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Review

Oxidative Stress as a Target for Medication of Influenza Virus Infection

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Abstract

During influenza pandemics in the last few years many people have died from severe complications associated with this pandemic despite receiving intensive care. This suggests that a definitive medical treatment for severe influenza-associated complications has not yet been established. About the pathogenicity of flu, many studies have shown that superoxide anion produced by macrophages infiltrated into the virus-infected organs is implicated in the development of severe influenza-associated complications. Selected antioxidants, such as alpha-tocopherol, N-acetyl-L-cysteine, glutathione, ascorbic acid, 5,7,4-trihydroxy-8-methoxyflavone, catechins, quercetin 3-rhamnoside, iso-quercetin etc. inhibit the proliferation of influenza virus and scavenge superoxide anions. The combination of antioxidants with antiviral drugs synergistically reduces the lethal effects of influenza virus infections. These results suggest that the combination of an agent with antiviral activities and an antioxidant could be a drug of choice for the treatment of patients with severe influenza-associated complications. Hopefully, this update of knowledge of antioxidant therapy of flu could be used as a potential approach to overcome the influenza-associated complications.
Key words: influenza virus infection, oxidative stress, antioxidants, specific inhibitors, combination therapy

Резюме

По време на грипна пандемия в последните години много хора са починали от тежки усложнения, въпреки получаване на интензивни грижи. Това предполага, че сериозна стратегия за медицинско лечение на тежко протичащия грип, все още не е установена. Много изследвания са показали, че супероксид анион произведени от макрофаги проникнали в инфектираните органи е свързан с развитието на тежки грипни усложнения. Някои антиоксиданти като α-токоферол, N-ацетил-L-цистеин, глутатион, аскорбинова киселина, 5,7,4-трихидрокси-8-метоксифлавон, катехини, кверцетин 3-рамнозид, изо- кверцетин др. инхибират пролиферацията на грипния вирус и улавят супероксидните аниони. Комбинацията от антиоксиданти със специфични антивирусни лекарства синергично намалява пагубните ефекти на грипните вирусни инфекции. Тези резултати предполагат, че комбинацията на специфичен антивирусен препарат и антиоксидант може да е успешна стратегия за лечение на пациенти с тежки усложнения, възникнали след грипна инфекция. Актуализирането на знанията за такива комбинации при профилактика и терапия на грип могат да бъдат потенциален подход за преодоляване на грип-свързаните усложнения.

Introduction

Influenza virus infection is a most widely spread illness and a major public health problem usually occurring in the northern hemisphere between the months of December and April. Epidemics of influenza are characterized by an increased morbidity and mortality in the community. Each year, seasonal influenza epidemics are responsible for 3–5 million cases of severe illness and 200 000–500 000 deaths worldwide. For this reason the prevention and treatment of influenza infections is of particular importance (Vlahos, 2012).

Influenza virus A is a member of the *Orthomyxoviridae* family of enveloped, segmented, negative-strand RNA viruses. This virus replicates in the epithelial cells lining the upper respiratory tract of humans, and in both the upper and lower respiratory tract of mice (Ghoshal *et al.,* 2001).

One of life's paradoxes is the fact that the

molecule supporting aerobic life –oxygen - is not just essential for energetic metabolism and for respiration, but almost equally involved in the ethiopathogenesis of numerous diseases and degenerative states due to oxygen-based reactive species called free radicals. Nature has selected and included, in an evolutionary manner, in the composition of living bodies, reactions generating free radicals with multiple roles: functional, intercellular communicational or destructive, cytolytic, etc. Free radicals occur in the body as the result of endogenous metabolic activity or of the local assimilation of some chemical pollutants at cellular level or at the level of several tissues, simultaneously or gradually. Due to their high reactivity, free radicals have been found responsible for many noxious effects on the living body. Oxidative stress is defined as an exaggerated production of oxygenated free radicals, accompanied by a dislocation of antioxidative agents. We cannot live without oxygen, since it is essential in the functioning of energy-producing cells. A body transforms and eliminates oxygen (as CO_2) properly almost entirely (98%). Free radicals produced by one's own body play a role in the cell defense system, destroying bacteria and viruses, decomposing chemical pollutants, and neutralizing toxins (Peterhans, 1997a,b; Ghoshal *et al*., 2001; Vlahos, 2012).

The purpose of this work is to analyze the role of reactive oxygen species and development of oxidative stress in the pathogenesis of influenza viral infection, as an area of target for medication of flu. Attention should be focused on: (i) the effect of the virus on activation of phagocytic cells to release of free radical generation and pro-oxidant cytokines such as tumor necrosis factor; (ii) the effect of the virus on the pro-/antioxidant balance in host cells; (iii) effects of the redox state of the cell on the genetic composition of the virus as well as ROS-mediated release of host cell nuclear transcription factor-kappa-B, resulting in increased viral replication; and (iiii) efficacy of antioxidants as therapeutic agents administered individually and in combination with specific inhibitors of viral replication (Peterhans, 1997a; Peterhans, 1997b; Vlahos, 2012).

Oxidative stress as a key of influenza virus pathogenesis

The influenza infection starts with virus infusion in the lungs (Fig. 1). A cascade of processes follows:

• Massive infiltration of the infected tissue by leukocytes, mainly PMNs, in the alveolar space, after the inoculation of influenza virus;

• Decrease in the partial pressure of oxygen and the development of hypoxia;

• Increase in the partial pressure of CO_2 and development of metabolic acidosis;

• Release of cytokines and prostaglandin E2, and an enhanced immune response;

• Reducing the levels of endogenous lipo- and water-soluble antioxidants, as well as compensatory changes in the activity of antioxidant enzymes, massive bronchitis and pneumonia (Peterhans, 1997a,b; Mileva *et al*., 2000, 2002a,b).

This concept of viral pathogenesis was put to the test using a mouse model of influenza (Hennet *et al.,* 1992; Peterhans, 1997; Lui *et al*., 2003). Animals infected intranasally develop severe systemic symptoms, including a decrease in body temperature and weight, and anorexia at days five and seven post infection. The infection remains restricted to the airways and lungs, but the symptoms involve the whole infected organism. Viruses are parasitic pathogens that replicate in living cells, exploiting multiple intracellular pathways in their hosts for their own advantage and growth. As a rule, viruses cause a wide array of inflammatory and neurological diseases, leading to serious consequences (Fig. 2). Both viral products and host mediators can cause tissue damage, and depending on the cell type and

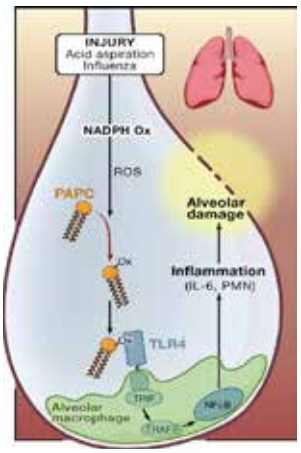

Fig. 1. Influenza A enters the lungs where it infects epithelial cells and then replicates and infects other cells (e.g. alveolar macrophages). Activation of these cells leads to the induction of the master transcription factors NFkB and results in the production of various cytokines (e.g. TNF-a) and chemokines that serve to perpetuate the inflammatory response through the recruitment of peripheral blood monocytes, macrophages, neutrophils and T cells into the airways. Activated macrophages and epithelial cells also release ROS, which cause lung inflammation, edema, tissue injury and lung dysfunction

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site of nervous system involvement, this damage can lead to such varied pathological processes as meningitis, neuritis, myelitis, encephalitis, vasculitis, or demyelinating disease (reviewed by Tyler and Nathanson, 2001; Love and Wiley, 2002).

The acute form of influenza was shown to result in the release of several biogenic amines (Han *et al*., 2000; Jabody, 2002), production of interferon (Samuel, 1991; Cox and Hughes, 1999), activation of nitric oxide synthase and xanthine oxidase (Oda *et al.,* 1989; Akaike *et al.,* 1996; Peterhans, 1997a; Murphy *et al.,* 1998), stimulation of respiratory burst in phagocytic cells (Peterhans, 1997a, b) and accumulation of lipid peroxidation products in blood and target organs (Oda *et al.,* 1989; Schwarz, 1996; Peterhans, 1997a,b; Murphy *et al.,* 1998; Mileva *et al.,* 2000). Influenza infection activates resident phagocytes in the tissues to generate reactive oxygen species as part of the host's defense mechanism against virus replication. In order to protect the cells from virus-induced oxidative processes, host cells must have evolved some antioxidant mechanism that effectively scavanges the reactive oxygen species (Fig. 3).

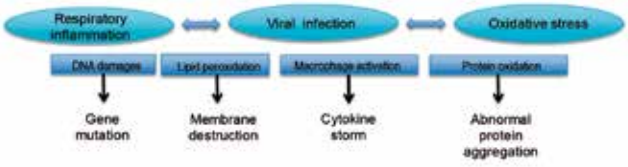

Fig. 2. Consequences caused by viral infections

The lipid-rich lung cell membranes are particularly susceptible to lipid peroxidation, an autocatalytic process that damages lipid-containing structures and yields reactive by-products as malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE), which can covalently modify and damage cellular macromolecules. Their levels in the body are an adequate marker of oxidative damage (Fig. 4).

HNE and MDA are electrophilic species that can covalently modify and damage cellular macromolecules. At physiologic concentrations, HNE and MDA are potent regulators of cell growth and differentiation, affecting both cellular transcription and cell cycle progression (Ji *et al*., 1998; Keller and Mattson, 1998; Poli and Schaur, 2000; Kakashita and Hattori, 2001). Oxidative cellular injury can cause cellular dysfunction and, when severe, this form of injury can cause cell death. Steady-state levels of oxidative tissue damage rep-

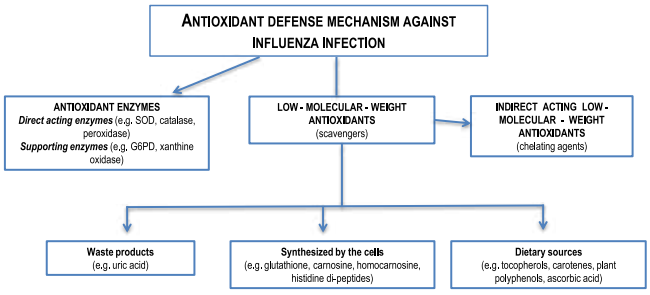

Fig. 3. Antioxidant defence mechanisms against influenza varus infection

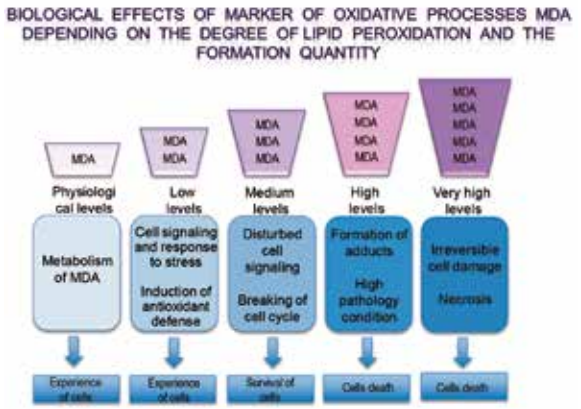

Fig. 4. Biological effects of biochemical marker of oxidative processes malondialdehyde (MDA) depending on the degree of lipid peroxidation and the formation quantity

resent a balance between rates of damage caused by pro-oxidant stimuli and rates of antioxidant and tissue repair mechanisms that decrease ROS/RNS levels and remove oxidatively damaged molecules (reviewed by Halliwell and Gutteridge, 1999; Halliwell and Whiteman, 2004).

The production of reactive oxygen species (ROS), particularly superoxide, is an important host defense mechanism for killing invading pathogens. However, excessive superoxide may be detrimental following influenza A virus infection. Influenza A virus infection causes a rapid influx of inflammatory cells, resulting in increased reactive oxygen species production, cytokine expression, and acute lung injury.

This is one of life's paradoxes: (i) molecular oxygen supports aerobic life: it plays an essential role for energetic metabolism and for respiration; (ii) oxygen radicals produced by one's own body are responsible for the cell defense system, destroying bacteria and viruses, decomposing chemical pollutants, and neutralizing toxins; (iii) almost equally they are involved in the ethiopathogenesis of numerous diseases and degenerative states due to oxygen-based reactive species called free radicals.

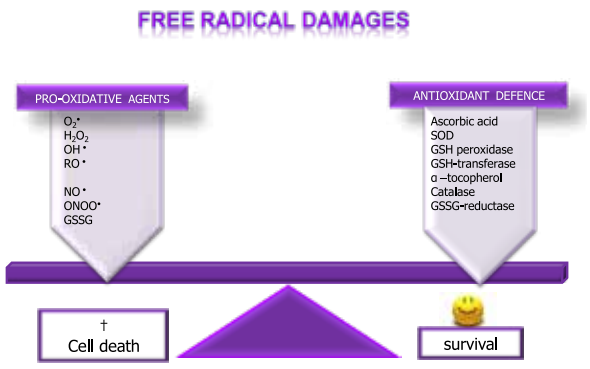

Fig. 5. Oxidative stress is defined as a state of:

- Abnormal generation of free radicals;
- Compensatory changes in the activities of antioxidant enzymes;
- Decreased levels of endogenous low molecular antioxidants (vitamin E, glutathione);
- Disturbed anti-oxidant/pro-oxidant balance;
- Disturbed redox state of organism.

Therefore, ROS are a necessary evil of aerobic life, being generated continuously during the process of respiration, but with the potential to cause oxidative deterioration of protein, lipid and DNA (Fig. 2). A range of different stress conditions elevates ROS generation. ROS damage is linked to serious degenerative conditions in humans. The cumulative production of ROS/RNS through either endogenous or exogenous ways is an important element of the condition called oxidative stress (Fig. 5). Oxidative stress corresponds to an imbalance between the rate of oxidant production and that of their degradation (Fig. 4). It is implicated as a pathogenic factor in a number of viral infections, including influenza viral infection (Oda *et al.,* 1989; Schwarz, 1996; Peterhans, 1997a,b; Murphy *et al*., 1998; Mileva *et al.,* 2000). Oxidative stress caused by influenza virus increased vascular permeability and increased "fragility" of vessels leading to edema of the airway mucosa and lung tissue, multiple hemorrhages in the alveolar area, and interstitial lung as well as in virtually all the internal organs. The extent to which oxidative damage plays a beneficial role for the host by limiting viral replication is largely unknown. An enhanced understanding of the role of oxidative damage in influenza viral infection may lead to therapeutic strategies to reduce tissue damage during viral infection without impeding the host antiviral response (Valyi-Nagy *et al*., 2005).

New therapeutic approaches for treatment of influenza

Early treatment of influenza might fail to prevent complications in some patients, particularly those infected with novel viruses such as the 2009 pandemic influenza A H1N1, avian influenza A H5N1 virus subtype, or the avian influenza A H7N9 virus subtype. Furthermore, treatment with one antiviral drug might promote the development of antiviral resistance, especially in immunocompromised hosts and critically ill patients. An obvious strategy to optimize antiviral therapy is to combine drugs with different modes of action. Because host immune responses to infection might also contribute to illness pathogenesis, improved outcomes might be gained from the combination of antiviral therapy with drugs that modulate the immune response in an infected individual.

Preventing lung injury induced by influenza with CR2 targeting complement inhibitor

The overreaction of the immune system is an important cause of patient mortality. Oda *et al.* pointed out in 1989 that symptoms of influenza are inflammatory injury as a result of immune activation by influenza virus, instead of being directly induced by influenza virus. The immune system is activated in case of invasion by influenza virus.

Complement is an important and conservative system of natural immunity, and provides pathways for rapid and effective elimination of invasive micro-organisms (Walport, 2001; Stoiber *et al.*, 2008). It is a "bridge" between natural immunity and acquired immunity. If the complement system is over activated, many complement components will be consumed, and the anti-infection ability of the body will be reduced; many active substances derived from the activation will induce a severe inflammatory reaction or tissue injury, resulting in a pathological process (Blach-Olszewska *et al.*, 2007). CR2 is the central molecule for the immune response regulation by the complement system. An effective CR2 targeting complement inhibitor can reduce mortality; significantly improve clinical symptoms (decreased weight, lung index and hemagglutination titer) and lung tissue inflammatory injury of a virus infected model group. Therefore in 2010, Zhang *et al.* hypothesized that a CR2 targeting complement inhibitor is expected to be an ideal drug for viral pneumonia.

Combination of neuraminidase inhibitor oseltamivir with immunomodulatory drugs

Sharma *et al*. (2013) evaluated the effect of the neuraminidase inhibitor oseltamivir in combination with immunomodulatory drugs *in vitro* and in a mouse model of influenza A H1N1 infection by determining survival rate, lung inflammation mark-

ers and histopathology. Sertraline and rolipram significantly improved survival in mice infected with a lethal dose of influenza A H1N1 virus. Prophylactic treatment resulted in survival rates of 40% (rolipram), 30% (oseltamivir), 0% (sertraline), 100% (rolipram/oseltamivir) and 70% (sertraline/oseltamivir). Treatment in a therapeutic setting (24 h post-infection) resulted in 80% (rolipram/oseltamivir) and 40% (sertraline/oseltamivir) survival. Sertraline and rolipram had no effect on virus replication *in vitro* and *in vivo*, but significantly reduced lung inflammation. A significant reduction in cellular infiltration (10-fold) along with inflammatory cytokines monocyte chemotactic protein-1 (10-fold), interleukin-6 (5-fold) and regulated on activation normal T cell expressed and secreted (5-fold) was observed in the animals treated with the combination compared to oseltamivir alone. Lung histopathology of mice treated with combinations revealed significantly reduced consolidation, infiltration and alveolitis compared to oseltamivir alone. Rolipram and sertraline reduced H1N1 virus-induced lung inflammation and mortality. These data support further development of immunomodulatory agents for severe influenza.

Combination of neuraminidase inhibitor oseltamivir with low molecular antioxidants

The combined therapy of influenza is a question of particular interest. Garozzo *et al*. (2007) achieved up to 100% survival rate in mice infected with influenza virus and treated with combination of N-acetylcysteine (NAC) as a precursor of glutathione in a dose of 1000 mg/kg and oseltamivir in a dose of 1 mg/kg. The glutathione system is especially important for cellular defense against ROS. GSH reacts directly with radicals in non-enzymatic reactions and is the electron donor in the reduction of peroxides catalyzed by glutathione peroxidase (GPx) (Dringen *et al.,* 2000). Glutathione serves as the major scavenger of reactive oxygen species. Certain lymphocyte functions, such as the DNA synthetic response, are exquisitely sensitive to reactive oxygen species and, therefore, are favored by relatively high levels of glutathione. Even a moderate depletion of the intracellular glutathione pool has dramatic consequences for a variety of lymphocyte functions (Droge and Breitkreutz, 2000).

According to Dimitrova *et al*. (2016), in an experimental mice model of influenza type H3N2, the virus infection with 10 LD50 causes 70% lethality. The monotherapy with oseltamivir in a dose of 2.5 mg/kg increases the survival rate from 30 to 70%. The monotherapy with S-adenosyl-L-methionine **(**SAM) in a dose of 50 mg/kg and 100 mg/kg does not increase the survival rate. It is a matter of interest that the combined therapy - SAM in both doses applied intraperitoneally combined with oseltamivir in a dose of 2.5 mg/kg daily in two intakes applied orally achieve 90% survival rate in mice infected with influenza virus. This work demonstrates the advantage of combining agents acting through different mechanisms – the antiviral drug oseltamivir and SAM as a precursor of the main antioxidant - glutathione.

The present study showed the positive effect of combining oseltamivir as a specific neuraminidase inhibitor of the influenza virus replication with S-adenosyl-L-methionine as a precursor of glutathione - the most abundant antioxidant in the body. The good results of the combination could be explained by the modulation of the host defense mechanisms and by the direct antioxidant effect of increased glutathione against oxidative stress associated with influenza infection.

These findings suggest that therapy with molecules converted into antioxidants in the body increases survival by modulating the host defense mechanisms and by a direct antioxidant effect against oxidative stress associated with viral infections. Those studies demonstrated the effectiveness of combining agents that act through different mechanisms - antiviral drug oseltamivir as specific neuraminidase inhibitor of influenza virus, and SAM and NAC as precursors of the most important antioxidant - glutathione.

To target the main processes involved in influenza pathogenesis, Galabov *et al.* (2016) studied the effects of oseltamivir and α-tocopherol combinations against influenza A/Aichi/2/68(H3N2) virus infection in mice. Oseltamivir was applied orally at three daily doses, 2.5, 1.25, and 0.625 mg/kg, in 5-day course post-infection. α-Tocopherol (120 mg/kg) was administered intraperitoneally. The results demonstrated pronounced dose-dependent synergistic antiviral effect of the combination α-tocopherol/0.625 mg/kg oseltamivir when α-tocopherol was administered simultaneously with oseltamivir: a 78% protection effect and lengthening of mean survival time by 3.2–4 days. Lung parameters showed a substantial decrease in infectious virus content (Δlogs=3.8/4.1) and a marked diminishment of lung index and pathology. The combination with 1.25 mg/kg oseltamivir manifested a marked protection, but less effect on lung parameters. The combination α-tocopherol /2.5 mg/

kg oseltamivir did not surpass the monotherapeutic effect of oseltamivir. When α-tocopherol was applied 5 or 2 days before infection, its combination with oseltamivir was ineffective.

Finally, oxidative stress and stress-mediated complications of influenza viral infection successfully respond to antioxidants or their precursors, including in monotherapy. However, it should be kept in mind that antioxidants are not antivirals, their function is more auxiliary, particularly in combination therapy with specific inhibitors of influenza infection.

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