ACTA MICROBIOLOGICA BULGARICA

Therapeutic Strategy for Survival of Mice Infected with Influenza Virus by Combination of S-Adenosyl-L-Methionine and Oseltamivir

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Abstract

Influenza is a highly contagious viral infection of the respiratory system. Many studies provide compelling evidence that the abnormal production of reactive oxygen species is a crucial mediator of acute lung injury in influenza A virus infection. Therefore, antioxidants are potentially useful against this ongoing clinical problem. Our studies showed that S-adenosyl-L-methionine (SAM) has a protective effect in a model of influenza infection in mice. This substance converts into glutathione - the main antioxidant in the body, through a multistep biochemical cycle. In the present study, we report the effect of combined treatment with SAM and the antiviral agent oseltamivir in infected with influenza A virus mice. SAM was given as a single daily dose of 50 and 100 mg/kg in different mice groups, starting from 5 days before infection until day 4 after infection. Oseltamivir was given in a dose of 2.5 mg/kg daily in two intakes for 5 days, starting from 4 h before infection. End-point evaluation was 14 - day survival. Survival was 70% in the treatment with oseltamivir and rose to 90% in the treatment with oseltamivir and SAM in both doses. SAM alone did not show any antiviral activity. The present findings suggest that therapy with molecules converted into antioxidants in the body increases survival by modulating the host defense mechanisms and by a direct antioxidant effect against oxidative stress associated with viral infections. This study demonstrated the effectiveness of combining agents that act through different mechanisms - antiviral drug oseltamivir as a specific neuraminidase inhibitor of influenza virus, and SAM as a precursor of the most important antioxidant - glutathione.

Key words: influenza, oxidative stress, antioxidant, glutathione, S-adenosyl-L-methionine, oseltamivir

Резюме

Грипът е остра инфекциозна вирусна инфекция на дихателните пътища. Много изследвания предоставят убедителни доказателства, че абнормната генерация на активни форми на кислорода е фактор от изключителна важност като медиатор на острото белодробно увреждане при грипната инфекция. Затова антиоксидантите са потенциално полезни при този клиничен проблем. Нашите изследвания показват, че S-аденозил-L-метионин (SAM) има протективно действие при експериментален модел на грипна инфекция в мишки. Чрез многоетапен биохимичен цикъл SAM се превръща в глутатион - основния антиоксидант в живата. В настоящото проучване ние отчитахме ефекта на комбинираното действие на SAM и антивирусния препарат оселтамивир в инфектирани с грипен вирус тип А мишки. SAM се прилагаше под формата на единични дневни дози от 50 и 100 мг/кг, започвайки 5 дни преди заразяването и продължавайки 4 дни след него. Оселтамивир се прилагаше в дневна доза от 2.5 мг/кг, разделена в 2 приема, в продължение на 5 дни, като се започваше 4 часа преди заразяването. Референтен пункт в оценката беше 14 дневната преживяемост на мишките, която беше 70% при индивидуалното прилагане на оселтамивир и достигаше до 90% при комбинация на оселтамивир и SAM и в двете дози. SAM не показа антивирусна активност. Получените резултати подсказват, че лечението с молекули, които се превръщат в антиоксиданти в организма, увеличава преживяемостта чрез модулиране на защитните механизми на гостоприемника. Изследването

демонстрира ефективността на комбинирането на средства, които действат чрез различни механизми – антивирусния препарат оселтамивир като специфичен инхибитор на невраминидазата на грипния вирус, и SAM като прекурсор на най-важния антиоксидант - глутатион.

Introduction

Influenza virus infection is a major public health problem, occurring typically in the Northern Hemisphere between the months of December and April. Epidemics of influenza are characterized by an increased morbidity and mortality in the community (Monto *et al.,* 2000). Influenza viruses encompass a major group of human and animal pathogens belonging to enveloped, segmented, negative-strand RNA viruses (Biswas *et al.,* 1998). They have a multipartite, negative-sense, single-stranded RNA genome and a lipid envelope (Beigel and Bray, 2008) and are one of the rare RNA viruses to replicate in the nucleus (Boulo *et al*., 2007). Current pharmacological strategies to control influenza A virus - induced lung disease are problematic owing to antiviral resistance and the requirement for strain-specific vaccination. The production of reactive oxygen species (ROS), particularly superoxide, is an important host defense mechanism for killing invading pathogens. However, excessive superoxide may be detrimental following influenza A virus infection (Vlahos, 2012).

Oseltamivir is a potent and selective inhibitor of the neuraminidase enzyme of the influenza viruses A and B. The neuraminidase enzyme is responsible for cleaving sialic acid residues on newly formed virions and plays an essential role in the release and spread of progeny virions (Kamps and Hoffman, 2006).

S-Adenosyl-L-methionine (SAM, also known as AdoMet and SAMe) is an important molecule that is found in all living organisms. The importance of SAM stems from the fact that SAM is the principal biological methyl donor, the precursor of aminopropyl groups utilized in polyamine biosynthesis and, in the liver, SAM is also a precursor of glutathione (GSH) through its conversion to cysteine via the transsulfuration pathway (Lu, 2000). The tripeptide glutathione is the most abundant thiol present in mammalian cells. GSH has important functions as an antioxidant. The glutathione system is especially important for cellular defense against ROS. GSH reacts directly with radicals in nonenzymatic reactions and is the electron donor in the reduction of peroxides catalyzed by glutathione peroxidase (GPx) (Dringen *et al.,* 2000). Glutathione serves as the major scavenger of reactive oxygen species. Certain lymphocyte functions, such as the DNA synthetic response, are exquisitely sensitive to reactive oxygen species and, therefore, are favored by relatively high levels of glutathione. Even a moderate depletion of the intracellular glutathione pool has dramatic consequences for a variety of lymphocyte functions (Droge and Breitkreutz, 2000).

Our previous research confirmed that using S-adenosyl-L-methionine (SAM) did not have a positive response in influenza infected mice.

The combined therapy of influenza is a question of particular interest. Garozzo *et al.* (2007) achieved up to 100% survival rate in mice infected with influenza virus and treated with a combination of N-acetylcysteine as a precursor of glutathione in a dose of 1000 mg/kg and oseltamivir in a dose of 1 mg/kg.

Hence, the present study focuses on the analysis of the positive effect when combining oseltamivir as a specific neuraminidase inhibitor of the influenza virus replication with S-adenosyl-L-methionine as a precursor of glutathione - the most abundant antioxidant in the body. This is a new approach which needs to be studied in details.

Materials and Methods

White male mice of the ICR line with body weight 14–16 g, obtained from Slivnitza Animal Pharm (Bulgarian Academy of Sciences (BAS), Bulgaria), were placed in specially designed, well-ventilated acrylic cage containers, with free access to water and food, and maintained in the Animal House facility of the Stephan Angeloff Institute of Microbiology, BAS. During a 3-day acclimation period (prior to experimental onset), they were observed for any signs of diseases and/or physical abnormalities. Animal husbandry and experiments were conducted in accordance with the guidelines of Bulgaria's Directorate of Health Prevention and Humane Behaviour toward Animals.

For the purpose of the experiment they were anaesthetized with ether and infected intranasally with $10\times LD_{50}$ of an influenza virus strain adapted for mice: A/Aichi/2/68 (H3N2). The experimental groups were designed as follows:

I. Healthy, non-infected animals;

II. Mice infected with influenza virus, non-treated;

III. Mice infected with influenza virus and treated with oseltamivir in a dose of 2.5 mg/kg daily in two intakes, *per os*, for 5 days, starting 4 h before infection and for the subsequent 4 days;

IV. Mice infected with influenza virus and treated with SAM in a dose of 50 mg/kg, *i.p*, once a day starting 5 days before infection and for the subsequent 4 days after infection;

V. Mice infected with influenza virus and treated with SAM in a dose of 100 mg/kg, *i.p*, once a day starting 5 days before infection and for the subsequent 4 days after infection;

VI. Mice infected with influenza virus and treated with oseltamivir in a dose of 2.5 mg/kg daily in two intakes, *per os*, for 5 days, starting 4 h before infection and for the subsequent 4 days and SAM in a dose of 50 mg/kg, *i.p*, once a day starting 5 days before infection and for the subsequent 4 days after infection;

VII. Mice infected with influenza virus and treated with oseltamivir in a dose of 2.5 mg/kg daily in two intakes, *per os*, for 5 days, starting 4 h before infection and for the subsequent 4 days and SAM in a dose of 100 mg/kg, *i.p*, once a day starting 5 days before infection and for the subsequent 4 days after infection.

Mice were observed daily for 14 days for survival after infection.

Results and Discussion

Experimental data on the effect of the combination of oseltamivir plus SAM were compared against control uninfected and untreated animals of group I, and also compared to infected with influenza virus and non-treated animals of group II. The effect of treatment of influenza-infected mice with oseltamivir and SAM used alone and in combination is shown in Table 1.

Table 1. Results of treatment with oseltamivir and SAM on 14 days survival of influenza virus infected mice.

No Group	Experimental groups	Number of survivals/ total	Survival rate (%)
I	Healthy, non-infected animals	10/10	100
II	Mice infected with influenza virus	3/10	30
III	Mice infected with influenza virus and treated with oseltamivir 2.5 mg/kg *per os*	7/10	70
IV	Mice infected with influenza virus and treated with SAM in a dose of 50 mg/kg,*i.p*	1/10	10
V	Mice infected with influenza virus and treated with SAM in a dose of 100 mg/kg, *i.p*	2/10	20
VI	Mice infected with influenza virus and treated with oseltamivir 1.25 mg/kg, *per os*, and SAM in a dose of 50 mg/kg, *i.p*.	9/10	90
VII	Mice infected with influenza virus and treated with oseltamivir 1.25 mg/kg, *per os* and SAM in a dose of 100 mg/kg, *i.p*	9/10	90

The survival rate was estimated from the number of mice who survived till day 14 of the experiment calculated as a percentage of the total number of mice in the experimental group. The number of survivals shows the total number of mice who survived till day 14 of the experiment out of 10 in the experimental group. SAM was applied once a day intraperitoneally in doses of 50 and 100 mg/kg, starting 5 consecutive days before virus inoculation up to day 5 after infection. Oseltamivir was applied *per os* for 5 days after viral infection in a dose of 2.5 mg/kg daily in two intakes.

For statistical analysis of the results one-way ANOVA tests with Bonferroni's post-test were used.

The survival rate of the animals from the non-treated control group II, infected with influenza virus in a dose of 10 LD_{50} was 30%. Treatment with oseltamivir in a dose of 2.5 mg/kg increased the survival rate of the infected mice from 30% to 70%. SAM alone did not have any positive effect. SAM in a dose of 50 mg/kg showed a very low survival

rate - 10%, and in a dose of 100 mg/kg the survival rate slightly increased to 20%. The combination of oseltamivir and SAM showed significantly better protection in mice infected with influenza virus. In both groups – the combinations of oseltamivir in a dose of 2.5 mg/kg and SAM in a dose of 50 mg/kg; and oseltamivir in a dose of 2.5 mg kg and SAM in a dose of 100 mg/kg, 90% of the animals survived till day 14 of the experiment (Fig. 1).

The present study shows the positive effect of combining oseltamivir as a specific neuraminidase inhibitor of the influenza virus replication with S-adenosyl-L-methionine as a precursor of glutathione - the most abundant antioxidant in the body. As our previous research confirmed, SAM alone does not have a positive effect in influenza infected mice. The good results of the combination could be explained by modulation of host defense mechanisms and by direct antioxidant effect of increased glutathione against oxidative stress associated with influenza infection. Many authors indicate that glutathione plays a key role in airway function (Rahman and MacNee, 1995; Cai *et al.,* 2003; Fitzpatrick *et al.,* 2011; Kettle *et al*., 2014). It is also important for the normal function of the T-cell mediated immunity (Droge and Breitkreutz, 2000).

Oseltamivir (Tamiflu®, F. Hoffmann-La Roche Ltd.) is an orally administered antiviral drug that is approved for the treatment of influenza A and B in adults and children (including full term neonates) who present with symptoms typical of influenza when influenza virus is circulating in the community, and for the prophylaxis of influenza in patients aged 1 year or older. Oseltamivir is a prodrug that is administered as a phosphate salt (oseltamivir phosphate; OP). It is then converted by hepatic carboxylesterases to the active metabolite oseltamivir carboxylate (OC). In humans, OP is readily absorbed and converted to OC, which is detectable in plasma within 30 min, and the absolute bioavailability for OC is 80%. Peak plasma concentrations of OC are attained in about 3–4 h, and the apparent half-life is 6–10 h, with elimination primarily through renal excretion of OC (Reddy *et al.,* 2015). When exposed to oseltamivir, the influ-

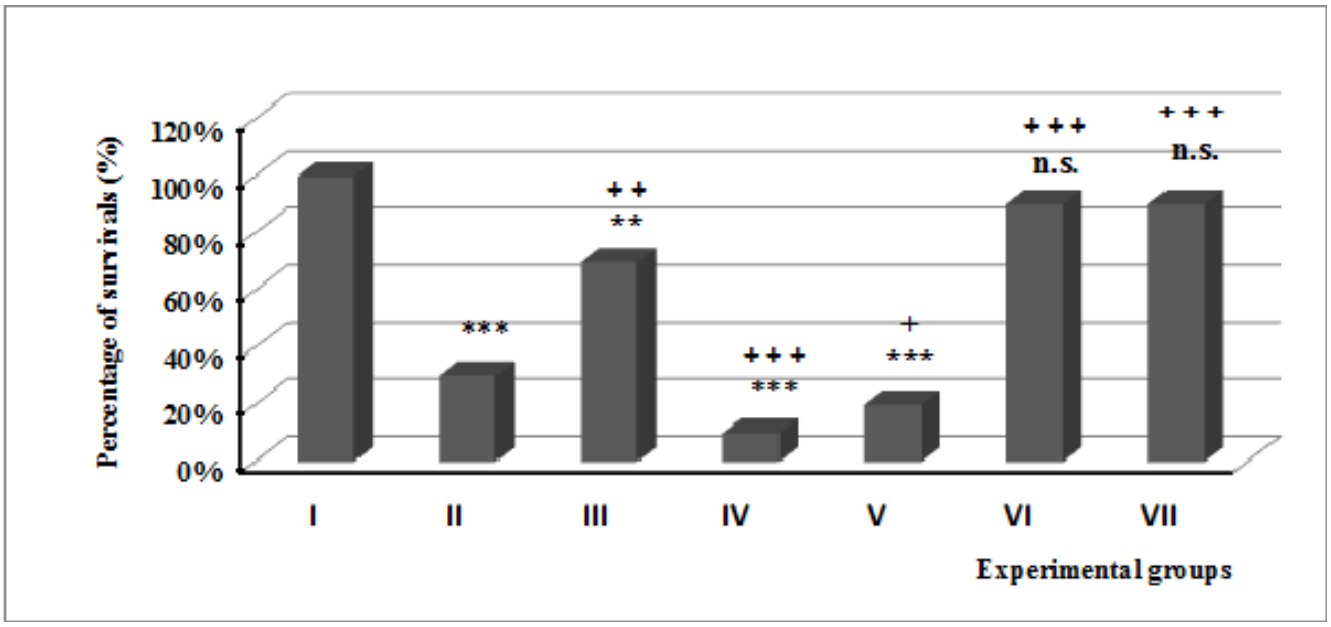

Fig. 1. Survival rate on day 14th after infection. Experimental groups are as follow:
I - Healthy, non-infected animals
II - Mice infected with influenza virus
III - Mice infected with influenza virus and treated with oseltamivir 2.5 mg/kg *per os*,
IV - Mice infected with influenza virus and treated with SAM in a dose of 50 mg/kg, *i.p.*
V - Mice infected with influenza virus and treated with SAM in a dose of 100 mg/kg, *i.p.*
VI - Mice infected with influenza virus and treated with oseltamivir 1,25 mg/kg, *per os*, and SAM in a dose of 50 mg/kg, *i.p.*
VII - Mice infected with influenza virus and treated with oseltamivir 1,25 mg/kg, *per os* and SAM in a dose of 100 mg/kg, *i.p.*
*** $p < 0.001$ vs group I; ** $p < 0.01$ vs group I;
+++ $p < 0.001$ vs group II;++$p < 0.01$ vs group II; +$p < 0.05$ vs group II
n.s. - non significant vs group I

enza virions aggregate on the surface of the host cell, thereby limiting the extent of infection within the mucosal secretions and reducing viral infectivity (Kamps and Hoffman, 2006).

SAM is synthesized in the cytosol of every cell, but the liver plays the central role in the homeostasis of SAM as the major site of its synthesis and degradation (Lu, 2000). SAM contains a high-energy sulfonium ion, which activates each of the attached carbons toward nucleophilic attack and confers on SAM the ability to participate in 3 major types of reactions: transmethylation, transsulfuration, and aminopropylation (Lieber and Packer, 2002). The methylation cycle involves the conversion of methionine via S-adenosylmethionine and S-adenosylhomocysteine into homocysteine, followed by reconversion of homocysteine into methionine. This cycle has three major cellular functions. First, it provides SAM, necessary for polyamine synthesis and for the methylation of numerous essential cell constituents, such as phospholipids, methyl-accepting proteins, CpG islands in DNA, adrenergic, dopaminergic and serotoninergic molecules. Second, it feeds the transsulphuration pathway that leads to the formation from homocysteine of glutathione, the main cellular antioxidant, required for the detoxification of various compounds and for the scavenging of free radicals (Fig. 2).

Finally, it recycles 5-methyltetrahydrofolate into tetrahydrofolate, a necessary cofactor for the synthesis of DNA and RNA. SAM plays a central role in the methylation cycle by controlling both the remethylation of homocysteine to methionine and its catabolism through the transsulfuration pathway. A normal adult makes about 8 g of SAM per day, the majority of it in the liver, where it is also mainly consumed (Mato *et al.*, 1997). It is the second most widely used enzyme substrate after ATP. SAM is biosynthesized during the reaction of methionine with ATP, which is catalyzed by SAM synthetase or methionine adenosyltransferase. SAM is recognized as the major methyl-donor reagent for essential methylation reactions that occur in all living organisms (Fontecave *et al*., 2004).

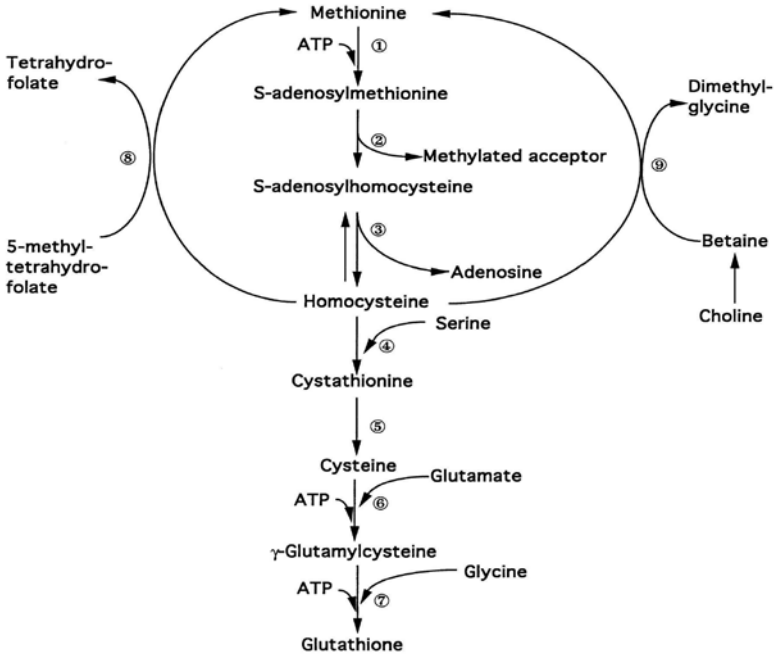

Fig. 2. Hepatic methionine metabolism and GSH synthesis. The transsulfuration pathway converts methionine to cysteine, which is then converted to GSH via the GSH synthetic pathway (Lu, 1999).

Conclusion

In our experimental mice model of influenza, the virus infection with 10 LD_{50} causes 70% lethality. The monotherapy with oseltamivir in a dose of 2.5 mg/kg increases the survival rate from 30 to 70%. The monotherapy with SAM in a dose of 50 mg/kg and 100 mg/kg does not increase the survival rate. It is a matter of interest that the combined therapy - SAM in both doses applied *i.p*. combined with oseltamivir in a dose of 2.5 mg/kg daily in two intakes applied *per os* achieve 90% survival rate

in mice infected with influenza virus. Our study demonstrates the advantage of combining agents acting through different mechanisms – the antiviral drug oseltamivir and SAM as a precursor of the main antioxidant - glutathione. Further experiments are necessary to clarify this statement.

Acknowledgments

The authors would like to express gratitude to Mrs. Kirilka Todorova for their excellent technical assistance and to Dr. Petya Stoyanova for supplying and housing the experimental animals.

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