



## Research Article

# Evaluation of Circ-Nrf2s Expression on Oxidative Stress Condition and Prediction Its Interaction with mi-RNAs and Proteins

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### Article Info

#### Article History:

Received: 17 Jan 2024

Accepted: 1 Jun 2024

ePublished: 25 Jun 2024

#### Keywords:

- Gene expression regulation
- Hippocampus
- Hydrogen peroxide
- Long non-coding RNA
- Nef2l2
- Oxidative stress

### Abstract

**Background:** Circular RNAs (Circ RNA) are a large class of non-coding RNAs which particularly stable RNAs and mostly originate from gene exons in animals. circRNAs are extremely abundant in the mammalian brains. They play role in the biological function and development of neurons. Nrf2 (nuclear factor erythroid 2-related factor 2) is a transcription factor that regulates cellular antioxidants and improves hippocampal synaptic plasticity, learning and memory. The aim of this study is the evaluation of Nrf2's circRNA expression in the hippocampus of rats during oxidative stress and the prediction of their function by bioinformatic tools.

**Methods:** After evaluation of Nrf2's circRNA prediction from circAtlas by BLAST, 3 out of 4 sequences with high homology were selected and circ-Nrf2s expression was evaluated by qPCR, the interaction with micro RNAs and proteins was evaluated by miRDB, TargetScan, catRAPID and HDOCK web server. Except control group, two groups of rats were treated with H<sub>2</sub>O<sub>2</sub> (1% and 5%) then rats were sacrificed and hippocampal tissue was separated. qPCR was performed for Nrf2 pathway gene expression and Nrf2 circRNAs. Then Circ-Nrf2-miRNA and 2circ-Nrf2-protein interaction were evaluated.

**Results:** The most important circRNA that origin from Nrf2 is circ-Nrf2-1. Circ-Nrf2-1 downregulates in oxidative stress and upregulates in 5% of H<sub>2</sub>O<sub>2</sub> compared to 1%. However, linear Nrf2 was upregulated in both 1% and 5% H<sub>2</sub>O<sub>2</sub>. Circ-Nrf2-1 can bind to several miRNAs including miR-144-3p, miR-148a-5p, miR-155-5p. It also interacts with Celf1 which plays a role in oxidative stress.

**Conclusion:** According to results, it is plausible to suggest that circNrf2 may play a regulatory role in modulating oxidative stress.

### Introduction

CircRNAs are a large class of particularly stable RNAs produced by the circularization of specific exons in animals.<sup>1</sup> CircRNAs were found extremely abundant in the mammalian brain.<sup>2</sup> The majority of circRNAs are derived from protein-coding genes and contain complete exons.<sup>1</sup> CircRNAs, characterized by their lariat structure can be made up by exons, introns, or a combination of the both.<sup>3,4</sup> CircRNAs are generated by a specific type of splicing known as back-splicing, which is catalyzed by canonical spliceosomal machinery. They are created when the 5' and 3' ends of transcribed exons and/or introns are covalently joined. In this process, the downstream splice site of the donor exon/intron joins the upstream splice site of the acceptor exon/intron in a pre-mRNA molecule. Depending on which exons are selected by alternative back-splice site selection during the back-splicing process, several splice

variants of circRNA transcripts could be generated from a single gene.<sup>5</sup>

According to high-throughput sequencing results, circRNAs have tissue- and developmental-specific expression, implying that they may play important roles in a variety of cellular processes.<sup>6</sup> More research has revealed that the deregulated expression of circRNAs plays a critical role in the development of a variety of human diseases.<sup>7</sup> Researchers have proposed three major functions for circRNAs including sponging miRNAs molecules, interaction with protein and translation into protein. Numerous studies have shown that circRNAs perform biological functions by acting as competing endogenous RNAs or miRNA sponges. For instance, CDR1as contain 63 conserved miR-7 binding sites and increase the levels of miR-7 target mRNAs, most of which are linked to tumor progression.<sup>8</sup> Circ RNAs can play a

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neuroprotective role in diseases by miRNAs regulation. For instance, Circ RNA, circDLGAP4 through regulation of miR-134-5p influences the expression of Brain Derived Neurotrophic Factor (BDNF), B-cell lymphoma 2 (Bcl2) and Peroxisome proliferator-activated receptor-gamma coactivator (PGC-1 $\alpha$ ) in Parkinson's disease.<sup>9</sup>

Spinal cord injury also changes circRNA expression especially circAbca1. Through selective binding to the miR-135b-5P/KLF4 axis, circAbca1 exerts a neuroinhibitory effect.<sup>10</sup> CircRNAs also serve as protein sponges, which have important physiologic functions of cells. A molecular reservoir of proteins were produced by binding specific proteins to circRNAs, which act as entrap to facilitate a quick response to extracellular stimuli.<sup>11</sup> Recent research has shown that a subset of endogenous circRNAs can be translated without the 5' 7methylguanosine cap. CircZNF609, for example, is related to heavy polysomes, exhibits cap-independently translation.<sup>12</sup>

Oxidativestressiscausedbyan imbalance in the production of reactive oxygen species (ROS) and antioxidant defenses, in which oxidative processes overcome antioxidant defenses.<sup>13</sup> Several studies have found that the presence of ROS is critical in initiating or acting as a secondary factor in a variety of pathologies, including metabolic and genetic diseases.<sup>14</sup> The mitochondrial respiratory chain is a major source of intracellular ROS generation as well as an important target for ROS-induced damage.<sup>15</sup> A man with average weight (70 kg) consumes about 250 mL oxygen per minute in the resting state. The normal weight of the brain is about 1400 grams that consumes about 49 mL or 20% of total body oxygen per minute at rest condition. Within the brain tissue, oxygen consumption is highly dynamic and region-specific.<sup>16</sup> Many studies have shown that oxidative stress has a pathogenetic role in some neuronal diseases such as aging, Alzheimer's disease, Parkinson's disease and Autism.<sup>17-19</sup>

The master regulator of cellular responses to environmental stresses is nuclear factor erythroid 2-related factor 2 (Nrf2) - also known as NFE2L2. Nrf2 promotes the expression of detoxification and antioxidant enzymes and also inhibits the expression of pro-inflammatory cytokine genes.<sup>20</sup> Nrf2 directly regulates the expression of Nqo1, Sod2 and Ho-1 genes and Nrf2 can protect cells from apoptosis. Upregulation of it elevates the expression of Nqo1, Sod2 and Ho-1.<sup>21</sup> Heterodimerization of Nrf2 with sMaf leads to a specific binding in the cis-acting enhancer region of DNA called an antioxidant-response element. Many cytoprotective genes have this element in their promoter or enhancer regions.<sup>22</sup> Ample evidence suggests that increasing Nrf2 signaling activation is neuroprotective in neuronal diseases such as neurodegeneration.<sup>23</sup> Nrf2 also has an accelerating effect on learning and memory through regulation of circ-Vps41 which increases hippocampal synaptic plasticity.<sup>24</sup> Some miRNAs including miR-155 and miR-144 inhibit Nrf2 mRNAs and play a role in oxidative stress.<sup>25</sup>

circNrf2 RNAs can influence Nrf2 expression regulation.

They may also have important roles in oxidative stress and neuronal disease regulation. Hippocampus is vulnerable to oxidative stress and reduced antioxidant capacity.<sup>26</sup> It can significantly reduce new neuron production and alter dendritic structures in the hippocampus which leads to impair hippocampal-dependent learning and memory functions.<sup>27</sup> Based on the mentioned studies, we used the rat's hippocampus tissue in the present study. Therefore, our purpose in this study is evaluate circRNAs which origin from Nrf2 gene and prediction whether they participate in oxidative stress pathway and neuronal functions.

## Methods

### Animals study

Twenty-one 5-month-old male rats (220–250 g) were purchased from the animal house of North Khorasan University of Medical Science. They were divided into three groups (n=7) including control and treatment groups. The animals were housed in standard laboratory conditions with 12-hour day and night cycles. The temperature and humidity in the laboratory were 25 °C and 55–65 percent respectively. All animals had free access to food and drinking liquid (water for control group and 1% and 5% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) for treatment groups).

Nrf2 is activated in oxidative stress and H<sub>2</sub>O<sub>2</sub> can induce oxidative stress in the brain.<sup>28,29</sup> We design a study for activation of Nrf2 gene expression in the brain tissue by induction of oxidative stress. The control group received normal food and water however, 1% and 5% H<sub>2</sub>O<sub>2</sub> solution were provided as drinking solution for second and third group respectively. Treatment was performed for 40 days and the liquid solution sources (plain water, 1% and 5% H<sub>2</sub>O<sub>2</sub>) were refreshed daily.<sup>30</sup>

### CircRNA prediction and primer designing

circAtlas 2.0 predicts circRNAs by using analysis of 1070 RNA-seq samples from 19 different tissues.<sup>31</sup> It has collected more than one million circRNAs from six organisms. Therefore, rat circRNAs were downloaded from circAtlas 2.0 then Nrf2 circRNAs were selected and aligned to rat Nrf2 mRNA. All or part of exons 2, 3, 4 and 5 were matched to circ-Nrf2-1, 2 and 3 so, it is possible to back splice perform the end of exons 3, 4 and 5. For testing this hypothesis three primers were designed for the junction site at the beginning of exon two with the end of exons 3, 4 and 5. Since Exon 2 was common to all three sequences, a common primer was designed for Exon 2. Nrf2 primer was designed for exon 1 which part does not participate in the circularization mechanism so it amplifies linear Nrf2 mRNA. Three primers also were designed for the downstream of the Nrf2 pathway including superoxide Dismutase-2 (Sod2), Nicotinamide Adenine Dinucleotide Phosphate (NAD(P)H), quinone dehydrogenase 1 (Nqo1) and heme Oxygenase-1 (Ho-1).<sup>32</sup> Gapdh was considered as a reference gene in the qPCR.<sup>33</sup> All primer were designed by Primer3plus and PrimerBLAST.<sup>34,35</sup>

### Real-time PCR and validation of circRNAs

Total RNA was extracted by “RNX-Plus” from hippocampus tissue (seven samples for each group) and 1000 ng of isolated RNAs were used for RNase R treatment (Lucigen RNase R). Total RNA from RNase treatment was used for reverse transcription using “AddPrep”. Finally real-time PCR was performed on three circRNAs for control and treatment groups after primer designing (Table 1). For this purpose SYBR green (RNX Plus) was used for the measurement of circNrf2s level transcription.

### CircRNA and miRNA interaction prediction

CircRNA and miRNA interaction was verified by miRDB and TargetScan.<sup>36,37</sup> In order to accomplish this objective, the sequences of three circRNAs that were confirmed using q-PCR were submitted to miRDB for analysis. There is an option in miRDB for submission as target sequence and prediction-related miRNAs. Therefore, miRNAs which can target these three circRNA sequences were predicted and downloaded from miRDB. In TargetScan, all miRNAs which target Nrf2's mRNA were extracted except exon 1 – exon 1 does not participate in Nrf2 circulation-. Eventually, the common results of miRDB and TargetScan were used for further analysis. The miRNA targets also were predicted by miRDB.

### Pathway analysis

Enrichr is a popular gene set search engine that contains a large collection of gene sets and gene set libraries for performing such analyses. Enrichr's large collection of annotated gene sets provides a valuable knowledge about gene pathway functions. Therefore, Enrichr was used to investigate the KEGG (Kyoto encyclopedia of genes and genomes) enrichment pathway (<http://amp.pharm.mssm.edu/Enrichr/>). The miRNAs targets were uploaded into Enrichr and pathways were selected based on KEGG results and statistical cutoff (P-value > 0.05).

### CircRNA and protein interaction prediction

catRAPID is an algorithm used for protein-RNA interaction prediction and to estimate the binding propensity of protein-RNA pairs.<sup>38</sup> This algorithm by using combining secondary structure, hydrogen bonding, and van der Waals forces predict protein- linear RNA and circRNA interaction. The catRAPID omics v2.1 tool was employed to predict the interaction between circNrf2 and proteins, with a propensity threshold of 75 utilized for this purpose. Subsequently, from the results obtained through catRAPID, a subset of proteins was selected based on their stronger association with neuronal function. The functional information of selected proteins was extracted from Uniprot.<sup>39</sup> Our predicted structures included Arid4b (PDB file: AF-Q9JKB5), Ccar (PDB file: AF-D4A2P1), Cdc5l (PDB file: AF-O08837), Ddx10 (PDB file: AF-D3ZBY5), Ddx46 (PDB file: AF-Q62780), Inpp5d (PDB file: AF-P97573), Nop14 (PDB file: AF-A0A0G2K0Z9) and Smg6 (PDB file: AF-D4A589). These protein interactions were confirmed by the HDOCK server. The HDOCK is a web server that uses a hybrid docking algorithm of template-based modelling and free docking to rescue cases with misleading templates. The server supports protein-protein and protein-DNA/RNA docking and accepts protein inputs in both sequence and structure.<sup>40</sup>

### Statistical analysis

In order to statistical analysis of data, GraphPad Prism version 8 was employed. Student's t-tests were performed to compare samples and a p-value less than 0.05 was considered as a statistical threshold.

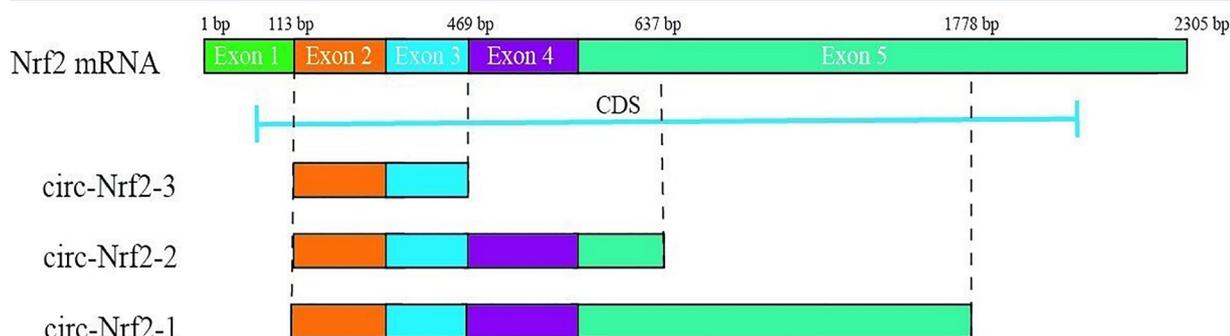
## Results

### CircRNA prediction

Four circRNA sequences for the Nrf2 of rat gene were obtained from the circAtlas 2.0 database including circNrf2-1 1666 bp, circNrf2-2 525 bp, circNrf2-3 357 bp and circ-Nrf2-4 179 bp. All four sequences were aligned to Nrf2's gene to check for sequence matching with Nrf2 mRNA.<sup>41</sup> All of the sequences were aligned with the Nrf2

**Table 1.** Real-time PCR primers were used in this study.

circRNA	Primer sequence	TM
circRNA1	F: TAGATGACCATGAGGACATGG	55°C
	R: GTCTCTTCATCCAGTTGTAAC (Common primer)	
circRNA2	F: CCGAGTTACAGGACATGGAT	55°C
circRNA3	F: GGAATATCATGACTTGACATGGA	55°C
Nrf2 (linear)	F: CTCACCTCTGCTGCCAGTA	55°C
	R: CCTTCTGTCTGCTGACTAAAGT	
Sod2	F: GGCCAAGGGAGATGTTAC	
	R: TCACGCTTGATAGCCTC	
Nqo1	F: TTCCAGAATAAGAAGACCTTG	55°C
	R: CTAAGACCTGGAAGCCAC	
Ho1	F: GCTTTAAGCTGGTGTATGG	55°C
	R: GTGGCTGGTGTGTAAGG	



**Figure 1.** BLAST results. Four Nrf2 circRNAs were BLASTed to Nrf2 mRNA three circRNAs including circ-Nrf2 1,2 and 3 were matched to Nrf2 mRNA but, circ-Nrf2 4 was not matched to Nrf2 mRNA. All three circRNA sequences were started at exon 2 and they were common in exon 2 and exon 3. No one of these circRNAs contained a CDS completely.

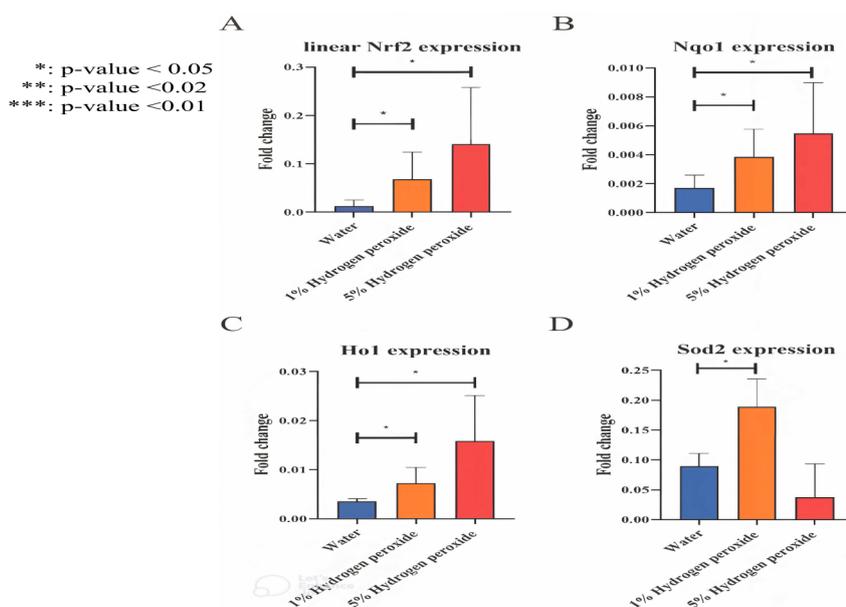
mRNA, with the exception of circ-Nrf2-4, as it was not taken into consideration for this analysis. All sequences started from exon 2, moreover exons 2 and 3 were common in all sequences. Therefore, it seems that Nrf2 mRNA produces three circRNA by a back splice at end of exons 3, 4 and 5 to the beginning of exon 2. None of these three sequences contains the CDS (coding sequences) region completely so, suggesting that they do not possess a functional translation capability. (Figure 1).

### Real-time PCR and validation of circRNAs

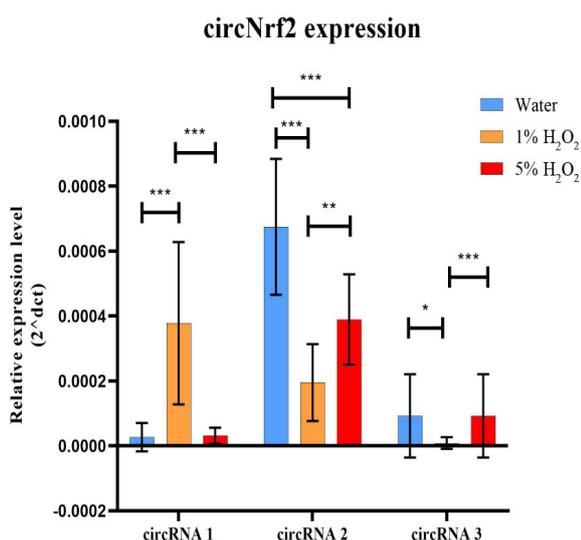
#### Nrf2 pathway gene expression

For the confirmation of Nrf2 pathway activation qPCR was employed for the expression of Nqo1, Sod2 and Ho-1. Linear Nrf2 expression was significantly increased by elevation of the H<sub>2</sub>O<sub>2</sub> level (Figure 2). Its expression was two and four times more, compared to control, in 1% (p-value

0.032) and 5% (p-value 0.041) H<sub>2</sub>O<sub>2</sub>, respectively. Both the expressions of Nqo1 and Ho-1 exhibited an increase in response to the elevated levels of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), along with Nrf2. Specifically, their expressions showed an approximately two-fold (p-value 0.029) and four-fold (p-value 0.038) increase in the 1% and 5% H<sub>2</sub>O<sub>2</sub> groups, respectively. Additionally, Sod2 demonstrated an upregulation in the 1% H<sub>2</sub>O<sub>2</sub> group; however, it exhibited a downregulation in the 5% (p-value 0.021) H<sub>2</sub>O<sub>2</sub> group. Figure 2. Expression of linear Nrf2 and its downstream genes. A) linear Nrf2 was significantly upregulated with 1% H<sub>2</sub>O<sub>2</sub> (p-value 0.032) and it increased more by 5% H<sub>2</sub>O<sub>2</sub> (p-value 0.041). B) Nqo1 was upregulated with the raise of the H<sub>2</sub>O<sub>2</sub> level (p-value 0.029 and 0.038). C) Ho-1 expression elevated with 1% (p-value 0.045) and 5% H<sub>2</sub>O<sub>2</sub> (p-value 0.039). D) Sod2 expression increased by 1% H<sub>2</sub>O<sub>2</sub> (p-value 0.021) while it decreased by 5% H<sub>2</sub>O<sub>2</sub>.



**Figure 2.** Expression of linear Nrf2 and its downstream genes. **A)** Linear Nrf2 was significantly upregulated with 1% H<sub>2</sub>O<sub>2</sub> (p-value 0.032) and it increased more by 5% H<sub>2</sub>O<sub>2</sub> (p-value 0.041). **B)** Nqo1 was upregulated with the raise of the H<sub>2</sub>O<sub>2</sub> level (p-value 0.029 and 0.038). **C)** Ho-1 expression elevated with 1% (p-value 0.045) and 5% H<sub>2</sub>O<sub>2</sub> (p-value 0.039). **D)** Sod2 expression increased by 1% H<sub>2</sub>O<sub>2</sub> (p-value 0.021) while it decreased by 5% H<sub>2</sub>O<sub>2</sub>.

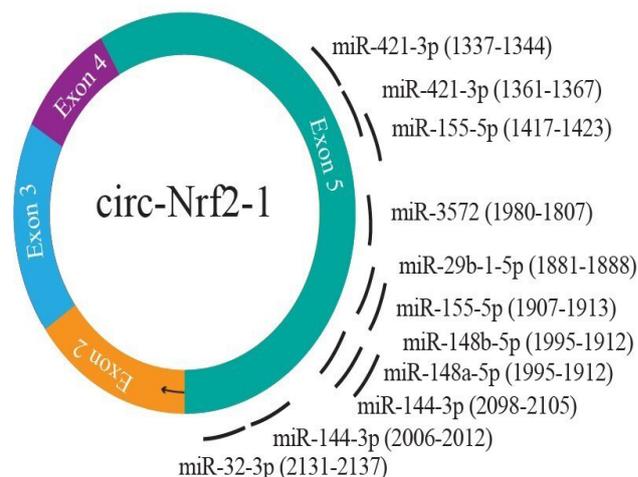


**Figure 3.** Oxidative stress changes circNrf2s expression. Although circNrf2-1's expression increased by 1% H<sub>2</sub>O<sub>2</sub> treatment (p-value 0.008), its expression was felled by 5% H<sub>2</sub>O<sub>2</sub> treatment (p-value 0.007). circNrf2-2/3 had the same behavior. Expression of both of them decreased by 1% H<sub>2</sub>O<sub>2</sub> (p-value 0.006 and 0.042) and rose by 5% H<sub>2</sub>O<sub>2</sub> (p-value 0.018 and 0.002).

#### Origination three circRNAs from the Nrf2 gene and regulation by oxidative stress

H<sub>2</sub>O<sub>2</sub> regulates circNrf2s expression. circNrf2-1's expression increased by 1% H<sub>2</sub>O<sub>2</sub> treatment (p-value 0.008), whereas circNrf2-2/3 (p-value 0.006 and 0.042) expression decreased in 1% H<sub>2</sub>O<sub>2</sub> treatment. circNrf2-1 expression felled significantly in 5% H<sub>2</sub>O<sub>2</sub> (p-value 0.007). Both circNrf2-2/3 are downregulated in 1% H<sub>2</sub>O<sub>2</sub>, but these counts were increased by 5% H<sub>2</sub>O<sub>2</sub> treatment (p-value 0.018 and 0.002) (Figure 3).

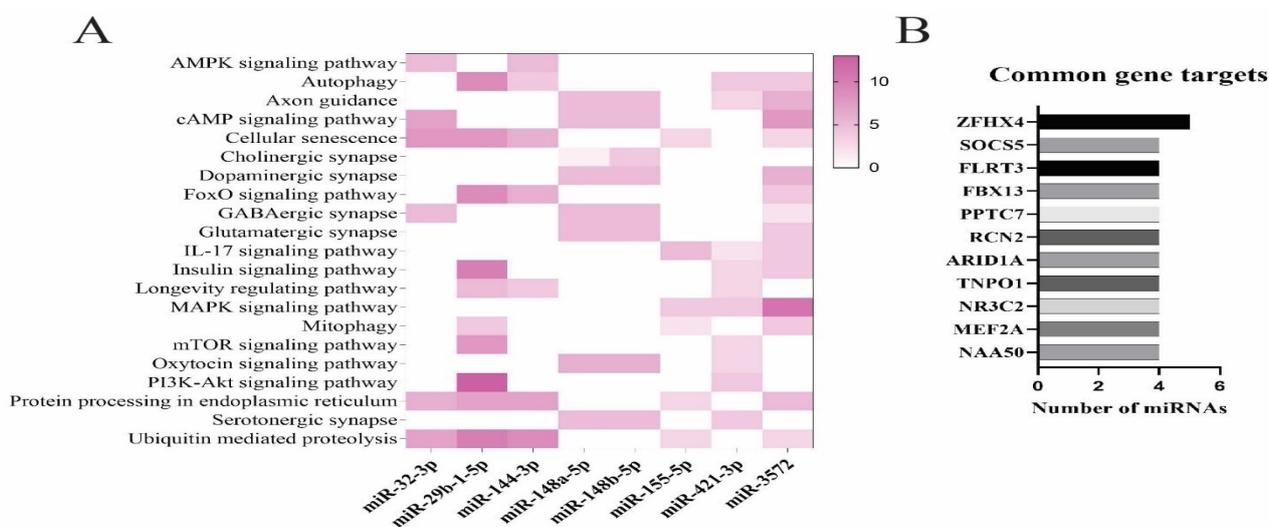
#### miRNA's binding sites



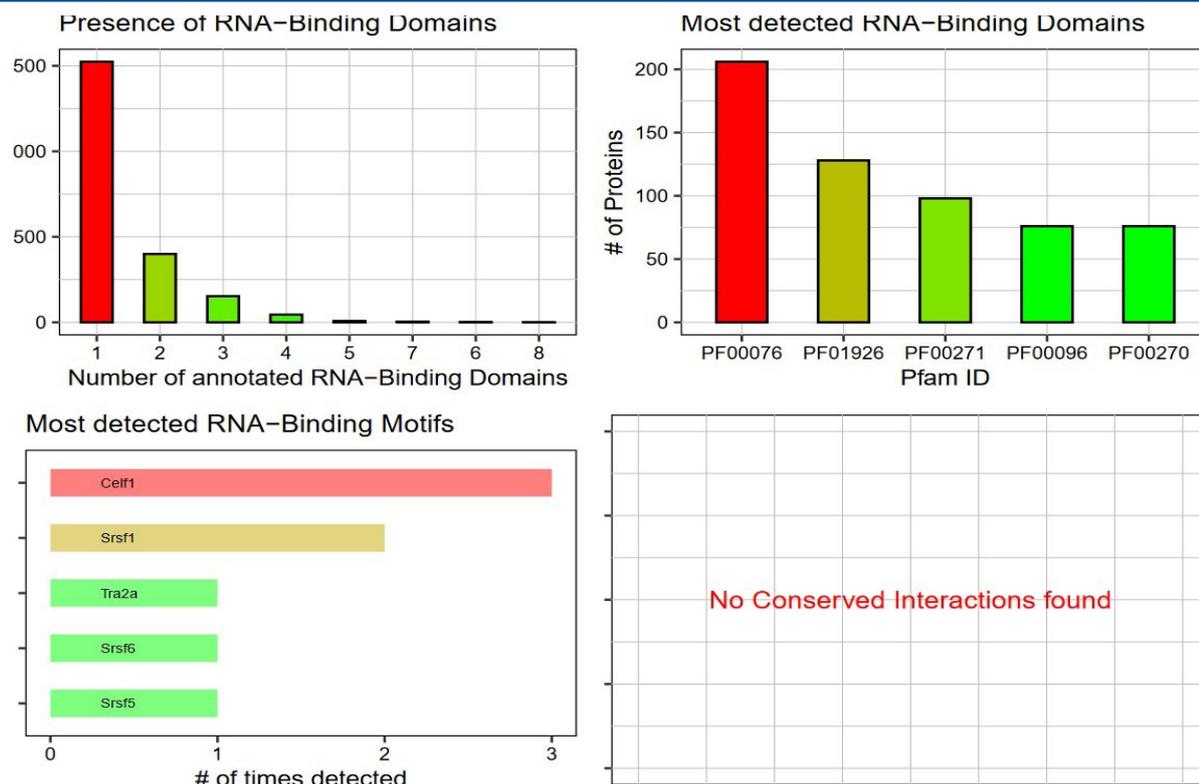
**Figure 4.** circRNA-miRNA interactions. circ-Nrf2-1 had the most interaction with miRNAs, which can absorb 18 different proteins. among 18 miRNAs, 13 miRNAs bind to exon 5, and exon 5 appears to be the most important part of the circ-Nrf2-1 for miRNAs absorption. Both circ-Nrf2-2 and circ-Nrf2-3 can absorb 4 miRNAs.

#### Circ-Nrf2-1 interaction with miRNAs and regulation inflammation and ubiquitin-mediated proteolysis

The sponging of miRNAs is one of the important circRNAs functions. Therefore, we employed miRDB for the prediction of circ-Nrf2-miRNAs interactions. Eleven loci of circRNA-miRNA interactions were found for circ-Nrf2-1, for 8 miRNAs including, miR-29b-1-5p, miR-32-3p, miR-144-3p, miR-148a-5p, miR-148b-5p, miR-155-5p, miR-421-3p and miR-421-3p. All miRNA interacts with exon 5 NFE2L2, but two interactions were obtained for miR-155 and miR-421(Figure 4).



**Figure 5.** CircNrf2-1's target miRNAs can target several mRNA and cellular pathways. A) ZFH4 was the most target gene for these miRNAs. B) circNrf2-1 can rescue cellular pathways by inhibiting miRNAs functions. The most cellular pathways targeted by these miRNAs were ubiquitin-mediated proteolysis, protein processing in the endoplasmic reticulum and cellular senescence.



**Figure 6.** High number of proteins were found which interact circ-Nrf2-1.

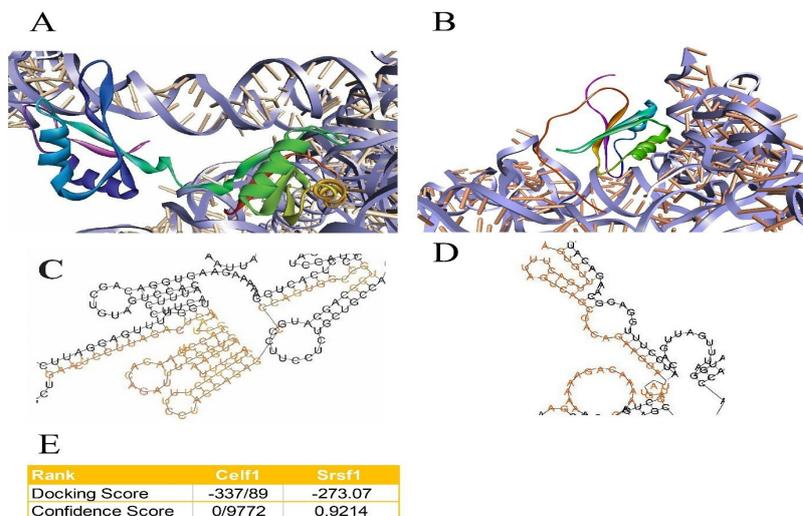
Nrf2/NFE2L2 and zinc finger homeobox 4 (Zfhx4) were the most target genes for 7 and 5 miRNAs respectively (Figure 5a).

CircNrf2-1 can sponge miRNAs and liberates cellular pathways which are aimed by these miRNAs. Here we verified these cellular pathways and the number of target genes that belong to each cellular pathway (Figure 5b). Ubiquitin-mediated proteolysis, protein processing in the endoplasmic reticulum and cellular senescence are targeted

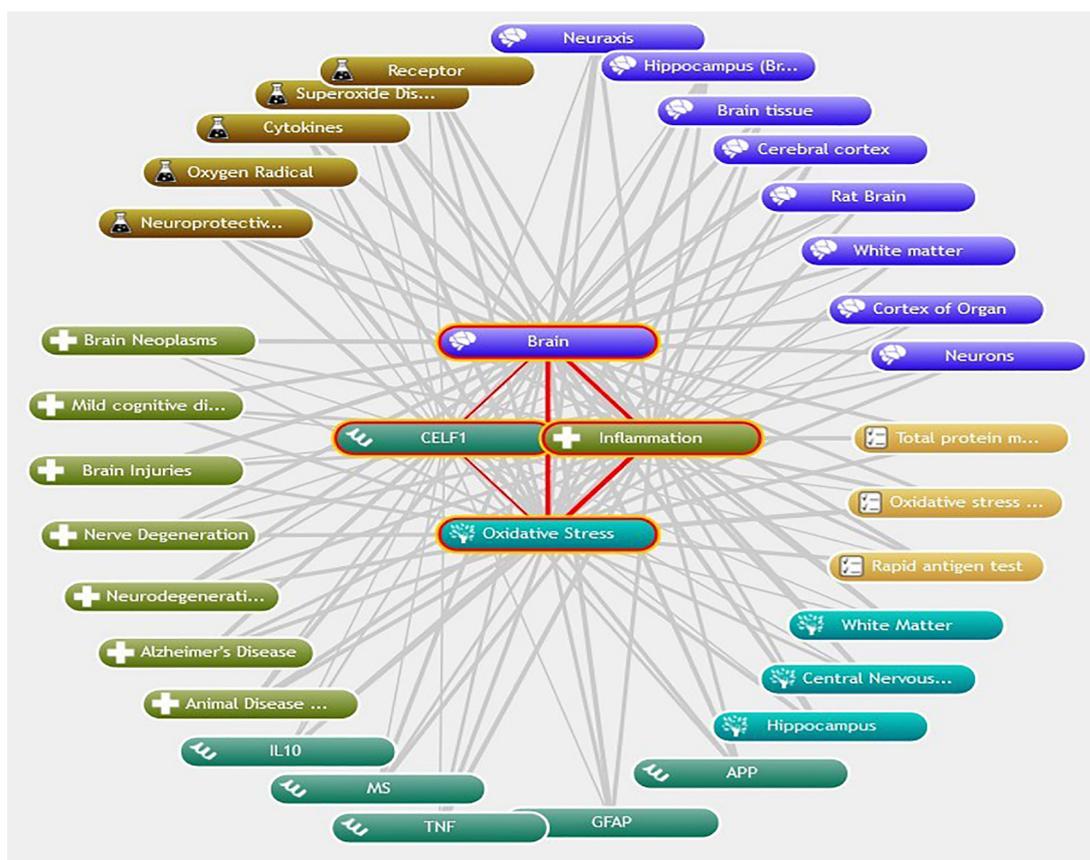
by five (the highest number) circNrf2-1 target-miRNAs.

*Circ-Nrf2-1 interacts with Celf1*

According to catRAPID results, circ-Nrf2-1 interact with a high number of proteins. PF00076 and PF01926 are the most RNA-binding domains (Figure 6). Celf1 and Srsf1 are the most important RNA-binding motifs. However, no conserved interactions found for protein-circ-Nrf2-1.



**Figure 7.** Interaction of circ-Nrf2-1 with Celf1 and Srsf1. A, B) Docking result reveal that Celf1 and Srsf1 surrendered by circ-Nrf2-1. C, D) docking values for Celf1-circ-Nrf2-1 interaction. E) circ-Nrf2-1 sequence which interact with Celf1 (orange sequence).



**Figure 8.** Celf1 relate to oxidative stress, inflammation and brain tissue. Moreover, it may have role in brain disease including brain neoplasms, brain injury and neurodegenerative disease.

Celf1-circ-Nrf2-1 docking was performed by HDOCK server (Figure 7A). According results Celf1 was surrendered with circ-Nrf2-1. Docking score, confidence score and ligand rmsd (Å) for Celf1-circ-Nrf2-1 were -337.89, 0.9772 and 363.37, respectively (Figure 7B). circ-Nrf2-1 with its 87 nucleotides interact with Celf1 (Figure 7C).

Base of medical information, Celf1 relate to oxidative stress, inflammation and brain tissue. It also may paly role in neuronal disease namely brain neoplasms, brain injuries and neurodegenerative disease (Figure 8).

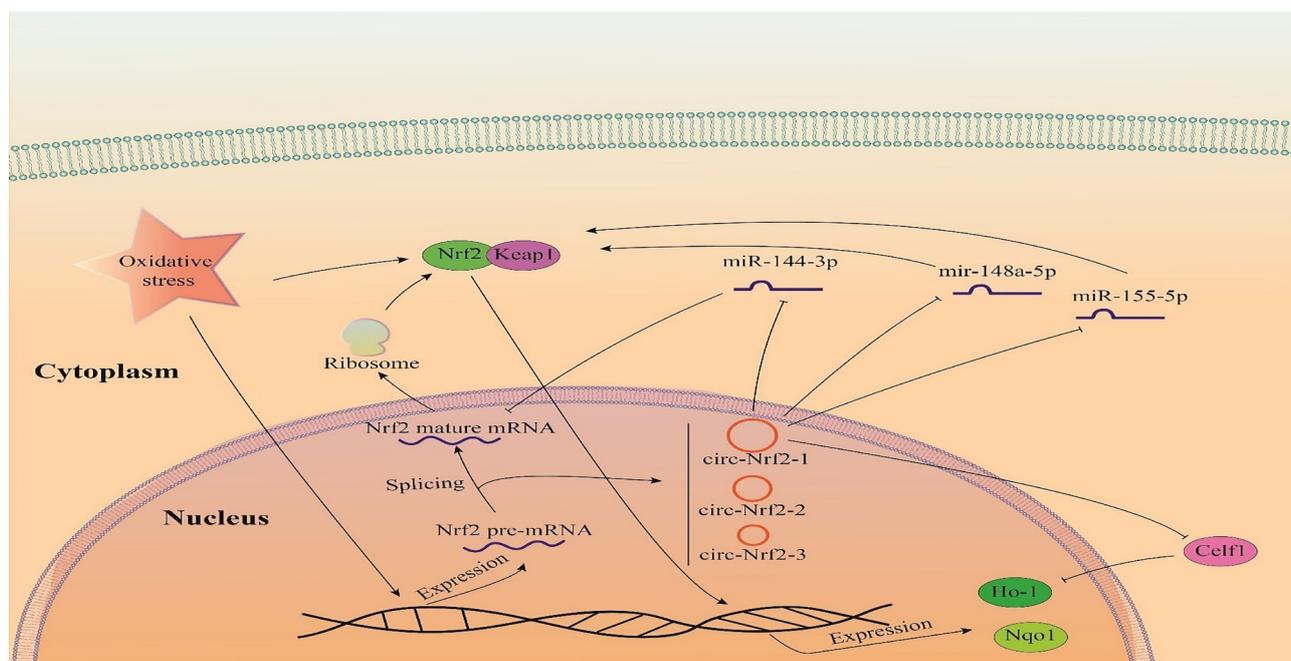
## Discussion

Recent research has found that the mammalian brain has preferential back-splicing of neural genes and increased expression of circRNAs in comparison to other tissues. Analysis of human and mouse neuronal cell lines, as well as ENCODE datasets, revealed that the mammalian brain have more circRNAs than other tissues including lungs, heart, kidney, testis, and spleen; in appropriate conditions circRNAs can produce 20% of the protein-coding genes in the brain.<sup>42</sup> circRNAs play an important role in gene expression regulation by acting as a microRNA sponge, RNA binding protein sponge and translational regulator. circRNAs have become a research hotspot due to their close association with disease development. Some circRNAs are expressed differently depending on the tissue and stage of development. Furthermore, due to other characteristics

of circRNAs such as stability, conservation, and high abundance in body fluids; circRNAs are thought to be potential biomarkers for a variety of diseases.<sup>43</sup>

Aim of this study was evaluation of circRNAs which origin from Nrf2's gene and prediction their roles in neuronal oxidative stress. The heightened generation of ROS serves as an inherent consequence of metabolic processes, often leading to detrimental effects on the integrity of the nervous system.<sup>44,45</sup> Nrf2 is a transcription factor that regulates cellular response to oxidative stress and has significant neuroprotective effects against central nervous system (CNS) diseases.<sup>46</sup> Many studies have revealed the beneficial effect of Nrf2 in neurodegenerative disease, traumatic brain injury and multiple sclerosis.<sup>46-48</sup>

Our data showed that circNrf2-1 and 2 were downregulated by low dosages of hydrogen peroxide, however, they were upregulated in high dosages compared to low dosages (Figure 2). It seems that a high level of oxidative stress stimulates circNrf2-1 and 2 expressions. circNrf2-1 and 2 expressions may follow a dose dependent manner. It has been reported that Nrf2 expression is H<sub>2</sub>O<sub>2</sub> dose-dependent, it means that increasing hydrogen peroxide concentration rises Nrf2 expression.<sup>49</sup> It seems that in the oxidative stress condition, Nrf2 was upregulated however, circNrf2s were downregulated which led to an increase in linear Nrf2, suggesting that this elevation caused more proteins expression.



**Figure 9.** Three circRNAs generates from Nrf2's gene. Cinrc-Nrf2-1 can sponge miR-144-3p, miR-148a-5p and miR-155-5p. It also interacts with Celf1's protein. In this way, circNrf2-1 can attenuates oxidative stress.

Nrf2 is a transcription factor known as the master regulator of multiple antioxidant enzymes including HO-1, NQO1, and SOD (Figure 9).<sup>50</sup> Our results showed that Nrf2's gene and its downstream genes upregulated in the rats' brains in oxidative stress conditions (Figures 2 and 3). In another study, scientists have shown that stress oxidative in rats' brains results in the elevation of Nrf2 and HO-1 proteins.<sup>51</sup> SOD expression also increases through a rise in the upregulation of Nrf2 by increasing oxidative stress, and the Nqo1 gene has the same behavior.<sup>52,53</sup> In human neurons (SH-SY5Y cells) Nrf2 protein increases follow by H<sub>2</sub>O<sub>2</sub> treatment.<sup>54</sup> In the PC12 cells also H<sub>2</sub>O<sub>2</sub> induces oxidative stress and activation of Nrf2 gene expression and its downstream genes including Ho-1 and Nqo1.<sup>55</sup> In our data, linear and circular form of Nrf2 mRNA upregulated in oxidative stress and Nrf2's downstream genes including Nqo1, Ho-1 and Sod genes that implies activation of Nrf2 pathway.

miRNAs interaction prediction shows that the circNrf2-1 can sponge miR-32-3p, miR-29b-1-5p, miR-144-3p, miR-148a (Figure 3) which plays role in oxidative stress. Overexpression of miR-144-3p downregulates Nrf2 expression and decreases antioxidant levels.<sup>56</sup> miR-155-5p is another target miRNA for circNrf2-1 that regulates inflammation in ischemic stroke. miR-155-5p knockdown inhibits cell injury, inflammation and apoptosis in the ischemia rat model.<sup>57</sup> miR-155-5, is associated with oxidative stress. Inhibition of miR-155-5p significantly decreases ROS generation (Figure 9).<sup>58</sup> Besides, miR-155 is an important inflammatory regulator. The deletion of miR-155 protects mice against cerebral ischemia-induced neuronal injury and hemorrhagic transformation. In addition, miR-155 by regulating inflammatory cytokines,

TNF- $\alpha$ , and IFN- $\gamma$  is one of the most crucial miRNAs in multiple sclerosis.<sup>59,60</sup> Studies have shown that downregulation of miR-148a decreases inflammation and oxidative stress.<sup>61</sup> miR-421 has the ability to alter the S-phase level of the cell cycle checkpoint. It targets caspase-3.<sup>62</sup> A study on MDA-MB-231 cells showed that upregulation of Nrf2, downregulates miR-29b-1-5p. On the other hand, miR-29b-1-5p overexpression decreases Nrf2 phosphorylation.<sup>63</sup> circNrf2-1 also can target miR-421 and regulates cell death in Parkinson's disease by regulation of the Bcl2/Bax system.<sup>64</sup>

According to result of molecular docking, circNrf2-1 interact with Celf1 (Figures 6 and 7). Celf1 relate to oxidative stress, inflammation and many brain's disease (Figure 8). Celf1 is an RNA binding protein that involve in various disease such as dilated cardiomyopathy, tumor metastasis and liver fibrosis.<sup>65</sup> It has demonstrated that Celf1 binds to 3'UTR of HO-1 mRNA and downregulate HO-1 expression.<sup>66</sup> HO-1 is an antioxidant, anti-inflammation and anti-apoptotic protein. It is positively regulated by the transcription factor Nrf2.<sup>67</sup>

Ubiquitin-mediated proteolysis is recognized as a molecular mechanism that controls a wide range of normal nervous system functions and is involved in the many neurodegenerative disorders. Neurodegenerative diseases are characterized by abnormal deposition of insoluble protein aggregation or inclusion bodies within neurons, which can be degraded by ubiquitin-mediated proteolysis.<sup>68</sup> Neurons are particularly sensitive to protein misfolding; therefore any dysfunction in protein processing in endoplasmic reticulum has been linked to a variety of neurodegenerative diseases.<sup>69</sup> Cellular senescence — a cell fate that includes a permanent cell cycle arrest and a pro-

inflammatory senescence-associated secretory phenotype — has recently emerged as a predisposing factor of Alzheimer's, dementia, and other age-related pathologies.<sup>70</sup>

### Conclusion

In summary, our findings demonstrate the presence of three distinct circRNA transcripts derived from the Nrf2 gene, each exhibiting different lengths. Among these circRNAs, circNrf-1 stands out as the largest, encompassing exon 2 to exon 5. Notably, circNrf2-1 has been identified as a sponge for miR-144-3p, miR-148a-5p, and miR-155-5p, and is also found to interact with Celf1. Given these interactions, it is plausible to suggest that circNrf2 may play a regulatory role in modulating oxidative stress. To gain further insights into the functional properties of circ-Nrf2-1, it is imperative to conduct transfection experiments utilizing animal and cellular models. Such investigations can provide valuable information regarding the precise role of circ-Nrf2-1 in various biological contexts.

### Ethical Issues

This study was performed base on ethical guidelines (ethical approval code: IR.NKUMS.REC.1399.014).

### Author Contributions

Javad Amini: Conceptualization, Investigation, Formal Analysis, Writing - Original Draft. Roghaye Arezumand: Conceptualization, Investigation, Formal Analysis, Resources, Funding, Writing - Review & Editing. Nima Sanadgol: Conceptualization, Investigation, Formal Analysis, Review & Editin. Peiman Alesheikh: Conceptualization, Investigation, Formal Analysis, Resources, Funding, Writing - Review & Editing.

### Conflict of Interest

The authors declared no potential conflicts of interest.

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