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Original article

THE INFLUENCE OF FLAVONOID COMPOSITIONS ON NRF2 TRANSCRIPTION FACTOR EXPRESSION IN CASE OF INFECTIONS TRIGGERED BY INFLUENZA A VIRUS AND TRANSMISSIBLE GASTROENTERITIS CORONAVIRUS

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Background. Viral infections trigger transcription factors, including Nrf2, which regulate the expression of genes related to cytokines, chemokines, and more. Nuclear factor erythroid 2-related factor 2 (Nrf2) participates in complex regulatory networks controlling the expression of cytoprotective genes and immune responses. Recently, new roles have been attributed to Nrf2, including the regulation of antiviral responses.
Objective. The aim of the study was to analyze Nrf2 activation in influenza virus infection *in vivo* and in transmissible gastroenteritis coronavirus (TGEV) infection *in vitro*, as well as the effects of the flavonoid compositions Proteflazidum® and Protoil on the Nrf2 expression in these two experimental models of viral infection.
Methods. Outbred white mice were infected with influenza A virus (A/FM/1/47/H1N1 strain). Jurkat cells were infected with TGEV, previously adapted to these cells. Flavonoid compositions Proteflazidum® and Protoil (Ecopharm, Ukraine), containing the mixture of tricin, luteolin, apigenin, quercetin, and rhamnosin, were used in corresponding dilutions. Real-Time PCR was employed to analyze Nrf2 RNA expression in the lungs of mice and in both uninfected and virus-infected cells. Additionally, ELISA was used to assess the expression of Nrf2 peptide.
Results. The Nrf2 expression in the lungs of influenza virus-infected mice showed a tendency to increase within a 100-fold range. In virus-infected mice treated with Proteflazidum® or Protoil, the level of Nrf2 expression in the lungs decreased about 10-fold compared to infected untreated mice. TGEV infection resulted in 100-fold increase in Nrf2 expression in Jurkat cells. Both Proteflazidum® and Protoil decreased Nrf2 expression in TGEV-infected cells, while their effects on Nrf2 expression in the intact cells on Day 1 were not detected.
Conclusions. Flavonoid compositions have only a slight effect on Nrf2 expression in intact cells. However, in case of virus infection, both *in vivo* and *in vitro*, they counteract the extensive up-regulation of Nrf2 expression due to viral infection.

Keywords: Nrf2; flavonoids; influenza virus; transmissible gastroenteritis coronavirus.

Introduction

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor initially discovered as a master regulator of redox homeostasis in cells [1]. Interplaying with NF-κB pathway, Nrf2 coordinates cellular response to oxidative stress and inflammation [2]. Under oxidative stress, Nrf2 dissociates from its suppressor Kelch-like-ECH-associated protein 1 (KEAP1) in cytoplasm and relocates to the nucleus. There, it binds to antioxidant response elements (AREs) in the genes encoding proteins that regulate oxidative stress, diminish inflammation, and confer other cytoprotective effects at the transcriptional level [3]. Keap1-Nrf2-ARE pathway interacts with NF-κB pathway regulating the response to stress and inflammation in such a way that the decreased expression of Nrf2 upregulates NF-κB expression by various mechanisms that is important for the balance of pro- and anti-inflammatory factors [2].

Nrf2, like other transcription factors, plays an important role in regulating the body's protective systems and maintaining cell homeostasis due to its versatile effects. It now became clear that the effects of Nrf2 are broader than its involvement in the antioxidant response. In particular, Nrf2 is essential for the regulation of innate immunity [4]. The experiments with Nrf2-deficient mice demonstrated Nrf2 involvement in regulation of neutrophil gene expression as well as genes related to phagocytosis, Fc receptor function, complement and immunoglobulin regulation during acute bacterial infections [5].

Recently, the new roles have been attributed to Nrf2 including regulation of antiviral responses [6]. Nrf2 activation has been demonstrated in many viral infections, and different mechanisms of its activation have been proved [7–9]. The mechanisms of pro- and antiviral activities of Nrf2 seem to be diverse. Nrf2's role in antiviral immunity seems to be partially associated with the regulation of the interferon response by inhibiting certain signaling components of interferon-inducing pathways [10, 11]. However, the prevailing belief is that Nrf2 primarily contributes to viral reproduction mostly by interferon-independent mechanisms [6].

Nrf2 seems to play a dual role in viral infections depending both on the type of the virus and the clinical or experimental setting. Many studies suggest a protective role of Nrf2 in viral infections. The sequels of the experimental viral infection were more pronounced in Nrf2-deficient mice [12]. The use of Nrf2 activators *in vitro* provides the cytoprotective effect and inhibits viral replication [13]. The *in vitro* antiviral effects associated with Nrf2 activation have been demonstrated for a broad range of viruses of different taxonomic groups such as influenza virus, Zika virus, herpes simplex virus, SARS-CoV2, etc. [6, 9, 10]. Nevertheless, the protective role of Nrf2 in viral infections cannot be considered universally consistent taking into account several reports associating Nrf2 activation with the progression of viral infection [9, 14].

Nrf2 transcription is induced by various substances of different classes. Various phenolic compounds including flavonoids are among the natural Nrf2 activators [13, 15]. Luteolin, apigenin, quercetin, myricetin, rutin, naringenin, epicatechin, and genistein have been experimentally proved as Nrf2 inducers [16]. On the other hand, these substances have proved to possess antiviral activity against the broad spectrum of viruses [17]. It is of interest to assess the Nrf2-inducing capacity in the experimental systems of virus infection where antiviral activity of these substances was demonstrated.

In Ukraine, a composition named Proteflazidum®, containing a sum of flavonoid glycosides and aglycons, extracted from wild gramineous plants *Deschampsia caespitosa* L. (tussock grass) and *Calamagrostis epigeios* L. (reed grass), has been developed [18]. Earlier, we have reported *in vitro* antiviral activity of Proteflazidum® against HIV and papillomavirus [19, 20].

The study of the dynamics of Nrf2 activation in viral infections associated with pneumonia is of particular importance taking into account the role of Nrf2 not only in viral infection but also in the accompanying pathological processes in the organs and tissues. Recently, we confirmed the antiviral effects of Proteflazidum® and its constituents *in vivo* in case of influenza virus-induced pneumonia in mice and in *in vitro* infection with transmissible gastroenteritis coronavirus (TGEV) that belongs to alpha-coronaviruses (unpublished data).

The aim of the present study was to research Nrf2 activation in influenza virus infection *in vitro* and TGEV infection *in vivo* and the effects of flavonoid compositions Proteflazidum® and Protoil on the Nrf2 expression in the setting of influenza virus infection *in vivo* and transmissible gastroenteritis coronavirus (TGEV) infection *in vitro*.

Materials and methods

Flavonoid compositions

Proteflazidum® composition, developed and produced by Ecopharm Research-and-Production Company, Ukraine, contains ethanol extract from wild gramineous plants *Deschampsia caespitosa L.* (tussock grass) and *Calamagrostis epigeios L.* (reed grass). The content of flavonoids is not less than 0.32 mg/ml calculated as rutin equivalent. The composition comprises O- and C-glycosides of tricin, lutheolin, apigenin, quercetin, rhamnasine as well as their aglycons) [18].

Protoil composition consists of the dense extract prepared via vacuum concentration of Proteflazidum, but ethanol here is substituted with the sunflower oil and polyethylene glycol 400. The content of flavonoids is not less than 3.5 mg/mL. The samples of Protoil were provided by Ecopharm Research-and-Production Company, Ukraine.

Viruses

Influenza virus A/FM/1/47 (H1N1) strain has been obtained from the Depository of Viruses of

L.V. Gromashevsky Institute of Epidemiology and Infectious Diseases. Infectious titer in Madin–Darby canine kidney (MDCK) cells – 5.0–9.0 lg ID50, hemagglutinin titer – 1:512 hemagglutination units (HAU)/0.2 ml. Virus was adapted to the lungs of white outbred mice with LD_{50} – 4.5 lg ID_{50} [21].

The TGEV strain $D_{52\text{-}5}$ (BRE_{79}) was provided generously by Dr. Hubert Laude (Molecular Virology Laboratory of Biotechnology Center, INRA, France). The attenuated TGEV variant was obtained following 100 passages in continuous porcine cell lines [22]. Prior to the study, the attenuated TGEV was adapted to Jurkat cells. All viral materials were stored at –70 °C prior to experimental studies.

Experimental animals

The outbred white mice, body weight – 14–18 g, sourced from the experimental animals' vivarium of the L.V. Gromashevsky Institute of Epidemiology and Infectious Diseases, were used in the study. The animals were kept under controlled conditions of temperature (22 to 24 °C), relative humidity (40% to 70%), lighting (12-hour light-dark cycle) and fed a standard pellet diet.

Design of in vivo experiment

The mice were infected intranasally with 10 LD_{50} of the mouse-adapted influenza virus A/FM/1/47 (H1N1). One hour after infection, the mice of experimental groups were exposed to flavonoid compositions. Proteflazidum® was administered intraperitoneally at a 1:100 dilution in a volume of 0.2 ml. Protoil was fed by gavage at a 1:100 dilution in a volume of 0.2 ml. Flavonoids were also administered to non-infected mice according to the same scheme. The mice of experimental groups as well as virus-infected and intact mice were sacrificed on Day 1 and Day 4. The experimental setting comprised 6 groups of animals with 6 mice in each group. Thus, at each experimental point (Day 1 and Day 4), studies were carried out on 3 mice.

RNA was isolated from lung homogenates of the untreated or flavonoid-treated virus-infected mice on Days 1 and 4 postinfection and Nrf2 expression was assessed by RT-PCR. RNA isolated from the lungs of the intact mice served as the negative control. The relative content of Nrf2-specific RNA was analyzed by the end-point dilution method. The log of the threshold dilution that did not reveal specific transcript was taken as a measure of its content in the initial sample.

Bioethical norms

The study was performed according to the guidelines of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Strasbourg, 1986) and was approved by the Institutional Animal Care and Use Committee. The experimental procedures with the animals were carried out in accordance with the provisions of the Law of Ukraine "On the Protection of Animals from Cruelty" (2006) and the provisions of the Directive 2010/63/EU of the European Parliament and the Council of the European Union "On the Protection of Animals Used for Scientific Purposes" (2010). All animal-related procedures were performed in accordance with the ethical standards of the Animal Ethics Committee, L.V. Gromashevsky Institute of Epidemiology and Infectious Diseases of the National Academy of Medical Sciences of Ukraine (No. AEC/25/2023).

Cell lines

Human Jurkat cell line of T-lymphoblastic leukemia was obtained from the Depository of Cell Lines and Tumor Strains of the R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology of the National Academy of Sciences of Ukraine. The cells were grown in RPMI-1640 medium with 10% fetal calf serum (FCS) (Sigma, USA) in a 5% CO_2-humidified atmosphere and subcultured twice a week. For experimental studies, the cells were seeded in 24-well microplates and each experimental point was repeated in four replicates.

RT-PCR analysis

The nucleotide sequences of the target genes (mouse and human Nrf2) were searched from GenBank database (https://www.ncbi.nlm.nih.gov/nucleotide/). The selected sequences were aligned and analyzed, and specific primers were construed based on Vector NTI v.10.0.1 software (Invitrogen, USA) and Basic Local Alignment Search Tool (BLAST) (National Center for Biotechnology Information (NCBI), USA), except for the sequences for reverse and forward primers for mouse Nrf2 that were taken from [23]. All the primers were synthesized by Generi Biotech (Czech Republic, http://www.generi-biotech.com/). All primers used in the study are listed in Table 1.

RNA from cells was isolated with "GeneJet RNA Purification Kit" (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. RNA concentration was measured on a Nano

Table 1: List of primers used in the study

Primer	Sequence (5'–>3')	Target gene
mNrf2-R	CCAGAGAGCTATTGAGGGACTG	
mNrf2-F	GCCCACATTCCCAAACAAGAT	Nrf2 (mouse)
mNrf2-Probe	FAM-TGACCATGAGTCGCTTGCCCTGG-BHQ1	
hNrf2-F	AGCCCAGCACATCCAGTCAG	
hNrf2-R	TGCATGCAGTCATCAAAGTACAAAG	Nrf2 (human)
hNrf2-Probe	FAM-ACTCCCAGGTTGCCCACATTCCCA-BHQ1	

Drop 1000C spectrophotometer (Thermo Fisher Scientific, USA).

TaqMan probe-based RT-PCR analysis was employed using "Luna Universal Probe One-Step RT-qPCR Kit" (BioLabs, Great Britain). The incubation mixture contained 10 μl of "Luna Universal Probe One-Step Reaction Mix" (2x); 0.8 μl of forward primer (10 μmol); 0.8 μl of reverse primer (10 μmol); 0.4 μl of the probe with fluorophore (FAM); 2 μl of nuclease-free water and 5 μl of RNA. RT-PCR was performed in microtubes in a CFX-96 thermal cycler (BioRad, USA). The reaction thermal profile was such as follows: reverse transcription (55 °C, 10 min) – initial denaturation (inactivation of revertase) (95 °C, 1 min) – denaturation/annealing/elongation (95 °C – 60 °C, 10 s – 30 s; 40 cycles). Fluorescence was measured once per cycle at the end of the 60 °C segment. The results were processed using Bio-Rad CFX Manager 2.1 (v. 3.1).

The relative content of the specific transcript in RNA samples was determined by end-point dilution method. For each sample, (real-time) RT-PCR was performed in tenfold serial dilutions, in four replicates each. The log of the threshold dilution of the initial RNA sample that did not produce the specific signal (Ct exceeding 38 in our experimental setting) was taken as the relative measure of the content of the specific transcript in the initial sample.

Nrf2 activation quantity assay

"Nrf2 Transcription Factor Assay Kit (Colorimetric)" (Abcam, USA) was used to quantify Nrf2 activation in nuclear extracts. This assay is a high throughput assay to quantify Nrf2 activation in nuclear extracts. It combines a quick ELISA (enzyme-linked immunosorbent assay) format with a sensitive and specific non-radioactive assay for transcription factor activation. The nuclear extracts were prepared from cell homogenate or cell culture with "Nuclear Extraction Kit" (Abcam, USA) according to the manufacturer's instructions. Protein concentration in the nuclear extract was measured by Bradford method [24] using Bradford Assay Kit (Sigma, USA). The absorbance was read on Bio-Tek Elx 800 ELISA reader at 450 nm vs. 655 nm [25].

Statistical processing

The data of ELISA analysis were expressed as mean ± standard error of the mean (M ± SEM). The groups compared were small and statistical power may not be sufficient to detect significant differences between groups. The non-parametric Mann–Whitney U test was used despite the normal distribution of the data. The significance of the differences was analyzed by Microsoft Excel and Microcal Origin software. The difference was considered as significant if $p < 0.05$.

The data for determining the Nrf2-specific RNA content (in the form of the logarithm of the threshold dilution) were expressed as mean ± standard error of the mean (M ± SEM) [26].

Results

Effects of flavonoid compositions and influenza virus infection on Nrf2 expression in lung of mice

The threshold points shown in Table 2 are indicative of the ballpark estimation of Nrf2 RNA expression changes due to virus infection and/or flavonoid treatment. On Day 1 after infection, level of Nrf2 in virus-infected mice's lungs tends to increase within a 100-fold range. Application of Protoil but not Proteflazidum® resulted in the increase of Nrf2 expression in the intact mice (the trend within 10-fold range). Notably, in virus-infected mice treated with Proteflazidum® or Protoil, the Nrf2 expression in lungs tended to decrease. On Day 4 after infection, the patterns were similar except for Proteflazidum®.

Effects of flavonoid compositions and TGEV infection on Nrf2 expression in Jurkat cells

The expression of Nrf2 was analyzed *in vitro* in Jurkat cells infected with TGEV and/or treated with Proteflazidum® or Protoil. Cells were infected

with 100 TCD_{50} TGEV via a standard technique. Following virus absorption, the medium containing Proteflazidum® at a 1:100 dilution or Protoil at a 1:1000 dilution was added to ensure equal concentrations of the active substances. Control cells were incubated in a standard RPMI-1640 medium. Following one-day culture, cells were collected and RNA was isolated. Ten-fold dilutions of the initial RNA were analyzed with RT-PCR with primers specific for human Nrf2. The threshold points are presented in Table 3. Proteflazidum® and Protoil had no impact on Nrf2 expression in intact Jurkat cells. TGEV infection led to the increase of Nrf2 expression within 100-fold range. Nevertheless, in TGEV-infected cells treated with either Proteflazidum® or Protoil the level of Nrf2 expression returned to the baseline level in the intact cells.

Effects of Proteflazidum® on Nrf2 activation in TGEV-infected or intact Jurkat cells

To confirm the data on the Nrf2 expression at the level of mRNA, the level of Nrf2 protein expression in TGEV-infected or intact Jurkat cells treated with Proteflazidum® was assessed by ELISA technique. Cells were infected with 100 TCD_{50} TGEV as described above and after 1-h virus absorption, RPMI-1640 medium with 2% FCS was added with or without Proteflazidum® at a 1:200 or 1:400 dilution. One day later, the cells were washed, the nuclear fraction was extracted and Nrf2 protein content was measured by ELISA technique. The results are presented in the Figure.

TGEV infection resulted in the substantial activation of Nrf2 protein. Proteflazidum® itself did

Table 2: Expression of Nrf2 gene in the lungs of mice following influenza virus infection and/or treatment with flavonoid compositions (Proteflazidum® or Protoil)

Treatment arm	−log of end-point (threshold) dilution	
	Day 1	Day 4
Non-infected mice (negative control)	4.00 ± 0.00	4.00 ± 0.00
Virus-infected mice	6.25 ± 0.71	6.00 ± 0.00
Non-infected mice + Proteflazidum®	4.00 ± 0.00	4.25 ± 0.71
Non-infected mice + Protoil	5.00 ± 0.00	5.25 ± 0.71
Virus-infected mice + Proteflazidum®	5.00 ± 0.00	7.25 ± 0.71
Virus-infected mice + Protoil	5.25 ± 0.71	5.00 ± 0.00

Table 3: Expression of Nrf2 gene in Jurkat cells infected with TGEV and treated with flavonoid compositions (Proteflazidum® or Protoil)

Treatment arm	−log of end-point (threshold) dilution
Non-infected cells (negative control)	3.00 ± 0.00
Virus-infected cells	5.25 ± 0.71
Non-infected cells + Proteflazidum®	3.00 ± 0.00
Non-infected cells + Protoil	3.25 ± 0.71
Virus-infected cells + Proteflazidum®	3.00 ± 0.00
Virus-infected cells + Protoil	3.25 ± 0.71

Notes. The relative content of the specific transcript in RNA samples is expressed as the log of end-point dilution of RNA in RT-PCR incubation mixture that did not detect the presence of the target gene sequences. All ten-fold dilutions of the initial RNA were assayed in four replicates.

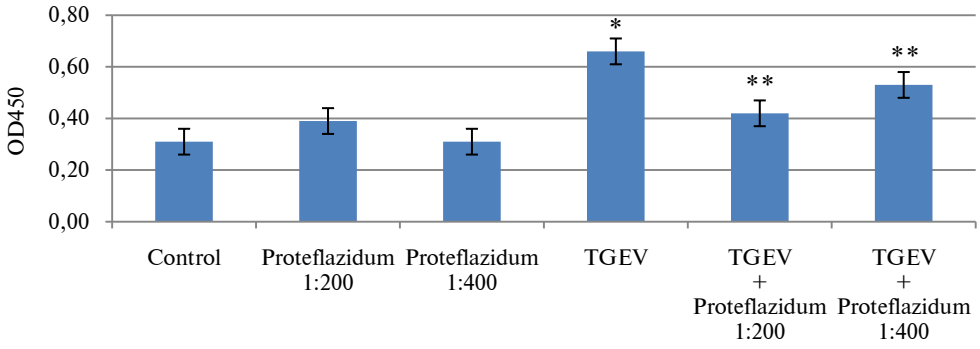

Figure: Expression of Nrf2 protein in nuclear extracts of Jurkat cells infected with TGEV and ciltured with/without Proteflazidum® for 1 day; * – the difference is significant compared to intact cells, $p < 0.05$; ** – the difference is significant compared to virus-infected cells, $p < 0.05$

not activate Nrf2 in our experimental setting. Nevertheless, similarly to the Nrf2 gene expression data, Proteflazidum® decreased significantly Nrf2 protein expression in TGEV-infected cells with a trend of the dose dependence.

Discussion

The Nrf2 pathway is an important cell signaling mechanism in maintaining redox homeostasis. Bacterial and viral infections are associated with oxidative stress contributing to their pathogenesis. Therefore, studying Nrf2 activation in the course of different viral infections could be important for outlining the dual role of this pathway in antiviral protection as well as in pathogenesis of viral infections at the level of tissues and organs of the host since Nrf2 activation influences the expression of various Nrf2-regulated genes, alterations in which are observed during viral infections.

While the role of Keap1-Nrf2-ARE pathway in the antioxidant protection of cells is well established, the recent studies explore its involvement in viral infection and immunity [6]. Sometimes this role may be dualistic depending on virus, cell type, or stage of infection. Infection by most viruses including influenza viruses induces Nrf2 expression [27]. Nrf2 not only exerts direct antiviral effect in influenza virus-infected cells but also protects the lungs from virus-induced lung injury [28]. Several viruses, however, promote Nrf2 degradation that facilitates viral replication [29]. Recent research has explored the role of Nrf2 activation in suppressing SARS-CoV2 replication, including dampening the response to pro-inflammatory cytokines [30]. In several viral infections, the dualistic role of Nrf2 activation is traced since it affects both virus replication and pathogenesis of viral infection. In general, the effect of virus infection on Nrf2 activation depends on the specific mechanisms of virus-cell interaction, in particular on the contribution of apoptosis as a factor of antiviral protection and the role of the inflammatory response that could be advantageous for viral propagation. Therefore, an analysis of the mechanisms of Nrf2 activation and the Keap1-Nrf2-ARE pathway modulation in different viral infections is important.

We have demonstrated Nrf2 activation in two different models: influenza virus infection *in vivo* and alphacoronavirus (TGEV) infection *in vitro*. In our study, as in [27], *in vivo* infection with accompanying pneumonia came amid with Nrf2 mRNA up-regulation in lung cells detected both on Day 1 and Day 4 of infection. It should be noted that in our experimental model, all infected mice died on Day 5 post-infection. The Nrf2 activation in our experimental model did not depend on the stage of influenza virus infection contrary to that for herpes virus infection stated in [31]. Our data correspond to the work [27], where it was shown that the influenza A virus increases the production of ROS and the expression of Nrf2, as well as its translocation to the nucleus. Note that experiments [27] were carried out *in vitro* in alveolar type II cells isolated from human lungs, and the Nrf2 expression assessment was carried out after 48 hours.

TGEV infection *in vitro* also was accompanied with Nrf2 mRNA up-regulation detected on Day 1 post-infection. The activation of Nrf2 in our study was confirmed also at the level of protein expression. The induction of Nrf2 in TGEV-infected cells *in vitro* was shown by Wang *et al.* [32]. It should be mentioned that in case of SARS-CoV-2, strong inhibition of Nrf2 in the lungs was demonstrated at the late stages of infection [33, 34].

Polyphenols including various flavonoids are among known Nrf2 inducers. On the other hand, many polyphenols possess antiviral activity when it comes to various viral infections [17]. Several different mechanisms of the antiviral effects of flavonoids in addition to their antioxidant activities have been studied. In particular, flavonoids can directly inhibit virus infection by impeding virus attachment, entry, replication, and release [35], although the mechanisms related to Nrf2 activation/inhibition are less researched. The possible mechanisms of Nrf2 activation include the disruption of interaction between Keap1 and Nrf2 [36] and different forms of Nrf2 protein modifications [16].

Anti-influenza activity of natural polyphenols including flavonoids has been reviewed elsewhere [37]. Such flavonoid substances as epigallocatechin, quercetin and its glycosides effectively inhibit replication of influenza virus *in vitro* [17]. Natural flavonoids effectively inhibit replication of coronaviruses [38]. The contribution of Nrf2 pathway to antiviral effects of flavonoids and various herbal phenolic compounds remains to be elucidated in detail. Recent findings support the role of various Nrf2 activators in inhibiting influenza virus replication [39]. Catechin flavonoids have been shown to inhibit TGEV-induced ROS and cytopathic effect [40]. The association of the anti-TGEV effects of the natural phenolic compound eugenol with the decreased Keap-1 expression and significant increase of Nrf2 expression has been recently demonstrated [32]. Note that the data [32], in contrast to the design of our study, relate to

in vivo conditions. That is why the comparison of our data and the data of [32] is extremely difficult: in terms of both the dose dependence and the complex effect of the viral infection and herbal medicine on the animal's body. Obviously, further *in vivo* studies of the anti-TGEV effects of the drugs under study should include different treatment conditions in order to determine the appropriate dose range and frequency of use. The effects of flavonoids on Keap1-Nrf2-ARE pathway depend on the concentration of these substances since high concentrations may cause the reverse effect, therefore inhibiting Nrf2 production [41]. Form of flavonoid substances and their compositions are also of a great significance. In our study, Protoil composition, not Proteflazidum®, increased Nrf2 expression in the lungs of mice. Nevertheless, in influenza virus-infected mice, on Day 1 post-infection, both Protoil and Proteflazidum®, decreased Nrf2 expression in the lungs as compared to virus-infected mice, which were not treated with these flavonoid compositions. The effects of both flavonoid compositions on Nrf2 expression in the intact Jurkat cells on Day 1 have not been detected, while both compositions down-regulated this expression in TGEV-infected cells. These trends have been confirmed when Nrf2 expression in this experimental system was analyzed at the protein level.

Conclusions

To sum up, we have studied the effects of two forms of flavonoid compositions with identical constituents and different solvents in two experimental models of viral infection (influenza virus infection *in vivo* and TGEV infection *in vitro*) on the Nrf2 expression. Both general and specific effects for these two experimental systems have been revealed. Both viruses up-regulate Nrf2 expression significantly. Flavonoid compositions have only slight effect on Nrf2 expression in the intact cells while in virus infection both *in vivo* and *in vitro* they counteract the extensive up-regulation of Nrf2 expression due to viral infection.

Interests disclosure

Alexander Galkin is the Editor-in-Chief and Svitlana Rybalko is the member of the editorial board of the journal *Innovative Biosystems and Bioengineering*, they did not participate in the editorial evaluation and decision-making on the publication of the article. Viktor Atamaniuk reports being employee of Ecopharm Research-and-Production Company; the author declares that he has no competing interests. The other authors have no conflicts of interest to disclose.

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References

[1] Moi P, Chan K, Asunis I, Cao A, Kan YW. Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the beta-globin locus control region. Proc Natl Acad Sci USA. 1994;91(21):9926-30. DOI: 10.1073/pnas.91.21.9926

[2] Gao W, Guo L, Yang Y, Wang Y, Xia S, Gong H, et al. Dissecting the crosstalk between Nrf2 and NF-κB response pathways in drug-induced toxicity. Front Cell Dev Biol. 2022;9:809952. DOI: 10.3389/fcell.2021.809952

[3] Saha S, Buttari B, Panieri E, Profumo E, Saso L. An overview of Nrf2 signaling pathway and its role in inflammation. Molecules. 2020;25(22):5474. DOI: 10.3390/molecules25225474

[4] Battino M, Giampieri F, Pistollato F, Sureda A, de Oliveira MR, Pittalà V, et al. Nrf2 as regulator of innate immunity: A molecular Swiss army knife! Biotechnol Adv. 2018;36(2):358-70. DOI: 10.1016/j.biotechadv.2017.12.012

[5] Gomez JC, Dang H, Martin JR, Doerschuk CM. Nrf2 modulates host defense during Streptococcus pneumoniae pneumonia in mice. J Immunol. 2016;197(7):2864-79. DOI: 10.4049/jimmunol.1600043

[6] Herengt A, Thyrsted J, Holm CK. NRF2 in viral infection. Antioxidants. 2021;10(9):1491. DOI: 10.3390/antiox10091491

[7] Lee J, Koh K, Kim YE, Ahn JH, Kim S. Upregulation of Nrf2 expression by human cytomegalovirus infection protects host cells from oxidative stress. J Gen Virol. 2013;94(Pt 7):1658-68. DOI: 10.1099/vir.0.052142-0

[8] Liu B, Fang M, He Z, Cui D, Jia S, Lin X, et al. Hepatitis B virus stimulates G6PD expression through HBx-mediated Nrf2 activation. Cell Death Dis. 2015;6(11):e1980. DOI: 10.1038/cddis.2015.322

[9] Ramezani A, Nahad MP, Faghihloo E. The role of Nrf2 transcription factor in viral infection. J Cell Biochem. 2018;119(8):6366-82. DOI: 10.1002/jcb.26897

[10] Olagnier D, Brandtoft AM, Gunderstofte C, Villadsen NL, Krapp C, Thielke AL, et al. Nrf2 negatively regulates STING indicating a link between antiviral sensing and metabolic reprogramming. Nat Commun. 2018;9(1):3506. DOI: 10.1038/s41467-018-05861-7

[11] Gunderstofte C, Iversen MB, Peri S, Thielke A, Balachandran S, Holm CK, Olagnier D. Nrf2 negatively regulates type I interferon responses and increases susceptibility to herpes genital infection in mice. Front Immunol. 2019;10:2101. DOI: 10.3389/fimmu.2019.02101

[12] Cho HY, Imani F, Miller-DeGraff L, Walters D, Melendi GA, Yamamoto M, et al. Antiviral activity of Nrf2 in a murine model of respiratory syncytial virus disease. Am J Respir Crit Care Med. 2009;179(2):138-50. DOI: 10.1164/rccm.200804-535OC

[13] Shoji M, Arakaki Y, Esumi T, Kohnomi S, Yamamoto C, Suzuki Y, et al. Bakuchiol is a phenolic isoprenoid with novel enantiomer-selective anti-influenza A virus activity involving Nrf2 activation. J Biol Chem. 2015;290(46):28001-17. DOI: 10.1074/jbc.M115.669465

[14] Lee C. Therapeutic modulation of virus-induced oxidative stress via the Nrf2-dependent antioxidative pathway. Oxid Med Cell Longev. 2018;2018:6208067. DOI: 10.1155/2018/6208067

[15] Robledinos-Antyn N, Fernбndez-Ginés R, Manda G, Cuadrado A. Activators and inhibitors of NRF2: a review of their potential for clinical development. Oxid Med Cell Longev. 2019;2019:1-20. DOI: 10.1155/2019/9372182

[16] Suraweera TL, Rupasinghe HPV, Dellaire G, Xu Z. Regulation of Nrf2/ARE pathway by dietary flavonoids: a friend or foe for cancer management? Antioxidants. 2020;9(10):973. DOI: 10.3390/antiox9100973

[17] Badshah SL, Faisal S, Muhammad A, Poulson BG, Emwas AH, Jaremko M. Antiviral activities of flavonoids. Biomed Pharmacother. 2021;140:111596. DOI: 10.1016/j.biopha.2021.111596

[18] Atamaniuk V. EA201700340A1 - Biological active substance of polypharmacological action of plant origin - Google Patents [Internet]. 2017 [cited 2023 Apr 19]. Available from: https://patents.google.com/patent/EA201700340A1/en

[19] Trokhymchuk T, Zavelevich M, Liulchuk T, Starosyla D, Rybalko S, Rudenko A. In vitro study of anti-HIV activity of Proteflazid herbal composition. Am J Fund Appl Env Res. 2017;4:87-91.

[20] Spivak MYa, Rybalko SL, Starosyla DB, Zavelevich MP, Oleksiienko IP, Diadiun ST, et al. Study of the effects of flavonoid-containing composition Proteflazid on modeled papillomavirus infection in vitro. Dopov Nac Akad Nauk Ukr. 2018;(10):103-11. DOI: 10.15407/dopovidi2018.10.103

[21] Zharkova L, Rybalko S, Panasenko G, Kovalenko E, Hetman K, Salkov S. Mechanism of adaptation of influenza viruses A/FM/1/47 (H1N1) and A/Port CHlamers/1/73 (H3N2) to the new host. Lab Diagnos. 2009;3:26-30.

[22] Deriabin ON, Deriabina EG, Krasnobaiev EA, Bobyk VI. Effect of culture system on the genetic features of TGEV. Vestnik Selskochoz Nauk. 1991;8:138-41.

[23] Kanzaki H, Shinohara F, Kajiya M, Kodama T. The Keap1/Nrf2 protein axis plays a role in osteoclast differentiation by regulating intracellular reactive oxygen species signaling. J Biol Chem. 2013;288(32):23009-20. DOI: 10.1074/jbc.M113.478545

[24] Pirog T, Paliichuk O, Lutsai D, Kliuchka L, Shevchuk T. Effect of cations on the activity of NAD^+P-dependent glutamate dehydrogenase in Acinetobacter calcoaceticus IMV B-7241, Rhodococcus erythropolis IMV Ac-5017 and Nocardia vaccinii IMV B-7405 grown on industrial waste. Ukr Food J. 2021;10(1):198-208. DOI: 10.24263/2304-974X-2021-10-1-17

[25] Galkin OY, Besarab AB, Lutsenko TN. Characteristics of enzyme-linked immunosorbent assay for detection of IgG antibodies specific to Chlamydia trachomatis heat shock protein (HSP-60). Ukr Biochem J. 2017;89(1):22-30. DOI: 10.15407/ubj89.01.022

[26] Kyryk V, Ustymenko A, Lutsenko T, Klymenko P, Tsupykov O. Regenerative effects of mouse aortic endothelial cells in a murine model of critical limb ischemia. Cell Organ Transpl. 2022;10(2):90-6. DOI: 10.22494/cot.v10i2.143

[27] Kosmider B, Messier EM, Janssen WJ, Nahreini P, Wang J, Hartshorn KL, et al. Nrf2 protects human alveolar epithelial cells against injury induced by influenza A virus. Respir Res. 2012;13(1):43. DOI: 10.1186/1465-9921-13-43

[28] Yageta Y, Ishii Y, Morishima Y, Masuko H, Ano S, Yamadori T, et al. Role of Nrf2 in host defense against influenza virus in cigarette smoke-exposed mice. J Virol. 2011;85(10):4679-90. DOI: 10.1128/JVI.02456-10

[29] Komaravelli N, Ansar M, Garofalo RP, Casola A. Respiratory syncytial virus induces NRF2 degradation through a promyelocytic leukemia protein - ring finger protein 4 dependent pathway. Free Radic Biol Med. 2017;113:494-504. DOI: 10.1016/j.freeradbiomed.2017.10.380

[30] Olagnier D, Farahani E, Thyrsted J, Blay-Cadanet J, Herengt A, Idorn M, et al. SARS-CoV2-mediated suppression of NRF2-signaling reveals potent antiviral and anti-inflammatory activity of 4-octyl-itaconate and dimethyl fumarate. Nat Commun. 2020 Oct 2;11(1):4938. DOI: 10.1038/s41467-020-18764-3

[31] Wyler E, Franke V, Menegatti J, Kocks C, Boltengagen A, Praktiknjo S, et al. Single-cell RNA-sequencing of herpes simplex virus 1-infected cells connects NRF2 activation to an antiviral program. Nat Commun. 2019;10(1):4878. DOI: 10.1038/s41467-019-12894-z

[32] Wang K, Tang Y, Wu X, Liang H, Chen D, Yu B, et al. Eugenol attenuates transmissible gastroenteritis virus-induced oxidative stress and apoptosis via ROS-NRF2-ARE signaling. Antioxidants (Basel). 2022;11(9):1838. DOI: 10.3390/antiox11091838

[33] Qu Y, Haas de Mello A, Morris DR, Jones-Hall YL, Ivanciuc T, Sattler RA, et al. SARS-CoV-2 inhibits NRF2-mediated antioxidant responses in airway epithelial cells and in the lung of a murine model of infection. Microbiol Spectr. 2023;11(3):e0037823. DOI: 10.1128/spectrum.00378-23

[34] Hamad RS, Al-Kuraishy HM, Alexiou A, Papadakis M, Ahmed EA, Saad HM, et al. SARS-CoV-2 infection and dysregulation of nuclear factor erythroid-2-related factor 2 (Nrf2) pathway. Cell Stress Chaperones. 2023 Oct 5. DOI: 10.1007/s12192-023-01379-0

[35] Ninfali P, Antonelli A, Magnani M, Scarpa ES. Antiviral properties of flavonoids and delivery strategies. Nutrients. 2020;12(9):2534. DOI: 10.3390/nu12092534

[36] Magesh S, Chen Y, Hu L. Small molecule modulators of Keap1-Nrf2-ARE pathway as potential preventive and therapeutic agents. Med Res Rev. 2012;32(4):687-726. DOI: 10.1002/med.21257

[37] Zhang ZJ, Morris-Natschke SL, Cheng YY, Lee KH, Li RT. Development of anti-influenza agents from natural products. Med Res Rev. 2020;40(6):2290-338. DOI: 10.1002/med.21707

[38] Yang JY, Ma YX, Liu Y, Peng XJ, Chen XZ. A Comprehensive review of natural flavonoids with anti-SARS-CoV-2 activity. Molecules. 2023;28(6):2735. DOI: 10.3390/molecules28062735

[39] Waqas FH, Shehata M, Elgaher WAM, Lacour A, Kurmasheva N, Begnini F, et al. NRF2 activators inhibit influenza A virus replication by interfering with nucleo-cytoplasmic export of viral RNPs in an NRF2-independent manner. PLoS Pathog. 2023;19(7):e1011506. DOI: 10.1371/journal.ppat.1011506

[40] Zhang X, Chen S, Li X, Zhang L, Ren L. Flavonoids as potential antiviral agents for porcine viruses. Pharmaceutics. 2022 Aug 26;14(9):1793. DOI: 10.3390/pharmaceutics14091793

[41] Paredes-Gonzalez X, Fuentes F, Jeffery S, Saw CL-L, Shu L, Su Z-Y, Kong A-NT. Induction of NRF2-mediated gene expression by dietary phytochemical flavones apigenin and luteolin. Biopharm Drug Dispos. 2015;36(7):440-51. DOI: 10.1002/bdd.1956

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ВПЛИВ ФЛАВОНОЇДНИХ КОМПОЗИЦІЙ НА ЕКСПРЕСІЮ ТРАНСКРИПЦІЙНОГО ФАКТОРА Nrf2 ПРИ ІНФЕКЦІЯХ, СПРИЧИНЕНИХ ВІРУСОМ ГРИПУ А ТА КОРОНАВІРУСОМ ТРАНСМІСИВНОГО ГАСТРОЕНТЕРИТУ

Проблематика. Вірусна інфекція активує кілька факторів транскрипції, що задіяні в індукції експресії генів цитокінів, хемокінів тощо. Серед них – ядерний фактор, похідний від еритроїдного-2 подібного (Nrf2), який бере участь у складних регуляторних мережах, що контролюють експресію цитопротекторних генів і генів імунної відповіді. Нещодавно у Nrf2 були виявлені нові функції, включаючи регуляцію противірусної відповіді.
Мета. Проаналізувати активацію Nrf2 при інфекції вірусом грипу *in vivo* і вірусом трансмісивного гастроентериту (TGEV) *in vitro* та вплив флавоноїдних композицій Протефлазід® і Протойл на експресію Nrf2 на цих двох експериментальних моделях вірусної інфекції.
Методика реалізації. Безпородних білих мишей інфікували вірусом грипу (штам A/FM/1/47/H1N1). Клітини Jurkat інфікували TGEV, попередньо адаптованим до цих клітин. Флавоноїдні композиції Протефлазід® та Протойл (Екофарм, Україна), що містять суміш трицину, лютеоліну, апігеніну, кверцетину та рамнозину, застосовували у відповідних розведеннях. Експресію РНК Nrf2 у легенях мишей, інтактних і вірус-інфікованих клітин аналізували за допомогою ЗТ-ПЛР у режимі реального часу. Експресію пептиду Nrf2 оцінювали методом імуноферментного аналізу.
Результати. У легенях мишей, інфікованих вірусом грипу, рівень експресії Nrf2 збільшувався приблизно в 100 разів. У інфікованих вірусом мишей, які отримали Протефлазід® або Протойл, рівень експресії Nrf2 у легенях знижувався приблизно в 10 разів порівняно з інфікованими мишами, які не отримували флавоноїдних композицій. Інфікування TGEV призвело до 100-кратного збільшення експресії Nrf2 в клітинах Jurkat. Протефлазід® і Протойл знижували експресію Nrf2 у клітинах, інфікованих TGEV, тоді як їхнього впливу на експресію Nrf2 в інтактних клітинах на першу добу виявлено не було.
Висновки. Досліджені флавоноїдні композиції слабо активують експресію Nrf2 в інтактних клітинах, тоді як за вірусної інфекції як *in vivo*, так і *in vitro* вони протидіють надекспресії Nrf2, обумовленій вірусною інфекцією.

Ключові слова: Nrf2; флавоноїди; вірус грипу; вірус трансмісивного гастроентериту.