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Evaluation of Changes in Biomarkers of Oxidative Stress Response in the Brain of Mice Infected with Street Rabies Virus

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ABSTRACT

BACKGROUND AND OBJECTIVE: The rabies virus infection leads to 60,000 deaths worldwide each year and is considered an economic and social hazard. Although our knowledge of the rabies virus replication and its interaction with the host cell is in its infancy, it helps us design optimal drugs and vaccines. Therefore, the aim of this study was to evaluate the changes in catalase and superoxide dismutase (SOD) enzymes, nitric oxide (NO) chemical composition and NF-κB gene activity as biomarkers of oxidative stress response in brain tissue of mice infected with street rabies virus.

METHODS: In this experimental study, 16 21-day-old outbred mice were divided into two groups of healthy and experimental (n=8). After determining the titer of rabies virus, the mice in the experimental group received 0.03 ml of rabies virus with LD50 intracerebrally (IC) using Hamilton 0.25 ml syringe. To evaluate the changes of catalase, NO, and SOD enzymes, test kit and colorimetric method were used and NF-kB activity was evaluated by Real-Time PCR according to Sdha gene.

FINDINGS: In this study, catalase activity in the brains of infected mice (0.14±0.007) was significantly reduced compared to healthy mice (control) (0.67±0.008) (p=0.01). In this experimental study, the SOD activity in the brains of infected mice (3±0.2 nmol/min/ml) was significantly reduced compared to healthy mice (control) (5.7 nmol/min/ml) (p=0.035). Evaluation of NO accumulation in the brain tissue of the infected group (9.0±0.04 mU/mg) was significantly increased compared to the healthy group (9.04±0.03 mU/mg) (p=0.025). Furthermore, the expression level of NF-κB in the brain of the mice infected with rabies virus was one tenth of the expression of this gene in the brain of healthy mice.

CONCLUSION: The results showed that rabies virus decreased the activity of catalase and SOD enzymes and increased nitric oxide. Furthermore, NF-κB expression decreased in the brain of mice infected with rabies virus, which may be due to the effect of the virus on the cellular signaling pathway.

KEY WORDS: *Rabies Virus, Oxidative Stress, Message Transmission.*

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Introduction

The rabies virus belongs to the lyssavirus, a genus of RNA viruses in the family Rhabdoviridae, order Mononegavirales (1). The virus causes acute disease of the central nervous system that causes fatal encephalitis in humans and animals (2). The rabies virus causes 60,000 deaths worldwide each year and is considered an economic and social hazard (3). The virus multiplies at the site of the bite in the muscle tissue and then travels to the peripheral nerves. The virus infects the peripheral sensory and motor nerves and binds specifically to the acetylcholine neurotransmitter and the neuromuscular junction (4).

The pathogenesis of street rabies virus differs from weaker strains such as CVS; street rabies virus, for example, prevents the induction of apoptosis in nerve cells and rarely infects glial cells. Weaker strains, however, cause apoptosis in these cells. Furthermore, the data show that strains of street rabies virus produce a moderate inflammatory response in the nervous system. It is proposed that regulatory mechanisms are established during the course of infection to reduce the induced inflammation of the nervous system (1, 2). Oxidative stress plays an important role in various viral diseases including rabies (4).

Oxidative stress is defined as an imbalance between the production of reactive oxygen species and the natural antioxidant defense mechanism, which can also lead to chronic inflammation. Oxidative stress, by activating various transcription factors, leads to differential expression of some genes involved in inflammatory pathways and can ultimately lead to dysfunction of nucleic acids, proteins and lipids (4). Catalase and superoxide dismutase (SOD) are among oxidative stress enzymes. Catalase is found in almost all living organisms and is one of the most important enzymes in protecting the cell against free radicals, which play an important role in inflammatory reactions (5).

SOD is an important type of antioxidant defense and is present in almost all cells exposed to oxygen. Superoxide is a byproduct of oxygen metabolism and, if left unchecked, can cause a variety of cellular damages (6). In addition, nitric oxide (NO) is an important molecule in cellular messaging that is involved in many physiological and pathological processes (7). Since rabies occurs in poor areas, few studies have been conducted on its pathogenicity. Further studies appear to be needed to identify pathogens of the virus; although knowing the replication cycle of rabies virus and its interaction with the host cell is at the beginning of the journey, it will help us design optimal drugs and vaccines. Therefore, the aim of this study was to evaluate the changes in catalase, SOD and NO enzymes as well as NF-κB activity as biomarkers of oxidative stress response in the brain tissue of mice infected with street rabies virus. It is hoped that the results presented is this study will be the beginning of a useful and effective step towards the treatment and prevention of rabies and control of this disease in Iran.

Methods

Animals: In this interventional study, after approval by the Ethics Committee of the Pasteur Institute of Iran with the code IR.PII.REC.1398.002, 46 NMRI outbred female mice with an average weight of 11-14 g were used. They were purchased from the laboratory animal center of Pasteur Institute of Iran. These types of mice are the strains found in nature (8). Mice were kept in ethical condition until the end of the experiment. Thirty mice were used to determine the viral titer and the rest of the animals were randomly divided into two groups of eight (the first group, control or healthy mice and the second group, test mice infected with rabies virus).

Production of rabies virus and determination of LD50 of produced virus: To perform the challenge, the viral titer was first determined. A sample of animal brain that was confirmed to be infected with rabies by FAT test was selected for further tests. A uniform suspension of 20% by weight of brain tissue was prepared in sterile diluent solution (or 2% horse serum in distilled water). Then, each of the serial dilutions 10^{-5}, 10^{-6} and 10^{-7} were prepared and each of these dilutions was injected intracerebrally (IC) into 10 mice. From the fifth day, the suspension-injected mice were monitored and the number of live and dead mice was recorded up to 14 days. The LD50 of virus was calculated by Spearman-Kärber method and appropriate statistical software based on the number of live and dead mice during this period (9).

Virus injection: After determining the titer of street rabies virus, the mice in the experimental group were challenged with 0.03 ml rabies virus with LD50 via IC and using Hamilton 0.25 ml syringe. Five days after the virus was injected, the mice that showed the three symptoms of rabies, namely paralysis, seizures and irritability, were sacrificed and their brains were extracted for further analyses and stored in a freezer at -70 °C. To evaluate the presence of rabies virus in the

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brains of symptomatic mice, FAT test was performed according to the instructions (10).

Evaluation of changes in biomarkers of oxidative stress response: Evaluation of the mentioned parameters of NO and catalase in the brain tissue of rabies-infected mice was done using Navand Salamat Co. Assay Kit (Natrix™, Iran) and SOD was done using Teb Pazhouhan Razi Kit (Iran) based on the instructions of the mentioned kits and based on the colorimetric method.

Evaluation of NF-κB gene expression by Real Time PCR

RNA extraction: TRIzol reagent (Invitrogen Life Technologies) was used to extract RNA in brain tissues according to the instructions provided by the manufacturer. Then, the RNA concentration was determined by Nanodrop 2000. Finally, the RNA was stored at -80 °C until the time of use.

CDNA synthesis: 5 µl RNA was used for cDNA synthesis using the Invitrogen kit (USA) and according to the instructions provided by the kit. The cDNA for the Real Time PCR was stored at -20 °C for later use.

Primer design: Primer design was performed using Primer3 software, its specificity was evaluated using NCBI BLAST website and its secondary structure was evaluated by Gene Runner software. Finally, it was synthesized by Gene Fanavaran Company (Iran). The sequence of primers used is shown in Table 1.

PCR polymerase chain reaction on Sdha gene: PCR was used to evaluate the quality of cDNA. According to the Taq DNA Polymerase Master Mix RED protocol with catalog number 180301 (Amplicon, Germany), all materials together with the designed primers were combined and placed in a thermal cycler (BIO RAD, USA). Then, electrophoresis of PCR products was performed on 1.5% agarose gel to confirm the reaction.

Relative Real Time PCR: In order to investigate the expression changes of NF-kB gene and compare it with Sdha housekeeping gene, the reaction was performed using Corbett RG-6000 device. All reaction steps were performed according to the instructions of the 5x HOT FIREPol® EvaGreen® qPCR Mix Plus kit (no ROX) with catalog number 08-25-00001.

Calculations for NF-kB gene expression: In order to calculate the amount of changes in NF-kB gene expression, ΔΔCT method was used. In this method, the difference between CT of the target gene and CT of the internal control gene in the target samples was calculated (ΔCT1 and ΔCT2). Finally, two ΔCTs were subtracted from each other and placed in the formula $2^{-\Delta\Delta CT}$ to calculate the relative quantity (RQ) of gene expression. Statistical analyses of various safety tests were performed using Statistical Package for the Social Sciences (SPSS) software and t-test with two independent samples.

Table 1. The primer sequences designed to study the expression of NF-kB gene and Sdha housekeeping gene

Primers Studied genes	Primer F	Primer R	Product length (nucleotide)
Sdha	5′-GGA GGT ATC AAT GCT GCT CTG-3′	5′-CTG TCA TGT AAT GGA TGG CGT-3′	118
NF-kB	CGCAAAAGGACCTACGAGAC	TGGGGGAAAACTCATCAAAG	193

Results

Results of evaluating changes in biomarkers of oxidative stress response

Catalase and SOD: In this study, a catalase and SOD activity test was performed on the homogeneous brain tissue of two groups of mice infected with rabies virus and healthy and the following results were obtained. The results showed a significant decrease in the activity of catalase (p=0.01) and SOD (p=0.035) in brain tissue of the experimental group compared to the control group. Catalase and SOD activity in the control group was 0.67±0.008 and 5.7±0.0001 nmol/min/ml, respectively, and in the experimental group was 0.14±0.007 and 3±0.2 nmol/min/ml, respectively.

NO: After measuring the accumulation of nitric oxide, it was found that free radical NO in the brain tissue of mice in the experimental group had a significant increase compared to the control group (p=0.025). Accumulation of NO in the control group was 9.4±0.03 mU/mg and in the experimental group was 9.8±0.04 µM.

NF-kB expression:

Results of RNA extraction of mice's brains using NanoDrop device: In order to control the quality of extraction and to determine the concentration of RNA extracted from the mentioned tissues, NanoDrop device was used. Protein contamination was measured based on 260/280 ratio and its optimal amount was considered close to 2 (11).

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Results of evaluating Sdha genes (PCR test results of Sdha gene) on 2% agarose gel to confirm RNA extraction and cDNA synthesis: Using 0.5 µl primer with a concentration of 10 pmol, nonspecific bands in this gene were removed. Sharp bands with high resolution were observed in the Sdha gene (Figure 1).

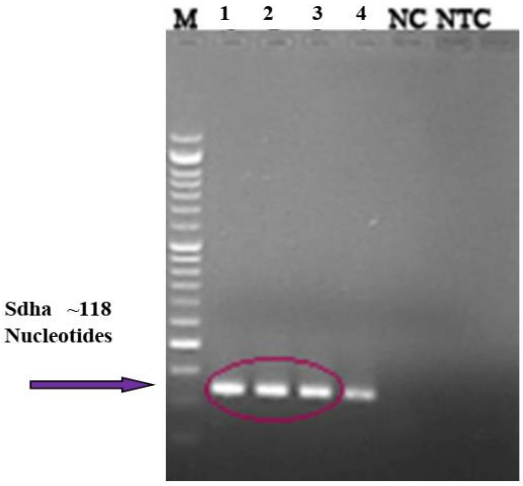

Figure 1. PCR product of Sdha gene with a size of 118 nucleotides. **Well M: 50 bp DNA Ladder, well 1: temperature of 59 °C, well 2: temperature of 60 °C, well 3: temperature of 61° C, well 4: temperature of 62 °C, well NC: Negative control sample (cDNA obtained from RNA extracted from mouse brain), and well NTC: Negative control sample (without sample). As shown in the figure, temperatures of 59-61 °C showed a clearer band**

Results of NF-κB gene expression compared to housekeeping gene in the brains of rabies-infected mice compared with healthy mice: Results showed decreased NF-κB gene expression in brain tissue after rabies infection compared with the control group (healthy mice). Using the formula described in the NF-κB gene expression calculation, the expression of the NF-κB gene in the brains of rabies-infected mice was one tenth of the expression of this gene in the brains of healthy mice (Figure 2).

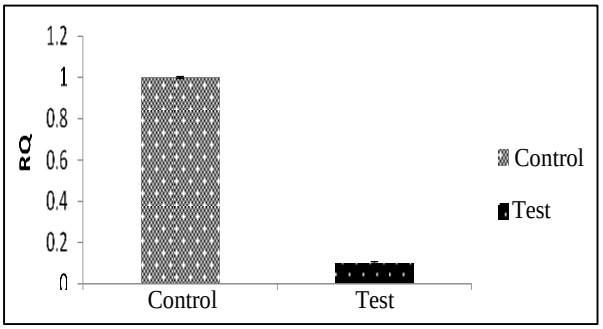

Figure 2. Bar chart of NF-κB gene expression in brain tissue of rabies mice. **The results of this study showed a significant decrease in NF-κB gene after the entry of rabies virus into brain cells ($p<0.05$); Test: Group of mice infected with rabies virus, Control: Group of healthy mice**

Results of melting curve of Sdha and NF-κB genes obtained from Real Time PCR: In order to ensure the absence of nonspecific peaks and the absence of contamination, it is necessary to study the melting curve of the genes (Figure 3 b and d). Figures 3 a and c are examples of the amplification curve of the Sdha and NF-κB genes.

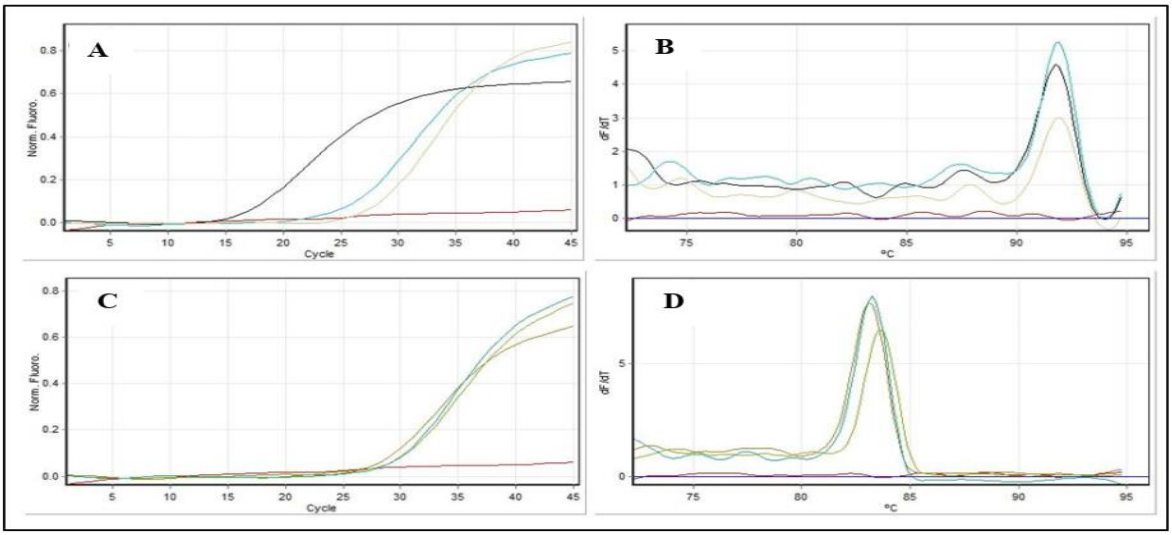

Figure 3. (A and B) amplification and melting curves of NF-κB gene along with negative control; (C and D) amplification and melting curves of the Sdha host gene along with negative control. **The presence of a melting curve at the same temperature for each of the genes indicates the absence of a non-specific product and represents the proper functioning of specific primers in this gene**

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Discussion

The results of this study showed that rabies virus decreased the activity of catalase and SOD enzymes and increased NO. A change in these biomarkers of the oxidative stress response is probably for the escape from the host's innate immune system. In this study, the expression of NF-κB in the brains of the mice infected with rabies virus was reduced, which could be due to the effect of the virus on the cellular signaling pathway. The first line of defense against the rabies virus is innate immunity. Studies have shown that inflammation is the main response of the innate immune system to an external infectious agent (12, 13). Enzymes such as catalase and SOD, as the first line of defense mechanism, play an important role against oxidative stress (14).

Oxidative stresses are involved in viral diseases (15). In street rabies virus, however, the virus inhibits inflammatory responses to prolong the life of infected cells and to replicate the virus (16, 17). Furthermore, oxidative damage has been shown to be an important component of acute experimental encephalitis due to herpes simplex virus type 1 (HSV-1) in mice (18) and in immunodeficiency virus infection (19), especially in people with dementia (20).

A study by Jackson et al. showed that rabies virus causes axonal damage to neurons through oxidative stress (21). In the inflammatory state, the production of NO as a key mediator by the arteries is significantly increased and contributes to oxidative stress (22, 23). The eNOS isoform produces NO in the nanomolar range and can regulate the expression of proinflammatory molecules such as nuclear factor NF-κB, cyclooxygenase, and proinflammatory cytokines (24). Numerous studies have shown that NO may play a key role in the pathogenesis of various neuroinflammatory disorders and degeneration of myelin in the central nervous system. In the central nervous system, oligodendrocytes are more sensitive to NO than other glial cells, and oligodendrocyte death may be the main mechanism in the pathophysiology of MS (25). In this study, in line with the study by Jackson et al. and other similar studies, NO levels increased (21-23). In a study, Ubol et al. showed that inhibition of nitric oxide synthases delayed mouse death from rabies (22).

Mitochondria have extensive antioxidant defense systems for ROS detoxification, including SOD, catalase, glutathione peroxidase, phospholipid hydroperoxide, and glutathione reductase (26). SOD activity leads to the transfer of an electron to a superoxide radical and its conversion to a hydrogen peroxide (H_2O_2) molecule. Hydrogen peroxide from SOD activity is itself an active species of oxygen that can lead to oxidative damage to biomolecules and is revived to H_2O by other antioxidant enzymes (27, 28). Moreover, the enzyme catalase is an antioxidant metalloprotein that leads to the transfer of electrons from one H_2O_2 molecule to another H_2O_2 molecule and their conversion to water and oxygen (29). To counteract the effects of oxidative stress, plant cells synthesize detoxifying enzymes of ROS such as SOD, peroxidase, and catalase. In many infectious diseases, defects in the production of these enzymes have been shown to increase pathogenicity (30).

Consistent with the few studies that have been performed on the effect of street rabies virus on the activity of catalase and SOD enzymes so far, this study showed that the activity of catalase enzymes decreased significantly in the brains of mice infected with rabies virus compared with the brains of healthy mice. In a study, the effect of oxidative stress on experimental autoimmune encephalomyelitis (EAE) was investigated, which showed that the activity of SOD enzyme decreased and NO had a significant increase, which is consistent with the results obtained in this study (31).

Consistent with the study of Habjan et al., findings obtained in the present study showed a significant decrease in NF-κB gene expression in the brains of rabies-infected mice compared to healthy mice (32). Kammouni et al. found that NF-κB acts as an important bridge between CVS infection and oxidative stress (33). In this study, rabies infection was shown to cause oxidative stress by inhibiting NF-κB nuclear activation. The phosphoprotein gene of rabies virus can directly inhibit NF-κB activity. In a study by Luco et al., RelAp43 was identified as a member of the NF-κB family responsible for the natural immune response against rabies (34).

The results showed that the activities of catalase and SOD enzymes and expression of NF-κB gene, as biomarkers of oxidative stress and inflammation, decreased significantly in the brain tissue of mice infected with street rabies virus compared to the brain of healthy mice. In this study, NO showed a significant increase in the brains of infected mice. Therefore, to design and produce therapeutic vaccines against rabies infection, we can use compounds that stimulate the expression of oxidative stress response pathways. Furthermore, for more detailed studies, the virus can be injected into a specific area of the brain of the studied mice by stereotactic surgery and electrode implantation.

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