inhibitory effect on antioxidant enzyme activity[42]. As an important antioxidant enzyme, MnSOD is primarily responsible for detoxifying ROS generated by mitochondrial oxidative stress[43]. Generally, the natural oxidative defense system in the body can prevent oxidative stress injury, but the antioxidant system is markedly depleted due to over production of ROS and MDA following ionizing irradiation[44], which is consistent with our finding. Izumi *et al.* reported that Suplatast tosilate can suppress ROS-mediated oxidative stress to alleviate radiation-induced lung

damage in mice [45]. Moreover, Hai *et al.* have demonstrated that rescuing irradiation-induced SG dysfunction by Sonic Hedgehog gene transfer is associated with increased DNA repair and reduced oxidative stress in SMGs[46]. For these reasons, we first evaluated the effects of LJP on antioxidant enzyme (MnSOD) and oxidative stress indicators (ROS and MDA) in SMG following irradiation. As expected, LJP showed its antioxidant effect by enhancing further upregulation of MnSOD expression and suppressing increase of ROS and MDA levels in mice after radiation. This also suggested that alleviating radiation-induced SMG impairment following LJP treatment might be ascribed with oxidative stress inhibition and antioxidant capacity elevation in SMG tissue.

Nrf2, widely expressed in mammals is a pivotal modulator of detoxification, drug metabolism and antioxidant[20]. Nrf2 is a key transcription factor that directly regulates expression of MnSOD, HO-1 and NQO1 to counter various oxidative stress injuries[47]. Among them, HO-1 and NQO1 are crucial antioxidant enzymes regulated by the Nrf2 pathway[48]. HO-1, alternatively referred to as heat shock protein-32, is engaged in antioxidant defense and anti-apoptosis[49]. NQO1 encodes a cytoplasmic 2-electron reductase that regulates redox status in the organism and is required for in maintaining cellular homeostasis[50]. It has been demonstrated that Nrf2 loss contributes to elevated ROS generation by promoting TGF-β/Smad signaling pathway, resulting in radiation-induced lung fibrosis[51]. Zhu *et al.* also have demonstrated that mitigation of X-rays radiation-induced murine embryonic fibroblast NIH3T3 cells damage is related to the Nrf2/ARE signaling pathway activation[52]. Moreover, LJP has been reported to alleviate oxidative stress via activation of antioxidative pathways[53–55]. Based on observation of these studies, we speculated that the Nrf2 signaling pathway might be engaged in the mechanisms of LJP protection against radiation-induced SMG oxidative damage. We investigated whether LJP can regulate the Nrf2 pathway-related genes expression in SMGs.

Our results of immunofluorescence and immunohistochemistry revealed that Nrf2 protein expression and its downstream antioxidant reductases (HO-1 and NQO1) were markedly increased in the irradiated mice, and levels of oxidative stress indicators (ROS and MDA) in SMGs were also significantly enhanced at this time compared to those non-irradiated mice. This implied that increased expression of Nrf2 pathway-related proteins might only play a compensatory role in these irradiated mice without LJP administration and was not sufficient to alleviate SMG oxidative stress damage after radiation. Encouragingly, we observed that treatment with LJP further increased Nrf2, HO-1 and NQO1 protein expressions, and mitigated SMG oxidative stress damage in mice post irradiation. The upregulation of Nrf2 pathway related genes expression in SMGs by LJP treatment was further confirmed by RT-PCR results. These results indicated that treatment with LJP promoted activation of Nrf2 signaling pathway to suppress irradiation-induced SMG oxidative stress injury. However, further study of the effect of LJP on radiation induced-SMG damage in vitro is still required and currently ongoing.

In conclusion, in this study, we demonstrated for the first time that the LJP treatment had protective effects on radiation-induced xerostomia in mice after radiation and further explored its relevant mechanisms that LJP treatment could alleviate SG oxidative stress damage mediated by irradiation via activation of Nrf2 signaling pathway in vivo (Fig. 6). Furthermore, previous published study has showed

that LJP effectually restrains the progress of nasopharyngeal carcinoma cells[56]. Collectively, our data suggest that LJP is a potential therapeutic agent for the prevention and treatment of radiation-induced xerostomia in head and neck cancer patients, even a SG function improving product for diet in future application.

Abbreviations

HNC: Head and neck carcinoma; SG: Salivary gland; SMG: Submandibular gland; LJP: *Laminaria japonica* polysaccharide; ROS: Reactive oxygen species; MDA: Malondialdehyde; Nrf2: Nuclear factor erythroid 2–related factor 2; ARE: antioxidant response element; HO-1: Heme oxygenase-1; NQO1: NADPH quinone oxidoreductase 1; MnSOD: Manganese superoxide dismutase; H&E: Hematein & eosin;

Declarations

Ethics approval and consent to participate

All protocols were conducted in accordance with the relevant guidelines and regulations required for animal studies. All experiments that used animals were approved by the Institutional Animal Care and Use Committee of Guangxi Medical University (No. 201806196, Nanning, China), in accordance with the ethical guidelines of Animal Care and Use and the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines. This is an animal study so consent to participate is not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during this study available from the corresponding author on reasonable request.

Competing interests

The authors declare that there is no conflict of interest.

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Author Contributions

SQZ, SYC designed and performed the experiments, researched the data and wrote the manuscript. PA, RC, YX and CC involved in the implementation of experiment, collected and analyzed the data. LW and XLN suggested the research point, supervised this study work and review the manuscript. All authors read and approved the final manuscript.

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Figures

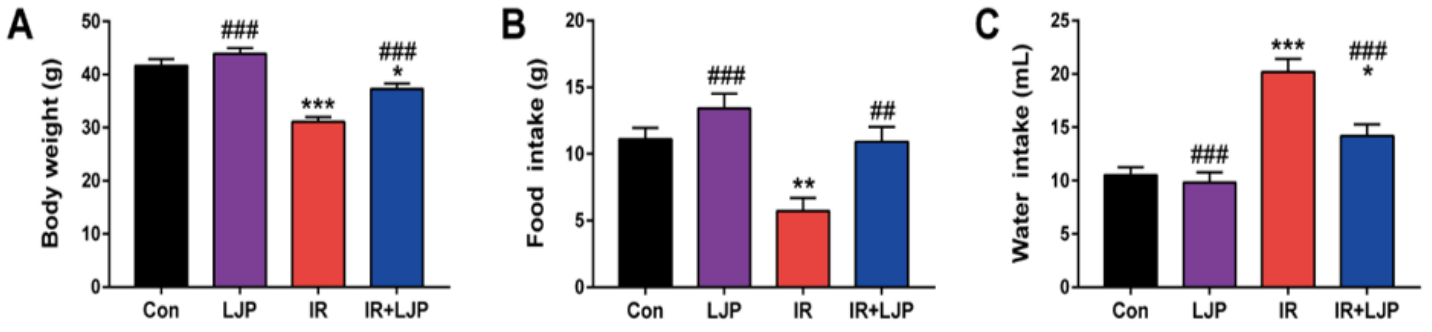

Figure 1

Effect of LJP on general conditions in mice after radiation. A Body weight, B Food intake, C Water intake. Con, normal control group; LJP, LJP control group; IR, irradiation group; IR+ LJP, irradiation with LJP treatment group. Data are presented as the mean ± SEM (SEM, Standard error of the mean, n = 8). * P < 0.05, ** P < 0.01, *** P < 0.001 versus the Con group; ## P < 0.01, ### P < 0.001 versus the IR group.

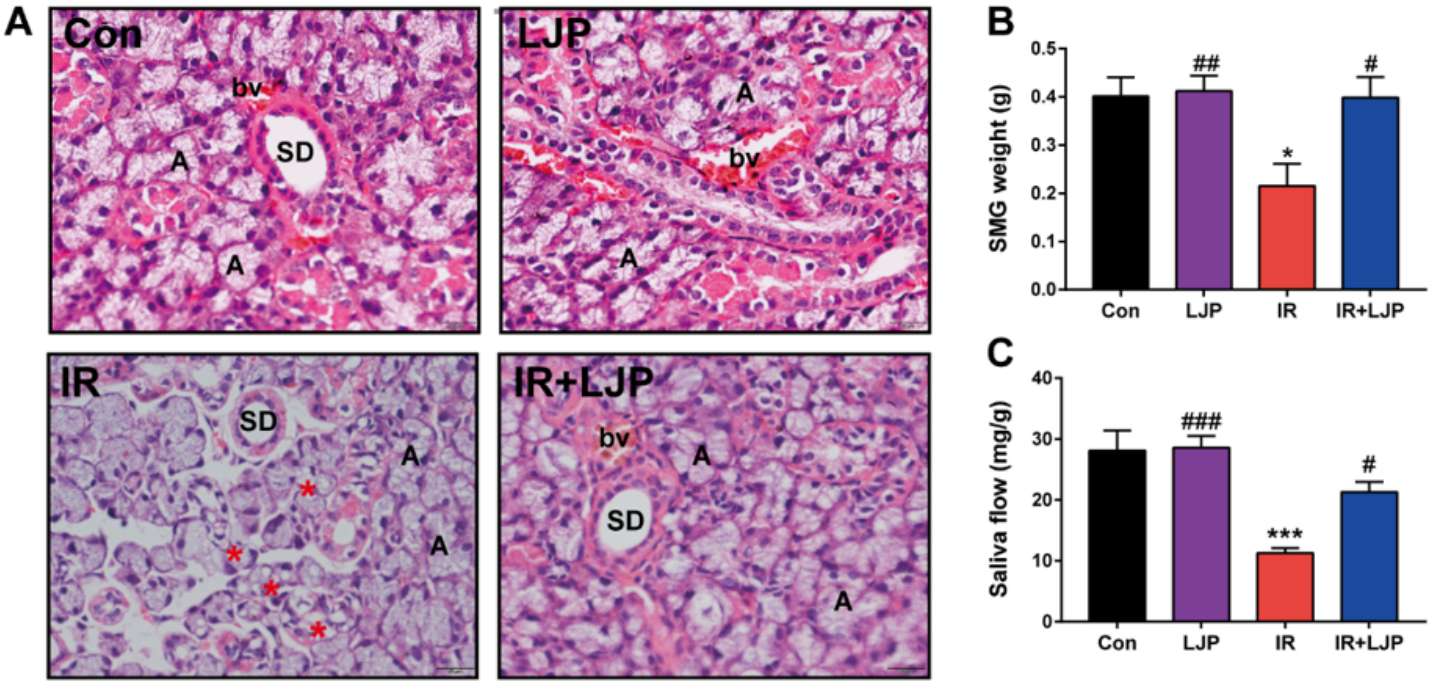

Figure 2

Effect of LJP on salivary glands' function in mice after radiation. A Hematoxylin and eosin staining of submandibular gland tissue in mice from four different group. The red pentangles present vacuolization and atrophy in acinus, bars = 20 μm (400×). SD, striated duct; bv, blood vessel; A, acinus. B Weight of SMG in mice from four different groups. C Saliva flow rate of mice in four different group. Con, normal control group; LJP, LJP control group; IR, irradiation group; IR+ LJP, irradiation with LJP treatment group. Data are presented as the mean ± SEM (SEM, Standard error of the mean, n = 4). * P < 0.05, *** P < 0.001 versus the Con group; # P < 0.05, ## P < 0.01, ### P < 0.001 versus the IR group.

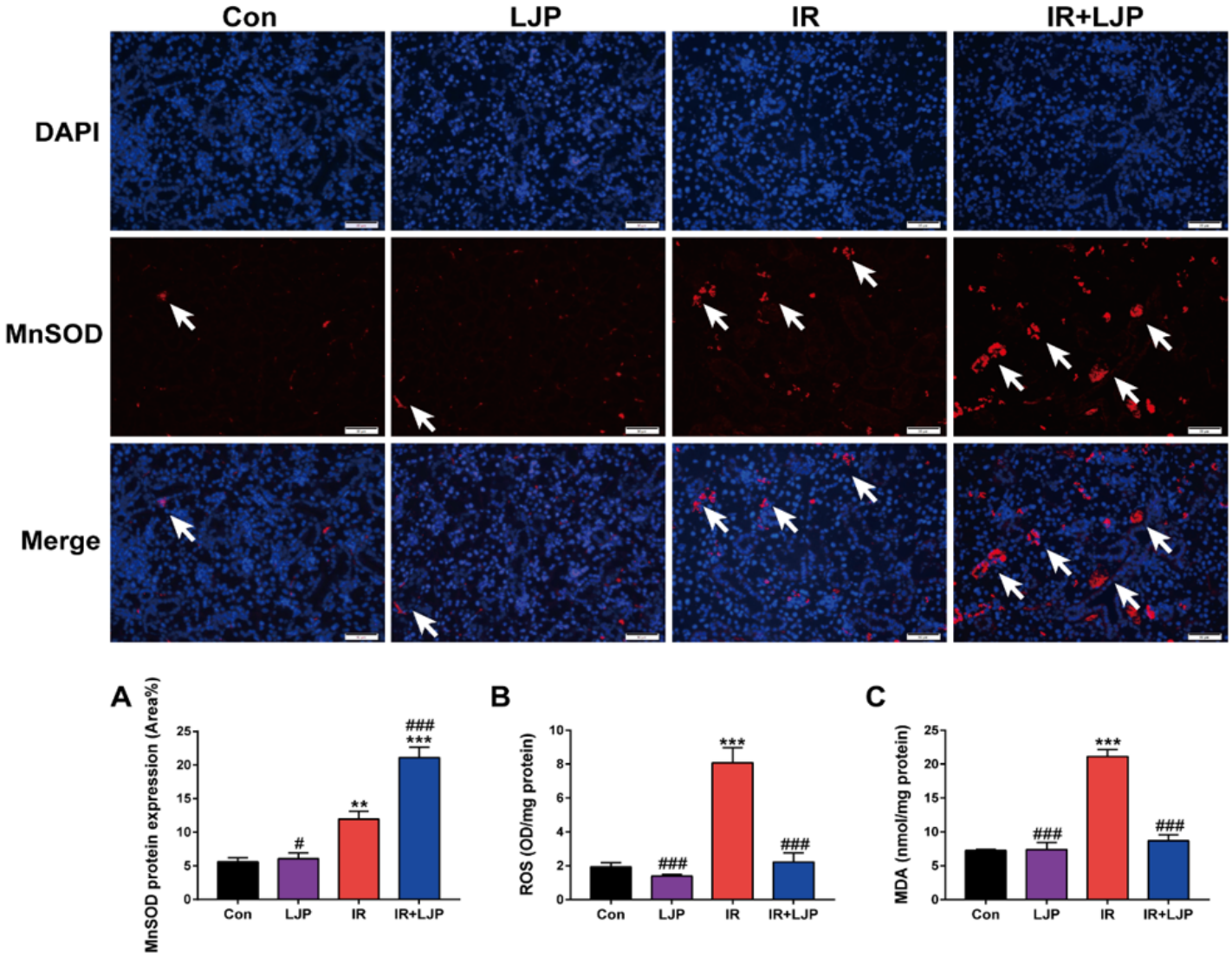

Figure 3

Effect of LJP on oxidative stress in submandibular gland tissues after radiation. A MnSOD, B ROS and C MDA levels in four different group. White arrows present positive expression of MnSOD, bars = 50 μm (200×). Con, normal control group; LJP, LJP control group; IR, irradiation group; IR+ LJP, irradiation with LJP treatment group. Data are presented as the mean ± SEM (SEM, Standard error of the mean, n = 4). * P < 0.05, *** P < 0.001 versus the Con group; # P < 0.05, ## P < 0.01, ### P < 0.001 versus the IR group.

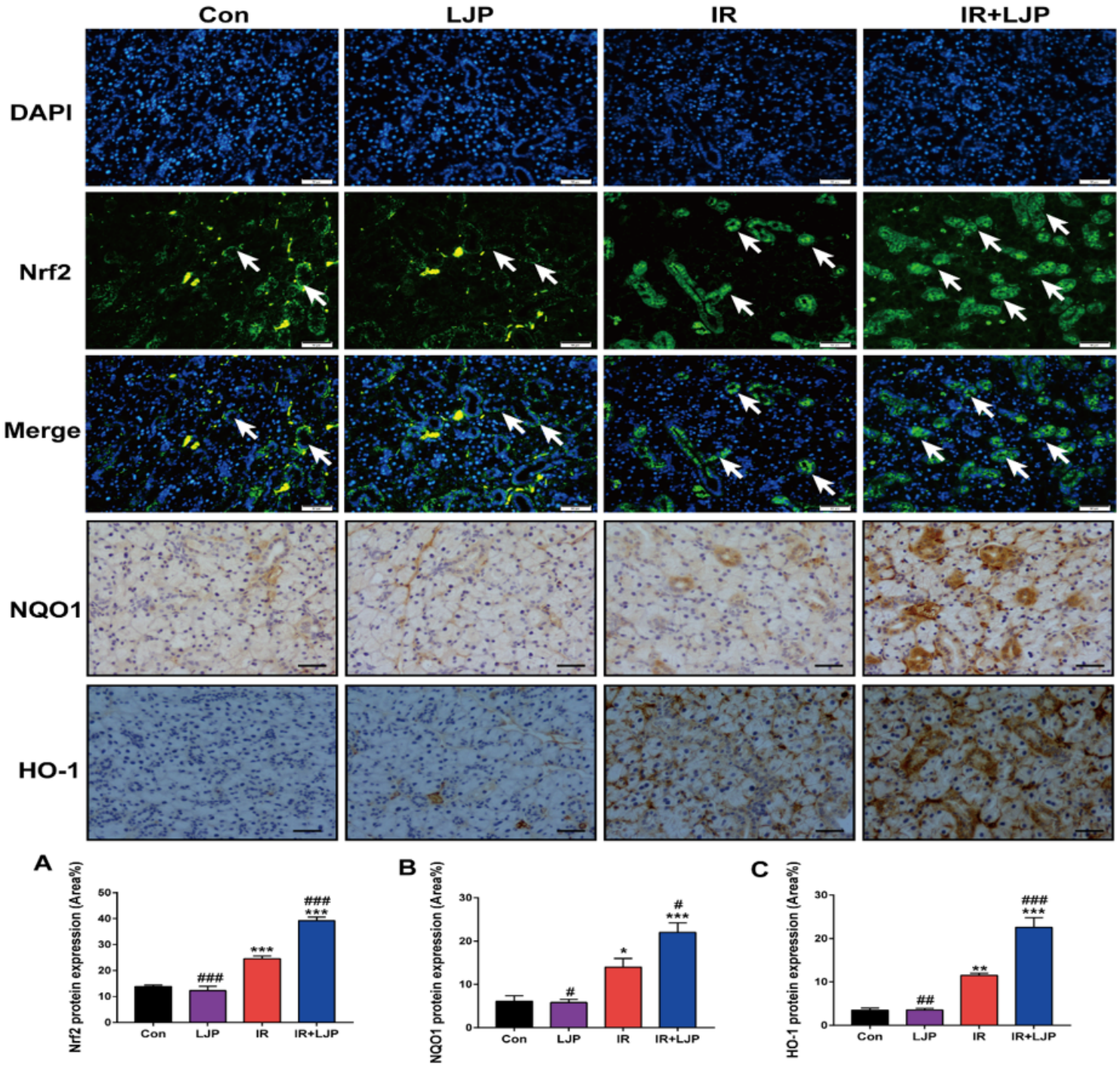

Figure 4

Effect of LJP on the Nrf2, NQO1 and HO-1 protein expression in submandibular gland tissues. A Nrf2, B HO-1 and C NQO1, bars = 50 μm (200×). White arrows present positive expression of Nrf2. Con, normal control group; LJP, LJP control group; IR, irradiation group; IR+ LJP, irradiation with LJP treatment group. Data are presented as the mean ± SEM (SEM, Standard error of the mean, n = 4). * P < 0.05, *** P < 0.001 versus the Con group; # P < 0.05, ## P < 0.01, ### P < 0.001 versus the IR group.

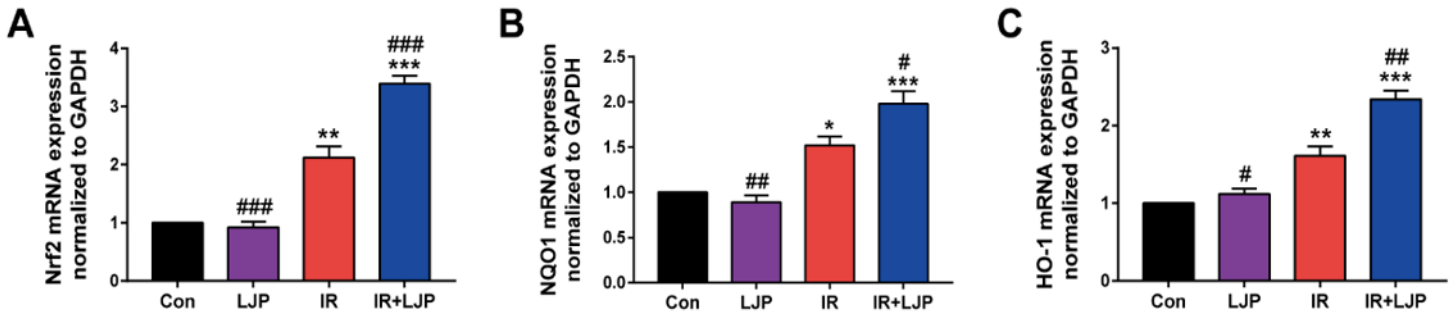

Figure 5

Effect of LJP on the Nrf2, NQO1 and HO-1 mRNA expression in submandibular gland tissues. A Nrf2; B NQO1; C HO-1. Con, normal control group; LJP, LJP control group; IR, irradiation group; IR+ LJP, irradiation with LJP treatment group. Data are presented as the mean ± SEM (SEM, Standard error of the mean, n = 4). * P < 0.05, *** P < 0.001 versus the Con group; # P < 0.05, ## P < 0.01, ### P < 0.001 versus the IR group.

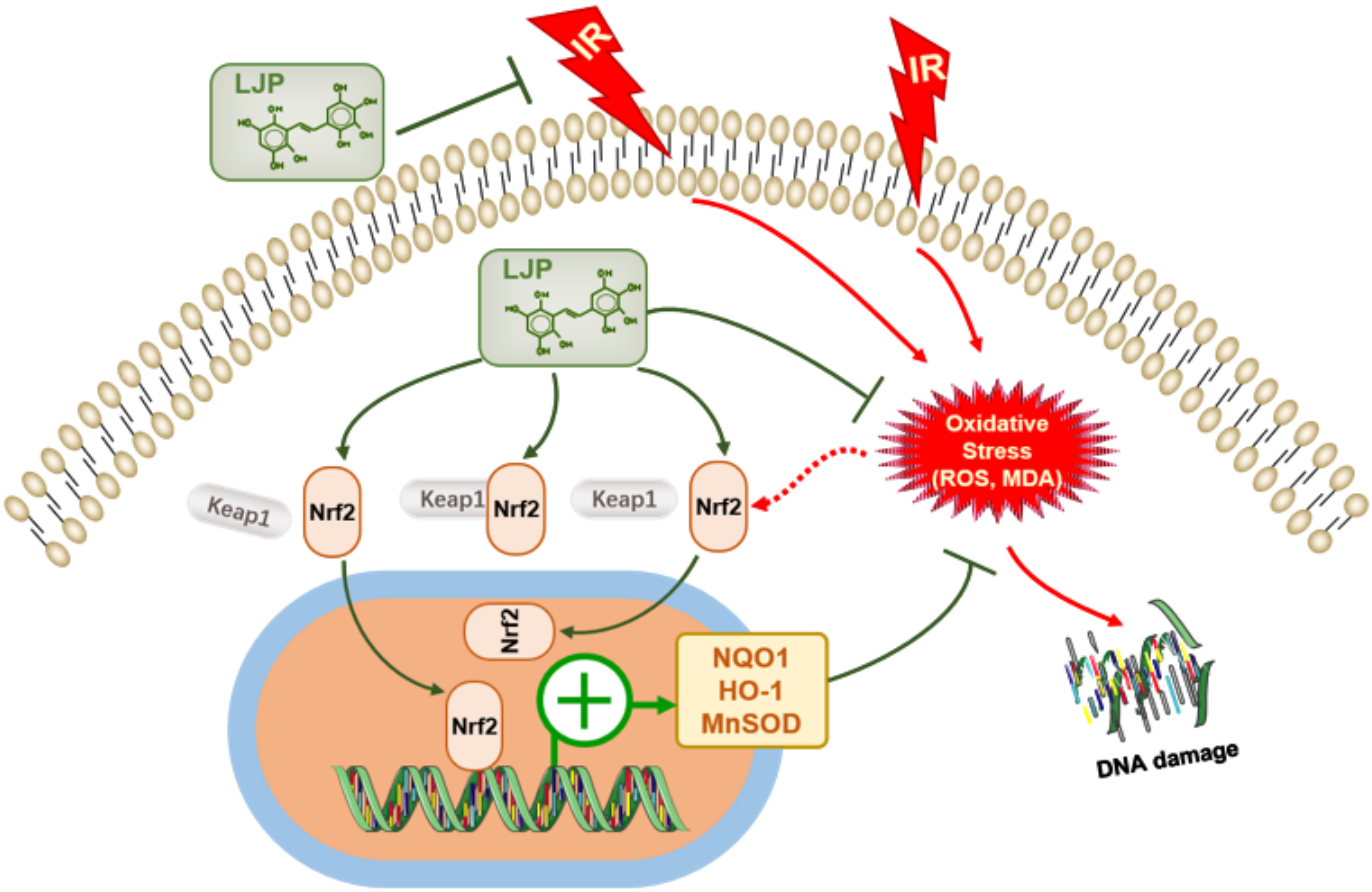

Figure 6

Schematic diagram of the proposed mechanisms of LJP protection against radiation-induced oxidative injury in submandibular gland by regulating Nrf2 pathway.