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Vitamin E and Influenza Virus Infection

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Abstract

Influenza is an infectious disease causing huge medical and economic losses. Influenza pathogenesis is associated with two processes in the human body: (i) lung damage due to viral replication in the columnar ciliary epithelium of bronchi and bronchioles and (ii) inflammatory burst inducing an increase in reactive oxygen species generation that causes extensive damage in cellular membranes of the small vessels. The oxidative stress in influenza virus-infected organism provokes free-radical oxidation of unsaturated lipid chains in the cell membranes. As vitamin E is a lipid-soluble substance and possesses a hydrophobic tail, it tends to accumulate within lipid membranes. There, it acts as the most important chain breaker, reacting with lipid peroxyl radicals much faster than they can react with adjacent fatty acid side chains. Among the antioxidants tested in influenza virus infections in mice, vitamin E occupies the leading position because of its efficacy in preventing oxidative damage through its free-radical scavenging activity. Although vitamin E is not possessing specific antiviral action, its antioxidant effect probably plays important role in lung and liver protection. Attention should be paid to the synergistic character of antiviral effect of the combination vitamin E and oseltamivir. Vitamin E could be recommended as a component in multitarget influenza therapy.

Keywords: flu pathogenesis, oxidative stress, vitamin E, combination with antivirals, influenza therapy

1. Influenza

Influenza is an acute infectious disease that exerts a very great effect on human society, causing huge medical and economic losses. Influenza usually occurs in annual seasonal (winter) outbreaks or epidemics (in moderate temperature climates). Moreover, influenza pandemics periodically attack the populations of all continents. People of all ages are affected, but the prevalence is greatest in school-age children. The disease's severe course, complications, and

mortality are greatest in infants, the elderly, and those with underlying illnesses—chronic pulmonary or cardiovascular diseases, and diabetes mellitus. The severe complications include hemorrhagic bronchitis or pneumonia (primary viral or secondary bacterial). In addition, fulminant fatal influenza viral pneumonia can occur, with death proceeding in as little as 48 hours after the initial flu symptoms [1]. The World Health Organization recommends influenza vaccines as a main tool for preventing infection and anti-influenza chemotherapeutics with antiviral drugs for treatment and/or prophylactically [1]. The antivirals effective against influenza are divided into two types based on their modes of action: (i) inhibitors of the neuraminidase—oseltamivir, zanamivir, peramivir, and related compounds, efficacious against influenza A and B virus infections, and (ii) blockers of the protein M2—rimantadine-HCl and amantadine-HCl, active against influenza virus A infections. Although both types of agents have proven antiviral effectivity, the rate of drug resistance is constantly increasing, especially for M2 blockers [2].

Two principal problems are related with vaccine prevention: (i) anti-influenza vaccines commonly demonstrate 70–90% effectivity in young persons, with rates markedly decreasing in the elderly; (ii) the protection length is limited to a few months or a season because of the continuous viral antigenic drift based on gradually accumulated mutations, requiring annual revaccination [3].

2. Influenza pathogenesis

The pathogenesis of influenza virus infection is associated with two general processes in the human body: (i) local lung damage due to viral replication in the columnar ciliary epithelium of bronchi and bronchioles, which leads to progressive damage of the alveolar cells, bronchopneumonia (viral or combined viral-bacterial), massive bronchitis (including bronchiolitis), and the like, as the major causes of death [4]; (ii) a dramatic inflammatory burst that induces among other processes an increase in reactive oxygen species generation, causing extensive damage in cellular membranes, predominantly in the small vessels, arterioles, and capillaries [5–8]. In addition, extrapulmonary complications affect many organs and tissues, such as heart, brain, middle ear, liver, and endocrines, and even stomach and kidneys, though that is rare [9–14].

2.1. Respiratory tract damages

Influenza virus replicates in the respiratory tracts of humans, mainly in the lungs. Extrapulmonary multiplication of this virus has not been proven in people with influenza, nor in experimental conditions in influenza virus-infected laboratory animals. Influenza virus replicates throughout the whole respiratory tree. Tracheobronchitis is the common clinical picture of influenza. In the acute stage, multifocal destruction and desquamation of the columnar epithelium of the trachea and bronchi accompanied with edema and congestion of the submucosa are characteristic. In about 50% of cases, tracheitis and bronchitis have a hemorrhagic character. Cell necrosis is the final stage of desquamation of the affected epithelium

with concomitant attainment of the mucus glands. Small- and medium-sized bronchioles are strongly affected by the processes seen in the larger airways, with an entirely necrotic bronchiolar wall associated with polymorphonuclear cell infiltrate. Influenza virus pneumonia very often proceeds to secondary bacterial pneumonias. Destruction of alveolar epithelium and endothelium can worsen the severity of lung injury [4, 15].

Lung disorders in influenza virus infection may be triggered by: (i) a massive infiltration of leukocytes, mainly polymorphonuclear leukocytes, into the alveolar space; (ii) a decrease in the partial pressure of oxygen, causing the development of hypoxia; (iii) an increase in the partial pressure of CO₂ and development of metabolic acidosis; (iv) a “cytokine storm” – a release of cytokines, eicosanoids and prostaglandin E₂ and an enhanced immune response; and (v) the development of oxidative stress [5, 7, 16, 17].

Our previous data and that of most of the literature showed that experimental influenza virus infection in susceptible laboratory animals (mice and ferrets) imitates the above influenza clinical picture: progressive damage of the alveolar cells, acute inflammatory reaction, and development of massive bronchitis and probably pneumonia, in parallel with a decrease in endogenous lipid- and water-soluble antioxidants levels, as well as the compensatory changes of antioxidant enzyme activities [18–21].

2.2. Oxidative stress in influenza virus infection

Lungs are the target organs of the influenza virus. However, in the course of influenza virus infection, dynamic changes in oxidative metabolism, provoked by the overgeneration of ROS (reactive oxygen species) and the activation of neutrophils, can reach the development of oxidative stress [5, 18, 19, 22–24]. Oxidative stress is defined as a disturbance of the prooxidant-antioxidant balance in favor of prooxidants. Influenza viruses are known to induce ROS-generating enzymes and to disturb antioxidant defenses [5, 18, 19, 25], causing changes in antioxidant enzyme activity [5] and decreases in endogenous low-molecular-weight antioxidants. Overgeneration of ROS may influence signaling pathways by activating “redox switches” [26]. In lungs, redox homeostasis is crucial in the pathology of influenza because it is associated with cytokine production, inflammation, cell death, and other pathological processes that could be triggered by enhanced ROS generation (**Figure 1**).

Since the products of oxidative stress possess high cytotoxic activity, it is very important to study mechanisms of detoxification in the infected body. After the epithelial cells are infected, tissue-resident alveolar macrophages are the first responders to viral infection in the lungs (**Figure 1**). They can promote viral clearance through the phagocytosis of viral particles or infected apoptotic cells (efferocytosis) and the release of a plethora of inflammatory cytokines and chemokines to initiate and drive the immune response [27–30]. Due to the ability of ROS to react with almost any kind of biological molecule, including proteins, lipids, and nucleic acids, their elevation is generally associated with genome instability, dysfunction of organelles, and apoptosis [31].

Antioxidant defense mechanisms, including enzymes like superoxide dismutase, catalase, and small molecules such as vitamins C and E and glutathione, protect tissues against oxidants [32].

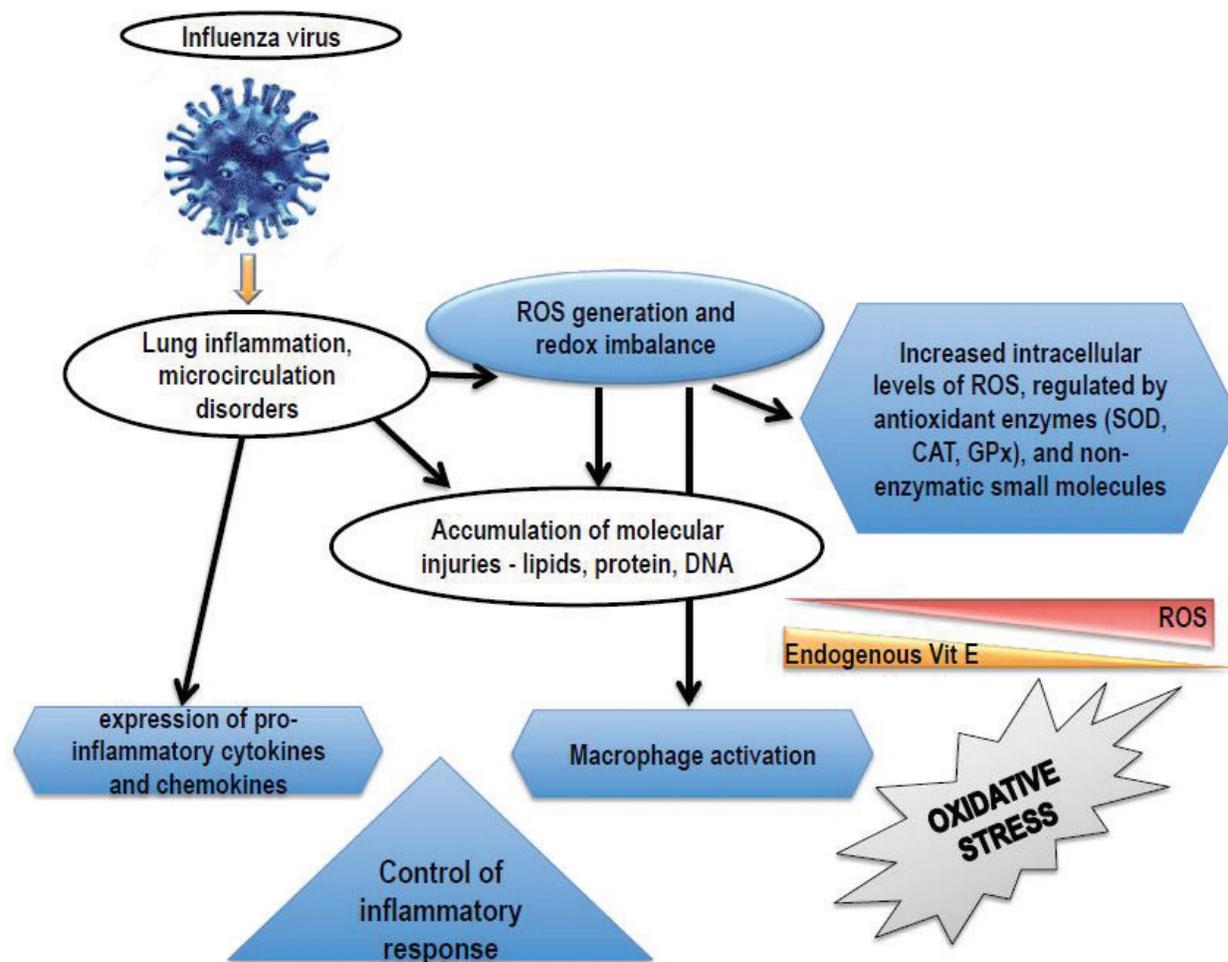


Figure 1. Respiratory tract damages, causes by influenza virus infection.

Vitamin E is the most active natural fat-soluble antioxidant capable of protecting unsaturated fatty acids in cellular membranes from peroxidation, thereby contributing to membrane stability [33]. Both human clinical trials and animal studies have shown a beneficial effect of supplemental vitamin E on the immune system [34].

Studies in the last decade established that the nuclear factor (erythroid-derived 2)-like 2 (NRF2) encoded in humans by the NSF2 gene, is a protein regulating the expression of antioxidant proteins that protect against oxidative damage triggered by injury and inflammation. NRF2 controls the basal and induced expression of antioxidant response element-dependent genes to regulate the physiological and pathophysiological outcomes of oxidant exposure. NRF2 has a substantial impact on oxidative stress and toxicity, regulating the antioxidant defense [35].

At this point of view, the oxidative stress is caused by the imbalance between production of reactive oxygen species (ROS) and the body's ability to detoxify the reactive intermediates.

Recent studies described the role of NRF2 gene coded protein in the development of oxidative stress. The antioxidant pathway controlled by NRF2 is pivotal for protection of lungs against the development of influenza virus infection-induced pulmonary inflammation and injury under oxidative conditions. The NRF2-mediated antioxidant system is essential to protect the lungs from oxidative injury and inflammation induced by influenza virus infection [36, 37].

3. Vitamin E and influenza virus infection

It has been proven that oxidative stress in the influenza virus-infected organism provokes free-radical oxidation of unsaturated lipid chains in the cell membranes (lipid peroxidation), which reduces their permeability as a whole. In the presence of antioxidant deficiency, as described below, when all cell membranes are exposed and/or damaged, influenza infection proceeds with severe pathology and results in serious damage at all levels in the body [38].

It was established that, during influenza infection in mice, the activity of antioxidant enzymes SOD and catalase were changed, along with a decrease of the amounts of endogenous low-molecular-weight antioxidants such as α -tocopherol (**Table 1**), glutathione, and ascorbate [19, 24, 39–41]. Endogenic levels of vitamin E were significantly decreased in lung, liver, and blood plasma [19, 23, 42]. In addition, changes in cytochromes were recorded as well as decreases in the activities of liver cytochrome P-450-dependent monooxygenases [18, 43]. Together, these facts indicate that, in the course of the disease, the buffering capacity of the organism's antioxidant protection diminished [18, 19, 22, 23, 25].

These data demonstrate that, during influenza virus infection, a decrease in natural antioxidant vitamin E was established, accompanied by a significant increase in endogenous lipid peroxidation products.

Oxidative damage in the course of influenza virus infection is quite large, even when registered in experimental animals (mice) at low virus-inoculation doses. In conditions involving non-infected animals with suppressed antioxidant defense systems, the consequent inoculation of influenza virus resulted in an acceleration of oxidative stress and graduated tissue damage.

Different conditions can favor the host's susceptibility to influenza virus infection; among them are cold exposure and stressors of physical, chemical, and psychological origin. For example, immobilization and cold-restraint stress are widely used experimental models that are accompanied by a considerable decrease in the antioxidative capacity of the animal organism; they are also used for the indirect modulation of antioxidant deficiency in experimental animals [19, 44–47].

Because of the significant role of oxidative stress in the pathogenesis of influenza virus infection, a lot of work has been done to test the influence of antioxidants on the course of influenza. Drugs stimulating NRF2 pathway are tested for treatment of diseases causing oxidative

Group	Lung		Liver		Blood plasma	
	5th day	7th day	5th day	7th day	5th day	7th day
I Control	2.2 ± 0.31	2.14 ± 0.26	4.94 ± 0.51	5.4 ± 0.42	1.8 ± 0.065	1.72 ± 0.07
II Flu	1.47 ± 0.14	1.7 ± 0.17	3.35 ± 0.42	3.12 ± 0.37	1.46 ± 0.035	1.2 ± 0.37

Values are expressed as means ± SEM [41].

Table 1. Endogenous content of vitamin E [nmol/mg protein] in lung, liver, and blood plasma of mice experimentally infected with influenza virus A/Aichi/2/68 H3N2 (1.5 MLD₅₀).

stress, influenza virus infection included [48]. Experiments on *in vivo* models, predominantly in mice, hold a significant place in such investigations.

Among the antioxidants tested against influenza virus infections in mice [17, 49–52] α -tocopherol (vitamin E) occupies the leading position. This is because of its efficacy in preventing oxidative damage through its free-radical scavenging activity [16, 18, 24, 42, 53–55].

Protein expression of NRF2 is found to be increased in both the cholesterol-fed and the vitamin E-supplemented rabbits via activation of NRF2 pathway, resulting in induction of several antioxidant genes. Vitamin E appeared to afford the protection effect of NRF2 [48, 55]. Besides, it was found that vitamin E prevents the NRF2 suppression by allergens in alveolar macrophages, proved for asthmatic model *in vivo* [56, 57].

These data clearly show the role of antioxidants, such as vitamin E, which can be manifested in several ways: (i) to capture free radicals in enzymatic or non-enzymatic mechanism(s), (ii) to suppress their generation, and (iii) to affect these processes in an indirect way, for example, by inhibiting viral replication.

As vitamin E is a lipid-soluble substance and possesses a hydrophobic tail, it tends to accumulate within the interior of lipid membranes. There, it acts as the most important chain-breaker, as it reacts with lipid peroxy radicals about four times faster than they can react with adjacent fatty acid side chains. It is well known that vitamin E is able to prevent oxidative damage [58–61], because its lipophilic structure contributes to easy and passive diffusion through the cell membranes, allowing it to reach the mitochondria and the single-plated reticulum. In this way, vitamin E protects them against lipid peroxidation and damage (**Figure 2**). Especially important is its termination of free-radical chain reaction, which protects membrane polyunsaturated fatty acids from oxidation involving reactive oxygen species [61].

Vitamin E is known to affect inflammatory responses in different tissues, including the lung, not only via direct quenching of oxidative stress [42, 62], but also through modulation of oxidative eicosanoid pathways and prostaglandin synthesis [58, 63, 64], inhibition of inflammatory mediators [59], and control of apoptotic lipid signaling [60]. A stabilizing role of Vitamin E has a stabilizing role for membrane phospholipids [61] (**Figure 2**).

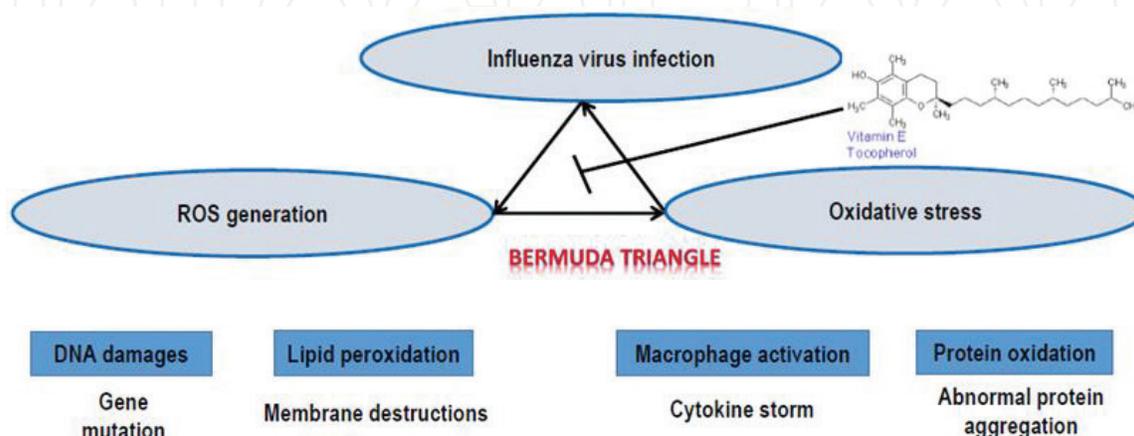


Figure 2. “Bermuda triangle” composed by the pathogenesis of influenza virus infection in the infected body. Vitamin E action is directed to the storm center.

Several different non-antioxidant functions of vitamin E may be essential for the maintenance of cell integrity and functions, such as its role as an anti-phospholipase A2 agent, that is, as a stabilizer of the lipid bilayer of membranes against hydrolyzed and oxidized lipids [65].

The *in vivo* investigations on influenza virus-infected laboratory animals and the clinical data on influenza patients revealed a negative correlation between pulmonary inflammations and endogenous levels of vitamin E in the body. Exogenous vitamin E supplementation has been tied to reducing severe symptoms of lung disease [16, 18, 24, 66].

It is clear that influenza virus infection is a powerful prooxidant that causes a significant increase in lipid peroxidation products in lung, liver, and blood plasma as well as a decrease in natural antioxidants (vitamin E, glutathione) and cytochrome P-450 (CYP). Moreover, in the liver, cytochrome *c* reductase and liver monooxygenases (aniline hydroxylase, ethylmorphin-N-demethylase, analgin-N-demethylase, and amidopyrine-N-demethylase) are inhibited as compared to their activity in control (non-infected) animals.

4. Effects of vitamin E supplementation

As mentioned above, investigations on mice experimentally infected with influenza virus found that endogenous vitamin E content was significantly decreased after influenza virus inoculation. In addition, the amount of cytochrome P-450 in the liver and the activity of cytochrome *c* reductase decreased by about two times on the 5th–7th days post virus inoculation. The decrease in cytochrome P-450 was found to correlate with increases in the concentration of lipid peroxidation products in liver, lung, and blood [18]. Influenza virus infection significantly inhibits liver monooxygenase activity. As a consequence, products from the decreased enzymatic function accumulate in the liver, resulting in the destruction of cytochrome P-450 and its transformation to the catalytically inactive P-420 form.

The effects of influenza virus infection on liver monooxygenases and lipid peroxidation are different from the effects of the hydrophobic xenobiotic substrates of cytochrome P-450. Evidently, the oxidative stress induced in the liver by hydrophobic xenobiotics is a consequence of enhanced oxidation by cytochrome P-450-dependent monooxygenases. The decrease in liver monooxygenase activity resulting from influenza virus infection is accompanied by increases in lipid peroxidation products in the liver, which is not a result of activation of cytochrome P-450-dependent monooxygenases. It may be presumed that influenza virus induces free-radical processes outside the liver, thus producing free radicals and/or activated oxygen species. These reactive compounds must diffuse or be transported over the hepatocyte barrier to initiate lipid peroxidation in the liver.

The protective effect of vitamin E against lipid peroxidation was dose-dependent and was more pronounced on the 5th day as compared to the 7th day after virus inoculation [18, 22]. This agrees with data from Peterhans [5] and Jacoby and Choi [38]. Vitamin E supplementation led to stabilization of cytochrome P-450. Concentrations of the hepatic cytochrome P-450 in infected mice reached the values found in control (non-infected animals) after vitamin E supplementation (120 or 240 mg/kg b.w.), because the monooxygenase activities were restored.

However, researchers have shown that endogenic levels of vitamin E are significantly decreased in lung, liver, and blood plasma (**Table 1**) during the course of flu infection [21, 23, 24, 42]. Animal and human studies have demonstrated a negative correlation between endogenous levels of vitamin E in the body and pulmonary inflammations, and exogenous vitamin E supplementation has been tied to reducing severe symptoms of lung disease [16, 18, 64].

It is well known that vitamin E is able to prevent oxidative damages [58–60].

5. Vitamin E in the influenza therapy

Currently, treatment of influenza is directed mainly at targeting the first pathogenetic component through administration of specific antivirals. Application of correctors of influenza pathogenesis that are associated with controlling inflammation and oxidative stress remains in the background.

Among the antioxidants tested in influenza virus infections in mice [17, 49–51, 65], α -tocopherol (vitamin E) occupies the leading position because of its efficacy in preventing oxidative damage through its free-radical scavenging activity [16, 18, 24, 42, 53–55, 66, 67].

Although vitamin E is not an agent with specific antiviral action, its antioxidant effect probably plays an important role in liver protection.

The most important question is whether vitamin E, as a natural antioxidant, could be used as anti-influenza agent.

In fact, the ideal protective agent against flu should fulfill several criteria: (a) it must not allow the formation of resistant viral strains; (b) it must have a general protective effect on the majority of organs; (c) it must have an acceptable toxicity profile and protective time-window effect; and especially importantly, (d) it must provide strong protection against the symptoms of emerging influenza, such as the oxidative state of the infected body.

Evidently, a more effective treatment strategy is needed. Immunomodulators have been proven to be highly successful in treating the flu, at least in mouse infection models [68–71]. Using antioxidative agents to act directly on downstream deleterious inflammation events is also of significant importance in flu therapy.

The preventive effects of vitamin E and vitamin C, alone and in combination, was tested on the damage caused by influenza virus infection [72]. Mice, infected with influenza virus A/2/68/(H3N2) (1.5 LD₅₀), were administered once-daily doses of vitamin E (60 mg/kg b.w.) and vitamin C (80 mg/kg b.w.) intraperitoneally (for 3 days before virus inoculation). Vitamin E effectively restored lipid peroxidation levels increased by influenza virus infection. The effect of vitamin C was similar, but slighter. The combination (vitamin E + C) had a greater effect on lipid peroxidation levels than did their separate administration. P-450-dependent monooxygenase activity was significantly restored, and more pronounced cytochrome P-450 content and NADPH-dependent cytochrome *c* reductase activity was noted. The preventive effect of vitamin E was stronger than that of vitamin C, but the combination (vitamin E + C)

had the strongest effect. The superior protective effect of the combination is probably due to the better interaction between hydrophobic and hydrophilic low-molecular-weight antioxidants against a free-radical disease like influenza. The mechanism of this interaction is related to vitamin C's ability (when situated in aqueous phase) to recycle vitamin E (located in membranes), repairing vitamin E's tocopheroxyl radical. Thus, vitamin C promotes the function of vitamin E as a free-radical scavenger [73, 74].

An underappreciated approach in flu therapy continues to be combination administration regimens of specific viral replication inhibitors together with antioxidants. Therefore, investigations on the combination effects of specific anti-influenza chemotherapeutic agents and antioxidants are of special interest. Previously, we established a favorable combination effect of the antioxidant 4-methyl-2,6-ditertbutylphenol (ionol) with M2-blocker rimantadine in mice infected with influenza virus A(H3N2). Ionol was administered intraperitoneally in a 3-day course (45 or 75 mg/kg daily) before virus inoculation, and rimantadine (oral application of 15 mg/kg) was administered for 5 days following the day of infection [75].

Recently, a strong beneficial effect of the combination of α -tocopherol (a component of vitamin E) and oseltamivir was demonstrated in the treatment of experimental infection with influenza virus A/H3N2 in mice [76]. The results showed that this combination of agents simultaneously suppressed the two main processes in the pathogenesis of influenza—the development of pulmonary lesions in the respiratory tract as a result of virus replication and the oxidative stress damage to membranes of small vessels and other tissues in the body—thus characterizing it as a very good prospect for flu therapy.

However, a question arose: Is oseltamivir an antioxidant? We used some model systems to test oseltamivir's ability to scavenge superoxide radicals, to inhibit their generation, and to influence Fe^{2+} or (Fe^{2+} -EDTA)-induced lipid peroxidation in liposomal egg suspension and in lung and liver microsomes [77]. We concluded that the reduction of oxidative stress in vivo is not connected with oseltamivir's effect on the development of free-radical processes in the organism. Oseltamivir's effect on oxidative stress in the course of viral infection could be explained by its specific therapeutic effect, which is connected with suppression of viral replication in the target organ.

The in vivo antiviral activity of the combination vitamin E + oseltamivir, expressed by a marked protective effect on the survival of influenza A virus-infected animals, was recorded when vitamin E was administered simultaneously with oseltamivir phosphate via a 5-day course post virus inoculation [76]. This effect was not observed when the vitamin E course started 120 or 48 hours before viral inoculation. According to this study, vitamin E applied individually had no effect on the course of influenza A virus infection caused by 10 MLD_{50} . Only a lower value of the lung index was registered. In our previous study, we established a protective effect of vitamin E at virus infection with 2 MLD_{50} [18, 24].

Special attention should be paid to the sharp synergistic character of the antiviral effect of the combination vitamin E and oseltamivir at a dose of 0.625 mg/kg administered simultaneously, which resulted in the following: (i) a pronounced increase in the protection index, attaining 76%, and a lengthening of the MSD by 3.2 and 4 days; (ii) a pronounced decrease in

lung infectious virus titer; and (iii) a strong reduction in lung lesions. Oseltamivir at the same dose applied separately did not manifest antiviral activity.

The observed phenomenon of a strong oseltamivir dose-dependence of the combined anti-flu effect attaining a pronounced synergism at the lowest tested dose of 0.625 mg/kg merits a special attention. One explanation for this phenomenon could be the interaction of these two agents related to their specific mechanisms of action on the viral target structures in the lung. It is well known that vitamin E is included in the cellular lipid bilayer, thus decreasing cellular membrane permeability [60, 62, 64]. Oseltamivir, for its part, mimics cellular neuraminic acid, thus interfering with the exit process of the new progeny virions [78, 79]. These two processes run in parallel, thus opposing the virus infection course on the cellular level. The two substances most likely compete in the modification of cellular membranes through their specific mechanisms of action. Therefore, the place of emerging synergism for oseltamivir and vitamin E is most likely the cell membrane in the viral target area in the lung.

The favorable (even synergistic) type of interaction between vitamin E and oseltamivir was absent when vitamin E was administered for 5 days before virus inoculation—that is, before the onset of the oseltamivir course.

The described results suggest that vitamin E has an important place as a component of the complex therapy of epidemic flu when administered simultaneously with chemotherapeutic agents, such as neuraminidase inhibitors. Moreover, in addition to its membrane protective effect in the influenza virus target area, vitamin E manifests pronounced activities as an antioxidant agent and as a protein kinase C inhibitor and a protector of lung tissue during inflammatory lung illnesses [59, 80]. The study discussed above [76] convincingly demonstrates a strong beneficial effect of the combination of vitamin E and oseltamivir in the treatment of experimental infection with influenza virus A/H3N2 in mice.

The results show that this combination of agents simultaneously suppresses the two main processes in the pathogenesis of influenza, the development of pulmonary lesions in the respiratory tract resulting from virus replication and the oxidative stress damage to the membranes of small vessels and other tissues in the body, thus characterizing it as a very likely prospect in the therapy of flu.

In summary, vitamin E could be recommended as a reliable agent, a component in multitarget influenza therapy.

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