Original Article

Nitrostative stress status during seasonal and pdmH1N1 infection in Iraq

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Abstract

Introduction: Influenza A virus infection is associated with oxidative and nitrosative stress. This study aimed to assess nitrosative stress in pandemic H1N1 (pdmH1N1) and seasonal influenza A infected patients.

Methodology: The study included the following subjects: 20 patients infected with seasonal (negative one-step probe RT-PCR) influenza and 12 patients infected with pdmH1N1 (positive, one-step probe RT-PCR) influenza during the 2009 pandemic in Iraq. Twenty healthy subjects served as controls. Serum nitric oxide using Greiss reagent and peroxynitrite were used to assess nitrosative stress status.

Results: Serum nitric oxide and peroxynitrite are significantly increased in patients infected with seasonal and pdmH1N1 influenza compared with the levels in healthy subjects. Infected patients with seasonal influenza showed significantly higher numbers of serum nitrogen species than corresponding pdmH1N1 infected patients. The turnover process reflected by the peroxynitrite/nitric oxide ratio was 0.177, 0.313 and 0.214 in healthy subjects, seasonal and pdmH1N1infected patients respectively.

Conclusions: Influenza A virus infection is associated with significant nitrosative stress activity which is more pronounced in seasonal than in pdmH1N1 infected patients. The determination of serum nitric oxide and peroxynitrite may serve as biochemical markers.

Key words: nitric oxide; peroxynitrite; pdmH1N1

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Introduction

Influenza A is a single-stranded negative sense RNA virus of the genus orthomyxiviridae that encodes eight major genes, including two major surface hemagglutinin (16) and nuraminidase (9) subtypes. From 19 April to 12 September 2009, the majority (60.6%) of all influenza specimens reported to World Health Organization (WHO) were pdmH1N1 or S-OIV, viruses [1]. As of 3 October 2009, a total of 12,848 laboratory-confirmed cases of pandemic A/H1N1 subtype were reported to the WHO by 21 out of 22 countries in the Mediterranean region, and185 cases including two deaths were reported from Iraq [2]. Influenza virus infection is characterized by the abrupt onset of constitutional and respiratory signs including fever, myalgia, headache, non-productive cough, sore throat and rhinitis. A current method used to detect A/H1N1 in the clinical practice is direct fluorescent antigen (DFA) [3], a rapid screening test based on visualization of a colorimetric disposable single use card.

Influenza virus directly infects human natural killer (NK) cells and induces cell apoptosis to counter their function [4]. Both the intact influenza virion and free hemagglutinin protein inhibit the cytotoxicity of fresh and interleukin-2 (IL-2)-activated primary human NK cells [5]. From the immunological point of the view, there is a link between the nitric oxide as an immune modulator and interleukin-2 as a proinflammatory marker. Cytokines secreted from IL-2-activated lymphocytes are involved in the production of nitric oxide which is responsible for macrophage apoptosis [6]. Influenza virus infection causes an intense infiltration of pulmonary tissues by macrophages, which abundantly generate a free radical, nitric oxide (NO), resulting in lung damage, and the antiviral neuraminidase inhibitors suppress the NO production [7]. Li *et al*. (2009) reported that the inducible nitric oxide synthetase (iNOS) was increased two- to four-fold in the samples of patients infected with influenza A virus in comparison with healthy individuals [8]. On the other hand, nitric oxide inhalation is one therapeutic model in the advanced treatment of severe respiratory failure attributable to pdmH1N1 [9,10].

This study aimed to determine the sera nitrogen species of suspect patients infected with

pdmH1N1and seasonal influenza A who were referred to Al-Yarmouk Teaching Hospital in Baghdad during October 2009.

Methodology

This study was conducted in the Department of Pharmacology, College of Medicine, in cooperation with Department of Biology, College of Science, Al-Mustansiriya University in Baghdad, Iraq, from January to March 2010. The study protocol was approved by the Scientific Committee of the College of Medicine, Al-Mustansiriya University. An informed consent form to participate in the study was obtained from patients or their proxies. Patients who referred to the Al-Yarmouk Teaching Hospital, the center that dealt with the diagnosis and management of pdmH1N1 infected patients during the pandemic of October 2009, were allocated randomly to enroll in this study. Each patient was thoroughly examined and subjected to laboratory and radiological examination. The diagnosis of pdmH1N1 infection was confirmed by positive RT-PCR testing sputum as well as oropharyngeal and nasopharyngeal swab specimens using the quantitative one-step RT-PCR probe (Invitrogen Superscript™111 Platinum® One-Step Quantitative Kit, Biosearch Technologies Inc, Novato, CA, USA) RT-PCR was performed in the Central laboratory of Health in Baghdad, Iraq. A total of 12 patients (male and female) who were positive by respiratory sample testing for pdmH1N1 and 20 patients (13 male and 7 female) who tested negative and therefore considered as having seasonal influenza A were admitted in the study. A group of 20 healthy subjects (male and female) served as controls. A venous blood sample was obtained under sterile conditions from each patient for determination of serum nitric oxide and peroxynitrite. Peroxynitrite mediated nitration of phenol was measured (an index of $ONOO^-$ release) as described by Beckman *et al.* [11] and cited by Van Uffelen *et al.* [12]. Briefly, 50 µL was added to 5mM phenol in 50 mM sodium phosphate buffer pH 7.4 in a final volume of 3 mL. After incubation for 2 hours at 37ºC, 50 µL of 0.1 M sodium hydroxide was added, and the absorbance at 412 nm of each sample was immediately recorded. The yield of nitrophenol was calculated from $\varepsilon = 4400\ M^{-1}.cm^{-1}$. All experiments were performed in duplicate.

Nitric oxide donating activity was determined as described by Newaz *et al.* [13] utilizing Greiss reagent. Briefly 0.5 mL serum was added to 50 µL HCl (6.5M) and 50 µL sulfunalic acid (37.5 mM). After incubation for 10 minutes, 50 µL of naphthylethylenediamine dihydrochloride (12.5 mM) was added and incubated for a further 30 minutes then centrifuged for 10 minutes at 1000 g. The reference nitric oxide donating compound was lithium nitrite. The absorbance at 540 nm was immediately recorded. All experiments were performed in duplicate.

Statistical analysis

The results were expressed as number, range, and median with a 95% confidence interval ($_{95\%}$ C.I.) and mean ± SD of number of observations. The data were analyzed using the two-tailed Student's unpaired "t" test taking $p \leq 0.05$ as the lowest limit of significance. Any value beyond the upper and the lower limits of $_{95\%}$ C.I. of the healthy subjects group was considered significant at the probability level of < 0.05.

Results

The clinical features of influenza A infection observed in both the seasonal and pdmH1N1 patients in our study were fever, myalgia, bone pain, sore throat, dyspnea. None of the patients suffered respiratory distress that necessitated admission to the intensive care unit. Table 1 shows the characteristics of the study. There was no significant difference between the groups regarding their ages and their residency. The median values of nitric oxide in patients with seasonal influenza A and patients with pdmH1N1 were three- and two-fold of those of the healthy subjects respectively. The mean serum level of nitric oxide was significantly ($p < 0.001$) higher in patients with influenza A virus infection whether it was pdmH1N1 or seasonal influenza (Table 2). Further analysis revealed significantly high serum nitric oxide levels among patients with seasonal influenza A compared with those of pdmH1N1 infected patients. Three out of twelve patients infected with pdmH1N1 had serum nitric oxide levels within the 95% confidence interval of seasonal influenza A, and 9 out of 12 patients had significantly lower levels (Table 2). The median serum peroxynitrite was higher than that of nitric oxide. The median serum peroxynitrite was approximately six- and three-fold times higher in patients infected with seasonal and pdmH1N1 respectively compared with that of the healthy subjects (Table3). The mean serum peroxynitrite was significantly higher in patients infected with seasonal influenza A than that in pdmH1N1 patients and healthy subjects (Table 3).

Table 1. The characteristics of the study

Variables	**Healthy subjects (n = 20)**	**Patients with seasonal influenza A (n = 20)**	**Patients with pdmH1N1 infleunza A (n = 12)**
Gender (male:female)	13:7	8:12	4:8
Age (year)			
Median	31	34	31
Mean ± SD	32.6 5± 7.32	32.95 ± 8.03	29.66 ± 10.94
Residency			
Baghdad	20	18	10
South of Iraq	0	2	2

Table 2. Serum nitric oxide level (μMol)

Patient number	**Healthy subjects (n = 20)**	**Seasonal influenza A patients (n = 20)**	**pdmH1N1 positive influenza A patients (n = 12)**
Min-Max	20.2-70.2	59.7-191	61-141
Median	45.05	134.9	96.45
Mean ± SD	45.89±13.09	127.44±39.23*†	97.89±22.80*
95% C.I.	40.159-51.531	110.253-144.637	75.089-120.693

***** $p < 0.001$ in comparison with healthy subjects, † $p < 0.01$ in comparison with pdmH1N1 positive influenza A patients

Table 3. Serum peroxynitrite level (μMol)

Patient number	**Healthy subjects (n = 20)**	**Seasonal influenza A patients (n = 20)**	**pdmH1N1 positive influenza A patients (n = 12)**
Min-Max	3.86-17.27	8.18-78.86	4.09-40.45
Median	7.38	42.38	20.11
Mean ± SD	8.136±3.63	39.89±21.05*§	20.98±11.96†
95% C.I.	6.545-9.727	30.669-49.125	14.215-27.753

***** $p < 0.001$ in comparison with healthy subjects, † $p < 0.004$ in comparison with healthy subjects, § $p < 0.003$ in comparison with pdmH1N1 positive influenza A patients

Four out of twelve patients infected with pdmH1N1 had serum peroxynitrite levels within the 95% confidence interval level of patients infected with seasonal influenza A, and only one case had a significantly higher level (Table 3). The turnover of peroxynitrite-nitric oxide cycle as expressed by the ratio of mean serum peroxynitrite to mean nitric oxide was accelerated in influenza A infection, which amounted 0.177, 0.313 and 0.214 for healthy subjects, seasonal and pdmH1N1 infected patients respectively.

Discussion

The present study clearly demonstrates the significant production of nitrogen species in influenza A infected patients, which tended to be higher in seasonal influenza patients compared to subjects with pdmH1N1. One study found that the signal magnitude of free radical detected by electron spin resonance was significantly higher in mice infected with influenza virus than in healthy control mice, denoting oxidative stress level [14]. This observation indicates that influenza A virus infection is associated with nitrosative stress. Further evidence of involvement of nitrosative stress in influenza A is the demonstration of strong 8-nitroguanosine immuno-staining in the cytosol of bronchial and bronchiolar epithelial cells of influenza virus-infected wild-type mice but not iNOS-deficient mice [15]. Moreover, a number of studies have demonstrated that reactive oxygen species contribute to the exacerbation of lung inflammation and lethality in influenza-infected mice, while the use of glutathione precursor promotes survival in influenza-infected mice [16,17]. The results reported in this work are in agreement with those of a study by Li *et al*., who demonstrated a 2.4-fold increase in nitric oxide production in patients infected with influenza A [8], and also showed that the production of nitric oxide in pdmH1N1 was significantly less than that of seasonal influenza A. The results of our study further show that the process of non enzymatic production of peroxynitrite from superoxide anion and nitric oxide is significantly accelerated in seasonal and to a lesser extent in pdmH1N1. The clinical utility of these results rely on the possible application of serum nitrogen species as a biomarker in a variety of influenza A infections.

One of the limitations of the current study is the small sample size of pdmH1N1 which represents 6.5% (12 out of 185) of all reported cases of swine influenza virus (A/H1N1 subtype) during 2009 in Iraq according to the WHO report. Therefore, further study involving a large sample size is recommended to look for the associated changes of serum cytokines.

We conclude that the influenza A virus infection is associated with nitrosative stress, which is more pronounced in seasonal than in pdmH1N1 and presents with significantly high levels of serum nitric oxide and peroxynitrite. The determination of serum nitrogen species may serve as biomarkers in influenza A virus infection.

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