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The induction of virus-neutralizing antibodies in influenza A-infected mice treated with Oseltamivir phosphate: effect of dosage and scheduling

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Summary. – Oseltamivir phosphate (OS) is currently the most frequently used influenza antiviral drug. It moderates the course of influenza virus type A (IAV) infection, however, its impact on the induction of virus-neutralizing antibodies (VNAbs) is not understood in details. Here, we examined the influence of low (10 mg/kg) or high (60 mg/kg) doses of OS on the viral titer in lungs of BALB/c mice infected with 0.5 LD_{50} of IAV and on the level of VNAbs. Prophylactic application of OS (6 h before the infection) delayed the increase of viral titer in lungs with a lower peak in comparison to non-treated control mice. After therapeutic OS application (44 h after the infection), maximum of virus titer did not significantly change. However, the induction of VNAbs strongly decreased, to 16.7%–18.1% of the control, after preventive application of high OS dose. A minimal decrease of VNAbs titers was observed in groups of mice treated with low dose of OS applied therapeutically. They lowered to 91.1% / 14 or to 94.1% / 21 days post infection (p.i.) of VNAbs titers of non-treated control mice. In all other groups, levels of VNAbs titers dropped to 26.5–53.7% of those of non-treated mice. It should be noted that VNAbs titers were in direct proportion to maximal virus titers in mouse lungs of corresponding groups. In summary, after OS application the clinical symptoms of the disease were milder or non-observable in all OS-treated groups, but the lowering of VNAbs titers was dependent on the OS dose and interval between drug application and the start of infection.

Keywords: influenza A virus; Oseltamivir; prophylactic treatment; therapeutic treatment; virus-neutralizing antibodies

Introduction

Influenza A viruses (IAV) cause a respiratory disease in humans, the course of which can be mild, severe or even life-threatening (Fislová *et al.,* 2009). The severity of the disease is influenced by many factors, including the virulence of the virus strain, which caused the infection, but especially by the status of the host immune system. These factors determine whether the common secondary bacterial infection, in praxis often suppressed by the application of antibiotics, will be associated with the primary viral infection (Mikušová *et al.,* 2022). However, antibiotics may not help to cure the bacterial co-infection if the infected individuals are immuno-compromised or suffer from other chronic diseases (McCullers, 2004, 2011). It was shown that a synergism occurs during influenza and bacteria coinfection (Kash and Taubenberger, 2015; Karlson *et al.,* 2017; Mikušová *et al.,* 2022). Because the influenza infection causes the damage of the respiratory epithelium, the accessibility of epithelial cell surfaces for bacterial infection is increased and, subsequently,

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Abbreviations: IAV = influenza virus(es) type A; OS = Oseltamivir phosphate; p.i. = post infection; PRNT = plaque reduction neutralization titer; VN = virus-neutralizing; VNAbs = VN antibodies

such dual infection can cause irreversible pathological changes in the lung tissue with a potentially fatal impact (Smith *et al.,* 2013; Gounder and Boon, 2019). Antiviral therapy or vaccination can prevent epithelial damage in the airways, inhibit the down-regulation of immune response triggered by influenza viruses and thus help to precede or prevent bacterial superinfection (Staneková and Varečková, 2010; Marois *et al.,* 2015; Kramer, 2017; Tomčíková and Varečková, 2019).

Currently, Oseltamivir phosphate (OS, trade name Tamiflu), the most frequently used influenza antiviral drug, is the inhibitor of the enzymatic activity of the IAV surface glycoprotein neuraminidase. OS blocks the viral neuraminidase-mediated cleavage of sialic acid from the cell surface receptors and from the HA of budding viral particles and thus prevents the virus release from the cell surface and its further spread (Li *et al.,* 2014; Hajzer *et al.,* 2017; Hooker and Ganusov, 2021).

It was observed that antiviral drug application suppresses proinflammatory cytokine expression, probably due to the reduced IAV replication (Walsh *et al.,* 2011; Bird *et al.,* 2015). It is, however, not known, how the induction of protective antibodies is modulated after the antiviral drug therapy and whether this antibody response could be sufficient for the prevention of the reinfection with the same or antigenically similar IAV virus.

In praxis, the therapy with OS is rarely applied immediately after the start of IAV infection. Therefore, a question arises, how the delayed application after the start of IAV infection influences its antiviral efficacy. From our point of view, other questions also arise: How does the prophylactic or therapeutic drug application influence the induction of VN antibodies? Will the patient after such cured infection acquire the protective antiviral antibodies?

Materials and Methods

Viruses. Influenza A virus of H3 subtype A/Mississippi/1/85(H3N2) – further only "A/Miss", adapted to mice, was used for the infection of mice, as well as for plaque reduction assay.

Cells. A stabilized cell line, Madin-Darby canine kidney (MDCK) cells, was used for the virus titration and for the estimation of the level of VNAbs in plaque assay (Hollý *et al.,* 2017). MDCK cells were cultured in DMEM medium containing 10% fetal calf serum at 37°C in a humid atmosphere containing 5% CO_2.

Monoclonal antibodies. Monoclonal antibody 107L, specific to the nucleoprotein of influenza A virus, was used for the staining of viral plaques in plaque reduction assay (Varečková *et al.,* 1995; Hollý *et al.,* 2017).

Animal experiments. BALB/c mice, feline (VELAZ, Praha) were used in all animal experiments. Five groups of six-week-old BALB/c mice (n = 15 mice/group for viral titer determination and n = 10 mice/group for serum examination) were intranasally infected with a dose 0.5 LD_{50} (23 PFU/40 µl/mouse) of mouse-adapted virus A/Miss under the isoflurane (3%) narcosis. Six hours before or 44 h after the infection, Oseltamivir phosphate (OS) was applied perorally at a dose of 10 mg/kg (low dose) or 60 mg/kg (high dose) in a volume 200 µl/mouse. In the control group (non-treated with OS), mice received qH_2O (200 µl/animal). OS or qH_2O were applied twice a day in a 12 hour-interval during 10 consecutive days.

Viral titer determination in lungs of infected mice. The 20 % suspension of lung cell homogenate was sedimented at 1000 g for 5 min at 4°C and 200 µl of the supernatant was serially two-fold diluted. The titer of the virus present in supernatant of lung-cell homogenate was evaluated by rapid culture assay in MDCK cell line on 96-well microtitration plates, as described before (Gocník *et al.,* 2007; Hollý *et al.,* 2017).

Serum samples. On days 14 and 21 after the infection, blood samples were collected and sera were prepared by the standard procedure. Serum samples were inactivated at 56°C for 30 min before testing.

Evaluation of virus-neutralizing antibody titer in sera. The level of antibodies reducing the virus titer was estimated in triplicates by plaque reduction assay. MDCK cells were seeded into 24-well plates (2x10^5 cells/well) and after 24 h cell monolayers were infected by a mix of virus with serum as follows: serum in 10-times dilutions ranging from 10^{-2} to 10^{-6} was mixed with the infectious virus A/Miss (40 PFU/well) at a ratio 1:1. After the incubation at 37°C for 30 min, mixtures of virus with the appropriate serum dilutions (200 µl/well) were applied on the cell monolayers to infect cells. After 60 min at 25°C, the supernatant was removed and a cover medium (containing 2xMEM; NEAA; Pen-Strep-AmpB; 2 mM L-glutamine; 0.1 % BSA; 1 µg.ml^{-1} TPCK trypsin and 2.5% Avicel) was added. Plates were incubated for 36 h at 37°C in a humid atmosphere with 5% CO_2. Viral plaques were evaluated by standard method using immunostaining with influenza A nucleoprotein-specific monoclonal antibody 107L and Anti-mouse IgG conjugated with horseradish peroxidase. The addition of peroxidase substrate AEC (aminoethylcarbazole) with H_2O_2 enabled the plaque visualization and viral plaques were evaluated as described before (Hollý *et al.,* 2017).

Mathematical evaluation of results. The levels of virus-neutralization antibodies in sera were evaluated by GraphPad Prism 7 regression analysis using non-linear regression. The analyses of the viral plaque inhibition curve enabled the expression of serum dilution, at which the 50% inhibition of viral plaques was calculated. The 50% Plaque Reduction Neutralization Titer of sera, designated as $PRNT_{50}$, was defined as the reciprocal value of serum dilution, at which the given virus titer was reduced to 50%.

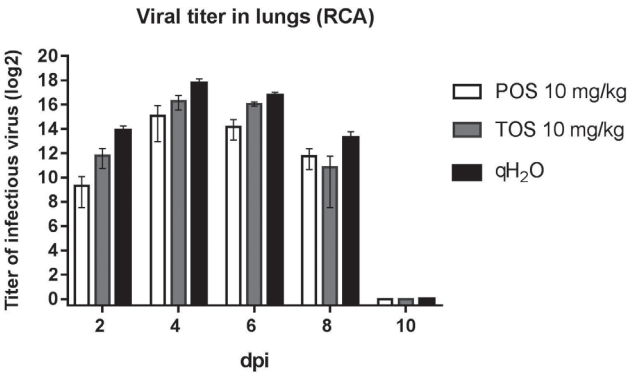

Fig. 1

Viral titer in lungs of mice treated with OS at the concentration 10 mg/kg

dpi = the day after virus infection; POS = OS administered prophylactically 6 h before virus infection; TOS = OS administered 44 h after virus infection; qH_2O = control group, only qH_2O administered after infection.

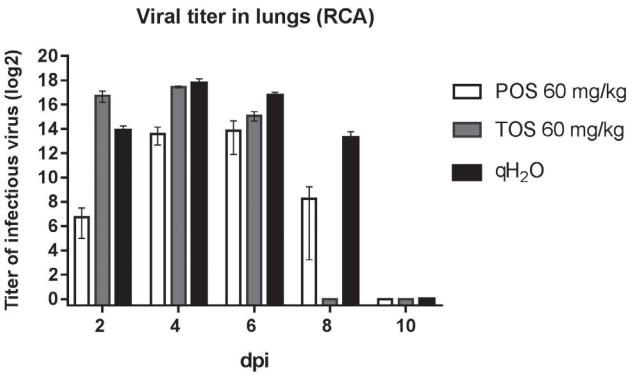

Fig. 2

Viral titer in lungs of mice treated with OS at the concentration 60 mg/kg

dpi = the day after virus infection; POS = OS administered prophylactically 6 h before virus infection; TOS = OS administered 44 h after virus infection; qH_2O = control group, only qH_2O administered after infection.

Results

The Oseltamivir therapy during influenza infection moderates the viral levels in mouse lungs

Five groups of mice (n = 15 mice/group) were infected with a dose of 0.5 LD_{50} of A/Miss virus. Mice in four groups were treated per os with antiviral drug OS at two concentrations (10 mg/kg or 60 mg/kg). The fifth group of mice was a control group of IAV-infected mice without OS treatment. The virus in lungs was detected in quadruplicates by rapid culture assay (RCA). We showed that the increase of virus titer in lungs of mice treated with the high dose of OS (prophylactically or therapeutically) was milder and its maximum was lower than in the control, non-treated mice. Moreover, the virus titer in lungs of mice in these groups decreased earlier than in the control, non-treated infected mice. After the application of the low dose of OS to IAV-infected mice therapeutically or prophylactically, similar results were obtained, but the differences among groups of mice were less evident (Figs. 1, 2).

Titers of VNAbs in sera of influenza A infected mice treated with OS depend on the time and the dose of its application

We compared the levels of antibodies neutralizing influenza virus in sera of mice infected with 0.5 LD_{50} of A/Miss virus, which were treated with OS at low (10 mg/kg) or high (60 mg/kg) doses. The virus-neutralizing activity of antibodies was measured by plaque reduction assay. As a reference value (100%) was used the number of viral plaques in a control without the specific antibody. All other values of plaque numbers obtained after the virus incubation with different serum dilutions were expressed as a percent of the reference sample. The reciprocal value of dilution of the examined serum, at which a 50% viral plaque reduction was observed, was estimated as the titer of virus-neutralizing antibodies in particular sera expressed as $PRNT_{50}$ (Figs. 3, 4, and 5, Table 1).

Titers of VNAbs in sera obtained from five groups of mice infected with the same dose of virus (0.5 LD_{50}), non-treated or treated with low or high doses of OS, applied prophylactically 6 h before the infection or therapeutically 44 h p.i., were compared. VN titers were examined at two intervals: 14 and 21 days p.i. The presence of VN antibodies was confirmed already on the 14th day p.i. However, their levels differed depending on the dose and time of OS application. The highest level of VN antibodies ($PRNT_{50}$: 8623) was reached in control mice without the OS treatment. The lowest titer was recorded in the group of mice after preventive application of the

Table 1. Titers of VN antibodies with 50% viral plaque reduction activity ($PRNT_{50}$)

$PRNT_{50}$ (%)	POS		TOS		qH_2O
OS (mg/kg)	10	60	10	60	–
14 days p.i.	4,636 (53.7%)	1,446 (16.7%)	7,857 (91.1%)	3,911 (45.3%)	8,623 (100%)
21 days p.i.	6,973 (34.5%)	3,669 (18.1%)	19,037 (94.1%)	5,376 (26.5%)	20,222 (100%)

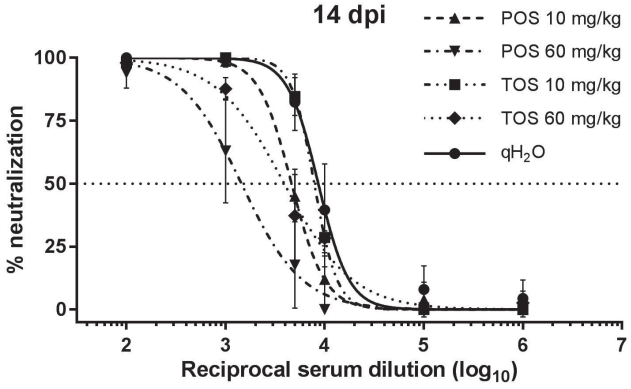

Fig. 3

Neutralization of the virus in the presence of sera obtained 14 days after IAV infection of mice

dpi = the day after virus infection; POS = OS administered prophylactically 6 h before virus infection; TOS = OS administered 44 h after virus infection; qH_2O = control group, only qH_2O administered after infection. Mouse serum from groups TOS 10 and qH_2O had the highest virus neutralizing ability – the ability to reduce the number of plaques.

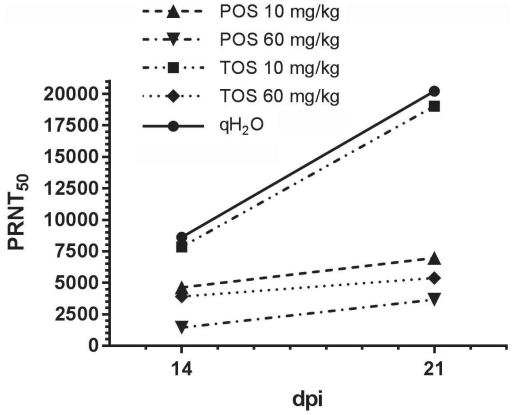

Fig. 5

Comparison of $PRNT_{50}$ of antibodies in sera of IAV-infected mice treated with OS at 14 and 21 dpi

dpi = the day after virus infection; POS = OS administered prophylactically 6 h before virus infection; TOS = OS administered 44 h after virus infection; qH_2O = control group, only qH_2O administered after infection.

high dose of OS, i.e. before the infection ($PRNT_{50}$: 1446). Medium, but comparable levels of VN antibodies were obtained in groups of mice, which received OS in the low dose preventively ($PRNT_{50}$: 4636) or in the high dose therapeutically ($PRNT_{50}$: 3911). The titer of VN antibodies measured in sera of mice treated with the low dose of OS therapeutically, i.e. 44 h after the viral infection started, reached the value ($PRNT_{50}$: 7857), which was very close to the level of antibodies in sera of non-treated mice ($PRNT_{50}$: 8623).

The differences in the induction of VN antibodies in sera of particular groups of mice obtained 21 days p.i. were more substantial. The titers increased and reached approximately 1.1–2.5 times higher values than on day 14 p.i. OS non-treated mice developed a strong antibody response with titer $PRNT_{50}$: 20 222, comparable to that after therapeutic administration of low dose of OS ($PRNT_{50}$: 19,037). However, in other groups, the titer ranged from the lowest value ($PRNT_{50}$: 3669) in serum of prophylactically treated mice with high dose of OS to the $PRNT_{50}$: 5,376 in sera of therapeutically treated mice with high dose of OS. In the group of mice, which received preventive low dose of OS, $PRNT_{50}$ reached the value 6,973.

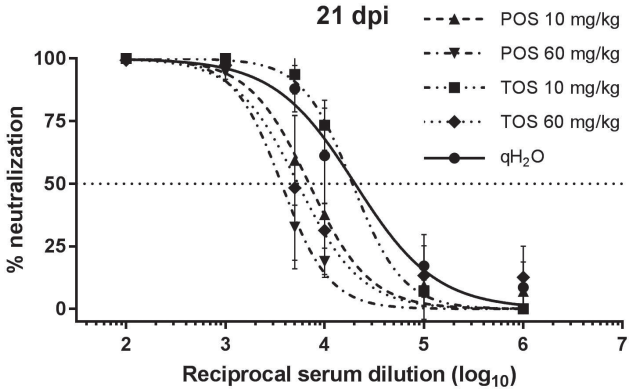

Fig. 4

Neutralization of the virus in the presence of sera obtained 21 days after IAV infection of mice

dpi = the day after virus infection; POS = OS administered prophylactically 6 h before virus infection; TOS = OS administered 44 h after virus infection; qH_2O = control group, only qH_2O administered after infection. Mouse serum from groups TOS 10 and qH_2O, similarly as in sera obtained at day 14 p.i., had the highest virus neutralizing ability – the ability to reduce the number of plaques.

Conclusion

We can conclude that the treatment of mice infected with influenza A virus with OS significantly moderates the clinical symptoms of infection. The progress of infection is delayed and clinical symptoms of disease depend on the dose of OS and also on the time of OS application. Disease manifestation corresponded with the viral titer in the lungs of mice. Viral titer increased more slowly in treated mice, especially after the prophylactic treatment, than in non-treated infected (control) mice. It should be emphasized that in any case, the treatment of mice did not prevent the induction of virus-specific

antibodies neutralizing infectivity of the virus. However, the antibody levels in sera of infected mice were lower and were in direct proportion to the viral titers in lungs of infected mice. We showed in this study that the prophylactic treatment of mice with low or high dose of OS causes lower induction of VNAbs than the therapeutic treatment with corresponding doses of OS. The titers of VNAbs after prophylactic application of OS at the higher dose (60 mg/kg) dropped to 16–18% of the control non-treated mice (100%). Thus, the therapy with high dose of OS markedly limited the production of VNAbs. A minimal decrease of titers of VNAbs was observed after the therapeutic application of OS at the dose 10 mg/kg, where $PRNT_{50}$ decreased to 91.1–94.1% of the control (100%). But in this case clinical symptoms of disease were similar to non-treated mice.

Thus, important conclusion of our experiments is that the therapeutic or prophylactic OS application at both high and low dose does not prevent the induction of VNAbs, but lowers their production. The question remains whether the limited concentration of VNAbs induced after IAV infection and treatment with OS is sufficient to prevent reinfection with the same or antigenically similar influenza virus. This will be the subject of our further studies.

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