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Research Article

lncRNA NONRATT021477.2 Interference Aggravates H_2O_2-Induced Oxidative Stress in BRL Cells

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Abstract

Long non-coding RNA (lncRNA) plays important biological regulatory functions at different levels. lncRNA NONRATT021477.2 (lncRNA77.2) was identified as a key gene under cold stress conditions in rats. From the literature, lncRNA77.2 may have important roles during antioxidant, but this conjecture requires investigation. To address this knowledge gap, we investigated the effects of lncRNA77.2 interference on H_2O_2-induced oxidative stress in rat liver cells (Buffalo rat liver, BRL).In the current study, H_2O_2 treatment simulated BRL cell oxidative stress. H_2O_2 treatment led to a significant increase in oxidative stress levels in BRL cells, whereas the gene expression levels of antioxidase and lncRNA 77.2 and cell viability were significantly reduced. Additionally, the expression of Nrf2 and Keap1 proteins decreased significantly compared to the control group. BRL cells were transfected with antisense oligonucleotides (ASO) or a negative control ASO (ASO-NC) and then treated with H_2O_2. The results showed lncRNA77.2 interference increased oxidative stress levels, reduced gene expression of antioxidant enzymes, Nrf2 and Keap1 expression levels. The results indicated that lncRNA77.2 interference aggravated H_2O_2-induced oxidative stress in BRL cells, which suggested that lncRNA77.2 is an antioxidant factor that plays an important role in the regulation of oxidative stress in BRL cells.

Keywords: BRL cells, lncRNA, Oxidative stress

Introduction

Long non-coding RNA (lncRNA) mediated gene regulation mechanisms are diverse, which may be attributed to their ability to interact with DNA, RNA, or proteins [1]. Despite the large proportion of lncRNAs in complex transcription processes, their functions remain poorly understood. LncRNA has a non-random small open reading frame, which can interact with ribosomes. LncRNA is involved in the regulation of mRNA prophase splicing, RNA editing, regulation of mRNA stability, translation activation, and miRNA sponge [2]. Additionally, lncRNAs play key regulatory roles in biological processes, including cell differentiation, proliferation, apoptosis, immune response, and in vivo homeostasis by forming RNA-DNA triplets and targeting specific DNA sequences [3]. Recent studies have found that certain lncRNAs can promote protein-protein interactions, such as epigenetic reprograming and signaling, to influence cancer occurrence [4]. LncRNAs can also interact with RNA or DNA, acting as the skeleton of subcellular domains or complexes to regulate protein activity. Abnormalities in lncRNAs are associated with oxidative stress in many human diseases, and there is a strong association between several human diseases and oxidative stress. Recent studies have shown that lncRNA plays an important role in the cellular response to oxidative stress by responding to different genes that regulate the expression of proteins [5].

Oxidative stress refers to an imbalance in the oxidative antioxidant system due to the accumulation of free radicals after internal and external environment stimulation [6]. Generally, the increased production of highly reactive oxygen species (ROS) and highly reactive nitrogen radicals (RNS) inhibits the function of normal substances in cells and destroys the structure of normal cells, leading to a decrease or loss of the activity of various enzymes, as well as damage to the cell structure. This imbalance can cause severe damage to some biomolecules and organs and may have systemic effects. Oxidative stress plays an important role in the pathogenesis of several diseases, including Alzheimer's disease and cancer. A previous study has demonstrated that long downregulation of strand-noncoding RNA SNHG1 can improve oxidative

stress and inflammation in Parkinson's disease models by inhibiting the miR-125b-5p/MAPK 1 axis [4]. Sunwoo et al.[3] evaluated the differential expression profile of lncRNA in Huntington's disease and demonstrated that transfection of NEAT 1 short isoforms into H_2O_2-treated Neuro-2a cells exhibiting oxidative stress caused cell death, demonstrating a neuroprotective role for lncRNA NEAT1 in the pathogenesis of Huntington's disease. Moreover, Kong et al.[7] revealed that the lncRNA LEGLTBC can function as a ceRNA to regulate SIRT 1 in glycolipid toxicity-induced oxidative stress and apoptosis in INS-1 β cells. Recent reports have also revealed that lncRNA plays an important role in response to oxidative stress, which implies that lncRNA, as a key molecule, may participate in oxidative stress [8]. The low conservation and tissue-specific features of most lncRNAs suggest that long non-coding RNAs can serve as specific biomarkers for oxidative stress-related diseases [9].

After preliminary laboratory identification, we selected the newly discovered *lncRNA NONRATT021477.2*, with a length of 746 bp. The subcellular localization of lncRNA77.2 in BRL cells was detected by FISH technology, and it was found to be mainly expressed in the nucleus of BRL cells [10]. Therefore, we interfered with the expression of *lncRNA77.2* to initially explore its regulatory role in the oxidative stress response in rat liver cells and build a foundation for the study of the mechanism of *lncRNA77.2* in regulating oxidative stress in rat hepatocytes.

Materials and Methods

Cell Culture and H_2O_2 Treatment

Buffalo rat liver (BRL) cell from Cell Bank, Chinese Academy of Sciences (Shanghai, China) were maintained in DMEM medium(Gibco, Carlsbad, CA, USA)with 10% fetal bovine serum (FBS, Gibco) and the optimal H_2O_2 treatment concentration using preliminary laboratory screening was 200 μ mol/L at a concentration of 200 μmol/L in a moist CO_2 incubator at 37°C under 5% CO_2 [11].

To construct an *in vitro* oxidative stress cell model and screen the optimal time point for hydrogen peroxide treatment, we stimulated BRL cells for 2h, 4h, and 6h respectively at 37°C using hydrogen peroxide at 200 μmol/L concentration.

Antisense Oligonucleotide(ASO) Interfere with Expression

The antisense oligonucleotide (ASO) of *lncRNA77.2* was designed and synthesized according to the full-length sequence of *lncRNA77.2*, whose sequence was: ASO: 5′-TCTCCTCTCTCAAATATCTG-3′, and a negative control ASO-NC was chemically synthesized. Antisense nucleotide sequences were designed and synthesized by Raybo Bio. The cells were coated on a 24-well plate, and when the cells reached 50% growth, BRL cells were transfected according to the instructions of the ASO kit (Ribo, Guangzhou, China) to construct an *lncRNA77.2* interference expression model. The experiments were divided into negative control group (NC group), H_2O_2 group, ASO interference expression group (H_2O_2+ ASO group), ASO no-load negative control group (H_2O_2 + ASO-NC group), and samples from each group were collected to detect oxidative stress related indicators.

Cell Viability Assays

CCK-8 assay was proceeded to detect cell viability. In brief, cells (100 μL) were seeded into 96-well plates and cultivated in a 5% CO_2 incubator for 48 h at 37°C. BRL cells were stimulated with H_2O_2 for 2 h, 4 h, and 6 h at 37°C, then CCK-8 reagent (Beyotime, China) was added to each well and incubated for 3 h. Finally, the absorbance at 450 nm was determined by a microplate reader (MR-96A, China Mindrary Mindray Medical Company, China).

Malondialdehyde (MDA) detection, Reactive Oxygen Species (ROS) Activity and Superoxide Dismutase (SOD) Activity Assay

The levels of MDA release, ROS and SOD were determined by the MDA Assay Kit (Beyotime, China), ROS Assay Kit (Sigma, Japan) and SOD Assay Ki (Beyotime, China), respectively, referring to the directions of manufacturers. The absorbance of samples at 450 nm, 532 nm and 530 nm was tested to reflect SOD, MDA and ROS activity via a microplate reader, respectively.

Reverse Transcription-Quantitative PCR (RT-qPCR)

Total RNA was extracted fromBRL cells and cDNA synthesis and qRT fluorescence PCR performed to quantify mRNA expression. β-actin was used to normalize gene

Table 1. *Primer sequences*

Gene	Primer Sequences	
	Forward Primer	**Reverse Prime**
GAPDH	CATCACCATCTTCCAGGAGCG	GAGGGGCCATCCACAGTCTTC
SOD	CCACGTCCATGCCTTTGG	TCAGCTGCTGCAGTCACGTT
CAT	CCGACCGTCCGTAAATGCTA	GCTTTTCAGATAGGCTCTTCATGTAA
GSH-Px	GATTCGTTCCAAACTTCCTGCTA	GCTCCCAGAACAGCCTGTTG

expression data. Relative quantification was calculated using 2-ΔΔCT comparison threshold formula. Primer sequences *SOD*, *CAT*, *GSH-Px* and *GAPDH* are listed in *Table 1*.

Western Blot

After cell lysis, the total protein concentration was determined according to the method described in the BCA kit (BioTeke, Beijing, China). Proteins were separated on 12% SDS-PAGE gels and then transferred to a polyvinylidene difluoride membrane. After sealing with 5% skim milk, the membranes were incubated with murine anti-Nrf 2, Keap 1, Bax, and Bcl-2 overnight at 4ºC. After 24 h, the PVDF membrane was washed with TBST and subsequently incubated with TB Green (cat: RR020A, fluorescence-conjugated secondary antibody, TAKARA) for 2 h. Finally, band intensity was quantified using Image J software.

Statistical Analysis

SPSS software was performed to analyze the experimental data, the data were displayed as mean ± standard deviation (SD). The GraphPad Prism 8.0 system was used to perform one-way ANOVA analysis. The t-test was used to reveal the pairwise differences between the samples, and the P less than 0.05 was considered statistically significant.

Results

In order to probe the impact of H_2O_2 on BRL cells, we first explored the level of ROS production of BRL cells. The level of intracellular ROS production at different times of H_2O_2 action (2 h, 4 h, 6 h) were detected, and the results showed that the level of ROS in BRL cells gradually increased with the duration of H_2O_2 stimulation in a time-dependent manner *(Fig. 1-A,B*, P<0.01). Meanwhile, similar results were obtained for the MDA level *(Fig. 1-C*, P<0.01). Moreover, the viability of BRL cells showed a gradual trend with the extension of H_2O_2 stimulation in a time-dependent manner *(Fig. 1-D*, P<0.01). TThe above results showed that ROS and MDA, the key indicators of oxidative stress, increased with the increase of H_2O_2 stimulation time, whereas cell viability decreased (P<0.01), indicating a time-dependent aggravation of oxidative stress.

The results of qRT–PCR experiments showed that the indicators of intracellular antioxidants in BRL antioxidant enzymes *(Fig. 2-A,B,C)* manifested a significant decline trend with the increase in H_2O_2 stimulation time (P<0.05), reaching a very significant decrease at 6 h. *lncRNA77.2* expression showed the same trend *(Fig. 2-D*, P<0.01). The above data indicated that the antioxidant capacity of BRL cells decreased with the prolongation of H_2O_2 stimulation time.

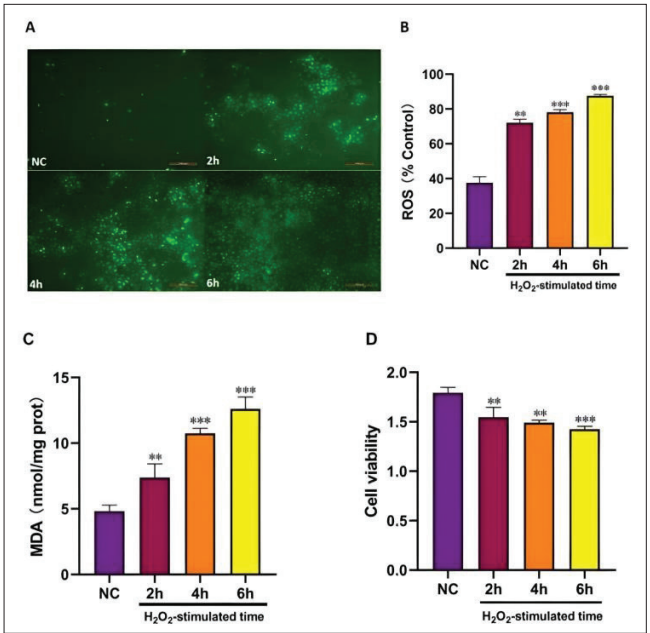

Fig 1. Effects of H_2O_2 action on ROS, MDA and cell proliferation at different time points. **A, B**- ROS analysis of BRL cells at different treatment times for NC, 2 h, 4 h, and 6 h; **C**- MDA levels in BRL; **D**- The effects of hydrogen peroxide action on BRL cell proliferation at different time points. Data are expressed as the ± standard error of the mean (n=3). * Compared with the NC group, * P<0.05, ** P<0.01

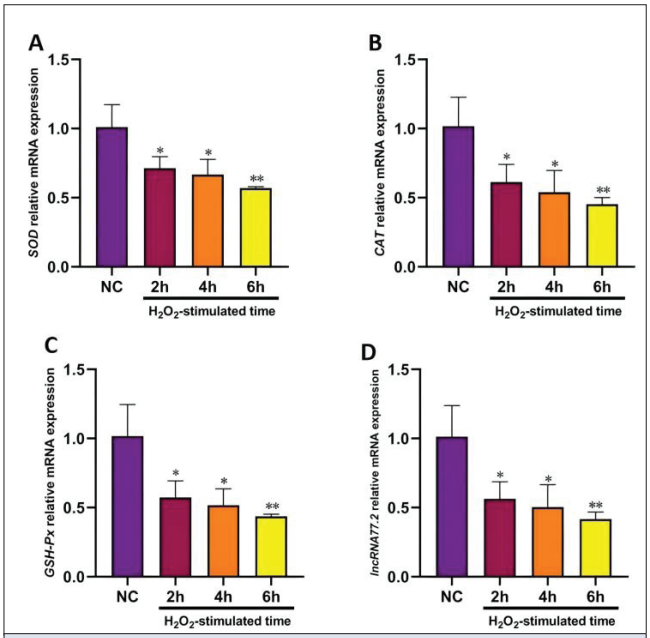

Fig 2. Effects of hydrogen peroxide action at different time points on intracellular antioxidant indexes in BRL. **A**- *SOD / GAPDH;* **B**- *CAT/ GAPDH;* **C**- *GSH-Px*/*GAPDH;* **D**- *lncRNA77.2*. Data are expressed as the ± standard error of the mean (n=3). * Compared with the NC group, * P<0.05, ** P<0.01

Moreover, the expression of Nrf2 and Keap1 in BRL was detected by western blot and found to be significantly decreased with increasing time of H_2O_2 stimulation starting from 2 h *(Fig. 3-A,C*, P<0.05), reaching the lowest value at 6 h (P<0.001), while the apoptosis-related proteins

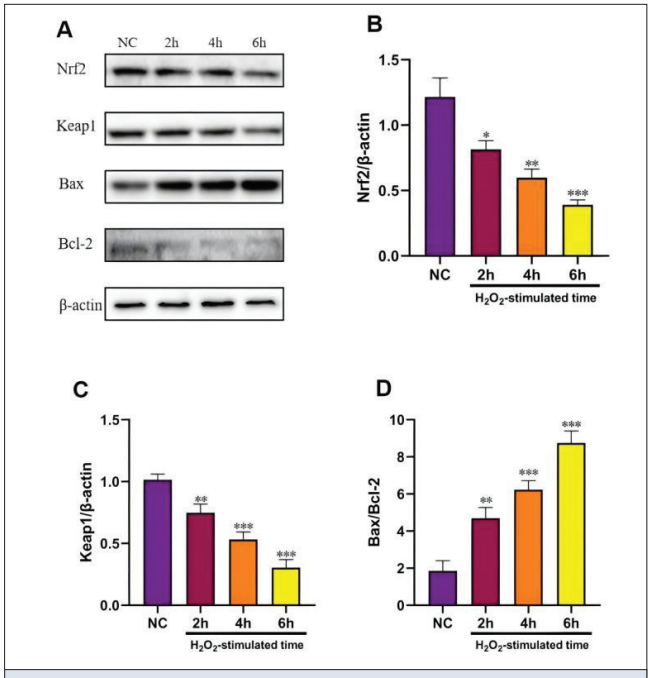

Fig 3. Effects of hydrogen peroxide on oxidation and apoptosis in BRL cells. **A-** Protein expression related to oxidation and apoptosis by Western blot; **B-** Nrf 2 expression analysis; **C-** Keap1 expression analysis; **D-** The analysis of Bax/Bcl-2 expression. Data are expressed as the mean ± standard error (n=3). * Compared with the NC group, * P<0.05, ** P<0.01, *** P<0.001

showed the opposite trend in our study *(Fig. 3-D)*. The above results indicated that oxidative stress decreased the antioxidant capacity and enhanced the apoptotic level in BRL cells. To further explore how *lncRNA77.2* functions as an antioxidant factor during oxidative stress in rat hepatocytes, the time point of 6 h, with the strongest oxidative stress, was selected for subsequent experimental studies.

To explore whether lncRNA77.2 participated in the regulation by H_2O_2-stimulated cytotoxicity, the effect of lncRNA77.2 knockdown was examined in BRL cells. Compared to the H_2O_2+ASO-NC group, the *lncRNA77.2* mRNA level was significantly reduced in the H_2O_2+ASO group, indicating that the *lncRNA 77.2* interference expression was successfully constructed *(Fig. 4-A*, P<0.01). The results of the CCK-8 assay showed that cell viability was significantly reduced in the H_2O_2+ASO group compared to the H_2O_2+ASO-NC group *(Fig. 4-B*, P<0.01). Additionally, the qRT-PCR results showed that the ROS levels in BRL cells were significantly higher in the H_2O_2+ASO group than that in the H_2O_2+ASO-NC group *(Fig. 4-C, D*, P<0.001). Moreover, the level of MDA in BRL cells was significantly increased after *lncRNA77.2* interference *(Fig. 4-E*, P<0.001), while knockdown of *lncRNA77.2* exacerbated H_2O_2-induced oxidative stress.

To verify whether the protective effect of lncRNA77.2 knockdown on H_2O_2-triggered oxidative stress was mediated by antioxidant genes, the expression levels of

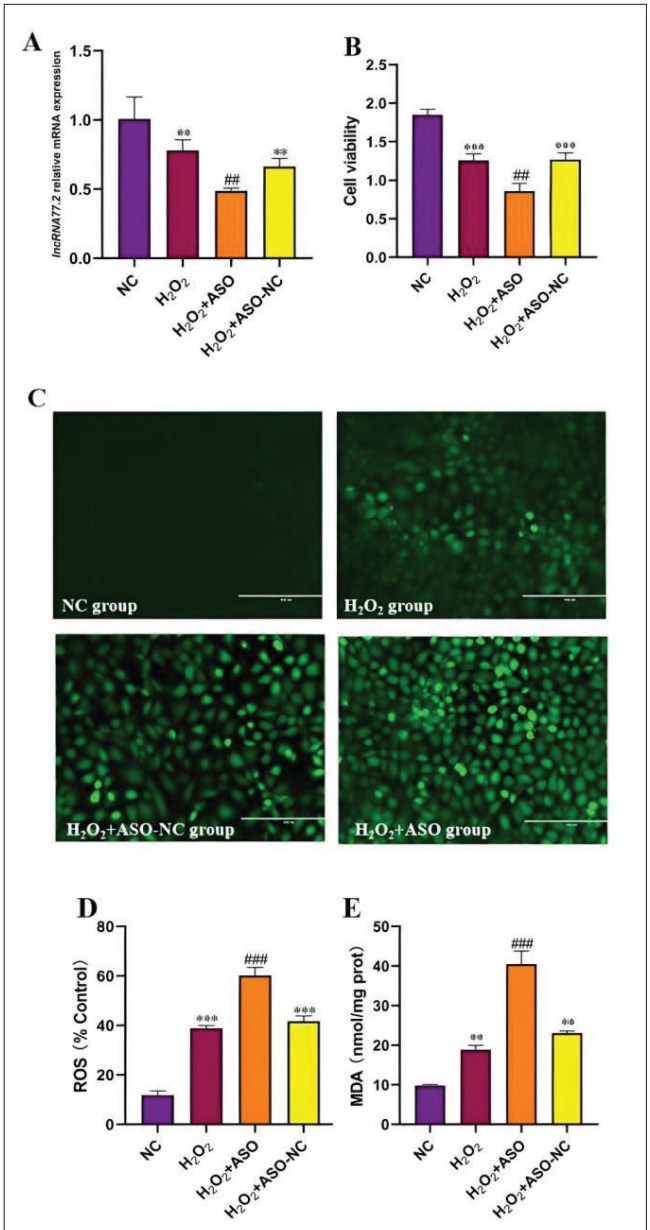

Fig 4. Effects of interfering *lncRNA 77.2* on intracellular cell proliferation and oxidative stress levels in BRL cells. **A-** The expression of *lncRNA77.2* in BRL cells was assessed by qRT-PCR analysis; **B-** Viability of BRL cells was detected using CCK-8 assay; **C-** ROS fluorescence staining, ×200; **D-** Fluorescence intensity of positive ROS staining; **E-** MDA results. Data are expressed as the mean ± standard error (n=3). * Compared with the NC group, # compared to the H_2O_2 + ASO-NC group, ** P<0.01, ##: P<0.01, *** P<0.001, ###: P<0.001

SOD, *CAT*, and *GSH-Px* in BRL cells were analyzed using qRT-PCR after exposure to H_2O_2 for 24 h. As shown in *Fig. 5-A,B,C*, compared to those in the control group, the expression levels of *SOD*, *CAT*, and *GSH-Px* were significantly downregulated in the H_2O_2+ASO group (P<0.01). In summary, *lncRNA77.2* has been shown to play an important role as an antioxidant factor during oxidative stress in BRL cells.

We explored whether *lncRNA77.2* interference affects the oxidative stress and apoptosis of BRL cells. Notably, the

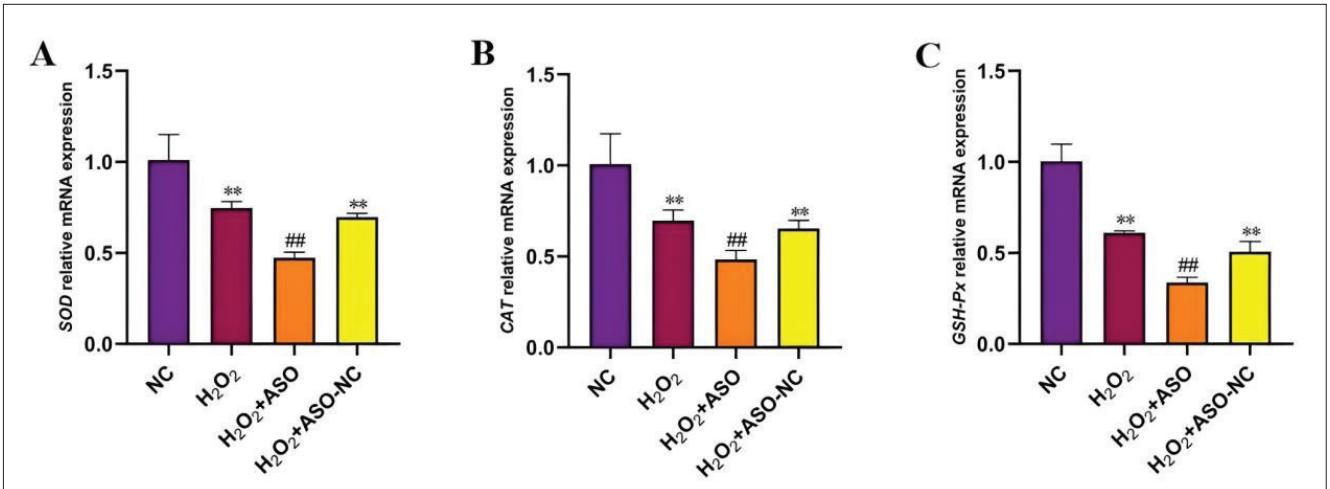

Fig 5. Effects of interfering *lncRNA77.2* on intracellular antioxidant genes in BRL. **A**- *SOD/GAPDH;* **B**- *CAT/GAPDH;* **C**- *GSH-Px/GAPDH.* Data are expressed as the mean ± standard error (n=3). * Compared with NC group, # compared with H_2O_2 + ASO-NC group, ** P<0.01, ##: P<0.01

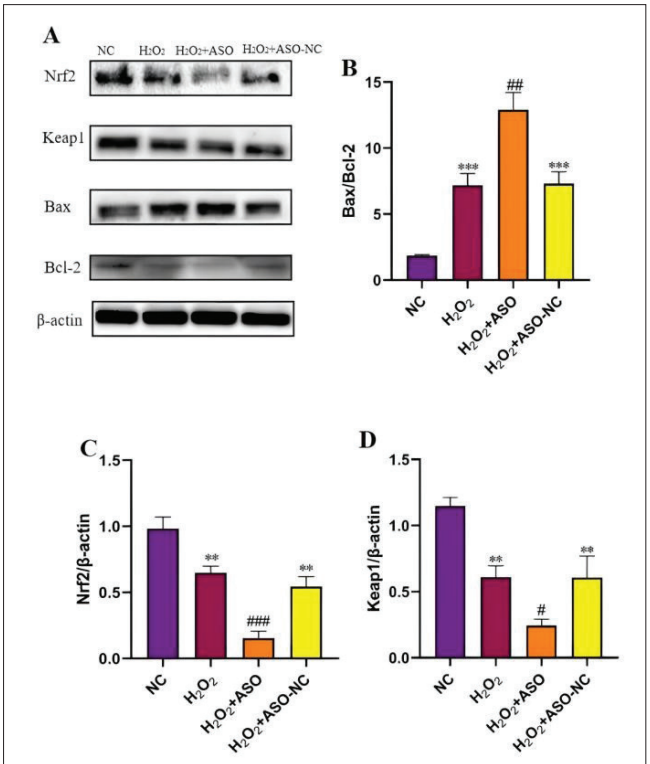

Fig 6. Effects of interfering *lncRNA77.2* on oxidation and apoptosis related proteins in BRL cells. **A**- Western Blot results of Nrf 2, Keap 1, Bax, Bcl-2, β-actin; **B,C,D**- Results of Bax/Bcl-2, Nrf 2/β-actin, Keap1/β-actin. Data are expressed as the mean ± standard error (n=3). * Compared with the NC group, # P<0.05, ** P<0.01, ## P<0.01, *** P<0.001

results of western blotting showed a significant decrease in Nrf2 and Keap1 protein expression and a significant increase in Bax/Bcl-2 protein expression after *lncRNA77.2* interference compared to the H_2O_2+ASO-NC group *(Fig. 6)*. These results suggest that H_2O_2-induced oxidative stress and apoptosis are exacerbated in cells with lncRNA77.2 interference.

The knockdown of *lncRNA77.2* in BRL cells aggravates its oxidative stress, and lncRNA 77.2 can act as an antioxidant factor and play an important role in the regulation of oxidative stress in BRL cells. This study highlights the unprecedented connection of *lncRNA77.2* in oxidative stress, a finding that deepens the understanding of the complex relationship between lncRNA and oxidative stress and provides new ideas and insights into the study of oxidative stress in animals.

Discussion

With the rapid development of science and technology, lncRNA has emerged from what was once regarded as the dark matter of the genome to become a key molecule in regulating gene expression. However, the biological function of lncRNA and stress has been poorly studied. Previous studies by our group have demonstrated that cold stress can induce oxidative stress in the organism. By constructing a cold stress rat model, we screened differentially expressed *lncRNA77.2* in the liver of cold-stress rats by high-throughput sequencing for functional studies. In this study, BRL cells were selected to construct an oxidative stress model via hydrogen peroxide treatment. *lncRNA77.2* interference was performed using antisense oligonucleotide plasmid expression, and the effects of interfering with lncRNA77.2 expression on oxidative stress, proliferation, and apoptosis in rat liver cells after oxidative stress were preliminarily investigated.

Oxidative stress is a hot research topic at present. Under physiological conditions, the oxidation and antioxidant systems of the body are in dynamic balance, and oxidation intermediates such as ROS are produced as a result of an imbalance in the antioxidant system. Excessive ROS can cause oxidative stress and impaired cell metabolic function and directly cause cell death by directly destroying essential proteins, DNA, or lipids [12].

Nrf2 has antioxidant detoxification and cytoprotective effects, and more than 90% of antioxidant genes are

regulated by Nrf2 [13]. Keap1 is a novel multiregional inhibitory protein of the KELCH family, and plays a key role in oxidation-reduction signaling [14]. Nrf2 is found in the cytoplasm with Keap1 and forms a complex after oxidative stress. Following dissociation of the Nrf2 and Keap1 complex, Nrf2 translocates to the nucleus and binds to the antioxidant response element (ARE), thus promoting the transcriptional activation of antioxidant enzymes (e.g. CAT GSH-Px and SOD) [15]. Some experiments have found that the Keap1-Nrf2/ARE signaling pathway during oxidative stress can induce the body to produce a series of free radical scavenging enzymes that are resistant to oxidative stress, which is the main mechanism to resist oxidative damage and enhance antioxidant ability [16]. Here, we used the rat hepatocyte oxidative stress model induced by H_2O_2, it was found that with the extension of H_2O_2 stimulation time, the levels of ROS and MDA gradually increased, the mRNA levels of antioxidant enzymes gradually decreased, and the protein expression levels of Nrf2, Keap1, and Bax/Bcl-2 gradually increased, indicating that the prolongation of H_2O_2 stimulation could increase the level of oxidative stress in BRL cells.

It Is generally accepted that there is a close relationship between oxidative stress and lncRNA. Xu et al.[17] found that knockdown of lncRNA KCNQ1OT1 increased miR-137 levels, inhibited the inflammatory response, and alleviated oxidative stress in ox-LDL-treated THP-1 macrophages, suggesting that silencing KCNQ1OT1 suppresses the inflammatory response and oxidative stress induced by ox-LDL through the miR-137/TNFAIP1 pathway in THP-1 macrophages. Furthermore, a study by Shen et al.[18] found that lncRNATUG1 was disrupted, increased cell viability, and downregulated apoptosis and caspase-3 levels. These results suggested that knocking down TUG1 expression may protect LECs from oxidative stress-induced apoptosis. Here, we used ASO to interfere with BRL cell *lncRNA77.2* expression, before treating BRL cells with H_2O_2. The results showed that antioxidant genes SOD, CAT, and GSH-Px relative mRNA expression indicated an obvious downward trend after interfering with *lncRNA77.2*. The protein expression associated with the antioxidant pathways Nrf2, Keap1 also showed the same trend. The above results suggest that different lncRNAs have different regulatory effects on oxidative stress.

In conclusion, our study found that disruption of *lncRNA77.2* accelerates the process of H_2O_2-induced oxidative stress. In conclusion, *lncRNA77.2* was found to be an antioxidant factor important in the regulation of oxidative stress in BRL cells. How signaling pathways are regulated to mitigate liver damage caused by oxidative stress during the antioxidant process requires further investigation. These data provide new insights into the regulatory mechanism of oxidative stress and provide favorable evidence for the functional diversity of lncRNA.

Declarations

Availability of Data and Materials: The data that support the findings ofthis study are available on request fromthe corresponding author (HJ). The data arenot publicly available due to privacy orethical restrictions.

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Author Contributions: HJ designed and supervised the study. YH for mplementation of the experiment and collection of specimens, analysis of data and drafting the paper. LCW, SZY, ZJJ, and YSQ for carrying out the statistical analysis and provided critical comments. HJ checked and improved the manuscript. All authors read and approved the final manuscript.

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