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Evaluation of the efficacy of vitamin C on the immune response after rabies virus vaccine in BALB/c mice

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Abstract. – OBJECTIVE: Rabies is a lethal zoonotic infection caused by the rabies virus. Interferon- (INF) and interleukins (ILs) are a cytokine that is primarily produced by cells of the immune system. Vitamin C is an essential micronutrient in various biological processes, especially immune responses, and plays an essential part. Vaccination can successfully activate immune responses to virus infection protection. This study aimed to investigate the effect of vitamin C administration on immune responses to an inactivated rabies vaccine.

MATERIALS AND METHODS: Thirty male Balb/c mice weighing between 25-30 gm (8 weeks old) were used in the current experimental study and randomly equally divided into three groups. Group I: untreated healthy control group was inoculated with PBS as a negative control. Group II: vaccinated intradermally with rabies vaccine alone using a dose of 4 ml/animal at 0, 7, 21 days. Group III: In addition to the dose of vaccine, mice were injected single intraperitoneally with 10 mg of vitamin C with each dose of vaccine on days 0, 7, 21. At experimental end, serum levels of IFN-γ, IL-4, and IL-5 were measured.

RESULTS: The results revealed that vitamin C supplementation significantly elevated IFN-γ, IL-4, and IL-5 levels in vaccinated mice and treated with vitamin C (group III) compared to vaccinated group II and healthy control group I. Similarly, vitamin C supplementation exhibited strong positive correlations between IFN-γ and both IL-4 and IL-5 level in all experimental group. Taken together, these results showed that vitamin C is an important stimulator of interferon, interleukin-4 and -5 during inactivated rabies vaccine vaccination in mice.

CONCLUSIONS: Our results supported the hypothesis that indicated the immunological improvement of vitamin C to the effectiveness of the inactivated rabies virus vaccination. High dose of vitamin C increases the levels of interferon and interleukin-4 and interleukin-5.

Key Words:
Eabies virus, Vaccine, Vitamin C, IFN-γ, IL-4, IL-5.

Introduction

Rabies virus is a single-stranded RNA virus belonging to the Lyssavirus class of *Rhabdoviridae*[1]. It is acute, progressive encephalitis, which causes more than 70,000 deaths each year and has a global public health issue[2]. The virus causes lethal encephalitis in both humans and animals, which is also a significant public health issue in developed countries in Asia, Africa, and Latin America[3]. Rabies can be avoided by vaccination and treated early after the infection. Pasteur's production of the first rabies vaccine effectively decreased rabies' occurrence, but the disease was not abolished because it is stored in certain repositories of animals[4]. *Via* the combination application of the rabies vaccine and a hyperimmune serum comprising virus-neutralizing antibodies or immunoglobulin, rabies is avoided by immediate post-exposure prophylaxis. Nevertheless, after patients have acquired clinical rabies, that is expressed by central nervous system infection, the virus cannot be cleared by either innate immunity or antibody administration[5].

It is considered that interferon (IFN) plays a critical role in the prevention of viral diseases as it rises within 1 to 2 days following infection[6,7]. IFN-stimulated genes exerted diverse IFN response as antiviral[8,9]. The potential of the Rabies virus to successfully subvert the host immune system by Toll-like receptor signaling evasion, IFN signaling down-regulation, and adaptive response avoidance by retaining decreased T-cell apoptosis induction of blood-brain barrier permeability exemplifies why early intervention is crucial[10-12]. For defense against rabies virus infection, interferon-mediated immune response is important[13]. Previous studies[14] have demonstrated that IFN production deficiency increased the mouse model's susceptibility to rabies virus.

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Vitamin C (L-ascorbic acid) is an essential nutrient necessary in the body for proper physiological processes. It is important for the health of cells in humans and animals. Vitamin C protects the body as an antioxidant from the harmful effects of oxidative stress, pathogens, and poisons[15]. Numerous research[15-17] have shown that vitamin C has anti-infective and immunomodulatory properties, this reduces the risk of infection and has immunomodulatory functions, especially at high levels. In addition, one explanation for the decrease of the vaccine's effectiveness could be a lack of vitamin C supplementation[18]. Many studies[19] have indicated that vitamin C could be used by catheterized patients with urinary tract infections or during bladder instrumentation in topical antibacterial applications or urinary bladder irrigation fluid. A recent study[20] demonstrated the efficacy of a high dose of vitamin C to decrease the Corona virus-2 (COVID-19) infectivity and influenza virus[21]. Many studies[12] have demonstrated that vitamin C, such as phagocytosis, neutrophil chemotaxis, and lymphocyte proliferation, can improve immune functions. However, the literature on vitamin C and rabies is scarce. Previous experiments[22,23] have shown that vitamin C improves the INF reaction to both the chemical inducers of interferon and certain viruses.

Therefore, the researchers need to identify inexpensive agents such as vitamin C to explore its impact on the immune responses against an inactivated rabies vaccine. This experimental study aimed to investigate vitamin C administration effects on immune responses to an inactivated rabies vaccine.

Materials and Methods

Materials

Inactivated rabies vaccine was supplied by Veterinary Serum and Vaccine Research Institute (VSVRI), Abasia, Cairo. This vaccine was used for the vaccination of experimental animals. Sodium L-ascorbate (Vitamin C) (Cat. No. 134-03-2) and Pentobarbital sodium (Cat. No. P3761) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Interferon-gamma (IFN-γ), Interleukin-4 (IL-4), and IL-5 were determined by ELISA commercial kits (Gen-Probe Diaclone, France).

Animals

In the current research, thirty male BALB/c mice (8 weeks old, 25-30 g) were used. The animals were divided into groups and allowed to stay in the animal house at the Medical Research Center for one week prior to the experiment conducted. The mice were given a regular pellet diet, and water *ad libitum* was given to them. Throughout the experimental time, the animals maintained a temperature of 22+3°C and a light/dark cycle of 12 h and steady relative humidity. The Animal Experimentation Ethics Committee accepted the research at the Faculty of Medicine, University of Ain Shams, Egypt (as this project is part of a big project held under the same ethical approval).

Experimental Protocol

These mice were randomly divided into three groups, each of which contained ten mice. Group I: untreated healthy control group was inoculated with PBS as a negative control. Group II: vaccinated intradermal with rabies vaccine alone using a dose of 4 ml/animal at 0, 7, 21 days. Group III: The mice were injected single intraperitoneally with 10 mg vitamin C in addition to the dose of vaccine with each dose of vaccine at day 0, 7, 21. Mice were shown to withstand a single intraperitoneal vitamin C dose equivalent to that used in a previous research article[15]. No apparent toxicity was detected, but animal skin cooling and decreased motor activity were detected for several hours after administration of the drug.

Blood Samples

The mice were anesthetized with pentobarbital sodium at a dosage of 40-45 mg/kg body weight on day 28, after the last vaccine dose on day 21. Blood samples were collected from the retro-orbital vein for biochemical study. Sera were separated by incubating the clotted blood samples at 4°C for 6 h, then centrifugation at 3000 rpm for 10 min. Serum samples were then inactivated at 56°C for 30 min and stored at -20°C until used. ELISA commercial kits determined serum concentrations of IFN-γ, IL-4, and IL-5 according to the manufacturer's instructions. In triplicate, each test was carried out. The ELISA kits' standard sensitivity curve ranges from 0.78-25 pg/mL, 0.31-10 pg/mL, and 4.7-300 pg/mL for IFN-γ, IL-4, and IL-5, respectively.

Statistical Analysis

Statistical analysis was conducted using the Statistical Package for Social Science package (Version 26, IMB Corp., Armonk, NY, USA). The results were presented as mean± standard error (SE). Using one-way analysis of variance (ANOVA) accompanied by the least significant difference (LSD) as a post hoc test to compare sample groups was made

between different groups. Correlations between parameters in each group were made using Pearson's correlation. $p < 0.05$ was considered significant. GraphPad Prism 9.00 for Mac (La Jolla, CA, USA) was used to create the graphs.

Results

IFN-γ Level

Inactivated vaccines against rabies showed a measurable amount of IFN-γ 28 days after vaccination. The current study showed that inactivated rabies vaccine significantly (p<0.0001) induced an increase in the IFN-γ level compared with the healthy control group. Also, it showed significantly (p<0.0001) decrease level com-pared with inactivated rabies vaccine treated animals with vitamin C. IFN-γ on the 28th day after vaccination with inactivated rabies vaccines (group II) was 40.30±4.97, in group III (inactivated vaccines treated with vitamin C) 139.14±2.38, and 12.33±1.35 for control healthy group I (Figure 1A).

Interleukin -4 (IL-4) Level

In the present study, IL-4 on the 28th day after vaccination with inactivated rabies vaccines was 13.21±.96, in group III (inactivated vaccines treated with vitamin C) 19.43±.34 3.62±.40 for control healthy group I. Inacti-vated rabies vaccines exhibited an increase in IL-4 level 28 days after vaccination. The results showed that in-activated rabies significantly (p<0.0001) increased IL-4 level in contrast to control healthy group I and significantly (p <0.0001) decreased level compared with inactivated rabies vaccine treated animals with vitamin C group III (Figure 1B).

Interleukin -5 (IL-5) Level

In the present study, IL-5 at the 28th day after vaccination with inactivated rabies vaccines (group II) was 711.40±42.05, in case of mice treated with inactivated rabies vaccines with vitamin C (group III) the IL-5 value was 1704.00±.58.55, and 16.83±1.21 for control healthy group I. Inactivated rabies vaccines showed increased IL-5 level 28 days post vaccination. In the present study, results demonstrated clearly that inactivated rabies vaccine (group II), relative to the control healthy group I, could significantly (p<0.0001) cause elevated IL-5 levels and significantly (p<0.0001) reduced levels as compared to inactivated rabies vaccine treated ani-mals with vitamin C (group III) (Figure 1C).

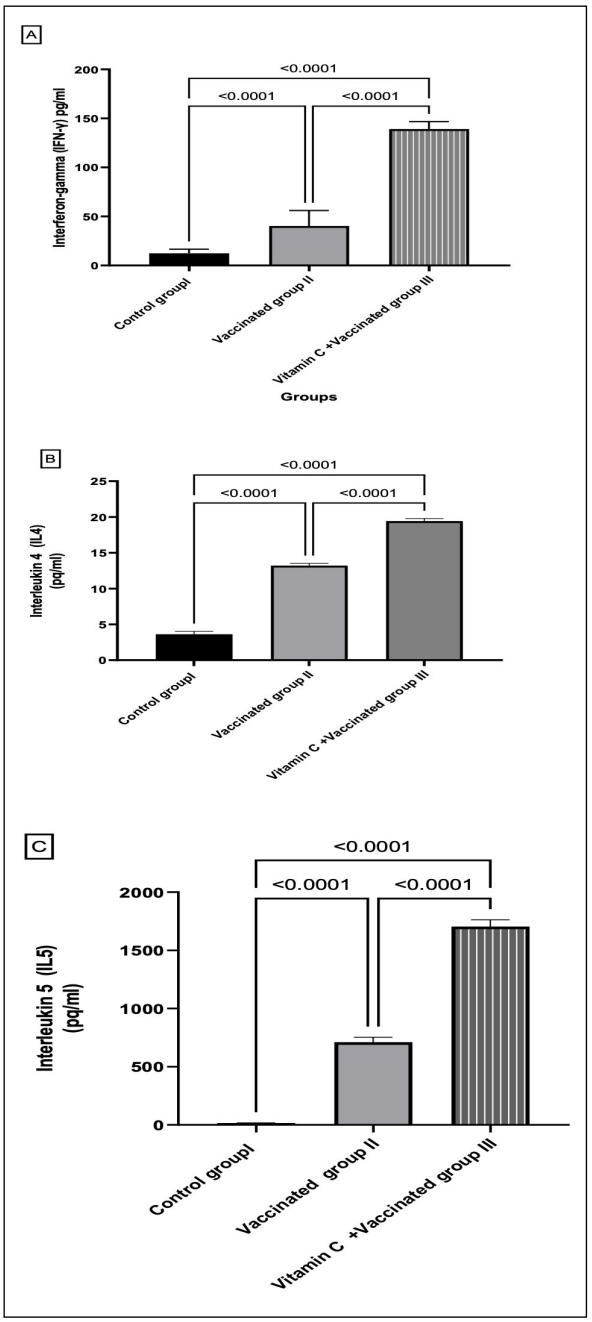

Figure 1. **A**, The level of Interferon-gamma (IFN-γ) in all experimental groups. **B**, The level of Interleukin-4 (IL-4) in all experimental groups. **C**, The level of Interleukin-5 (IL-5) in all experimental groups.

Pearson Correlations

In Figure 2, the current study showed a correlation parameters analysis in each group to investigate the correlations between IFN-γ levels and with the parameters of IL-4 and IL-5. The Pearson correlations between the IFN-γ and IL-4 and IL-5 in each group were demonstrated in Figure 3.

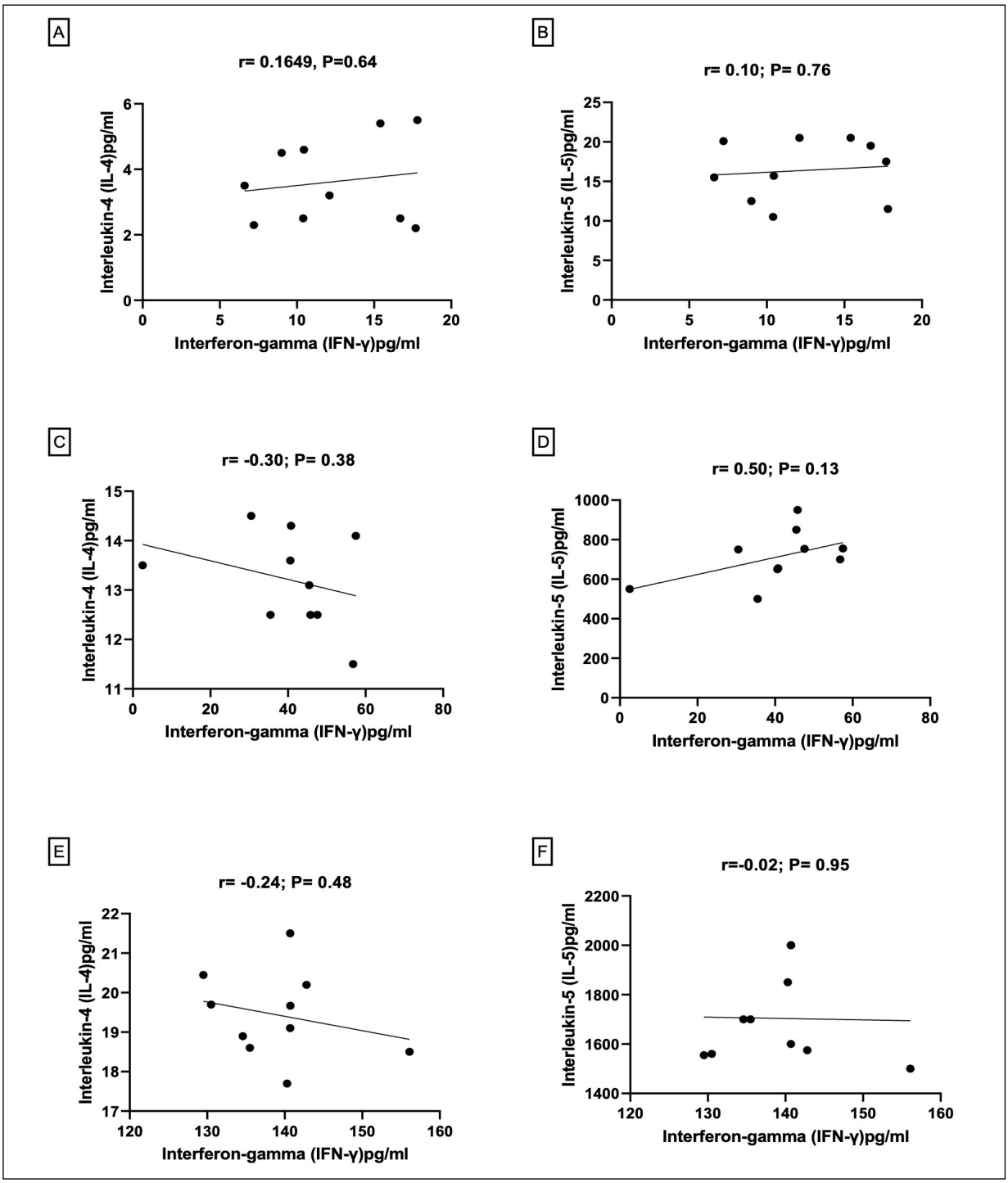

Figure 2. Pearson correlation between the level of IFN-γ in different groups with both IL-4 and IL-5. **A**-**B**, in group I, (**C**-**D**) in group II, and (**E**-**F**) in group III.

In the current study, the IFN-γ exhibited positive correlations with IL-4 (r=0.1649, *p*=0.64), and IL-5 (r=0.10, *p*=0.76) in control group I (Figure 2A-B). Also, the IFN-γ exhibited negative correlations with IL-4 (r=-0.30, *p*=0.38) but positive correlations with IL-5 (r= 0.50, *p*=0.13) in vaccinated group II (Figure 2C-D). Moreover, Figures 2E and 2F showed that the IFN-γ exhibited negative correlation with IL-4 (r=-0.24, *p*=0.48) and with IL-5 (r=0.02, *p*=0.95). Interestingly, the

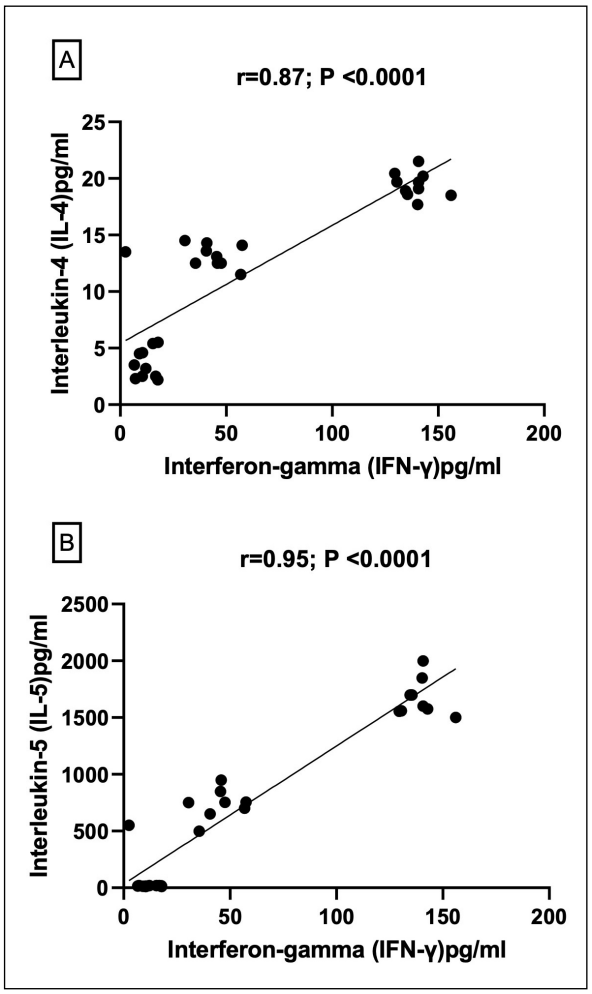

Figure 3. Pearson correlation between the level of Interferon-gamma (IFN-γ) in all different groups with both IL-4 (**A**) and IL-5 (**B**).

IFN-γ exhibited a significant positive correlation with IL-4 (r=0.87, p<0.0001). Moreover, IFN-γ showed significant positive correlation (r=0.95, p<0.0001) with IL-5 (Figure 3).

In general, our results highlighted that vitamin C is an effective stimulator of IFN-γ and IL-4, and IL-5 in mice vaccinated against rabies. There was also a strong positive association between IFN-γ, IL-4 and IL-5 in all study groups (Table I).

Discussion

Rabies is a fatal neurological disease and remains a global public health threat. Rabies virus causes human and animal fatal encephalitis[24-26]. Currently, while rabies is avoided by immediately giving post-exposure prophylaxis, curable care is lacking. However, there is substantial variation between individuals in the immune response to vaccination[27]. Moreover, vaccine after infection cannot offer enough immunity on its own. The development of antibodies is slow, the resultant antibody titer is weak, and the maintenance duration is minimal[28].

A basic concentration of vitamin C is needed for a natural and well-functional host defense system, and the pharmacological use of vitamin C is thought to enhance immune function[29,30]. The range of statistical relationship between the dosage of vitamin C and the concentration of immune cells emphasizes the unique role of vitamin C in the cellular immune response[31]. Even so, vitamin C deficiency that has been experimentally induced impairs cellular[31, 32] and humoral immune responses[29, 33]. Also, both research for both *in vivo* and humans' models have demonstrated the influence of vitamin C on diverse populations of immune cells[34-36]. In addition, high doses of vitamin C induced more distinct interleukin secretion not only by murine immune cells, mostly dendritic cells[37], but also activated functions of the T and B cells[38].

Table I. Evaluation of the level of cytokines in mice sera post-vaccination with rabies vaccines and vitamin C.

Parameters	Control healthy group I (n=10)	Vaccine only group II (n=10)	Vitamin C and Vaccine group III (n=10)
Interferon-gamma (IFN-γ) (pg/ml)	12.33±1.35	40.30±4.97	139.14±2.38
Significance	-	^1p<0.0001	^2p<0.0001
Interleukin-4 (IL-4) (pg/ml)	3.62±0.40	13.21±0.30	19.43±0.34
Significance	-	^1p<0.0001	^2p<0.0001
Interleukin-5 (IL-5) (pg/ml)	16.38±1.21	711.40±42.05	1704.00±58.55
Significance	-	^1p<0.0001	^2p<0.0001

Data were expressed as mean± standard error (SE), n= number of the mice in each group, One-way ANOVA followed by Least significant difference (LSD) comparison tests. ^1p: significance compared to control healthy group I. ^2p < significance compared to Vaccine treated only group II.

The Mice model is used to assess vaccine-induced immune responses and virus challenge pathogenesis before being tested in a human host[24]. There has been various pathogenesis of virus infections of different animal strains and vaccines. The mice model must continue to be used to prove and improve assessment methods[39].

In the present study, mice were injected with a single intraperitoneal dose of 10 mg vitamin C, at each dose of vaccination on day 0, 7, and 21. In 1975, Banic[40] reported that vitamin C prevented rabies in the guinea pig. Guinea pigs were inoculated intramuscularly with an emulsion of rabbit brain containing rabies virus. Starting at 6 h after inoculation, 100 mg/kg of vitamin C was injected twice a day intramuscularly for seven days. The efficacy of immunization with human diploid cell culture rabies vaccination in a group of guinea pigs vaccinated with the vaccine plus 10 mg/kg vitamin C was found to be greater than in a group treated with the vaccine alone[40]. Stantic-Pavlinic et al[23] reported that the rabies vaccine led to a substantially greater increase in INF-α level in humans who had been administered 2 g of vitamin C at the time of first vaccination, compared with the control group. Vitamin C is an efficient stimulator of human interferon formation and may thus be used to induce an increased interferon response to the rabies vaccine. They believed that a high dose of interferon could have a preventive function at the start of therapy where antibody levels against the rabies virus are not present or are not protective[23].

In order to facilitate proliferation and differentiation into cells containing immunoglobulins, IL-5 is produced mainly by activated T helper-2 (Th-2) and mast cells and acts on B cells. IL-5 is a significant cytokine[41]. IFN-τ has an antiviral effect by inducing lysis and destruction of cells infected with the viruses and by sup-pressing the activity and replication of the virus genes. The Th2 response, on the other hand, is characterized by the development of IL-4 and a high degree of rabies-specific antibodies after vaccination, which is commonly referred to as the hallmark of protective immunity to rabies infection[42,43].

A respected approach for researching cell-mediated immune responses following vaccination is to evaluate IFN-γ and IL-5 levels from antigen-stimulated cells of vaccinated individuals. This was based on the fact that cytokine recognition, like IFN-γ, is evidence of a type 1 cytokine reaction that promotes Th1 cells. An example of the type 2 cytokine reaction that is important for the creation of Th2 cells is IL-5. IL-5 demonstrates its ability to activate B cells for humoral immune response and its role in the promotion of immunopathology in viral infections[42].

In the present study, inactivated rabies vaccines exhibited increased INF-γ, IL-4, and IL-5 levels at the 28-day post vaccination. The results revealed that the inactivated rabies vaccine may significantly induce an increase in the levels of INF-γ, IL-4, and IL-5 in contrast with the healthy control group I and a significant decrease compared to the vitamin C treated group. These results agreed with those obtained by Olayan et al[44] who reported that ascorbic acid might produce complete inactivation of the rabies virus without affection antigenicity. In Norwegian rats, IFN-γ and IL-4 concentrations were elevated and reached the peak after 10 to 20 days from virus loaded, indicating that Th1 and Th2 reactions were promoted post infection[41,45]. Ze et al[28] recently observed that in conjunction with a quarter dose of vaccine, Golden03 adjuvant caused a four-fold higher number of IFN-γ developing cells relative to the initial vaccine population. They concluded that the adjuvant Golden03 retained the vaccine's stability against rabies, which helped the mice develop interferon rapidly. The resulting IFN benefited significantly from the synthesis of neutralizing antibodies against the rabies virus[28]. There was a strong positive association between IFN-γ and IL-4 and IL-5 in all experimental groups in this study. Similarly, Venkataswamy et al[42] reported a significant positive correlation between IFN-γ and IL-4. They explained that when viral antigen activates CD4 T cells, it will be induced stimulation of both T helper 1 and 2 cells. They considered the main cytokines IFN-γ, IL-4, and IL-5. In addition, in the pre-sent study, we used the intradermal injection of the vaccine. The World Health Organization has proposed intradermal route for vaccination[46].

Limitations

One of the limitations of the study is lack of determination of cytokines and interferon levels before administration of the vaccine. Another limitation is lack of determination of the genes that affected cytokines and interleukin levels.

Conclusions

Our data supported the hypothesis that indicated the immunological improvement of vitamin C to the effectiveness of the inactivated rabies virus vaccination. We demonstrated that the high dose of vitamin C increases the interferon and interleukin 4 and 5 levels.

Authors' Contributions

Conceptualization, N.S. and Y.Y.; methodology, N.S.; software, N.S.; validation, N.S., Y.Y. and Z.Z.; formal analysis, N.S.; investigation, N.S.; resources, N.S.; data curation, N.S.; writing—original draft preparation, N.S.; writing—review and editing, N.S.; visualization, N.S.; supervision, N.S.; project administration, N.S.; funding acquisition, N.S.

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Ethics Statement

The Animal Experimentation Ethics Committee accepted the research at the Faculty of Medicine, University of Ain Shams, Egypt (No. FMASU 1560/2018).

Data Availability Statement

All data are included within the article.

Acknowledgments

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Conflicts of Interest

The author declares no conflict of interest.

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