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Current and Future Anti-Influenza Virus Drugs

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Abstract: In 2009, we have been experiencing a new pandemic of novel influenza virus type A (H1N1) infection. The human beings still face the threat of highly pathogenic avian influenza A (H5N1) virus. Many patients with influenza virus infection have died due to severe complications even though receiving intensive care. This suggests the need for new treatment strategies of severe influenza-associated complications. In cases of severe influenza-associated complications, pathological manifestations are as a result of complex biological consequences, such as apoptosis induction, macrophage activation, oxidative damage and increased production of pro-inflammatory cytokines. Recent studies have revealed that the pathogenesis of severe influenza-associated complications involves not only the virus replication-mediated apoptotic cell death in the infected cells but also non-infected cell injury by toxicity of reactive oxygen species derived from macrophages phagocytosing apoptotic cells, and that pro-inflammatory cytokines produced by the virus-infected host cells play a critical role in the activation of macrophages. These findings provide a possibility that an agent with antiviral and antioxidant activities can be a drug of choice for the treatment of patients with severe influenza-associated complications. Selected antioxidants, such as pyrrolidine dithiocarbamate, *N*-acetyl-L-cysteine, glutathione, nordihydroguaiaretic acid, thujaplicin and certain types of flavonoids, possess both activities. The combination of antioxidants, such as superoxide dismutase and *N*-acetyl-L-cysteine, with antiviral drug ribavirin synergistically reduced the lethal effect of influenza virus infection. Accumulating a number of evidence highlights a potential of selected antioxidants for treatment of severe influenza-associated complications and a possibility that combination of antioxidants with current anti-influenza drugs can improve conventional influenza chemotherapy.

Keywords: Influenza virus, apoptosis, macrophage, reactive oxygen species, anti oxidant.

1. INTRODUCTION

In 2009, we have been experiencing a new pandemic of novel influenza A (H1N1) virus infection [1, 2], containing genes from avian, human and swine influenza viruses [3]. The clinical spectrum of pandemic influenza A (H1N1) 2009 virus infection is broad, from mild upper respiratory tract illness with or without fever and occasional gastrointestinal symptoms such as vomiting or diarrhea and exacerbation of underlying conditions, to severe complications such as pneumonia resulting in respiratory failure, acute respiratory distress syndrome, multi-organ failure, encephalopathy and death [4]. As of 17 October 2009, there have been more than 414,000 laboratory confirmed cases of pandemic A (H1N1) influenza 2009 and nearly 5,000 deaths (1.2%) reported to World Health Organization (WHO) [5]. Every year, the global burden of seasonal influenza epidemics is believed to be 3-5 million cases of severe illness and 300,000-500,000 deaths [6]. Beside seasonal influenza infection, we still face the threat of highly pathogenic avian influenza A (H5N1) virus infection.

In May 2009, WHO had reported that 2-6% of confirmed cases with pandemic A (H1N1) influenza virus infection had been admitted to hospital [4]. The main reason for hospitalization remains lower respiratory illness due to primary viral pneumonia, often described as "viral pneumonitis," reflecting direct viral invasion in lung tissues. The relative portion of hospitalized patients and approximately 80% of fatal cases have had underlying medical conditions considered high risk for seasonal influenza, such as chronic lung disease (including asthma), heart disease, kidney disease, immunosuppression and pregnancy. Severe cases and deaths have been reported in pregnant women from all sites, especially in their third trimesters, resulting in intrauterine fetal demise, spontaneous abortion, premature rupture of membranes and emergency cesarean section. Pediatric cases of pandemic A (H1N1) influenza 2009-associated encephalopathy have been reported in the USA, Chile and other countries. Since seasonal influenza virus infection has also been associated with severe complications, such as encephalopathy, transverse myelitis, myositis, myocarditis, pericarditis, Reye's syndrome, hepatitis and placentitis [6-9], it is reasonable to assume that the rate of such severe complications would be increased by the pandemic A (H1N1) influenza 2009.

Currently, three classes of anti-influenza drugs have been used for chemoprophylaxis and treatment for influenza infection [10]; (1) amantadine and rimantadine inhibit viral membrane protein (M2) of proton channel that is necessary for uncoating; (2) oseltamivir and zanamivir inhibit viral neuraminidase (NA) that is necessary for virion release; and (3) ribavirin inhibits enzyme activity for viral replication.

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Initial testing found that 2009 novel influenza virus was susceptible to NA inhibitors but resistant to M2 inhibitors [11]. Therefore, NA inhibitors have been used widely for treatment and chemoprophylaxis of pandemic A (H1N1) influenza [2]. Sporadic cases of oseltamivir-resistant pandemic A (H1N1) influenza virus have been reported worldwide [12]. In case of development of oseltamivir-resistance, treatment options are limited because zanamivir is not licensed for treatment of children under 7 years old and is contraindicated among persons with underlying airway disease. These results suggest that the need for development of new anti-influenza drugs utilizing alternative antiviral mechanisms and consideration of using anti-influenza drug combinations.

NA inhibitors have demonstrated efficacy in reducing the incidence of influenza-associated complications [13]. However, patients with severe influenza-associated complications have received conventional treatment with anti-influenza virus drugs available now, they have died due to organ failures. The mortality may attribute to the lack of appropriate treatment strategies for severe influenza-associated complications. Therefore, understanding the pathogenesis of severe influenza-associated complications is a serious issue to provide effective treatment strategies. In cases of severe influenza-associated complications, the pathological manifestations are result from complex biological consequences, such as apoptosis induction, macrophage activation, oxidative tissue damage and higher contents of pro-inflammatory cytokines [14, 15]. Many recent studies have clarified that the pathogenesis of severe influenza-associated complications involves not only the virus replication-mediated apoptotic cell death in the infected cells but also the injury of non-infected cells by reactive oxygen species (ROS) derived from activated phagocytes (i.e., macrophages and neutrophils) infiltrated into the virus-infected organs. These findings provide that an agent with antiviral and antioxidant activities can be a drug of choice for the treatment of patients with severe influenza-associated complications [16, 17]. This article reviews recent knowledge regarding: (1) pathogenesis of severe influenza-associated complications: focusing on apoptosis induction and macrophage activation, and (2) current and future anti-influenza drugs.

2. PATHOGENESIS OF SEVERE INFLUENZA-ASSOCIATED COMPLICATIONS: FOCUSING ON APOPTOSIS INDUCTION AND MACROPHAGE ACTIVATION

2.1. Lessons from Postmortem Study of Pandemic A (H1N1) Influenza

In a post mortem study, the pathology of lung and extrapulmonary organs in 21 fatal cases, including 5 pregnant cases, of pandemic A (H1N1) influenza virus infection has been described precisely for the first time [18]. In fatal cases, pathological findings revealed extensive diffused alveolar tissue damage, and variable degrees of pulmonary hemorrhage and necrotizing bronchiolitis. In infected cases, there was a marked Toll-like receptor (TLR)-3 protein expression in macrophages, in alveolar epithelial cells, in vascular endothelial cells and along the alveolar capillaries. A weak immunoreactivity against interferon (IFN)-γ protein was detected in alveolar macrophages and in endothelial cells in control lungs. In infected cases, an intensive immunoreactivity against IFN-γ protein was detected in macrophages, alveolar epithelial cells and vessels, suggesting that macrophages were activated by IFN-γ. $CD8^+$ T cells and granzyme B-positive cells were present surrounding airways and in alveolar walls in control lungs. The density of these cells was elevated in infected cases, and the cells tended to form small groups around small vessels and bronchioles. Tumor necrosis factor (TNF)-α protein was detected in alveolar macrophages and bronchial and vascular smooth muscle in both control and infected lungs. There were no signs of direct virus-induced injury in any organs examined other than the lungs. All patients had mild/moderate kidney acute tubular necrosis. All patients presented atrophic or non-reactive white pulp in the spleen. In the lymph nodes, non-reactive follicles and sinusoidal erythrophagocytosis were found. The liver showed erythrophagocytosis and a few mononuclear inflammatory cells in the sinusoids in all patients, and variable degrees of shock-related centrilobular necrosis. It should be noted that a massive hepatic necrosis was observed in a pregnant patient with hepatic failure. The placenta of the patient presented signs of intrauterine hypoxia without signs of infection. The fetus showed meconial aspiration in the lungs. No patients presented histological signs of encephalitis, myocarditis or myositis. These results suggest that in fatal cases with pandemic A (H1N1) influenza, the pathogenesis involves enhanced innate immune responses with sustained TLR-3 activation in macrophages, alveolar epithelial cells and vascular endothelial cells, and subsequent enhanced inflammation with large numbers of $CD8^+$/granzyme B^+ cytotoxic cells and local production of pro-inflammatory cytokines, such as IFN-γ and TNF-α.

2.2. Influenza Virus Infection During Pregnancy

2.2.1. Influence of Influenza Virus on Pregnant Woman and Fetus

Pregnant women are at increased risk for influenza-associated illness and death [19]. During pandemic A (H1N1) influenza 2009, pregnant women showed an increased rate of hospital admission than that of non-pregnant women, and pregnancy-associated complications, such as maternal death, premature rupture of membranes, vaginal bleeding, spontaneous abortion and emergency cesarean section, have been reported [20]. Similarly, the risk of maternal death, premature delivery, abortion and stillbirth has increased during the past pandemics of A (H1N1) Spanish virus in 1918 [21] and A (H2N2) Asian virus in 1957 [22-25], whereas no increase has been seen in the 1968-1969 pandemic of Hong Kong influenza (H3N2) [26].

During epidemics with seasonal influenza virus type A, pregnancy-associated complications have occurred. A cluster of spontaneous abortions and stillbirths has occurred within 2-4 weeks from the onset of influenza during an epidemic with seasonal influenza A (H3N2) virus in 1985-1986 [27]. In addition, some cases of pregnancy-associated complications were found. A pregnant woman at 29 weeks' gestation was hospitalized for pneumonia caused by influenza virus type A and developed uterine contractions within a week of hospitalization. Her cervix was 3 cm dilated, and an infant was delivered by emergency cesarean section, but mother became severely hypotensive and bradycardic and died [28]. In another case, a pregnant woman at 9 weeks' gestation was hospitalized for encephalopathy caused by influenza virus type A and underwent an abortion at 12 weeks' gestation; A (H3N2) Hong Kong virus was isolated from cerebrospinal fluid sample corrected at hospital admission [29]. In an additional case, a pregnant woman at 32 weeks' gestation was hospitalized for pneumonia caused by influenza virus and subjected to emergency cesarean section at 3 days later of admission; RNA for A (subtype H1) virus was detected in serum sample corrected at hospital admission [30].

Among pregnant women limited to under age 25 who would not have been previously exposed to A (H1N1) Russia virus, the rate of acute respiratory disease was increased after reappearance of the virus in Portland metropolitan area in the 1977-1978 [31]. An epidemiological study in the Tennessee from 1974 to 1993 estimated that 0.25% of pregnant women were hospitalized for the influenza infection [32]. Another epidemiological study in the Tennessee from 1985 to 1993 demonstrated that 6.0% of pregnant women with asthma were hospitalized during influenza season, which was significantly higher than 0.51% of pregnant women without asthma [33].

The above-mentioned evidences provide valuable information that pregnant women are at increased risk for severe disease associated with influenza, potentially resulting in pneumonia, premature delivery, spontaneous abortion and stillbirth, and moreover that influenza can be more severe in pregnant women with underlying medical conditions than those without, resulting in hospitalization.

2.2.2. Gestational Organ and Fetus Infection

Influenza A (H3N2) virus has been isolated from the placenta and amniotic fluid during the third trimester in fatal [34-36] and non-fatal cases [37], and from the lung of stillborn infant [24] and from fetal heart [36]. Placentitis caused by influenza virus type A and B has been observed in 32 of 186 placentas, which was characterized by hyperplasia and subsequent degradation of amnion cells, trophoblast cells, decidua cells and vascular endothelial cells and by the presence of viral proteins and fucsinophilic inclusions in the affected cells, and lymphoid cell infiltrations [35]. Such pathological changes were quite often observed in placentas obtained from patients with influenza virus infection [9]. Immunohistochemical analyses further demonstrated that influenza virus proteins were detected in astrocytes and neurons in the brain of infant who was delivered by emergency cesarean section [38]. Immunoglobulin M type antibody against an epidemic strain of virus has been found in umbilical cord blood sera after the community-wide epidemic of influenza virus type A [39], and lymphocytes isolated from umbilical cord blood were proliferated by the stimulation with epidemic strain of influenza A (H3N2) virus [40]. The occurrence of viremia with influenza virus has been substantiated as described in the next section. These results substantiate that influenza viruses spread from the maternal respiratory tract to the fetus, placenta and amniotic fluid *via* the bloodstream. By tradition, a marked maternal hypoxia due to pneumonia or febrile response has been considered as etiological factors for influenza-associated complications during pregnancy. However, results as described here clearly demonstrate that the direct virulence of influenza virus type A infection on gestational organs and fetus implicates in the etiology of pregnancy-associated complications, such as premature rupture of membranes, premature delivery due to emergency cesarean section, spontaneous abortion and stillbirth.

Both the placenta and the 4-month-old fetus have been studied on a fatal case of 24-year-old woman infected with highly pathogenic avian influenza A (H5N1) virus [41, 42]. The placenta showed scattered foci of syncytiotrophoblast necrosis as well as necrotizing deciduitis, and diffuse villitis. Both negative- and positive-strand viral RNAs for hemagglutinin (HA) and nucleoprotein (NP) were detected in Hofbauer and cytotrophoblast cells but not syncytiotrophoblast cells in the placenta by *in situ* hybridization, and HA and NP proteins were consistently detected by immunohistochemical analysis. Both negative- and positive-strand viral RNAs for HA were amplified from the placental tissue using reverse transcriptase-polymerase chain reaction (RT-PCR) techniques and detected in fetal bronchi, alveolar pneumocytes, Kupffer cells, and circulating mononuclear cells by *in situ* hybridization. The fetal tissues mostly showed no specific histopathological features, except for some edema and a few scattered interstitial neutrophils in the lungs. These results suggest that influenza A (H5N1) virus proliferates in Hofbauer and cytotrophoblast cells but not syncytiotrophoblast cells, and support a view that the infection of gestational organs and fetus with influenza virus type A implicates in the etiology of pregnancy-associated complications.

In swine, some reports suggest that abortion and stillbirth can be associated with epidemics of swine influenza [43, 44]. Transplacental transmission of swine influenza virus has been also observed in pregnant gilts [45]. Influenza virus has been isolated from amnion and chorion tissues in pregnant guinea-pig models [46] and amniotic fluid in pregnant ferret models [47] after intracardial inoculation with the virus. These results demonstrate the occurrence of fetal membrane infection with this virus. In addition, influenza virus infection may preferably spread to chorion tissues adhered to decidua tissue *via* the bloodstream, since human endometrial and decidua tissues provide a preferable environment for influenza virus replication than placental tissues [48].

2.3. Viremia and Systemic Infection

Human influenza A viruses have been isolated directly from blood [49-52] or serum [53] and from various extrapulmonary tissues, fluids and excreta, such as placenta [34, 35], amniotic fluid [34, 36, 37], brain [36], meninges [54], spinal cord [36], cerebrospinal fluid [29, 53, 55-57], heart [34, 36, 58], liver [36, 50, 58], kidney [36, 58], adrenal [54], urine [57], spleen [36, 49, 58], tonsil [58], lymph node [36, 58], muscle [55] and feces [53]. Immunochemical analysis has demonstrated that viral proteins were detected in Purkinje cells [59], neurons [59], ependymocytes of plexus choriodeus [9] in the brain, β cells [59] in the pancreas, $CD8^+$ T lymphocytes [59] in the spleen, hepatocytes [9] and stellate endothelial cells [9] in the liver, epithelial cells of convoluted tubules [9] of the kidney and cerebrospinal fluid [56] obtained from patients with influenza virus infection. Viral RNAs were also detected in various tissues and fluids, such as brain [29, 59], cerebrospinal fluid [59], heart [29], diaphragm [29], liver [29, 59], kidney [59], lymph node [29], peripheral blood mononuclear cells [60] and serum [30] obtained from patients with influenza virus infection. Furthermore, in mouse model virus particles and mRNAs have been detected in blood (both red blood cells and plasma), brain, liver, spleen, pancreas, salivary gland, kidney, heart and skeletal muscle after intranasal inoculation with influenza virus [61, 62]. These results substantiate the occurrence of viremia and systemic infection with human influenza A virus.

The virological consequence of avian influenza A (H5N1) virus in human body is incompletely characterized. In a patient with fatal A (H5N1) influenza infection, viral RNA was detected by RT-PCR in the lung, intestine and spleen tissues, but viral replication was confined to the lung and intestine by the detection of positive-stranded viral RNA [63]. A case report showed that influenza A (H5N1) virus was isolated from the serum, cerebrospinal fluid and fecal samples in addition to the respiratory secretions [53]. Viral antigen was detected in pneumocytes by immunohistochemical tests [63]. These studies suggest that influenza A (H5N1) virus replicates in the respiratory and gastrointestinal tracts.

2.4. Apoptosis Induction and Macrophage Activation

An increased number of activated macrophages was found in the brain containing neurons and glial cells undergoing apoptosis in the patients with influenza-associated encephalopathy [55]. The co-existence of macrophages and degraded cells, containing viral proteins, and pycnotic and fragmented nuclei, was also observed in other extrapulmonary tissues, such as liver, kidney and intestine, obtained from patients with influenza virus infection [9]. The same phenomena were observed in mice infected with influenza virus intranasally [9]. Moreover, intranasal influenza virus infection induced apoptosis in neurons in mouse brain, in which the virus protein-positive apoptotic bodies were phagocytosed by activated macrophages [64]. Thus the co-existence of influenza virus-infected cells undergoing apoptosis and activated macrophages is observed as a common pathological manifestation of various severe complications [8, 9, 65].

Most patients with influenza virus A (H5N1) infection have initial symptoms of high fever and an influenza-like illness with lower respiratory tract symptoms, and diarrhea, vomiting, abdominal pain and pleuritic pain. Bleeding from the nose and gums have also been reported early in the course of illness in some patients [66, 67]. The pathological findings including apoptotic cell death and macrophage activation are observed in the lung and some extrapulmonary tissues obtained from patients infected with influenza A (H5N1) virus [41, 68-73]. Apoptosis was observed in alveolar epithelial cells, and numerous numbers of apoptotic leukocytes were observed in the lung of a patient [71]. Macrophages appeared to be the predominant cells within the alveoli, and T lymphocytes accompanying with or without the existence of neutrophils in the interstitium. Scattered histiocytes with hemophagocytic activity have been observed in the lungs of some cases. Reactive histiocytes with hemophagocytic activity have been noted in the spleen, lymph node, bone marrow, lungs, and liver. In liver tissue specimens, necrosis, activated Kupffer cells, cholestasis, and fatty changes have been observed. In most instances the brain was edematous without any significant histopathological change, whereas reactive histiocytes and foci of necrosis have been observed in demyelinated lesions in two cases. In other organs no remarkable histological changes have been observed. Mice infected with highly pathogenic H1N1 and H5N1 viruses exhibit significantly high numbers of macrophages and neutrophils in the lungs compared to mice infected with low pathogenic viruses [74].

2.5. Phagocytosis of Influenza Virus-Infected Cells by Macrophages

Apoptosis, known as a programmed cell death, is involved not only in the physiological processes of development and tissue homeostasis but also in the pathological processes of a number of human diseases including influenza virus infection [75, 76]. Apoptotic cell death occurs sporadically during the development and tissue homeostasis [77]. Resident macrophages present in normal, non-inflamed tissues in limited numbers and undertake to scavenge scattering corpses of apoptotic cells as well as non-professional phagocytes such as fibroblasts [77, 78]. In contrast, apoptotic cell death induced by viral pathogens occurs focally and extensively in order to destruct the harmful cells producing infectious virus particles and block the spread of virus infection [79, 80]. It has been observed that a plenty of professional phagocytes (i.e., macrophages and neutrophils) are recruited into the site of infection with influenza virus in order to scavenge a large number of corpses of apoptotic cells resulting from the virus infection [81].

Recent studies suggest that the phagocytosis of influenza virus-infected cells undergoing apoptosis by macrophages plays a critical role in the presentation of viral antigen to T lymphocytes [82], the abortion of virus growth [83] and the prevention of virus dissemination in the infected organs [64]. The virus-infected cells were phagocytosed by macrophages anchored with phosphatidylserine expressing on the surface of infected cells during the process of apoptosis [84, 85]. The phosphatidylserine-mediated phagocytotic reaction was stimulated through the desialylation of macrophage surface by viral NA of the virus-infected cells [86]. Two types of phagocytes (i.e. macrophages and granulocytes) equally contributed to the phagocytotic elimination of apoptotic cells in the lung of mice infected with the virus, and the administration of annexin V, phosphatidylserine-binding protein, reduced the level of phagocytosis by alveolar macrophages, resulting in the augmentation of lethality in mice and inflammation in the lung [81]. Alveolar macrophages prepared from the virus-infected mice showed greater phagocytotic activity than those from uninfected mice [87]. Moreover, Fc receptor-mediated phagocytosis by macrophages contributed to the elimination of the virus particles *in vivo* [88]. Depletion of alveolar macrophage increased the virus titers in the lung of mice infected with the virus as compared with those of control the virus-infected mice [89]. Thus phagocytosis of influenza virus-infected cells undergoing apoptosis by macrophages is recognized as a protective host response to eliminate the virus-infected harmful cells and the viral pathogens from the body.

2.6. Superoxide Production by Activated Macrophages

Subsequent to phagocytosis by macrophages, an abrupt increase in superoxide production by macrophages, known as the oxidative burst, occurs, which is catalyzed by reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme complex [90]. The production of superoxide by phagocytes is necessary for remodeling tissues damaged by infectious agents [91]. However, it has been evidenced that superoxide is one of molecules responsible for death in individuals infected with influenza virus [92, 93], and that a controlled superoxide production by phagocytes is critical for the pathogenesis of influenza virus infection as evidenced by a study using mouse model lacking functional phagocyte NADPH oxidase [94]. It is most likely that necrotic focus in the virus-infected organs is formed by the cytotoxic effect of superoxide. When a wide area in organ is infected with the virus, massive necrosis occurs, resulting in organ failure. Therefore, phagocytosis of influenza virus-infected cells undergoing apoptosis by macrophages appears to implicate in the development of severe influenza-associated complications resulting from organ failure.

2.7. Macrophage Activation Factors Derived from Influenza Virus-Infected Cells Undergoing Apoptosis

It has been postulated that immature monocytes circulating in the bloodstream are able to infiltrate into the site of infection and differentiate to mature macrophages according to the theory of mononuclear phagocyte system [95]. Interaction of peripheral blood mononuclear cells with vessel wall involves initial tethering, rolling and firm adhesion to the endothelium, followed by their extravasation to the subendothelial space [96]. Following transendothelial migration, monocytes reside in close proximity to subendothelial matrix macromolecules. It has been demonstrated that monocytes are differentiated to macrophages by contacting with matrix proteins, such as type I and IV collagen and fibronectin [97]. This suggests that macrophages are recruited into normal tissues under physiological conditions. In contrast, various infectious agents induce the expression of pro-inflammatory cytokine genes. It has been demonstrated that pro-inflammatory cytokines, such as interleukin (IL)-1α and TNF-α, stimulate the adhesion and transendotherial migration of monocytes [98]. Interestingly, we found that influenza virus infection induced apoptosis in cultured chorion cells, and the secretion of a factor with monocyte differentiation-inducing (MDI) activity (MDI factor) from the virus-infected cells. Monocytes were differentiated to mature macrophages by the MDI factor, which is composed of IL-6, TNF-α and IFN-β at least, without the contact with matrix protein. Accordingly, in pathological conditions, it is likely that many matured macrophages are recruited into the inflamed tissues infected with influenza virus infection by the MDI factor secreted from the host cells. Consequently, *in vitro* influenza placentitis model using chorion cells is valuable to investigate the interaction between monocytes/macrophages and the virus-infected cells undergoing apoptosis.

A comprehensive study of *in vitro* influenza placentitis model using chorion cells has revealed various following pathological findings: Influenza virus type A replicates in both chorion cells and amnion cells [99]. Apoptotic cell death is induced in only chorion cells by the virus infection [99]. The virus infection results in intracellular lactate dehydrogenase (LDH) leakage, caspase-3 protein cleavage, and oligonucleosomal DNA fragmentation, all of which were inhibited by the presence of a general caspase inhibitor, *N-t*-Boc-Asp(OMe)-fluoromethylketone, except for the virus proliferation [100]. These results suggest that the LDH leakage results from the cellular degradation mediated through apoptosis induced by influenza virus infection [100]. LDH level in amniotic fluid is known to be one of markers for predicting fetal membrane damage [101, 102]. Therefore, these studies provide a possible diagnostic application of LDH level to predict the extent of tissue damage of fetal membranes *via* apoptosis induced by influenza virus infection. MDI activity is simultaneously increased in an extracellular medium of the virus-infected cells undergoing apoptosis [103]. Pro-inflammatory cytokines, such as IL-6, TNF-α and IFN-β, are identified as a member of MDI factor [104-106, our unpublished data]. P38 mitogen-activated protein (MAP) kinase is implicated in the process of MDI factor production induced by the virus infection [107]. Furthermore, mature macrophages induced by the MDI factor phagocytose corpuses of chorion cells resulting from apoptosis induced by the virus infection [108-110]. It should be noted that these phenomena are not observed in amnion cells where apoptosis is not induced by the virus infection [99, 100, 103-106]. Therefore, it is possible that influenza virus-infected cells secrete MDI factor in order to facilitate phagocytosis of cell corpses by macrophages. It is notable that T lymphocytes secrete lymphokine with powerful MDI activity by stimulation with mitogen, but apoptosis is not induced in the cells. Therefore, it is interesting to study the biological significance of MDI factor derived from cells undergoing influenza virus-mediated apoptosis. Our previous reports have suggested a possibility that the secretion of MDI factor from fetal membrane chorion cells is implicated in the pathogenesis of premature delivery during influenza virus infection [105, 111, 112].

As listed in Table **1**, in certain types of cells, such as monocytes/macrophages [113-115] or bronchial epithelial

Table 1. Induction of Cytokine Gene Expression by Influenza Virus Infection in Cells Undergoing Apoptosis

Cell Types	Cytokines
Chorion cells	Pro-inflammatory cytokines: IL-6, TNF-α, IFN-β C-C chemokines: MCP-1, MIP-1β, RANTES C-X-C chemokines: IL-8, GRO-α, GRO-β, ENA-78, IP-10
Monocytes or macrophages	Pro-inflammatory cytokines: IL-1, IL-6, TNF-α, IFN-α/β C-C chemokines: MCP-1, MIP-1α, MIP-1β, RANTES C-X-C chemokines: IP-10
Bronchial epithelial cells	Pro-inflammatory cytokine: IL-6 C-C chemokine: RANTES C-X-C chemokine: IL-8

cells [116, 117] as well as chorion cells [99, 104, 105], our unpublished data], it has been demonstrated that influenza virus infection induces apoptosis and the gene expression of pro-inflammatory cytokines (e.g., IL-1, IL-6, TNF-α), antiviral cytokines (e.g., IFN-α/β), monocyte directive cysteine-cysteine (C-C) chemokines (e.g., monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1α/β, regulated on activation, normal T cell expressed and secreted (RANTES)) and neutrophil directive cysteine-X-cysteine (C-X-C) chemokines (e.g., IL-8, growth-regulated gene (GRO)-α, GRO-β, epithelial cell-derived neutrophil-activating protein (ENA)-78, interferon inducible protein (IP)-10). These results suggest that influenza virus-infected host cells secrete a set of pro-inflammatory, antiviral and monocyte chemoattractive cytokines in order to attract immature monocytes circulating in the bloodstream into the site of infection and differentiate them to mature macrophages.

3. CURRENT AND FUTURE ANTI-INFLUENZA VIRUS DRUGS

3.1. Prophylaxis and Treatment of Influenza

Influenza vaccines are successful in preventing viral transmission. The efficacy of vaccines in preventing laboratory-confirmed illness is around 70%-90% both in children and in adults but is substantially lower in elderly people [118]. Therefore, the use of anti-influenza virus drugs is receiving much greater attention as a first-line defense against a new pandemic of influenza virus infection [119, 120]. Currently, two classes of anti-influenza virus drugs are available for chemoprophylaxis and treatment of influenza [121, 122].

The first-generation, amantadine (1-adamantanamine hydrochloride; known under several brand names) and rimantadine hydrochloride (α-methyl-1-adamantane-methyamine hydrochloride; brand name Flumadine) (Fig. **1**), compounds 1 & 2, respectively) target the viral M2 proton channel. The adamantanes, amantadine and rimantadine, exert their antiviral activity by blocking the M2 ion channel, preventing virion uncoating and the release of viral genome segments into the cytoplasm. They have been used almost exclusively to prevent infection or to reduce the duration of uncomplicated seasonal influenza, but their efficacy for the treatment of severe disease cases has not been defined. The adamatanes are inexpensive and highly stable in storage, but treatment is frequently complicated by side effects observed in gastrointestinal and central nervous system. Their use has also been significantly restricted by the rapid emergence of drug-resistant viruses that retain full virulence and transmissibility [123].

The second-generation, the NA inhibitors oseltamivir ((3R, 4R, 5S)-4-acetylamino-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylic acid ethyl ester; brand name Tamiflu) and zanamivir (5-acetamido-4-guanidino-6-(1, 2, 3-trihydroxypropyl)-5, 6-dihydro-4H-pyran-2-carboxylic acid; brand name Relenza) (Fig. **1**), compounds 3 & 4, respectively) impair the release of virus particles from infected cells. The NA inhibitors are effective against all NA subtypes of influenza, whereas the M2 inhibitors are effective only against influenza A virus because influenza B virus does not have M2 protein [122].

Ribavirin (1-β-D-ribofuranosyl-1, 2, 4-triazole-3-carboxamide) (Fig. **1**), compound 5) is a guanosine analogue that inhibits influenza virus ribonucleoprotein synthesis through reducing the size of the cellular guanosine 5'-triphosphate pool and by directly affecting viral replicative enzymes [124]. Ribavirin has been used in the treatment of human influenza A virus infections, usually administered orally or by aerosolization, and occasionally by the intravenous route for severe infections or in immunocompromised hosts [125]. However, ribavirin is not considered to be a drug of choice for influenza virus type A infection, since satisfactory clinical efficacy has not been achieved [125].

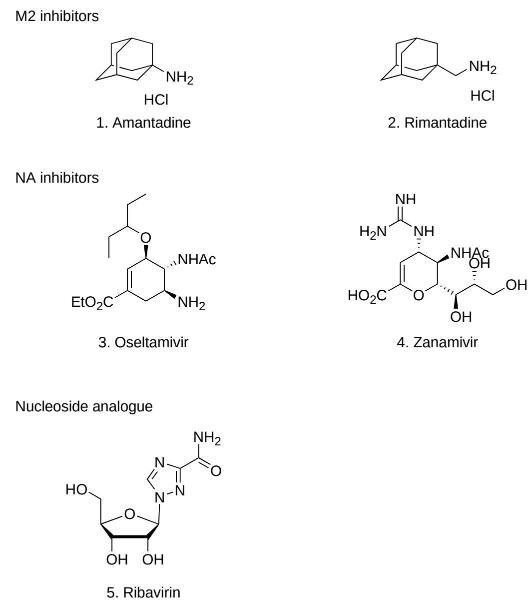

Fig. (1). Current available anti-influenza drugs.

3.2. Emergence of Anti-Influenza Drug Resistant Viruses

3.2.1. M2 Inhibitor-Resistant Viruses

Influenza viruses resistant to both types of anti-influenza drugs are emerged by a single amino acid substitution in the M2 protein and the NA protein [120]. A worldwide surveillance among >7,000 isolated influenza A viruses has demonstrated that the number of M2 inhibitor-resistant viruses has significantly increased from 0.4% in 1994/1995 to 12.3% in 2003/2004; especially viruses isolated from Hong Kong and China were resistant as high as 70% and 74%, respectively [126]. M2 inhibitor-resistant viruses are transmissible and able to cause influenza-like illness in humans [127, 128]. During the initial months of the 2005/2006 influenza season in the United States, 92% of the isolated influenza A (H3N2) viruses tested contained a mutation in the M2 gene known to be correlated with resistance to M2 inhibitors [129].

To investigate the frequency of amantadine-resistance among influenza A viruses isolated in Korea during the 2003-2009 seasons, 369 of 2199 A (H1N1) viruses and 780 of 5263 A (H3N2) viruses were randomly selected [130]. The results showed that the resistance rate to amantadine among A (H1N1) viruses increased significantly from 2004-2005 (33.3%) to 2007-2008 (97.8%) and then decreased dramatically in 2008-2009 (1.9%). The A (H1N1) isolates recently detected in 2008-2009 turned amantadine-sensitive containing two new substitutions at specific sites (serine-to-asparagine substitution at position of 141 (S141N), and glycine-to-alanine substitution at position of 185 (G185A)) in HA1. Compared with A (H1N1) viruses, the amantadine-resistance among the A (H3N2) viruses increased from 2003-2004 (9.7%) to 2005-2006 (96.7%) and decreased in 2006-2007 (57.4%). During 2006-2007, both of amantadine-resistant and -sensitive A (H3N2) viruses co-circulated but clustered in different branches phylogenetically. All of A (H3N2) isolates tested during 2007-2009 appeared to cluster in the same amantadine-resistant group. Reversion of A (H1N1) and (H3N2) viruses from amantadine-resistant to amantadine-sensitive has also observed in Thailand [131]. Phylogenetic analysis based on the viral genome demonstrated that the amantadine-resistant A (H1N1) isolates had been produced by genetic reassortment [131, 132].

3.2.2. NA Inhibitor-Resistant Viruses

The mechanism of the development of oseltamivir-resistance has been considered as follows [133]. The influenza NA releases newly formed viruses from infected cells, allowing them to spread from cell to cell. The inhibitor molecules mimic the natural substrate of the influenza NA (the sialic acid receptors) and bind to the active site, preventing NA from cleaving host-cell receptors and releasing new virus. All the resistant variants thus far have contained specific mutations in the NA molecule. To accommodate the bulky side chain of oseltamivir in the active site, the NA molecule must undergo rearrangement to create a pocket. Zanamivir, by contrast, binds to the active site without any rearrangement of the molecule. Several mutations that limit the necessary molecular rearrangement may diminish the binding of oseltamivir. Molecular analysis shows that glutamic acid at position of 276 (E276) must rotate to bind with arginine at position of 224 (R224) in order to form a pocket for the side chain of oseltamivir. The mutations (arginine-to-leucine substitution at position of 292 (R292K), asparagine-to-serine substitution at position of 294 (N294S), and histidine-to-tyrosine substitution at position of 274 (H274Y)) inhibit this rotation and prevent forming the pocket, resulting in resistance to oseltamivir. The mutations nonetheless allow the binding of natural sialic acid substrate, so mutated virus can survive and propagate. In contrast, the binding of zanamivir does not require any reorientation of amino acids, so these mutated viruses remain sensitive to that drug. A mutation (glutamic acid-to-valine substitution at position of 119 (E119V)) also interferes only with oseltamivir binding, possibly because a water molecule can fit between oseltamivir and valine at the active site but cannot insinuate itself between zanamivir and valine at residue 119.

Among 2,287 viruses isolated globally during the first 3 years of NA inhibitors use (1999 to 2002), eight viruses had a >10-fold decrease in susceptibility to oseltamivir, 0.22% in 1999/2000, 0.36% in 2000/2001, and 0.41% in 2001/2002 [134]. Two strains (A/New York/24/2001 and A/Hokkaido/15/02) were resistant to both oseltamivir and zanamivir [134]. A 2004 study in Japan demonstrated that 9 of 50 children (18%) with influenza A (H3N2) virus infection, who had been treated with oseltamivir, had a virus with a drug-resistance mutation in the NA gene [135]. Oseltamivir-resistant viruses were transmissible in ferrets [136]. Oseltamivir-resistant A (H5N1) viruses with an amino acid substitution in the NA were isolated from two of eight patients during oseltamivir treatment, and both patients died [137]. Moreover, an oseltamivir-resistant A (H5N1) virus has been also isolated from a Vietnamese girl [138]. Initial testing found that 2009 novel influenza virus was susceptible to NA inhibitors but resistant to M2 inhibitors [11]. Therefore, NA inhibitors have been used widely for treatment and chemo-prophylaxis of pandemic A (H1N1) influenza. Sporadic cases of oseltamivir-resistant pandemic A (H1N1) influenza virus have been reported worldwide [12].

A frequent emergence of influenza viruses resistant to the M2 and NA inhibitors during the treatment with current drugs used suggests the need for development of the third-generation anti-influenza virus drugs with alternative antiviral mechanisms.

3.3. Potential of Selected Antioxidants as Future Anti-Influenza Virus Drugs

3.3.1. Superoxide Dismutases

Intravenous injection of pyran polymer conjugated with copper/zinc (Cu/Zn)-superoxide dismutase (SOD) protected mice against a potentially lethal influenza virus infection [92]. Intravenous injection of manganese (Mn)-SOD to mice with influenza virus infection at a lethal dose mildly increased mean days of survival, lessened arterial oxygen saturation decline, and lowered lung consolidation [139]. A combination of Mn-SOD and ribavirin, each of which was administered with small-particle aerosol, resulted in a generally mild improvement of the disease induced by the influenza A virus compared with use of either material alone [139]. A combined application of Cu/Zn-SOD and rimantadine hydrochloride in doses, which by themselves did not protect significantly mice against the infection, resulted in a synergistically decrease in lung virus titers, lung weights and consolidation and mortality rates [140]. Treatment with allopurinol, an inhibitor of xanthine oxidase capable of generating superoxide anion, improved the survival rate of influenza virus-infected mice [141]. Thus the treatment with SOD decreased the lethal or toxic effect of influenza virus infection in mouse models but did not inhibit the virus proliferation [92, 139-141]. Transgenic mice carrying overexpressed extracellular SOD exhibited less severe lung injury after influenza virus infection [93]. Mice lacking functional phagocyte NADPH oxidase exhibited the augmentation of macrophages and the reduction of apoptosis in macrophages and virus titer in bronchoalveolar space after influenza virus infection as compared to those of wild-type animals [94]. Therefore, these studies suggest that superoxide anion produced by phagocytes, especially macrophages, play a critical role in the pathogenesis of influenza virus infection.

3.3.2. Thiol Antioxidants

(a) Pyrrolidine Dithiocarbamate

Pyrrolidine dithiocarbamate (PDTC) (Fig. **2**), compound 6) has been shown to scavenge hydroxyl and superoxide anion radicals, the effect of which is comparable to other free radical scavengers, such as ascorbate and glutathione [142]. Both PDTC and trolox (6-hydroxy-2, 5, 7, 8-tetramethyl-chroman-2-carboxylic acid; a water-soluble vitamin E analogue) suppressed the induction of ROS production in chorion cells by influenza virus infection [143, 144]. PDTC inhibited both apoptosis induction and virus proliferation in chorion cells infected with influenza virus, whereas no such

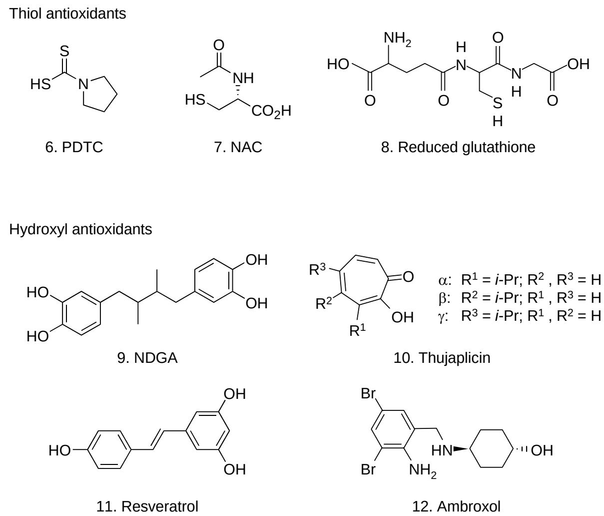

Fig. (2). Thiol and hydroxyl antioxidants with anti-influenza virus activity.

inhibitory effect was observed by trolox [143, 144]. PDTC also inhibited the cytopathic effect of influenza virus infection on the other types of cells, such as human pulmonary epithelial A549 cells and murine macrophage J774.1 cells [145-147]. The studies using J774.1 cells also demonstrated that various other antioxidants, such as trolox, deferoximine nesyiate, dithiothreitol, *N*-methyl-D-arginine, catalase and SOD, did not inhibit the cytopathic effect of influenza virus infection [146, 147]. These results suggest that the inhibition of influenza virus-induced apoptosis by PDTC is attributable to its antiviral activity rather than its antioxidant property. That is, ROS may not be responsible for apoptosis induction by influenza virus infection [143, 144]. However, as described in the previous section, it has been suggested that organ injury was occurred by ROS derived from phagocytes during influenza virus infection. Therefore, these evidence reveals that the pathogenesis of influenza virus infection involves not only the virus replication-mediated apoptotic cell death in the infected cells irrespective of ROS, but also the injury of non-infected cells by ROS derived from macrophages and neutrophils infiltrated into the virus-infected organs. The findings provide a possibility that an agent with antiviral and antioxidant activities can be a drug of choice for the treatment of patients with severe influenza-associated complications. PDTC is one of the drug candidates. Since PDTC is well tolerated *in vivo* at doses by 100 mg/kg (intraperitoneal injection) and exhibits the therapeutic effect on animal inflammation and tissue injury models [148-151], further studies on the therapeutic effect of PDTC on animal influenza models are warranted.

The mode of inhibitory effect of PDTC on influenza virus proliferation has been investigated. PDTC inhibited the synthesis of negative-strand virion RNA (vRNA) and positive-strand complementary and/or messenger RNA (c/mRNA) for influenza virus HA gene [144]. Therefore, it is likely that the inhibition of influenza virus gene replication and transcription contributes to the inhibition of virus proliferation. Dithiocarbamate can chelate various divalent metal ions, leading to the formation of a lipophilic dithiocarbamate-metal complex, and rapid transport *via* a lipophilic complex by PDTC has been proposed to explain the intracellular recruitment of copper and zinc ions from the extracellular medium [152]. It has been demonstrated that copper and zinc ions inhibit influenza virus RNA-dependent RNA polymerase activity, and that the inhibitory effect of bathocuproine-copper and bothoquproine-zinc complexes is greater than that of bathoquproine itself [153]. Moreover, PDTC-copper or PDTC-zinc complex inhibited the replication of coxsackievirus [154] and rhinovirus [155]. Conceivably, it is possible that PDTC inhibits influenza virus gene replication and transcription through the inhibition of viral RNA-dependent RNA polymerase activity by increasing the amount of intracellular copper and zinc ions or intracellular PDTC-copper and PDTC-zinc complexes. Further study is needed to elucidate the precise mechanism of inhibitory effect of PDTC on influenza virus gene replication and transcription.

(b) N-Acetyl-L-Cysteine

N-Acetyl-L-cysteine (NAC) (Fig. **2**, compound 7), the acetylated variant of the amino acid L-cysteine, is an excellent source of thiol groups, and is converted into metabolites in the body capable of stimulating glutathione synthesis, promoting detoxification, and acting directly as free radical scavengers [156]. NAC inhibited the induction of apoptosis [157-159] and pro-inflammatory cytokines and chemokines, such as IL-6, IL-8, RANTES and IP-10, by influenza virus infection [159]. NAC inhibited the proliferation of influenza virus at an early, but not later, stage of infection [158, 159]. Administration of the NAC significantly decreased the mortality in mice infected with influenza virus [160], and combination of NAC and ribavirin synergistically reduced the lethal effect [161]. These results suggest that combination of antioxidants with current drugs used can improve the treatment for influenza virus infection.

Administration of NAC appears to reduce symptomatic conditions associated with influenza virus infection. A total of 262 subjects of both sexes were given either placebo or NAC (600 mg) orally twice daily for six months. Although incidents of seroconversion towards A (H1N1) Singapore 6/86 influenza virus was similar in the two groups, NAC treatment decreased both the incidents and severity of influenza-like episodes, and the length of time confined to bed. The authors concluded that NAC did not prevent influenza A

(H1N1) virus infection but significantly reduced the incidence of clinically apparent disease [162].

(c) Glutathione

Reduced glutathione (Fig. **2**), compound 8) has an anti-influenza activity *in vitro* and *in vivo* [163]. The addition of reduced glutathione into culture medium exogenously blocked the induction of apoptosis through the inhibition of viral macromolecule synthesis in Madin-Darby canine kidney (MDCK) cells after influenza virus infection. The antiviral effect of reduced glutathione on influenza virus proliferation was also observed in normal human small airway epithelial cells. In BALB/c mice, inclusion of reduced glutathione in the drinking water decreased viral titer in both lung and trachea homogenates at 4 days after intranasal inoculation with a mouse-adopted influenza strain A/X-31. Moreover, both the levels of Bcl-2 expression and the content of intracellular reduced glutathione contribute to the ability of host cells for down-regulating influenza virus replication, although their effects are exerted at different stages of the viral life-cycle [164].

3.3.3. Hydroxyl Antioxidants

(a) Nordihydroguaiaretic Acid

Nordihydroguaiaretic acid (NDGA; 1, 4-bis (3, 4-dihydroxyphenyl)-2,3-dimethylbutane) (Fig. **2**), compound 9) occurs in the resinous exudates of the creosote bush *Larrea divaricata*. NDGA scavenges oxygen radicals, such as peroxynitrite, singlet oxygen, hydroxyl and superoxide anion radicals [165]. The treatment with NDGA inhibited apoptotic DNA fragmentation and virus proliferation in chorion cells infected with influenza virus [166]. The maximum inhibition against DNA fragmentation was observed with 500 μM NDGA. The antiviral activity of NDGA against influenza virus was more potent than that of PDTC. This study, therefore, has suggested for the first time that NDGA, a known antioxidant reagent, inhibits the induction of apoptosis in chorion cells infected with influenza virus through the more potent antiviral activity than that of PDTC. In this regard, it should be noted that Erimos Pharmaceuticals and North Carolina State University have filed a joint patent for the use of a developmental Erimos product, EM-1421 (tetra-*O*-methyl NDGA).

Recently, it has been reported that several methylated derivatives of NDGA possessed an inhibitory activity on the expression of reporter genes driven by some viral promoters of herpes simplex virus, human papillomavirus and human immunodeficiency virus, which was resulting from the inhibitory effect on the binding of cellular transcription factor Sp-1 to viral gene promoters [167]. NDGA derivatives did not affect the expression of reporter genes driven by the adenovirus major late promoter and the cytomegalovirus promoter [168]. It is predicted that the antiviral activity of NDGA derivatives is selectively depending on the virus types. Influenza virus has viral RNA-dependent RNA polymerases, which contribute to the replication and transcription processes of the viral genes, probably irrespective of cellular transcription factor Sp-1. An additional mechanism of NDGA for the inhibition of influenza virus proliferation has been proposed. NDGA is shown to inhibit the intracellular transport of vesicular stomatitis virus glycoproteins [168]. Conceivably, NDGA may inhibit influenza virus proliferation *via* inhibition of intracellular transport of viral glycoproteins.

(b) Thujaplicin

Thujaplicins, including α-thujaplicin (2-hydroxy-3-isopropyl-2, 4, 6-cycloheptatrien-1-one), β-thujaplicin (2-hydroxy-4-isopropyl-2, 4, 6-cycloheptatrien-1-one) and γ-thujaplicin (2-hydroxy-5-isopropyl-2, 4, 6-cycloheptatrien-1-one) (Fig. **2**), compound 10), are tropolone-related compounds found in the heartwood of several cupressaceous plants, such as western red cedar (*Thuja plicata*), eastern white cedar (*Thuja occidentalis*) and hinoki crypress (*Chamaecryprais obtusa*) [169]. All complexes of α-thujaplicin-copper, β-thujaplicin-copper and γ-thujaplicin-copper blocked the induction of apoptosis in MDCK cells by influenza virus infection through their antiviral effects [170]. While thujaplicin-ferrous, thujaplicin-ferric, thujaplicin-magnesium and thujaplicin-manganese complexes showed no inhibition of influenza virus-induced apoptosis [170]. Thujaplicin scavenges hydroxyl radical, *tert*-butyl peroxyl radical, hydrogen peroxide, superoxide anion radical and singlet oxygen [171].

(c) Resveratrol

Plant polyphenol resveratorol (3, 5, 4'-trihydroxy-*trans*-stilbene) (Fig. **2**, compound 11) inhibited the progressive effects of superoxide anion and hydrogen peroxide radicals on arachidonic acid production and cyclooxygenase-2 induction in macrophages [172]. Resveratrol inhibited the replication of influenza virus in MDCK cells, as a result of the blockade of the nuclear-cytoplasmic translocation of viral ribonucleoproteins and the reduced expression of late viral protein, such as HA and matrix protein [173]. Resveratol also improved survival and decreased pulmonary viral titers in influenza virus-infected mice [173].

(d) Ambroxol

Ambroxol (2-amino-3, 5-dibromo-*N*-[trans-4-hydroxycyclohexyl]benzylamine) (Fig. **2**), compound 12), known as a mucolytic agent, has been used for the treatment of chronic bronchitis and neonatal respiration distress syndrome [174]. Ambroxol suppressed the proliferation of influenza virus in the mouse airway and improved the survival rate of mice [175]. Antioxidant activity of ambroxol is related to the direct scavenging effect for ROS, such as superoxide anion and hydroxyl radicals [176, 177].

3.3.4. Flavonoids

Flavonoids are a ubiquitous group of polyphenolic substances which are present in most plants, concentrating in seeds, fruit skin or peel, bark, and flowers. The structural components common to these molecules include two benzene rings on either side of a 3-carbon ring. Multiple combinations of hydroxyl groups, sugars, oxygens, and methyl groups attached to these structures create the various classes of flavonoids: flavanols, flavanones, flavones, flavan-3-ols (catechins), anthocyanins, and isoflavones. Flavonoids have been shown in a number of studies to be potent antioxidants, capable of scavenging hydroxyl radicals, superoxide anions, and lipid peroxy radicals [178].

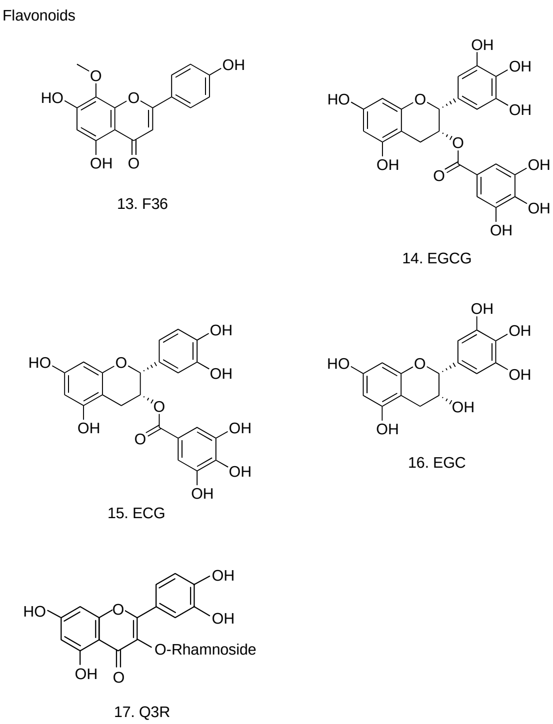

Fig. (3). Flavonoids with anti-influenza virus activity.

(a) 5, 7, 4'-Trihydroxy-8-Methoxyflavone

5, 7, 4'-trihydroxy-8-methoxyflavone (F36) (Fig. **3**, compound 13) isolated from the roots of *Scutellaria baicalensis* was shown to have a specific inhibitory activity against influenza virus NA because it did not affect the mouse liver NA activity [179, 180]. F36 inhibited the proliferation of influenza virus in MDCK cells, in the allantoic sac of embryonal chicken egg and *in vivo* using BALB/c mice [180-182]. Immunoelectron microscopic analysis revealed that F36 inhibited the budding of progeny influenza virus particles from MDCK cell surface and microvilli [183].

(b) Catechins

Catechins, (-)-epigallocatechin gallate (EGCG), (-)-epicatechin gallate (ECG) and (-)-epigallocatechin (EGC) (Fig. **3**, compounds 14, 15 and 16, respectively), from green tea have been evaluated for their ability to inhibit influenza virus replication in cell culture [184]. Among the test compounds, the EGCG and ECG were found to be potent inhibitors of influenza virus replication in MDCK cell culture, and this effect was observed in all influenza virus subtypes tested, including A (H1N1), A (H3N2) and B virus. The 50% effective inhibition concentration of EGCG, ECG, and EGC for influenza A virus were 22-28, 22-40 and 309-318 μM, respectively. EGCG and ECG exhibited inhibitory activity of hemagglutination, suppressed viral RNA synthesis in MDCK cells, and inhibited the NA activity, however, the effects of EGC were much lesser. The results show that the 3-galloyl group of catechin skeleton plays an important role on the observed antiviral activity, whereas the 5'-OH at the trihydroxy benzyl moiety at 2-position plays a minor role. Catechins have been shown to possess the ability to scavenge for superoxide anion and hydroxyl radicals [185]. Gargling with tea catechin extracts prevented influenza virus infection in elderly nursing home residents [186]. The introduction of long alkyl chains enhances anti-influenza virus activity 24-fold relative to native EGCG [187].

(c) Quercetin 3-Rhamnoside

Quercetin 3-rhamnoside (Q3R) (Fig. **3**, compound 17) from *Houttuynia cordata* possessed strong anti-influenza A/WS/33 virus as well as oseltamivir [188]. The mode of action of Q3R involved the inhibition of virus replication in the initial stage of virus infection by indirect interaction with virus particles.

4. CONCLUSION

As illustrated in Fig. (**4**), host cell secretes monocyte directive C-C chemokines (e.g., MCP-1, RANTES and MIP-1α/β) and MDI factor (i.e. IL-6, TNF-α and IFN-β) in response to influenza virus infection prior to undergoing apoptotic cell degradation. The C-C chemokines act on immature monocyte circulating in the bloodstream, resulting in recruitment of monocyte into the site of infection. The MDI factor acts on the recruited monocyte, resulting in differentiation into well-matured macrophage capable of phagocytosing and producing superoxide. The activated macrophage move to the virus-infected host cell and phagocytoses apoptotic cell debris resulting from the virus infection. An abrupt increase in superoxide production occurs during phagocytosis. The superoxide induces injury in non-infected cell. These MDI factor-relating pathways represent a part of mechanisms of tissue injury during severe influenza-associated complications.

Scavenge of superoxide is an important aspect to develop new strategies for prevention of organ failure during severe influenza-associated complications. Since the most important aspect in viral disease treatment is to inhibit virus replication, an agent with antiviral and antioxidant activities should be a drug of choice for the treatment of patients with severe

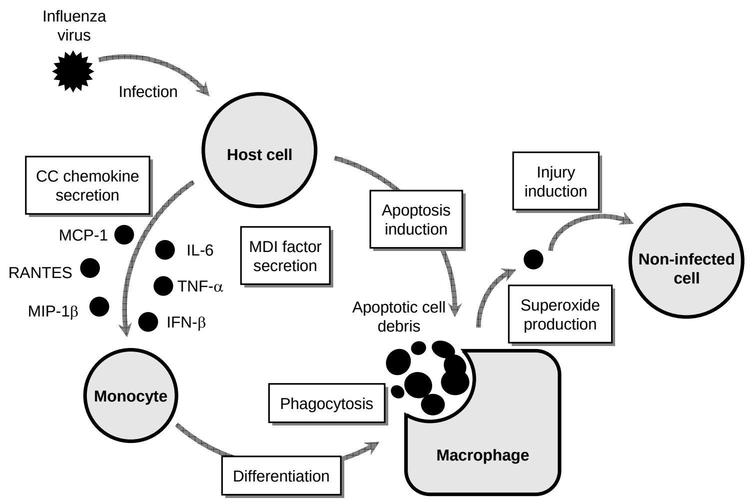

Fig. (4). Tissue injury model during influenza virus infection.

influenza-associated complications. Selected compounds, such as PDTC, NAC, glutathione, NDGA, thujaplicin, resveratrol, ambroxol, F36, EGCG, ECG and Q3R, possess both antiviral and antioxidant activities. Consequently, they are potential drugs of choice for severe influenza-associated complications. In theory, combination of these antioxidants with current anti-influenza drugs can improve conventional chemotherapy for severe influenza-associated complications.

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REFERENCES

[1] Peiris JS, Tu WW, Yen HL A novel H1N1 virus causes the first pandemic of the 21st century. Eur J Immunol 2009; 39: 2946-54.
[2] Chang LY, Shih SR, Shao PL, Huang DT, Huang LM. Novel swine-origin influenza virus A (H1N1): the first pandemic of the 21st century. J Formos Med Assoc 2009; 108: 526-32.
[3] Shinde V, Bridges CB, Uyeki TM, *et al*. Triple-reassortant swine influenza A (H1) in humans in the United States, 2005-2009. N Engl J Med 2009; 360: 2616-25.
[4] World Health Organization. Strategic Advisory Group of Experts on Immunization - report of the extraordinary meeting on the influenza A (H1N1) 2009 pandemic, 7 July 2009. Wkly Epidemiol Rec 2009; 84: 301-4.
[5] World Health Organization. Pandemic (H1N1) 2009 - update 71 [website on the internet]. [updated 2009 Oct 23; cited 2009 Oct 26]. Available from: http://www.who.int/csr/don/2009_10_23/en/index.html
[6] Kamps BS, Hoffmann C, Preiser W, Eds. Influenza Report [book on the internet]. Flying Publishers; 2006 [cited 2009 Oct 26]. Available from: http://www.influenzareport.com/ir/overview.htm
[7] Kuiken T, Taubenberger JK. Pathology of human influenza revisited. Vaccine 2008; 26 (Suppl 4): D59-66.
[8] Adams DH, Hubscher SG. Systemic viral infections and collateral damage in the liver. Am J Pathol 2006; 168: 1057-9.
[9] Zinserling AV, Aksenov OA, Melnikova VF, Zinserling VA. Extrapulmonary lesions in influenza. Tohoku J Exp Med 1983; 140: 259-72.
[10] Beigel J, Bray M. Current and future antiviral therapy of severe seasonal and avian influenza. Antiviral Res 2008; 78: 91-102.
[11] Centers for Disease Control and Prevention (CDC). Update: drug susceptibility of swine-origin influenza A (H1N1) viruses, April 2009. MMWR Morb Mortal Wkly Rep 2009; 58: 433-5.
[12] World Health Organization. Pandemic (H1N1) 2009 - update 60 [website on the internet]. [updated 2009 Jul 31; cited 2009 Oct 29]. Available from: http://www.who.int/csr/don/2009_08_04/en/index.html
[13] Ruf BR, Szucs T. Reducing the burden of influenza-associated complications with antiviral therapy. Infection 2009; 37: 186-96.
[14] Tumpey TM, García-Sastre A, Taubenberger JK, *et al*. Pathogenicity of influenza viruses with genes from the 1918 pandemic virus: functional roles of alveolar macrophages and neutrophils in limiting virus replication and mortality in mice. J Virol 2005; 79: 14933-44.
[15] Kash JC, Basler CF, García-Sastre A, *et al*. Global host immune response: pathogenesis and transcriptional profiling of type A influenza viruses expressing the hemagglutinin and neuraminidase genes from the 1918 pandemic virus. J Virol 2004; 78: 9499-511.
[16] Uchide N, Toyoda H. Potential of selected antioxidants for influenza chemotherapy. Anti-Infect Agents Med Chem 2008; 7: 73-83.
[17] Uchide N, Toyoda H. Future target molecules for influenza treatment. Mini-Rev Med Chem 2008; 8: 491-5.
[18] Mauad T, Hajjar LA, Callegari GD, *et al*. Lung pathology in fatal novel human influenza A (H1N1) infection. Am J Respir Crit Care Med 2009; in press. [cited 2009 Oct 29]. Available from: http://ajrccm.atsjournals.org/cgi/reprint/200909-1420OCv1
[19] Rasmussen SA, Jamieson DJ, Bresee JS. Pandemic influenza and pregnant women. Emerg Infect Dis 2008; 14: 95-100.
[20] Jamieson DJ, Honein MA, Rasmussen SA, *et al*. Novel Influenza A (H1N1) Pregnancy Working Group. H1N1 2009 influenza virus infection during pregnancy in the USA. Lancet 2009; 374: 451-8.
[21] Harris JW. Influenza occurring in pregnant women: a statistical study of thirteen hundred and fifty cases. J Am Med Assoc 1919; 72: 978-80.
[22] Hardy JM, Azarowicz EN, Mannini A, Medearis DN, Cooke RE. The effect of Asian influenza on the outcome of pregnancy, Baltimore, 1957-1958. Am J Public Health 1961; 51: 1182-8.
[23] Freeman DW, Barno A. Death from Asian influenza associated with pregnancy. Am J Obstet Gynecol 1959; 78: 1172-5.
[24] Greenberg M, Lacobziner H, Pakter J, Weist BAG. Maternal mortality in the epidemic of Asian influenza, New York city, 1957. Am J Obstet Gynecol 1958; 76: 897-902.
[25] McDonald AD. Maternal health in early pregnancy and congenital defect. Final report on a prospective inquiry. Br J Prev Soc Med 1961; 15: 154-66.
[26] Sterner G, Grandien M, Enocksson E. Pregnant women with acute respiratory illness at term. Scand J Infect Dis 1990; 71: 19-26.
[27] Stanwell-Smith R, Parker AM, Chakraverty P, Soltanpoor N, Simpson CN. Possible association of influenza A with fetal loss: investigation of a cluster of spontaneous abortions and stillbirths. Commun Dis Rep CDR Rev 1994; 4: R28-32.

[28] Kort BA, Cefalo RC, Baker VV. Fatal influenza A pneumonia in pregnancy. Am J Perinatol 1986; 3 179-82.
[29] Hakoda S, Nakatani T. A pregnant woman with influenza A encephalopathy in whom influenza A/Hong Kong virus (H3) was isolated from cerebrospinal fluid. Arch Intern Med 2000; 160: 1041-5.
[30] Parkins MD, Fonseca K, Peets AD, Laupland KB, Shamseddin K, Gill MJ. A potentially preventable case of serious influenza infection in a pregnant patient. CMAJ 2007; 177: 851-3.
[31] Mullooly JP, Barker WH, Nolan TF. Risk of acute respiratory disease among pregnant women during influenza A epidemics. Public Health Rep 1986; 101: 205-11.
[32] Neuzil KM, Reed GW, Mitchel EF, Simonsen L, Griffin MR. Impact of influenza on acute cardiopulmonary hospitalizations in pregnant women. Am J Epidemiol 1998; 148: 1094-102.
[33] Hartert TV, Neuzil KM, Shintani AK, *et al.* Maternal morbidity and perinatal outcomes among pregnant women with respiratory hospitalizations during influenza season. Am J Obstet Gynecol 2003; 189: 1705-12.
[34] Jewett JF. Influenzal pneumonia at term. N Engl J Med 1974; 291: 256-7.
[35] Mel'nikova VF, Tsinzerling AV, Aksenov OA, Vydumkina SP, Kalinina NA. Involvement of the afterbirth in influenza. Arkh Patol 1987; 49: 19-25.
[36] Yawn DH, Pyeatte JC, Joseph MJ, Eichler SL, Garcia-Bunuel R. Transplacental transfer of influenza virus. J Am Med Assoc 1971; 216: 1022-3.
[37] McGregor JA, Burns JC, Levin MJ, Burlington B, Meiklejohn G. Transplacental passage of influenza A/Bangkok (H3N2) mimicking amniotic fluid infection syndrome. Am J Obstet Gynecol 1984; 149: 856-9.
[38] Conover PT, Roessmann U. Malformational complex in an infant with intrauterine influenza viral infection. Arch Pathol Lab Med 1990; 114: 535-8.
[39] Ruben FL, Winkelstein A, Sabbacha RE. In utero sensitization with influenza virus in man. Proc Soc Exp Biol Med 1975; 149: 881-3.
[40] Ruben FL, Thompson D. Cord blood lymphocyte *in vitro* responses to influenza A antigens after an epidemic of influenza A/Port Chalmers/73 (H3N2). Am J Obstet Gynecol 1981; 141: 443-7.
[41] Gu J, Xie Z, Gao Z, *et al.* H5N1 infection of the respiratory tract and beyond: a molecular pathology study. Lancet 2007; 370: 1137-45.
[42] Shu Y, Yu H, Li D. Lethal avian influenza A (H5N1) infection in a pregnant woman in Anhui Province, China. N Engl J Med 2006; 354: 1421-2.
[43] Vannier P. Infection causes of abortion in swine. Reprod Dom Anim 1999; 34: 367-76.
[44] Wesley RD. Exposure of sero-positive gilts to swine influenza may cause a few stillbirth per litter. Can J Vet Res 2004; 68: 215-7.
[45] Wallace GD, Elm JL. Transplacental transmission and neonatal infection with swine influenza virus (Hsw1N1) in swine. Am J Vet Res 1979; 40: 1169-72.
[46] Sweet C, Collie MH, Toms GL, Smith H. The pregnant guinea-pig as a model for studying influenza virus infection in utero: infection of foetal tissues in organ culture and *in vivo*. Br J Exp Pathol 1977; 58: 133-9.
[47] Rushton DI, Collie MH, Sweet C, Husseini RH, Smith H. The effects of maternal influenzal viremia in late gestation on the conceptus of the pregnant ferret. J Pathol 1983; 140: 181-91.
[48] Rosztoczy I, Sweet C, Toms GL, Smith H. Replication of influenza virus in organ cultures of human and simian urogenital tissues and human foetal tissues. Br J Exp Pathol 1975; 56: 322-8.
[49] Lehmann NI, Gust ID. Viremia in influenza. A report of two cases. Med J Aust 1971; 4: 1166-9.
[50] Naficy K. Human influenza infection with proved viremia. N Engl J Med 1963; 269: 964-6.
[51] Ritova VV, Schastnyi EI, Ratushkina LS, Shuster IY. Investigation of the incidence of influenza A viremia caused by virus strains circulating among children in 1968-1977. J Hyg Epidemiol Microbiol Immunol 1979; 23: 35-41.
[52] Stanley ED, Jackson GG. Viremia in Asian influenza. Trans Assoc Am Physicians 1966; 79: 376-87.
[53] de Jong MD, Bach VC, Phan TQ, *et al.* Fatal avian influenza A (H5N1) in a child presenting with diarrhea followed by coma. N Engl J Med 2005; 352: 686-91.
[54] Roberts GT, Roberts JT. Postsplenectomy sepsis due to influenzal viremia and pneumococcemia. Can Med Assoc J 1976; 115: 435-7.
[55] Nakai Y, Itoh M, Mizuguchi M, *et al.* Apoptosis and microglial activation in influenza encephalopathy. Acta Neuropathol 2003; 105: 233-9.
[56] Salonen O, Koshkiniemi M, Saari A, *et al.* Myelitis associated with influenza A virus infection. J Neurovirol 1997; 3: 83-5.
[57] Zhdanov VM, Ritova VV. On the pathogenesis of influenza. Klin Med (Mosk) 1959; 37: 45-8.
[58] Kaji M, Oseasohn R, Jordan WS Jr, Dingle JH. Isolation of Asian virus from extrapulmonary tissues in fatal human influenza. Proc Soc Exp Biol Med 1959; 100: 272-5.
[59] Takahashi M, Yamada T, Nakashita Y, *et al.* Influenza virus-induced encepharopathy: Clinicopathologic study of an autopsied case. Pediatr Int 2000; 42: 204-14.
[60] Tsuruoka H, Xu H, Kuroda K, *et al.* Detection of influenza virus RNA in peripheral blood mononuclear cells of influenza patients. Jpn J Med Sci Biol 1997; 50: 27-34.
[61] Frankova V, Rycheterova V. Inhalatory infection of mice with influenza A0/PR8 virus. II. Detection of the virus in the blood and extrapulmonary organs. Acta Virol 1975; 19: 35-40.
[62] Mori I, Komatsu T, Takeuchi K, Nakakuki K, Sudo M, Kimura Y. Viremia induced by influenza virus. Microb Pathog 1995; 19: 237-344.
[63] Uiprasertkul M, Puthavathana P, Sangsiriwut K, *et al.* Influenza A H5N1 replication sites in humans. Emerg Infect Dis 2005; 11: 1036-41.
[64] Mori I, Goshima F, Imai Y, *et al.* Olfactory receptor neurons prevent dissemination of neurovirulent influenza A virus into the brain by undergoing virus-induced apoptosis. J Gen Virol 2002; 83: 2109-16.
[65] Taubenberger JK, Morens DM. The pathology of influenza virus infections. Annu Rev Pathol 2008; 3: 499-522; Adams DH, Hubscher SG. Systemic viral infections and collateral damage in the liver. Am J Pathol 2006; 168: 1057-9.
[66] Beigel JH, Farrar J, Han AM, *et al.*Writing Committee of the World Health Organization (WHO) Consultation on Human Influenza A/H5. Avian influenza A (H5N1) infection in humans. N Engl J Med 2005; 353: 1374-85.
[67] Tran TH, Nguyen TL, Nguyen TD, *et al.* World Health Organization International Avian Influenza Investigative Team. Avian influenza A (H5N1) in 10 patients in Vietnam. N Engl J Med 2004; 350: 1179-88.
[68] Ungchusak K, Auewarakul P, Dowell SF, *et al.* Probable person-to-person transmission of avian influenza A (H5N1). N Engl J Med 2005; 352: 333-40.
[69] Peiris JS, Yu WC, Leung CW, *et al.* Re-emergence of fatal human influenza A subtype H5N1 disease. Lancet 2004; 363: 617-9.
[70] To KF, Chan PK, Chan KF, *et al.* Pathology of fatal human infection associated with avian influenza A H5N1 virus. J Med Virol 2001; 63: 242-6.
[71] Uiprasertkul M, Kitphati R, Puthavathana P, *et al.* Apoptosis and pathogenesis of avian influenza A (H5N1) virus in humans. Emerg Infect Dis 2007; 13: 708-12.
[72] Chotpitayasunondh T, Ungchusak K, Hanshaoworakul W, *et al.* Human disease from influenza A (H5N1), Thailand, 2004. Emerg Infect Dis 2005; 11: 201-9.
[73] Korteweg C, Gu J. Pathology, molecular biology, and pathogenesis of avian influenza A (H5N1) infection in humans. Am J Pathol 2008; 172: 1155-70.
[74] Perrone LA, Plowden JK, García-Sastre A, Katz JM, Tumpey TM. H5N1 and 1918 pandemic influenza virus infection results in early and excessive infiltration of macrophages and neutrophils in the lungs of mice. PLoS Pathog 2008; 4: e1000115.
[75] Thompson CB. Apoptosis in the pathogenesis and treatment of disease. Science 1995; 267: 1456-62.
[76] Mori I, Komatsu T, Takeuchi K, Nakakuki K, Sudo M, Kimura Y. *In vivo* induction of apoptosis by influenza virus. J Gen Virol 1995; 76: 2869-73.
[77] Wyllie AH, Kerr JF, Currie AR. Cell death: the significance of apoptosis. Int Rev Cytol 1980; 68: 251-306.
[78] Parnaik R, Raff MC, Scholes J. Differences between the clearance of apoptotic cells by professional and non-professional phagocytes. Curr Biol 2000; 10: 857-60.
[79] Hardwick JM. Virus-induced apoptosis. Adv Pharmacol 1997; 41: 295-336.
[80] Young LS, Dawson CW, Eliopoulos AG. Viruses and apoptosis. Br Med Bull 1997; 53: 509-21.

[81] Watanabe Y, Hashimoto Y, Shiratsuchi A, Takizawa T, Nakanishi Y. Augmentation of fatality of influenza in mice by inhibition of phagocytosis. Biochem Biophys Res Commun 2005; 337: 881-6.
[82] Albert ML, Sauter B, Bhardwaj N. Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. Nature 1998; 392: 86-9.
[83] Fujimoto I, Pan J, Takizawa T, Nakanishi Y. Virus clearance through apoptosis-dependent phagocytosis of influenza A virus-infected cells by macrophages. J Virol 2000; 74: 3399-403.
[84] Shiratsuchi A, Kaido M, Takizawa T, Nakanishi Y. Phosphatidylserine-mediated phagocytosis of influenza A virus-infected cells by mouse peritoneal macrophages. J Virol 2000; 74: 9240-4.
[85] Watanabe Y, Shiratsuchi A, Shimizu K, Takizawa T, Nakanishi Y. Role of phosphatidylserine exposure and sugar chain desialylation at the surface of influenza virus-infected cells in efficient phagocytosis by macrophages. J Biol Chem 2002; 277: 18222-8.
[86] Watanabe Y, Shiratsuchi A, Shimizu K, Takizawa T, Nakanishi Y. Stimulation of phagocytosis of influenza virus-infected cells through surface desialylation of macrophages by viral neuraminidase. Microbiol Immunol 2004; 48: 875-81.
[87] Hashimoto Y, Moki T, Takizawa T, Shiratsuchi A, Nakanishi Y. Evidence for phagocytosis of influenza virus-infected, apoptotic cells by neutrophils and macrophages in mice. J Immunol 2007; 178: 2448-57.
[88] Huber VC, Lynch JM, Bucher DJ, Le J, Metzger DW. Fc receptor-mediated phagocytosis makes a significant contribution to clearance of influenza virus infections. J Immunol 2001; 166: 7381-8.
[89] Tumpey TM, García-Sastre A, Taubenberger JK, *et al*. Pathogenicity of influenza viruses with genes from the 1918 pandemic virus: functional roles of alveolar macrophages and neutrophils in limiting virus replication and mortality in mice. J Virol 2005; 79: 14933-44.
[90] Park JB. Phagocytosis induces superoxide formation and apoptosis in macrophages. Exp Mol Med 2003; 35: 325-35.
[91] Cohen MS, Isturiz RE, Malech HL, *et al*. Fungal infection in chronic granulomatous disease. The importance of the phagocyte in defense against fungi. Am J Med 1981; 71: 59-66.
[92] Oda T, Akaike T, Hamamoto T, Suzuki F, Hirano T, Maeda H. Oxygen radicals in influenza-induced pathogenesis and treatment with pyran polymer-conjugated SOD. Science 1989; 244: 974-6.
[93] Suliman HB, Ryan LK, Bishop L, Folz RJ. Prevention of influenza-induced lung injury in mice overexpressing extracellular superoxide dismutase. Am J Physiol Lung Cell Mol Physiol 2001; 280: L69-78.
[94] Snelgrove, RJ, Edwards, L, Rae, AJ, Hussell, T. An absence of reactive oxygen species improves the resolution of lung influenza infection. Eur J Immunol 2006; 36, 1364-73.
[95] Hume DA. The mononuclear phagocyte system. Curr Opin Immunol 2006; 18: 49-53.
[96] Butcher EC. Leukocyte-endothelial cell recognition: three (or more) steps to specificity and diversity. Cell 1991; 67: 1033-6.
[97] Jacob SS, Shastry P, Sudhakaran PR. Monocyte-macrophage differentiation *in vitro*: modulation by extracellular matrix protein substratum. Mol Cell Biochem 2002; 233: 9-17.
[98] Kindle L, Rothe L, Kriss M, Osdoby P, Collin-Osdoby P. Human microvascular endothelial cell activation by IL-1 and TNF-α stimulates the adhesion and transendothelial migration of circulating human $CD14^+$ monocytes that develop with RANKL into functional osteoclasts. J Bone Miner Res 2006; 21: 193-206.
[99] Uchide N, Ohyama K, Bessho T, Yuan B, Yamakawa T. Apoptosis in cultured human fetal membrane cells infected with influenza virus. Biol Pharm Bull 2002; 25: 109-14.
[100] Uchide N, Ohyama K, Bessho T, Toyoda H. Lactate dehydrogenase leakage as a marker for apoptotic cell degradation induced by influenza virus infection in human fetal membrane cells. Intervirology 2009; 52: 164-73.
[101] Kidokoro K, Furuhashi M, Kuno N, Ishikawa K. Amniotic fluid neutrophil elastase and lactate dehydrogenase: association with histologic chorioamnionitis. Acta Obstet Gynecol Scand 2006; 85: 669-74.
[102] Madazli R, Atiş A, Uzun H, Aksu F. Mid-trimester amniotic fluid angiogenin, lactate dehydrogenase and fibronectin in the prediction of preterm delivery. Eur J Obstet Gynecol Reprod Biol 2003; 106: 160-4.
[103] Uchide N, Ohyama K, Yuan B, Bessho T, Yamakawa T. Differentiation of monocytes to macrophages induced by influenza virus-infected apoptotic cells. J Gen Virol 2002; 83: 747-51.
[104] Uchide N, Ohyama K, Yuan B, Sano T, Bessho T, Yamakawa T. Differential mRNA expression of inflammatory cytokines in cultured human fetal membrane cells responding to influenza virus infection. Biol Pharm Bull 2002; 25: 239-43.
[105] Uchide N, Suzuki A, Ohyama K, Bessho T, Toyoda H. Secretion of bioactive interleukin-6 and tumor necrosis factor-α proteins from primary cultured human fetal membrane chorion cells infected with influenza virus. Placenta 2006; 27: 678-90.
[106] Uchide N, Tadera C, Sarai H, Ohyama K, Bessho T, Toyoda H. Characterization of monocyte differentiation-inducing (MDI) factors derived from human fetal membrane chorion cells undergoing apoptosis after influenza virus infection. Int J Biochem Cell Biol 2006; 38: 1926-38.
[107] Uchide N, Ohyama K, Bessho T, Toyoda H. Effects of mitogen-activated protein kinase inhibitors on tumor necrosis factor-α gene expression and apoptosis induction in cultured human fetal membrane chorion cells infected with influenza virus. Intervirology 2007; 50: 99-107.
[108] Uchide N, Toyoda H. In: Demasi AR, Ed. Cellular Signaling and Apoptosis Research. New York: Nova Science Publishers Inc. 2007; pp. 91-128.
[109] Uchide N, Toyoda H. Current status of monocyte differentiation-inducing (MDI) factors derived from human fetal membrane chorion cells undergoing apoptosis after influenza virus infection. Gene Regul Systs Biol 2007; 1: 295-302.
[110] Uchide N, Toyoda H. Significance of monocyte differentiation-inducing (MDI) factor in phagocytosis of apoptotic cells induced by influenza virus infection. Curr Topics Virol 2008; 7: 33-46.
[111] Uchide N, Ohyama K, Bessho T, Toyoda H. Induction of pro-inflammatory cytokine gene expression and apoptosis in human chorion cells of fetal membranes by influenza virus infection: possible implications for maintenance and interruption of pregnancy during infection. Med Sci Monit 2005; 11: RA7-16.
[112] Uchide N, Toyoda H. In: Canfield RN, Ed. Infectious Pregnancy Complications. New York: Nova Science Publishers Inc. 2009; pp. 111-38.
[113] Hofmann P, Sprenger H, Kaufmann A, *et al*. Susceptibility of mononuclear phagocytes to influenza A virus infection and possible role in the antiviral response. J Leukoc Biol 1997; 61: 408-14.
[114] Sprenger H, Meyer RG, Kaufmann A, Bussfeld D, Rischkowsky E, Gemsa D. Selective induction of monocyte and not neutrophil-attracting chemokines after influenza A virus infection. J Exp Med 1996; 184: 1191-6.
[115] Fesq H, Bacher M, Nain M, Gemsa D. Programmed cell death (apoptosis) in human monocytes infected by influenza A virus. Immunobiology 1994; 190: 175-82.
[116] Matsukura S, Kokubu F, Noda H, Tokunaga H, Adachi M. Expression of IL-6, IL-8, and RANTES on human bronchial epithelial cells, NCI-H292, induced by influenza virus A. J Allergy Clin Immunol 1996; 98: 1080-7.
[117] Brydon EW, Smith H, Sweet C. Influenza A virus-induced apoptosis in bronchiolar epithelial (NCI-H292) cells limits pro-inflammatory cytokine release. J Gen Virol 2003; 84: 2389-400.
[118] Harper SA, Fukuda K, Uyeki TM, Cox NJ, Bridges CB; Advisory Committee on Immunization Practices (ACIP), Centers for Disease Control and Prevention (CDC). Prevention and control of influenza. Recommendations of the Advisory Committee on Immunization Practices (ACIP). MMWR Recomm Rep 2005; 54: 1-40.
[119] Hayden FG. Perspectives on antiviral use during pandemic influenza. Philos Trans R Soc Lond B Biol Sci 2001; 356: 1877-84.
[120] Regoes RR, Bonhoeffer S. Emergence of drug-resistant influenza virus: population dynamical considerations. Science 2006; 312: 389-91.
[121] Hayden FG. Antivirals for influenza: historical perspectives and lessons learned. Antiviral Res 2006; 71: 372-8.
[122] Stiver G. The treatment of influenza with antiviral drugs. CMAJ 2003; 168: 49-56.
[123] Hayden FG. Antiviral resistance in influenza viruses – implications for management and pandemic response. N Engl J Med 2006; 354: 785-8.

[124] Wray SK, Gilbert BE, Noall MW, Knight V. Mode of action of ribavirin: effect of nucleotide pool alterations on influenza virus ribonucleoprotein synthesis. Antiviral Res 1985; 5: 29-37.
[125] Wong SS, Yuen KY. Avian influenza virus infections in humans. Chest 2006; 129: 156-68.
[126] Bright RA, Medina MJ, Xu X, *et al*. Incidence of adamantane resistance among influenza A (H3N2) viruses isolated worldwide from 1994 to 2005: a cause for concern. Lancet 2005; 366: 1175-81.
[127] Hayden FG, Belshe RB, Clover RD, Hay AJ, Oakes MG, Soo W. Emergence and apparent transmission of rimantadine-resistant influenza A virus in families. N Engl J Med 1989; 321: 1696-702.
[128] Mast EE, Harmon MW, Gravenstein S, *et al*. Emergence and possible transmission of amantadine-resistant viruses during nursing home outbreaks of influenza A (H3N2). Am J Epidemiol 1991; 134: 988-97.
[129] Bright RA, Shay DK, Shu B, Cox NJ, Klimov AI. Adamantane resistance among influenza A viruses isolated early during the 2005-2006 influenza season in the United States. JAMA 2006; 295: 891-4.
[130] Choi WY, Kim S, Lee N, *et al*. Amantadine-resistant influenza A viruses isolated in South Korea from 2003 to 2009. Antiviral Res 2009; 84: 199-202.
[131] Bai GR, Chittaganpitch M, Kanai Y, *et al.* Amantadine- and oseltamivir-resistant variants of influenza A viruses in Thailand. Biochem Biophys Res Commun 2009; 390(3): 897-901.
[132] Furuse Y, Suzuki A, Kamigaki T, Shimizu M, Fuji N, Oshitani H. Reversion of influenza A (H3N2) virus from amantadine resistant to amantadine sensitive by further reassortment in Japan during the 2006-to-2007 influenza season. J Clin Microbiol 2009; 47: 841-4.
[133] Moscona A. Oseltamivir resistance – disabling our influenza defenses. N Engl J Med 2005; 353: 2633-6.
[134] Monto AS, McKimm-Breschkin JL, Macken C, *et al*. Detection of influenza viruses resistant to neuraminidase inhibitors in global surveillance during the first 3 years of their use. Antimicrob Agents Chemother 2006; 50: 2395-402.
[135] Kiso M, Mitamura K, Sakai-Tagawa Y, *et al*. Resistant influenza A viruses in children treated with oseltamivir: descriptive study. Lancet 2004; 364: 759-65.
[136] Herlocher ML, Truscon R, Elias S, *et al*. Influenza viruses resistant to the antiviral drug oseltamivir: transmission studies in ferrets. J Infect Dis 2004; 190: 1627-30.
[137] de Jong MD, Tran TT, Truong HK, *et al*. Oseltamivir resistance during treatment of influenza A (H5N1) infection. N Engl J Med 2005; 353: 2667-72.
[138] Le QM, Kiso M, Someya K, *et al.* Avian flu: isolation of drug-resistant H5N1 virus. Nature 2005; 437: 1108.
[139] Sidwell RW, Huffman JH, Bailey KW, Wong MH, Nimrod A, Panet A. Inhibitory effects of recombinant manganese superoxide dismutase on influenza virus infections in mice. Antimicrob Agents Chemother 1996; 40: 2626-31.
[140] Serkedjieva J, Roeva I, Angelova M, Dolashka P, Voelter WG. Combined protective effect of a fungal Cu/Zn-containing superoxide dismutase and rimantadine hydrochloride in experimental murine influenza a virus infection. Acta Virol 2003; 47: 53-6.
[141] Akaike T, Ando M, Oda T, *et al*. Dependence on O_2^- generation by xanthine oxidase of pathogenesis of influenza virus infection in mice. J Clin Invest 1990; 85: 739-45.
[142] Shi Y, Niculescu R, Wang D, Patel S, Davenpeck KL, Zalewski A. Increased NAD(P)H oxidase and reactive oxygen species in coronary arteries after balloon injury. Arterioscler Thromb Vas Biol 2001; 21: 739-45.
[143] Uchide N, Ohyama K. Antiviral function of pyrrolidine dithiocarbamate against influenza virus: the inhibition of viral gene replication and transcription. J Antimicrob Chemother 2003; 52: 8-10.
[144] Uchide N, Ohyama K, Bessho T, Yuan B, Yamakawa T. Effect of antioxidants on apoptosis induced by influenza virus infection: inhibition of viral gene replication and transcription with pyrrolidine dithiocarbamate. Antiviral Res 2002; 56: 207-17.
[145] Knobil K, Choi AM, Weigand GW, Jacoby DB. Role of oxidants in influenza virus-induced gene expression. Am J Physiol 1998; 274: L134-42.
[146] Lowy RJ, Dimitrov DS. Characterization of influenza virus-induced death of J774.1 macrophages. Exp Cell Res 1997; 234: 249-58.
[147] McKinney LC, Galliger SJ, Lowy RJ. Active and inactive influenza virus induction of tumor necrosis factor-α and nitric oxide in J774.1 murine macrophages: modulation by interferon-γ and failure to induce apoptosis. Virus Res 2003; 97: 117-26.
[148] Chen K, Long YM, Wang H, Lan L, Lin ZH. Activation of nuclear factor-kappa B and effects of pyrrolidine dithiocarbamate on TNBS-induced rat colitis. World J Gastroenterol 2005; 11: 1508-14.
[149] Cuzzocrea S, Chatterjee PK, Mazzon E, *et al*. Pyrrolidine dithiocarbamate attenuates the development of acute and chronic inflammation. Br J Pharmacol 2002; 135: 496-510.
[150] Mallick IH, Yang WX, Winslet MC, Seifalian AM. Pyrrolidine dithiocarbamate reduces ischemia-reperfusion injury of the small intestine. World J Gastroenterol 2005; 11: 7308-13.
[151] Yang CH, Fang IM, Lin CP, Yang CM, Chen MS. Effects of the NF-kappaB inhibitor pyrrolidine dithiocarbamate on experimentally induced autoimmune anterior uveitis. Invest Ophthalmol Vis Sci 2005; 46: 1339-47.
[152] Kim CH, Kim JH, Hsu CY, Ahn YS. Zinc is required in pyrrolidine dithiocarbamate inhibition of NF-kappaB activation. FEBS Lett 1999; 449: 28-32.
[153] Oxford JS, Perrin DD. Inhibition of the particle-associated RNA-dependent RNA polymerase activity of influenza viruses by chelating agents. J Gen Virol 1974; 23:59-71.
[154] Si X, McManus BM, Zhang J, *et al*. Pyrrolidine dithiocarbamate reduces coxsackievirus B3 replication through inhibition of the ubiquitin-proteasome pathway. J Virol 2005; 79: 8014-23.
[155] Krenn BM, Holzer B, Gaudernak E, Triendl A, van Kuppeveld FJ, Seipelt J. Inhibition of polyprotein processing and RNA replication of human rhinovirus by pyrrolidine dithiocarbamate involves metal ions. J Virol 2005; 79: 13892-9.
[156] Kelly GS. Clinical applications of *N*-acetylcysteine. Altern Med Rev 1998; 3: 114-27.
[157] Saito T, Tanaka M, Yamaguchi I. Effect of brefeldin A on influenza A virus-induced apoptosis *in vitro*. J Vet Med Sci 1996; 58: 1137-9.
[158] Lin C, Zimmer SG, Lu Z, Holland RE Jr, Dong Q, Chambers TM. The involvement of a stress-activated pathway in equine influenza virus-mediated apoptosis. Virology 2001; 287: 202-13.
[159] Geiler J, Michaelis M, Naczk P, *et al*. *N*-acetyl-l-cysteine (NAC) inhibits virus replication and expression of pro-inflammatory molecules in A549 cells infected with highly pathogenic H5N1 influenza A virus. Biochem Pharmacol 2009; 199: 93-101. [cited 2009 Nov 18]. Available from: http://www.sciencedirect.com/science?_ob=ArticleURL&_udi=B6T4P-4X4RWMX-2&_user=999637&_rdoc=1&_fmt=&_orig=s earch&_sort=d&_docanchor=&view=c&_acct=C000050100&_version=1&_urlVersion=0&_userid=999637&md5=5a709cc2a979dfe1ec5e2c78c4fa8276
[160] Ungheri D, Pisani C, Sanson G, *et al*. Protective effect of *N*-acetylcysteine in a model of influenza infection in mice. Int J Immunopathol Pharmacol 2000; 13: 123-8.
[161] Ghezzi P, Ungheri D. Synergistic combination of *N*-acetylcysteine and ribavirin to protect from lethal influenza viral infection in a mouse model. Int J Immunopathol Pharmacol 2004; 17: 99-102.
[162] De Flora S, Grassi C, Carati L. Attenuation of influenza-like symptomatology and improvement of cell-mediated immunity with long-term *N*-acetylcysteine treatment. Eur Respir J 1997; 10: 1535-41.
[163] Cai J, Chen Y, Seth S, Furukawa S, Compans RW, Jones DP. Inhibition of influenza infection by glutathione. Free Radic Biol Med 2003; 34: 928-36.
[164] Nencioni L, Iuvara A, Aquilano K, *et al*. Influenza A virus replication is dependent on an antioxidant pathway that involves GSH and Bcl-2. FASEB J 2003; 17: 758-60.
[165] Floriano-Sanchez E, Villanueva C, Medina-Campos ON, *et al*. Nordihydroguaiaretic acid is a potent *in vitro* scavenger of peroxynitrite, singlet oxygen, hydroxyl radical, superoxide anion and hypochlorous acid and prevents *in vivo* ozone-induced tyrosine nitration in lungs. Free Radic Res 2006; 40: 523-33.
[166] Uchide N, Ohyama K, Bessho T, Toyoda H. Inhibition of influenza virus-induced apoptosis in chorion cells of human fetal membranes by nordihydroguaiaretic acid. Intervirology 2005; 48: 336-340.
[167] Craigo J, Callahan M, Huang RC, DeLucia AL. Inhibition of human papillomavirus type 16 gene expression by nordihydroguaiaretic acid plant lignan derivatives. Antiviral Res 2000; 47: 19-28.

[168] Tagaya M, Henomatsu N, Yoshimori T, Yamamoto A, Tashiro Y, Mizushima S. Inhibition of vesicle-mediated protein transport by nordihydroguaiaretic acid. J Biochem 1996; 119: 863-9.

[169] Nozoe T. Uber die farbstoffe im holzteile des "Hinoki" baumes. I. hinokitin und hinokitiol. Bull Chem Soc Jpn 1936; 11: 295-8.

[170] Miyamoto D, Kusagaya Y, Endo N, *et al*. Thujaplicin-copper chelates inhibit replication of human influenza viruses. Antiviral Res 1998; 39: 89-100.

[171] Arima Y, Hatanaka A, Tsukihara S, Fujimoto K, Fukuda K, Sakurai H. Scavenging activities of α-, β- and γ-thujaplicins against active oxygen species. Chem Pharm Bull 1997; 45: 1881-6

[172] Martinez J, Moreno JJ. Effect of resveratrol, a natural polyphenolic compound, on reactive oxygen species and prostaglandin production. Biochem Pharmacol 2000; 59: 865-70.

[173] Palamara AT, Nencioni L, Aquilano K, *et al*. Inhibition of influenza A virus replication by resveratrol. J Infect Dis 2005; 191: 1719-29.

[174] Germouty J, Jirou-Najou JL. Clinical efficacy of ambroxol in the treatment of bronchial stasis. Clinical trial in 120 patients at two different doses. Respiration 1987; 51: 37-41.

[175] Yang B, Yao DF, Ohuchi M *et al*. Ambroxol suppresses influenza-virus proliferation in the mouse airway by increasing antiviral factor levels. Eur Respir J 2002; 19: 952-8.

[176] Gillissen A, Bartling A, Schoen S, Schultze-Werninghaus G. Antioxidant function of ambroxol in mononuclear and polymorphonuclear cells *in vitro*. Lung 1997; 175: 235-42.

[177] Gillissen A, Schärling B, Jaworska M, Bartling A, Rasche K, Schultze-Werninghaus G. Oxidant scavenger function of ambroxol *in vitro*: a comparison with *N*-acetylcysteine. Res Exp Med (Berl) 1997; 196: 389-98.

[178] Robak J, Gryglewski RJ. Bioactivity of flavonoids. Pol J Pharmacol 1996; 48: 555-64.

[179] Nagai T, Miyaichi Y, Tomimori T, Yamada H. Inhibition of mouse liver sialidase by plant flavonoids. Biochem Biophys Res Commun 1989; 163: 25-31.

[180] Nagai T, Miyaichi Y, Tomimori T, Suzuki Y, Yamada H. Inhibition of influenza virus sialidase and anti-influenza virus activity by plant flavonoids. Chem Pharm Bull (Tokyo) 1990; 38: 1329-32.

[181] Nagai T, Miyaichi Y, Tomimori T, Suzuki Y, Yamada H. *In vivo* anti-influenza virus activity of plant flavonoids possessing inhibitory activity for influenza virus sialidase. Antiviral Res 1992; 19: 207-17.

[182] Nagai T, Suzuki Y, Tomimori T, Yamada H. Antiviral activity of plant flavonoid, 5,7,4'-trihydroxy-8-methoxyflavone, from the roots of Scutellaria baicalensis against influenza A (H3N2) and B viruses. Biol Pharm Bull 1995; 18: 295-9.

[183] Nagai T, Moriguchi R, Suzuki Y, Tomimori T, Yamada H. Mode of action of the anti-influenza virus activity of plant flavonoid, 5,7,4'-trihydroxy-8-methoxyflavone, from the roots of Scutellaria baicalensis. Antiviral Res 1995; 26: 11-25.

[184] Song JM, Lee KH, Seong BL. Antiviral effect of catechins in green tea on influenza virus. Antiviral Res 2005; 68: 66-74.

[185] Kashima M. Effects of catechins on superoxide and hydroxyl radical. Chem Pharm Bull (Tokyo) 1999; 47: 279-83.

[186] Yamada H, Takuma N, Daimon T, Hara Y. Gargling with tea catechin extracts for the prevention of influenza infection in elderly nursing home residents: a prospective clinical study. J Altern Complement Med 2006; 12: 669-72.

[187] Mori S, Miyake S, Kobe T, *et al*. Enhanced anti-influenza A virus activity of (-)-epigallocatechin-3-O-gallate fatty acid monoester derivatives: effect of alkyl chain length. Bioorg Med Chem Lett 2008; 18: 4249-52.

[188] Choi HJ, Song JH, Park KS, Kwon DH. Inhibitory effects of quercetin 3-rhamnoside on influenza A virus replication. Eur J Pharm Sci 2009; 37: 329-33.

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