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Original article

Antiviral properties of yolkin in a homologous hen model

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Abstract

Eggs are among the most nutritious and accessible products that have been an important part of human diets for thousands of years. Well digestible proteins belong to the main nutritional components of eggs. Evolutionary old vitellogenin in serum of laying hens is enzymatically degraded to vitally important proteins, including phosvitin and yolkin. The latter protein has documented immunoregulatory properties and improves cognitive function. Yolkin isolated from hen eggs was tested for its potential virucidal and antiviral properties against Rous-associated virus-2 (RAV-2) using DF-1 hen fibroblasts, defined as a reduction in virus titer and the cytopathic effect, following 96 h culture in DF-1 infected cells. The mixing of yolkin with the virus led to concentration-dependent virus inactivation by 2-log at 50 μg/mL – and by 1-log at 10 μg/mL of yolkin – virucidal properties. On the other hand, the treatment of infected DF-1 cells with yolkin caused a diminution of the virus titer by 3-log for a concentration of 50 μg/mL and by 1-log at 10 μg/ml of yolkin – an antiviral effect. The results indicate that yolkin may exhibit both a direct virucidal property and the capability to interfere with virus replication in the homologous model. The involvement of ERK/MAPK signaling in RAV-2 replication in DF-1 cells, by using specific inhibitor PD98059, was also demonstrated. In conclusion, yolkin may play an antiviral role in the development of bird embryos. The accumulated data on the actions of yolkin in rodent models strongly suggest benefit of egg consumption in human everyday diet.

Keywords: DF-1 cell line primary chicken embryonic fibroblasts, D98059 inhibitor, RAV-2 virus Rous-associated virus-2, Yolkin

Introduction

Viral infections pose serious worldwide social and economic consequences in humans and animals, and are also a cause of malignant diseases. Viruses enter cells by a variety of receptors (Montefiori et al. 1993, Haan et al. 2000, Yang et al. 2000, Akula et al. 2001, Colin et al. 2005, Erlenhoefer et al. 2005). Antiviral therapeutic strategies include application of drug combination (Kainov et al. 2025), peptides (Boas et al. 2019), proteins (Kazana et al. 2020, Shafqat et al. 2025), lectibodies (Celis and Matoba 2024) or flavonoids (Spedding et al. 1989). Natural flavonoids, such as amentoflavone, scutellarein and quercetin inhibit reversed transcriptase in several types of viruses (Spedding et al. 1989). Another flavonoid, baicalin, demonstrated effective antiviral properties in avian leukosis virus J (ALV-J) by affecting virus entry and replication (Kun et al. 2028). The inhibitory effects of Taishan Pinus massoniana pollen polysaccharide (TPPPS) on ALV-J poultry virus was also investigated (Wang et al. 2019B). Polysaccharides inhibited virus replication during adsorption and early period of infection. In turn, the antiviral actions of lactoferrin (LF) include neutralization of virus particles (Välimaa et al. 2009), competition with cell receptors for viruses (Di Biase et al. 2003) and interference with viral replication (Picard-Jean et al. 2014). Activation of mitogen activated protein kinases (MAPK) contributes to replication of RNA and DNA viruses, multiplying both in the nucleus and in the cytoplasm. This phenomenon was found in infections by many types of viruses such as human immunodeficiency (Jacqué et al. 1998), influenza A and B (Ludwig et al. 2004, Pleschka, 2013), mouse coronavirus (Cai et al. 2007), Born disease (Planz et al. 2001), Coxsackie B3 (Luo et al. 2002), human cytomegalovirus (Johnson et al. 2001), herpes simplex (Zhang et al. 2010), vaccinia (Andrade et al. 2004) or avian leucosis virus (Dai et al. 2016). Infection with exogenous ALV viruses from J, A or B subgroups may activate the ERK/AP1 signaling pathway associated with viral gp85 and gag proteins, predominantly in early and late phases of infection (Dai et al. 2016). Virus-host cell interactions are reflected by activation of signaling pathways in infected target cells and determine the efficacy of virus propagation. Infection with ALV-A and ALV-B viruses elicited phosphorylation of ERK/MAPK in DF-1 cells, which could be significantly diminished by PD98059, a specific ERK inhibitor, leading to an exceptionally low level of virus transcription and protein synthesis (Dai et al. 2016). Avian leukemia encapsulated one stranded RNA viruses belong to *Alpharetrovirus* genera from the *Retroviridae* family. These retroviruses are responsible for various avian diseases, including cancer, or immunosuppression (Fadly 1997). Among the most frequently, naturally distributed avian retroviruses are ALV, reticuloendotheliosis virus (REV) and lymphoproliferative disease virus (LPDV) (Niebora et al. 2024). ALV causes carcinogenesis, delayed development, lower fertility and immune suppression increasing susceptibility to other infections (Niebora et al. 2024). The *Retroviridae* family consists of 11 subgroups (A-K) according to the structure of the capsule glycoproteins, which play a key role in host-virus interactions. Viruses from the B, D and F subgroups cause general cytopathic effects in avian cells. The DF-1 cell line of hen embryo fibroblasts displayed a higher susceptibility to the ALV-B virus than primary hen embryo fibroblasts (CEF) (Himly et al. 1998, Maas et al. 2006).

Eggs are among the most nutritious and accessible food products belonging to human diet for thousands of years. Easily digestible, high-quality proteins, omega-3 and omega-6 acids, carotenoids, as well as A, B2, B6, B12 vitamins and minerals, such as phosphorus, iron and selenium, belong to egg components essential for human health (Formisano et al. 2025). The development of biochemistry and medicine, has led to characterization of the egg components and established a benefit of egg's consumption. Vitellogenin, the evolutionary old precursor protein, present in serum of laying hens, is enzymatically cleaved during transport to oocyte to vitally important proteins, including phosvitin and yolkin contained in egg's yolk. Recently, yolkin was subjected to a series of in vitro studies and in studies resulting in determination of its structure and immunoregulatory properties (Zambrowicz et al. 2023, Zambrowicz et al. 2024). Our recent studies in mice revealed beneficial effects of yolkin by reconstitution of the immune function in immunocompromised mice (Zimecki et al. 2025B) and mice subjected to psychic stress (Zimecki et al. 2025A), as well as by organ protection in endotoxemic mice (Zimecki et al. 2026A). Yolkin also regulated the immune status of young mice (Zimecki et. al. 2024**).** Our in vitro studies revealed several aspects of its mode of action including effects on cytokine production, cell signaling and cell maturation (Zimecki et al. 2025C, Zimecki et al. 2026B). The effects of yolkin on the cognitive function in old rats was also demonstrated (Lemieszewska et al. 2016). Therefore, we and others (Zambrowicz et al. 2023) envisage a potential of this protein as a preventive or a therapeutic preparation in metabolic disorders in humans.

Through its ability to induce a number of cytokines, yolkin was shown to exert an indirect antiviral effect on bone marrow derived macrophages infected with the Vesicular Stomatitis Virus (Kazana et al. 2020).

To evaluate the potential, direct antiviral action of yolkin in the homologous model, we chose the Rous-associated virus-2 (RAV-2), based on the original findings (Weller et al. 1980, Weller and Temin 1981), due to its strong property of inducing a cytopathic effect in DF-1 cells. Of importance, DJ-1 cells express TLR-4 receptors (Jang and Song 2020) for which yolkin is a ligand (Kazana et al. 2022). The specific inhibitor PD98059 of ERK/MAPK activity was also used to confirm involvement of this signaling pathway in replication of RAV-2 virus in DF-1 cells.

Materials and Methods

Reagents

Dulbecco Modified Eagle Medium (DMEM) and Eagle's Medium Essential Medium (EMEM) were from ATCC (St. Manassas, VA, USA); fetal bovine serum (FBS) was from HyClone (Pittsburgh, PA, USA), penicillin and streptomycin, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), dimethylsulfoxide (DMSO), sodium dodecyl sulphate (SDS) and N,N-dimethylformamide (DMF) were from Sigma-Aldrich (St. Louis, MO, USA). Sodium chloride buffered with phosphates (PBS) and trypsine were obtained from Biowest (Nuaillè, France); the ERK2/MAPK specific inhibitor PD98059 was from Cell Signaling Technology (Danvers, MA, USA).

DF-1 cell line

DF-1 (ATCC CRL-3586TM) – primary chicken embryonic fibroblasts were grown in DMEM supplemented with 10% FBS, 100 U/mL of penicillin and 100 g/mL of streptomycin at 39°C in a 5% CO2 and 95% humidified atmosphere.

Virus propagation

VR-1828™ – Rous-associated virus-2 (RAV-2) (ATCC CRL-12203), *Retroviridae, Alpharetrovirus, avian leukosis (subgroup B) was used. The virus* was propagated in DF-1 cells in a culture medium consisting of DMEM containing 2% FBS and antibiotics. DF-1 cells, known to be susceptible only to exogenous ALV (Maas et al. 2006) after attaining 80% confluence, were infected for 2 h at 37°C in 5% CO_2. After the inoculum was removed, the maintenance medium containing 2% DMEM was added and incubated for 7 days. Next, DF-1 cells were centrifuged at 4°C and 300 × g for 10 min to separate the supernatants, which were then stored as viral stocks at -80°C until use. The virus titer was determined using the Tissue Culture Infective Dose 50 (TCID50) assay.

Determination of multiplicity of infection (MOI)

A cytopathic effect after virus infection depends on MOI. An optimal MOI value in ALV-B infection of DF-1 cells as 1 was previously established (Diaz-Griffero et al. 2003). Of interest, the cell number at MOI=10 increased the viability of target cells, indicating that high MOI values are not cytopathic. Therefore, the model of DF-1 cells infected with the RAV-2 virus MOI=1.0 was selected for our studies on the antiviral properties of yolkin.

Yolkin Preparation

Yolkin was isolated from hen egg yolks as described elsewhere (Polanowski et al. 2013). A detailed procedure of isolation and Sodium Dodecyl Sulfate–Polyacrylamide Gel (SDS-PAGE) analysis of the yolkin preparation was recently described (Obmińska-Mrukowicz et al. 2020). The yolkin preparation consisted of three main proteins with Molecular Weight (MWs) lower than 45 kDa. Yolkin used in this study derived from a specially prepared big batch, according to the original isolation procedure, for the performance of the National Science Centre (NSC), Poland, no. DEC-2021/41/B/NZ6/01167 grant which generated a number of articles cited in this manuscript and proved expected activity in each model. The preparation was stored in a refrigerator as a lyophilized powder for the whole investigation period**.**

The preparation was dissolved in Eagle's Medium Essential Medium (EMEM), without or with 1% addition of FBS, and passed through 0.22 μm filters. The preparation was prepared de novo before each experiment.

Dilution of the specific inhibitor PD98059

DMSO was used as a solvent for the initial dissolution for the PD98059 inhibitor, then PD98059 was further diluted in the EMEM medium containing 1% FBS, referred below to as 1% EMEM. Respective dilutions of DMSO in the culture medium, corresponding to appropriate concentrations of PD98059, were used as control cultures.

Colorimetric MTT assay

Briefly, 25 μL of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) from stock solution (5 mg/mL) was added per well to 96 well plates at the end of the cell incubation period and the plates were incubated for an additional 3 h in a cell culture incubator. Then, 100 μL of the extraction buffer (20% SDS with 50% DMF, pH 4.7) was added. After overnight incubation, the optical density (OD) was

measured at 550 nm with the reference wavelength of 630 nm (Hansen et al. 1989).

Evaluation of yolkin cytotoxcity

DF-1 cells were incubated in the 1% EMEM culture medium at a density of 1 × 10^4 cells/mL in 96 well plates for 96 h with yolkin at a concentration range of 0.8-100 μg/mL. After the incubation, the viability of cells was determined (Hansen et al. 1989).

Evaluation of PD98059 cytotoxicity

DF-1 cells, at a density of 1 × 10^4 cells/mL, were incubated for 24 h. After the incubation, PD98059, at a concentration range of 0.08-10 μM, was added to the cell monolayers and incubated for an additional 96 h. Control cultures contained corresponding dilutions of DMSO. Cell growth, morphology and viability were analyzed to determine its cytotoxicity. The degree of cytotoxicity was defined as the highest dilution of the compounds that causes 50% or greater cell death. The viability of cells was determined as above.

Yolkins virucidal activity

Yolkin, at a concentration of 10 and 50 μg/mL, as well as RAV-2 (10^5 TCID50) in 500 μL aliquots, were incubated at room temperature for 2 h. The samples were then kept frozen at -80°C for further tests. To evaluate virus activity, the supernatants were transferred to a 24 h culture of target DF-1 cells, and after the subsequent 4 day incubation the cytopathic effect of the virus was analyzed in an inverted microscope. The calculations were performed with a Kárber test (Lei et al. 2021).

Anti-viral effect of yolkin at different stages of viral replication

In order to determine antiviral activities, yolkin (at 50 μg/mL) was tested using DF-1 cells infected with the RAV-2 virus. Briefly, the DF-1 cells were seeded onto 96-well culture plates at a density of 1 × 10^4 cells/mL, followed by incubation at 37°C in a cell culture incubator for 20 h to form a semi-confluent monolayer. To examine the mechanism underlying viral inhibition by yolkin, an experiment determining drug dose dependent efficacy was carried out, as described previously with minor modifications (Qian et al. 2018).

In the first protocol (**P1**): DF-1 cells, in 96-well plates were pre-incubated with yolkin for 20 h at 37°C before viral infection. After incubation, cells were washed four times with 0% EMEM and DF-1 cells were infected with RAV-2 for 3 h at 37°C. After virus adsorption, the cells were washed four times with 1% EMEM to remove the medium (virus inoculum) and incubated at 37°C for 4 days in 1% EMEM. Following the incubation, the supernatants were collected and stored at -80°C for virus titration using a standard Tissue Culture Infective Dose 50 (TCID50) method with a five-fold serial dilution.

In the second protocol (**P2**): a mixture of the virus suspension and yolkin was added to cell monolayers and incubated for 3 h at 37°C. After removing the medium, the cells were washed four times with 1% EMEM and then incubated in 1% EMEM without yolkin at 37°C in a cell culture incubator. After 4 days, the virus titer was determined using the Tissue Culture Infective Dose 50 (TCID50) assay.

In the third protocol (**P3**): DF-1 cells were infected with the virus in the absence of yolkin. After adsorption for 3 h at 37°C the virus was removed. The cells were washed four times with 1% EMEM and subsequently incubated in 1% EMEM containing yolkin. Following 96 h of incubation, the supernatants were collected and stored at -80°C for virus titration using a standard TCID50 method with a five-fold serial dilution. Briefly, the supernatants were diluted and adsorbed to DF-1 cells at a density of 1 × 10^4 cells/mL in 96-well culture plates. Following 96 h of incubation, the viral cytopathic effect (CPE) was analyzed in an inverted microscope (magnification x300). The TCID50 was calculated by determining the end-point dilution of the virus where 50% of the infected cells displayed CPE. The antiviral activity of the tested compound was determined by comparing the logarithmic reduction factor (log 10) of the viral titer with the control (cells treated only with RAV-2 served as control).

Effect of ERK2/MAPK pathway inhibitor PD98059 on RAV-2 replication

The ERK/MAPK specific inhibitor PD98059 (1.0 μM) or solvent (0.1% DMSO) were added to DF-1 cells for 20 h at 37°C before viral infection (P1). After incubation, the cells were washed four times with 1% EMEM and the DF-1 cells were infected with the RAV-2 (MOI=1.0) for 3 h at 37°C. After virus adsorption, the cells were washed four times with 1% EMEM to remove the medium (virus inoculum) and incubated at 37°C for 4 days in 1% EMEM. Following 96 h of incubation, the supernatants were collected and stored at -80°C for virus titration using a standard TCID50 method with a five-fold serial dilution.

Statistical analyses

The Brown-Forsythe test was used to determine the homogeneity of variance between groups. When the variance was homogenous, analysis of variance

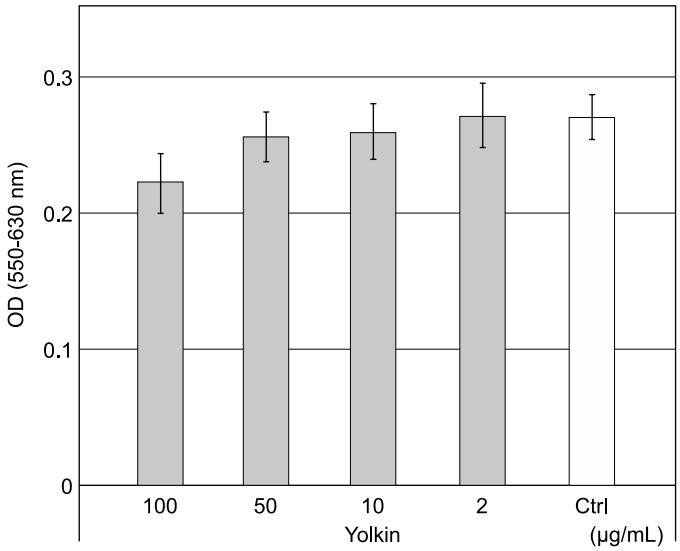

Fig. 1. The effect of yolkin on viability of DF-1 cells. A monolayer culture of cell line DF-1 was incubated at a density of 1×10^4 cells/mL in 96 well plates for 96 h. Yolkin was tested at a 2-100 µg/mL concentration range. Control cultures contained corresponding dilutions of the solvent (1% EMEM medium only) – Ctrl. Cell viability was determined by the MTT colorimetric method. The results are presented as OD mean values ± SD from six determinations.

(One-way ANOVA) was applied, followed by post hoc comparisons with the Tukey's test to estimate the significance of the difference between groups. Non-parametric data were compared using the Kruskal-Wallis test with Mann-Whitney U test to estimate the significance of the difference between groups with the Benjamini-Hochberg correction for performing multiple comparisons. Significance was determined at $p<0.05$. The results are presented as mean values ± standard deviation (SD). The statistical analysis was performed using STATISTICA 7.0 for Windows.

Results

Effect of yolkin on viability of DF-1 cells

The metabolic activity of the DF-1 cell line was evaluated using yolkin (Fig.1) at a concentration range of 2-100 µg/mL and applying relevant dilutions of the solvent (1% EMEM) in control cultures. The results revealed no sign of toxicity up to 50 µg/mL concentration of yolkin as evaluated by MTT colorimetric method and cell morphology. A small toxic effect of yolkin was only observed at 100 µg/mL concentration. No significant changes in cell morphology or reduction in OD 550-630 values was observed for yolkin-treated DF-1 cells at yolkin concentrations of 2-50 µg/mL, whereas yolkin at a concentration of 100 µg/mL caused microscopically detectable alterations (data not shown), correlated with the decreased OD value reflecting a slower cell metabolism. For this reason, the 50 µg/mL concentration was used for the determinations of antiviral and virucidal properties of yolkin. The minimal concentration, toxic to approximately 50% of the cells, was accepted as the tissue culture cytotoxic dose (TCCD50). The degree of cytotoxicity was determined by measuring the growth of the DF-1 cells for 96 h at 37°C. The conditions of the cultures incubated with the tested compound were evaluated using image analysis (Fig. 1).

The effect of PD98059 inhibitor on viability of DF-1 cells

The cytotoxicity of the inhibitor PD98059 was tested against the DF-1 cell line as described (Dai et al. 2016). The ERK/MAPK specific inhibitor PD98059 (0.08-10 µM) or solvent (0.1%) DMSO v/v) were added to DF-1 cells for 96 h. The PD98059 inhibitor was non-toxic at compound concentrations from 0.07 to 1.0 µM as assessed by the colorimetric MTT method and cell morphology. The toxic effect was observed at concentrations ranging from 2 to 10 µM (data not shown).

PD98059 inhibits the replication of RAV-2 virus in DF-1 cells

The inhibitory effect of PD98059 (ERK/MAPK inhibitor) on the replication of the RAV-2 virus in DF-1 cells was investigated. The virus titer was determined by the TCID50 assay. The results showed that PD98059, preincubated with DF-1 cells for 20 h, significantly inhibited the replication of the RAV-2 virus in a dose-dependent manner at a concentration of 1 and 0.5 µM (Fig. 2).

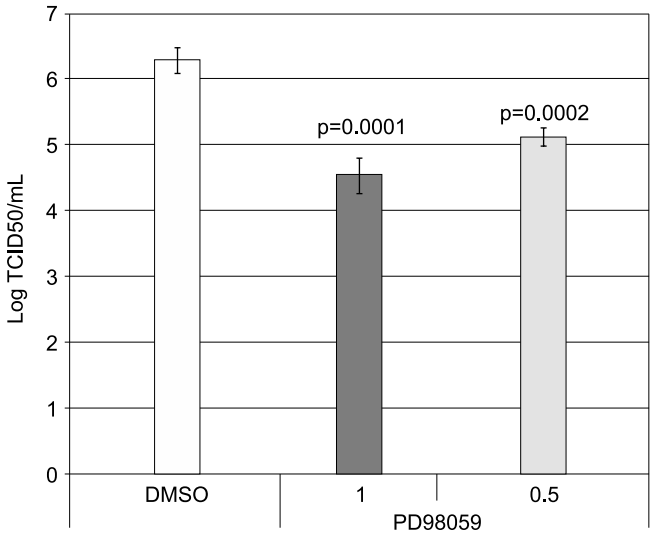

Fig. 2. Inhibitory effect of PD98059 on replication of RAV-2 virus in DF-1 cells. DF-1 cells were pre-incubated with PD98059 (1 and 0.5 μM) or DMSO (0.01% v/v) for 20 h at 37°C and after incubation the DF-1 cells were infected with the RAV-2 (MOI=1.0) for 3 h at 37°C. After virus adsorption, the cells were washed four times with 1% EMEM to remove the medium (virus inoculum) and incubated at 37°C for 4 days in 1% EMEM. The culture samples were kept frozen at -80°C until use. Viral load was measured after 96 hours of DF-1 cell culture, the cythopathic effect of the virus was analyzed in an inverted microscope. The calculations were performed by a Kárber test cited by (Lei et al. 2021). The results, obtained from 4 independent experiments (6 wells per probe in individual experiments), are presented as the logTDIC50 values (mean ± SD) in relation to DMSO control. $p<0.05$ statistically significant as compared with DMSO. One-way ANOVA was applied, followed by post hoc comparisons with the Tukey's test to estimate the significance of the difference between groups.

The inhibitory effect of the yolkin and virus RAV-2 mixtures on viral replication in DF-1 cells – antiviral activity yolkin

We wished to test whether yolkin has an antiviral effect against virus particles with a co-culture of yolkin and the virus. Yolkin at concentrations of 10 and 50 μg/mL was incubated with the RAV-2 virus. As shown in Fig. 3, yolkin co-incubated with the virus causes a dose-dependent loss of the virus replication. Yolkin, at a concentration of 50 μg/mL, reduced the virus titer from 5.47-log to 3.36-log (a statistically significant effect), and at a concentration of 10 μg/mL from 5.47-log to 4.45-log (Fig. 3).

The inhibitory effect of yolkin on the replication of the RAV-2 virus

To determine whether yolkin has antiviral activity against the RAV-2 virus, DF-1 cells were infected with RAV-2 (MOI=1) and then treated with 50 and 10 μg/mL concentrations of yolkin for 96 h. In this model, yolkin had the highest effect on virus replication, since already at a concentration of 50 μg/mL it significantly reduced the virus level from 7.39- to 4.5-log and at a concentration of 10 μg/mL from 7.39- to 6.08-log (effect not significant) (Fig. 4).

Inhibitory Effect of Yolkin on Different Stages of Viral Replication

The inhibitory effect of yolkin on RAV-2 virus replication was tested at different stages of viral replication using three different infection protocols (P), as shown in Fig. 5A. A marked inhibitory effect of yolkin was observed at a concentration of 50 μg/mL when yolkin was added after viral infection – **P3** protocol. The viral load significantly decreased from 7.77-log to 4.83-log (Fig. 5B). The virus titer significantly dropped from 7.56- to 6.08-log when yolkin was added to the cell cultures during virus adsorption, i.e., at the time of virus inoculation – **P2** protocol. The weakest (not significant) protective effect of yolkin was observed when DF-1 cells were treated with yolkin for 20 h before infection. The virus titer decreased from 7.91- to 6.88-log – **P1** protocol (Fig. 5A-5B).

When testing the antiviral properties of different substances, reference drugs are used as controls, e.g., Acyclovir in infections of human herpes simplex virus type 1 or 2 (HSV-1 and 2) or Cidofovir as a reference drug for human adenovirus. In the absence of reference drugs/substances for infections caused by ALV group viruses, the specific ERK/MAPK inhibitor PD98059 was used in our studies as a reference (control) substance. Infections with exogenous viruses from ALV subgroups J, A or B can activate the ERK/AP1 pathway associated with viral gp85 and gag proteins, mainly in the early and late stages of infection (Dai et al. 2016).

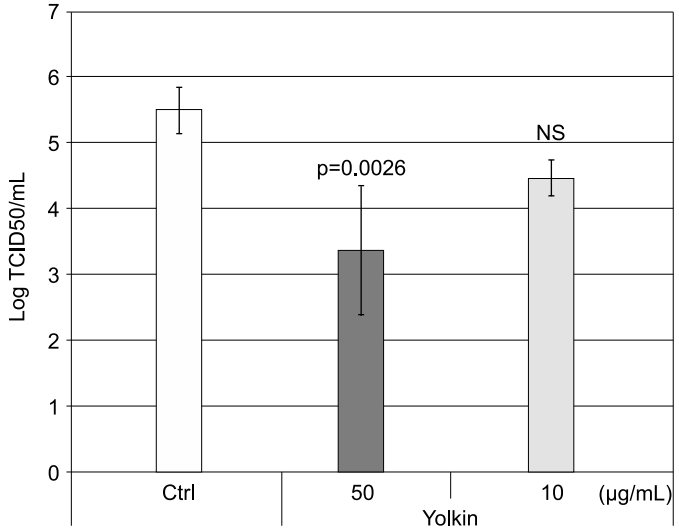

Fig. 3. The antiviral activity of egg yolk – effect of preincubated mixtures of virus and egg yolk on virus replication in DF-1 cells. Egg yolk at concentrations of 50 and 10 µg/mL was incubated for 2 hours at room temperature with the same volume (500 µL) of virus (10^5 TCID50/mL). Culture samples were stored frozen at -80°C until use. Viral load was measured after 96 hours of DF-1 cell culture, the cythopathic effect of the virus was analyzed in an inverted microscope. The calculations were performed by a Kárber test cited by (Lei et al. 2021). Results obtained from 4 independent experiments (6 wells per probe in individual experiments) are presented as the logarithm of the TCID50 value (mean ± SD) relative to the medium control (Ctrl). $p<0.05$ statistically significant as compared with Ctrl. NS denotes not statistically significant. The Kruskal-Wallis test with Mann-Whitney U test to estimate the significance of the difference between groups with the Benjamini-Hochberg correction for performing multiple comparisons was applied.

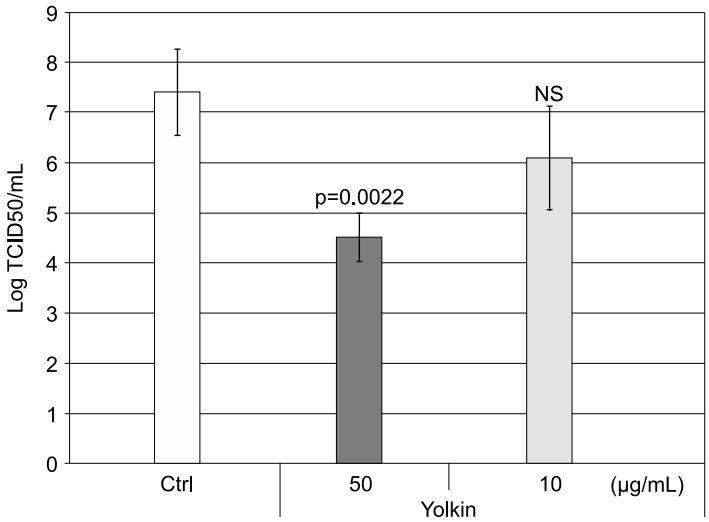

Fig. 4. The virus-inhibiting properties of yolkin against the RAV-2 virus in the DF-1 cell line. DF-1 cells were infected with RAV-2 (MOI=1) and, after 3 h of virus absorption at 37°C, the virus inoculum was removed and the infected cells were incubated for 96 h with yolkin (50 and 10 µg/mL). The culture samples were kept frozen at -80oC until use. The viral titer was expressed with reference to the TCID50 value, based on the cytopathic effect caused by this virus in approximately 50% of infected cells. The calculations were performed by a Kárber test cited by (Lei et al. 2021). The results, obtained from 3 independent experiments (6 wells per probe in individual experiment 6), are presented as the logTDIC50 values (mean ± SD) in relation to medium control (Ctrl). $p \leq 0.05$ statistically significant as compared with Ctrl. NS denotes not statistically significant. One-way ANOVA was applied, followed by post hoc comparisons with the Tukey's test to estimate the significance of the difference between groups.

Based on these results, reporting that the specific ERK inhibitor PD98059 significantly inhibited ALV virus replication, we also used this inhibitor and showed that it inhibited RAV-2 virus replication in DF-1 cells at a concentration of 1 µM from 7.89- to 6.29-log – P1 protocol (Fig. 5B).

Discussion

In this investigation we demonstrated that yolkin inhibited to various degrees viral replication of the RAV-2 avian virus in DF-1 hen fibroblasts, depending on the applied experimental protocols. In addition,

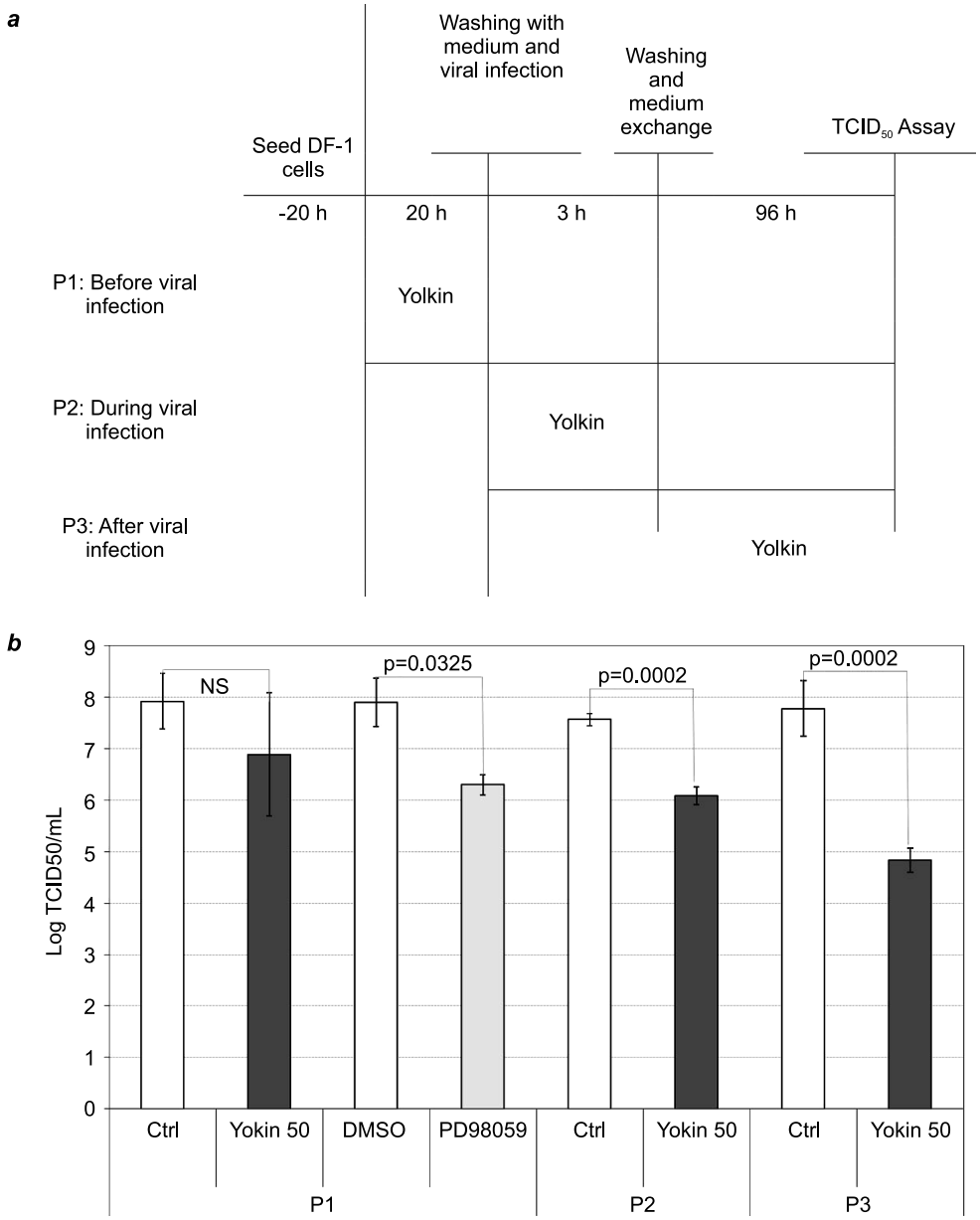

Fig. 5a–5b. Inhibitory effect of yolkin on different stages of viral replication. The DF-1 cells were infected with the RAV-2 virus and treated with yolkin with different protocols: P1 – before viral infection; P2 – during viral infection and P3 – after viral infection (Fig. 5A). Effects of yolkin on RAV-2 replication (Fig. 5B). DF-1 cells were infected with the RAV-2 virus (MOI=1) followed by treatment of 50 µg/mL of yolkin at the indicated time points. The culture samples were kept frozen at -80°C until use. The virus titers were determined by using the TCID50 assay, based on the cytopathic effect caused by this virus in approximately 50% of infected cells. The cytopathic effect of the virus was analyzed in an inverted microscope. The calculations were performed with the Kárber test. The results, obtained from 3 independent experiments (6 wells per probe in individual experiments) are presented as the log TCID50 values (mean ± SD) in relation to medium control (Ctrl) or DMSO $p<0.05$ statistically significant as compared with Ctrl or DMSO. NS denotes not statistically significant. One-way ANOVA was applied, followed by post hoc comparisons with the Tukey's test to estimate the significance of the difference between groups.

application of the ERK/MAPK inhibitor PD98059 showed that the RAV-2 virus uses this cell activation pathway for replication in DF-1 cells.

Previous studies showed that yolkin may exhibit, beside other immunoregulatory activities, antiviral properties (Kazana et al. 2020). Although a direct treatment of VSV-infected bone marrow derived macrophages (BMDM) with yolkin did not lower the virus titer, a decrease of the virus titer was observed when the cells were cultured with supernatants from yolkin treated BMDM. The results indicated that yolkin indirectly inhibited replication of the virus via induction of suppressive mediators such as nitric oxide, TNF α and interferons (IFNs) type I (α/β) (Kazana et al. 2020).

The remarkably strong inhibition of RAV-2 replication upon constant exposure of cells to yolkin could be explained by its effects on expression of MAPK and cyclooxygenases (COXs) demonstrated in mouse splenocyte cultures (Zimecki et al. 2025C). The expression of ERK1, ERK2 and JNK after 24 h incubation with 20 μg/mL of yolkin represented 0.33, 0.53 and 0.04 values of the control GAPDH protein. In addition, the expression of COX-1 in the splenocyte cultures increased several hundred times, with an absence of COX-2 expression. Hence, the inhibitory effect of yolkin on viral replication in DF-1 cells seems to be consistent with the role of ERK (Luo et al. 2002, Andrade et al. 2004, Seamone et al. 2010, Dai et al. 2016), JNK kinases (He et al. 2019, Yuan et al. 2021, Jones et al. 2024) and COX-1 (Rahmouni et al. 2004, Carey et al. 2005, Depboylu et al. 2011) in viral infection and replication. Of importance, by using the ERK/MAPK inhibitor PD98059, we demonstrated that this signaling pathway enables replication of RAV-2 virus in DF-1 cells.

The lack of a parallel determination of MAPK production in this model is a limitation of the study. Nevertheless, the strong indirect evidence (Zimecki et al. 2025C), confronted with the above cited literature, indicates that MAPK levels are inhibited by yolkin during viral infection also in our model. We showed (Zimecki et al. 2025C) that yolkin strongly downregulates cell activation in several models such as: mitogen-induced cell proliferation, LPS-enhanced cell viability, LPS-induced TNF α and COX-2 production, and growth of L-1210 leukemia. Thus, yolkin directs cell metabolism or cell cycle to an unfavorable status for viral replication. Taken together, based on the accumulated literature, we cautiously propose that yolkin, acting via TLR4 receptors in infected cells, modifies the cell metabolism to augment antiviral response by lowering MAPK activity, induction of type I interferons and pro-inflammatory cytokines and inducible nitric oxide production. The latter cell mediator is particularly effective in combat of viral infection (Abdul-Cader et al. 2016). Nevertheless, potential inhibitory effects of yolkin in this or a similar model should be experimentally proved. However, it is difficult to interpret the mechanism of the inhibitory effects of yolkin on virus replication when DF-1 cells were preincubated with yolkin or with a mixture of RAV-2 and yolkin. Regarding the first protocol, no data are available, in contrast to lactoferrin (LF) (Di Biase et al. 2003), indicating that yolkin molecules may contain a fragment with a blocking property for a putative cell receptor for viruses. Although 20 h pre-incubation of the cells with yolkin could affect the phenotype and metabolic activity of DF-1 cells, it had no effect on virus replication. In the second protocol, a possible direct inactivation of the virus particles is possible, although it cannot be proved. On the other hand, a direct effect of yolkin on MAPK activation after virus infection is highly probable since in ALV-J CHN06 strain infection of DF-1 cells, ERK2 activation occurred as early as 15 min following infection (Dai et al. 2016). Therefore, during 2 h pre-incubation of DF-1 cells with yolkin, the activation of ERK2 kinase could already be strongly downregulated. These effects are weaker in comparison to those found in conditions of constant exposure of cells to yolkin, but it should also be noted that yolkin loses some activity upon freezing and storage at -20°C (Zambrowicz et al. 2024).

Due to differences in evaluation of antiviral activity, e.g. molecular methods versus determination of cytopathic effects, and ways of their measurement (TCID, logarithmic scale titer, percent inhibition of reversed transcriptase, numbers of RNA copies) it is difficult to compare efficacy of known natural antiviral compounds with yolkin. In addition, the antiviral compounds may act using different mechanisms. Among known antiviral flavonoids (Spedding et al. 1989) quercetin at 50 μM inhibited activity of RAV-2 reversed transcriptase by 23% but amentoflavone and scutellarein showed higher potency. In turn, a fraction of TPPS pollen polysaccharide (Yang et al. 2015) inhibited ALV-B avian virus titer in DF1 cells by almost 2 log at 50-100 μg/mL. A fine example of a protein with antiviral properties, combing all manners of antiviral actions like direct neutralization, block of adsorption and replication inhibition, is milk derived lactoferrin. Thanks to its carbohydrate moiety and cationic nature it is very efficient in inhibition HDV-1 virus in Vero cells (Valimae et al. 2009). Nevertheless, we do not envisage promotion of yolkin as a potential natural therapeutic for use in humans. Rather, we postulate that yolkin may share antiviral function with its other roles played in developing avian embryo such as promotion of immune and nervous system development.

The ongoing studies on yolkin may reveal in its molecule structures that has the property of directly binding to viruses or their cell receptors. Of interest, yolkin contains interesting glycan structures, resembling in part, those of the strong antiviral compound TPPPS (Wang et al. 2019B). In practice, antiviral therapies are initiated following diagnosis of viral infection so preventive approaches are not expected in designing antiviral therapeutics. Nevertheless, yolkin may still augment innate antiviral immune status in humans, thanks to its ability to induce production of type I and II IFNs. It should be underlined that DF-1 cells represent embryonic fibroblasts. Therefore, our presumption, resulting from this work, is that yolkin

may play a key antiviral, protective role for birds developing embryos.

In summary, the present investigation revealed yet another activity of yolkin, besides the already described immunoregulatory and precognitive properties of the protein. Thus, a potential use of yolkin preparation as a nutraceutical in amelioration of viral infections cannot be excluded (Zambrowicz et al. 2023).

Conclusion

In conclusion, this is a first demonstration of antiviral activity in a homologous avian model. It should be underlined that DF-1 cells represent embryonic fibroblasts. Therefore, we propose that yolkin may play a key antiviral, protective role for birds' developing embryos. In summary, the present investigation revealed yet another activity of yolkin, besides its already described immunoregulatory properties and the capability to improve animal cognitive function. The accumulated evidence on yolkin actions *in vivo* predispose the protein for designing clinical trials for patients with immune and cognitive deficits. Furthermore, yolkin requires more research on its mechanism of action, including changes in gene expression, and determination of its exact role in a developing avian embryo**.**

Author Declarations

Ethics approval

In our investigation, involving only an established tumor cell line, an agreement of a local ethics committee is not required.

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Use of generative artificial intelligence (AI)

Al was not used to prepare the manuscript or its figures.

Conflicts of interest

The authors declare no conflict of interest.

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