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The effect of citrus flavanones on the redox homeostasis in cells exposed to oxidative stress – studies *in vitro*

Wpływ flawanonów cytrusowych na homeostazę redoks komórek narażonych na stres oksydacyjny – badania *in vitro*

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(a) *concept*
(b) *collecting materials*
(c) *experimental section*
(d) *statistics*
(e) *text and bibliography compilation*

ABSTRACT

Introduction: Flavanones (an important group of antioxidants in citrus fruits) are beneficial for health, which is connected with their anti-inflammatory, anti-atherogenic and anti-carcinogenic properties. The present study was undertaken to investigate whether – and in what way – the presence of flavanones influences the redox homeostasis of fibroblasts and alleviates the effects of oxidative stress.

Material and methods: The study was conducted on murine fibroblast cell cultures with the addition of flavanones (hesperidin, hesperetin, naringin, naringenin), exposed to oxidative stress (Fe/Asc). In cell homogenates, the activity of superoxide dismutase (SOD) and glutathione peroxidase (GPx) was measured; in the medium, the concentration of nitric oxide was measured.

Results and conclusion: Our results demonstrate that the addition of naringenin, hesperetin, naringin and hesperidin has a protective effect on cells subjected to oxidative stress The changes observed are particularly visible in the case of aglycone forms of both compounds. Despite the protective properties against oxidative stress which flavanones display, we determined distrubances in redox homeostasis in comparison to the control culture.

Keywords: cell culture, flavanones, oxidative stress, redox homeostasis

STRESZCZENIE

Wstęp: Flawanony, będące głównymi związkami bioaktywnymi cytrusów, wykazują działanie prozdrowotne, co wynika z ich właściwości przeciwzapalnych, przeciwmiażdżycowych i przeciwnowotworowych. Celem badania była ocena wpływu flawanonów na homeostazę redoks fibroblastów oraz czy ich obecność łagodzi skutki stresu oksydacyjnego.

Materiały i metody: Badanie przeprowadzono na hodowlach mysich fibroblastów z dodatkiem flawanonów (hesperydyna, hesperetyna, naringina, naringenina) poddanych stresowi oksydacyjnemu (Fe/Asc). W homogenatach komórkowych oznaczono aktywność dysmutazy ponadtlenkowej (SOD) i peroksydazy glutationowej (GPx), a bezpośrednio w pożywce stężenie tlenku azotu.

Wyniki i wnioski: Wyniki naszej pracy wskazują, że dodatek naringeniny, hesperetyny, naringiny i hesperydyny działa ochronnie na komórki poddane stresowi oksydacyjnemu. Pomimo stwierdzonego ochronnego działania flawanonów zaobserwowaliśmy również zaburzenie homeostazy redoks w odniesieniu do hodowli kontrolnej.

Słowa kluczowe: hodowle komórkowe, flawanony, stres oksydacyjny, homeostaza erdoks

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INTRODUCTION

Free radicals and their effect on the human body are important health issue. Both technological processes or wrong heat treatment of food, environmental pollution and exposure to the magnetic field can cause the formation of reactive oxygen and nitrogen species, which leads to many diseases. Eating foods rich in antioxidant compounds is very important in the fight with free radicals. Flavanones are an important group of antioxidants; they belong to flavonoids and are major bioactive compounds in citrus fruits.

Flavanones belong to a numerous group of flavonoid compounds, which are diverse in structure and antioxidative activity. In the last 15 years the total number of known flavanones increased so much that they are considered one of the main classes of flavonoids [1]. So far, 350 flavanones have been identified in the form of aglycones and 100 of them in the form of glycosides [2].

Among the best known flavanones there are naringenin and hesperetin and their glycosides: naringin and hesperidin. Flavanones are present in many plants, especially those from *Compositae*, *Leguminosae* and *Rutacea* families, but their main source are fruits and citrus fruit juices [3]. Greater concentrations of flavanones are present in the skin than in the flesh of the citrus fruit [4]. Hesperetin and its glycosides are the flavanones characteristic of sweet oranges, tangerines, lemons and limes. Considerable amounts of hesperetin are also present in grapefruit [5, 6]. Naringin is the main flavonoid of grapefruit and bitter oranges. Its content in the fruit depends on the variety of the plant [7]. Sweet oranges, tangelo, lemons and limes contain little naringin. However, naringenin is present in sweet oranges, tangerines and tangelo [6]. As it has been shown in many studies, flavanones are beneficial for health, which is connected with their anti-inflammatory, anti-atherogenic and anti-carcinogenic properties. Their potential beneficial influence on the body is connected with their antioxidative activity and regulating gene expression [8-10].

The present study was undertaken to investigate whether - and in what way - the presence of flavanones influences the redox homeostasis of fibroblasts and alleviates the effects of oxidative stress.

MATERIAL AND METHODS

Chemical substances

All tested flavanones (hesperidin - HG, hesperetin - HA, naringin - NG, naringenin - NA) were purchased from Sigma-Aldrich (USA).

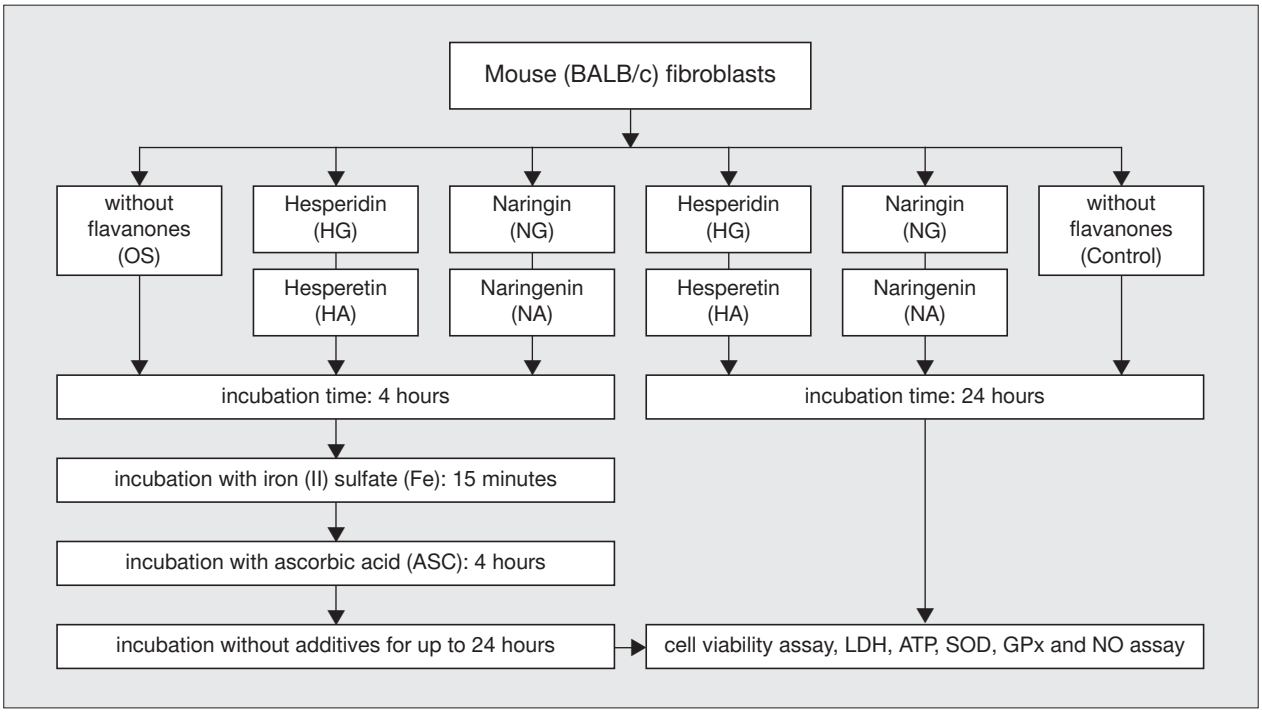


Fig. 1. The experimental procedure
Ryc.1. Schemat eksperymentu

Fibroblast cell culture

Fibroblasts were isolated from the skin of the tail and belly of 60-day mouse (BALB/c). Animals come from a breeding colony at the Center for Experimental Medicine at the Medical University of Silesia. The permission of the Local Animal Experimentation Ethics Committee had been granted.

Cell culture

The freshly isolated fibroblasts were incubated and suspended in the fresh medium with the addition of flavanones (hesperidin, hesperetin, naringin, naringenin); in the case of control experiment carried out at the same time, they were suspended in the medium alone (Fig. 1). The flavanones in DMSO were dissolved. The concentration of DMSO in the medium did not exceed 0.1%. The results obtained for the fibroblast cultures incubated only with the addition of DMSO at 0.1% did not differ from the results obtained for control cultures and were therefore left out in the results presentation. The final concentration of flavanones was 10^{-5} mol/L. The flavanones concentration (10 µM) was selected in a pilot study with 10–1000 µM flavanones concentration. Cell viability was measured and concentration 10 µM was chosen because it had no cytotoxic effect on fibroblast. The fibroblasts incubations time with flavanones addition was 4 hours. After this time oxidative stress (by Fe/ASC) was induced. Iron (II) sulfate was added to the medium and after 15 minutes the medium was changed for one with ascorbic acid addition for free radical production. The final concentrations of iron (II) sulfate and ascorbic acid were 2×10^{-6} mol/L and 10^{-6} mol/L respectively [11]. The fibroblasts incubations time with ascorbic acid addition was 4 hours. After this time the medium was changed to medium without additives. The total incubation time of the cells was 24 hours. The incubation of fibroblasts was carried out for 24 hours in the atmosphere containing 5% v/v CO_2, at the temperature of 37°C, in Heraeus incubator. After the suitable incubation time, the fibroblasts were percolated and rinsed with PBS solution.

Cell viability assay

To assess cell viability, the fibroblasts were stained with 0.4% trypan blue and counted. Cell viability was measured by analysing the trypan blue uptake [12] (data not shown).

Lactate dehydrogenase (LDH) release assay

LDH activity was measured using an assay kit (Sigma-Aldrich, USA) according to the manufacturer's instruction. The reduction of NAD^+ to NADH, which is catalysed by lactate dehydrogenase, was exploited in this assay. The LDH activity is reported as the percentage of the control value (data not shown).

Cell homogenate preparation

At the termination of each treatment, the cells were washed twice with ice-cold phosphate-buffered saline (PBS). The fibroblasts were mechanically homogenised using an Ultra-Turrax T8 homogeniser (IKA Laboratechnik, Germany) in a flask placed on ice. The resultant homogenates were then used in subsequent analyses.

Biochemical analysis

All studied biochemical parameters (ATP concentration, SOD, GPx activities and NO concentration) were recalculated to 10^6 cells.

Superoxide dismutase (SOD) activity assay

SOD activity was estimated, in accordance with the method of Beauchamp and Fridovich [13]. This method employs xanthine and xanthine oxidase to generate superoxide radicals, which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a red formazon dye. The SOD activity is measured by the degree of inhibition of this reaction. Percent of inhibition was converted to enzyme activity from a calibration curve with the standard solution.

Glutathione peroxidase (GPx) activity assay

GPx activity was measured according to the method of Paglia and [14]. In this method, GPx catalyses the oxidation of glutathione by cumene hydroperoxide. In the presence of GR and NADPH, the oxidized glutathione is immediately converted to the reduced form with a concomitant oxidation of NADPH to $NADP^+$.

The determination of nitric oxide (NO) concentration

Nitric oxide concentration was measured in the medium by means of Nitric Oxide Assay Kit, Colorimetric, Calbiochem® (Merck KGaA, Darmstadt, Germany). The measurement was based on reactions with Griess 1 and 2 reagents, as a result of which a coloured compound appears.

ATP concentration assay

The measurement of the ATP concentration was performed using the ATPlite 1step test (PerkinElmer, USA). The ATPlite 1step assay system is based on the production of light caused by the reaction of ATP with added luciferase and D-luciferin (Table I).

Table I. ATP concentration [µmol/10^6 cells] in fibroblast cultures
Tabela I. Stężenie ATP [µmol/10^6 komórek] w hodowlach fibroblastów

Group	C	NA	NG	HA	HG	OS	NA+OS	NG+OS	HA+OS	HG+OS
ATP	4,01 ±0,11	4,23 ±0,16	3,96 ±0,11	4,16 ±0,13	3,89 ±0,14	3,26 ±0,10[a]	3,97 ±0,13[b]	3,79 ±0,12[b]	3,93 ±0,12[b]	3,76 ±0,15[b]

Experimental conditions as per that described in Figure 4.
Warunki eksperymentu takie jak opisano przy rycinie 4.

Statistical analysis

All data are expressed as the mean ± standard deviation of five separate experiments. An ANOVA and Tukey's post-hoc test were used to evaluate the results of the experiments. The statistical calculations were performed using Statistica 10.0 (StatSoft, Poland), and the statistical significance was defined at $p<0.05$.

RESULTS

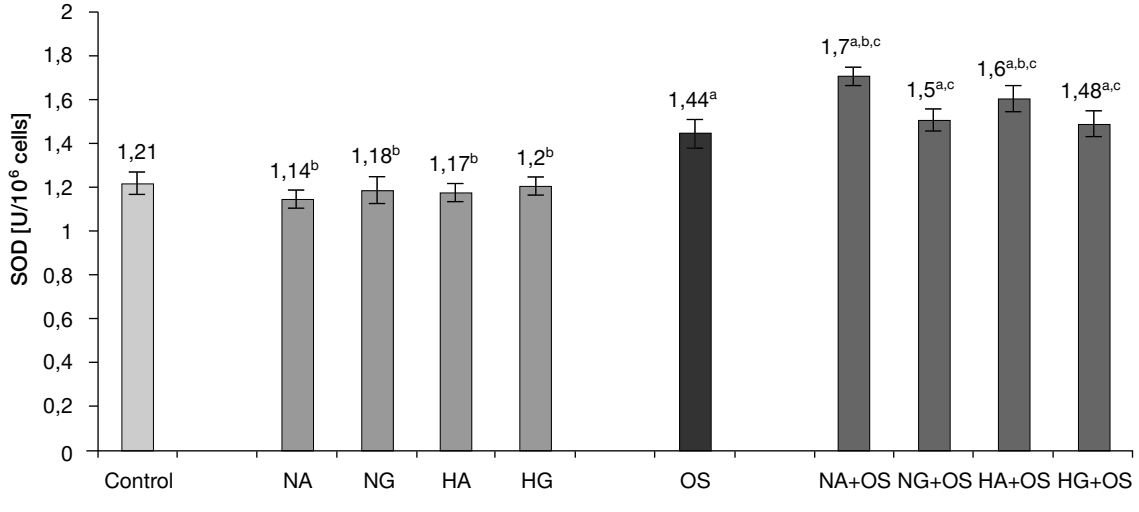
The cells viability in all fibroblast cultures was not lower than 95%. Flavanones did not cause cell membrane integrity damage, as demonstrated by the presence of an equally low LDH level in the test and control cultures. The LDH and ATP concentration results for fibroblast cell cultures with flavanones and exposed or not exposed to oxidative stress did not differ from the results obtained for control cultures and were therefore left out in the result presentation. ATP concentration for fibroblast cell cultures exposed to oxidative stress (OS) was lower than the results obtained for control cultures and for cultures with flavanones not exposed to oxidative stress (NA, NG, HA, HG) (Table I).

The effects of oxidative stress on defence parameters in fibroblasts

The introduction of Fe/ASC into the fibroblast medium in order to cause oxidative stress caused about 20% increase in the activity of SOD (Fig. 2) and a decrease in the activity of GPx by about 30% (Fig. 3). The measured concentration of NO, on the other hand, increased more than twice (by 120%) (Fig 4.)

The effects of flavanones on defence parameters in fibroblasts

The introduction of hesperidin (HG), hesperetin (HA), naringin (NG) and naringenin (NA) to the fibroblast culture did not cause statistically significant changes in the activity of SOD and GPx in com-



Experimental conditions as per that described in Figure 4.
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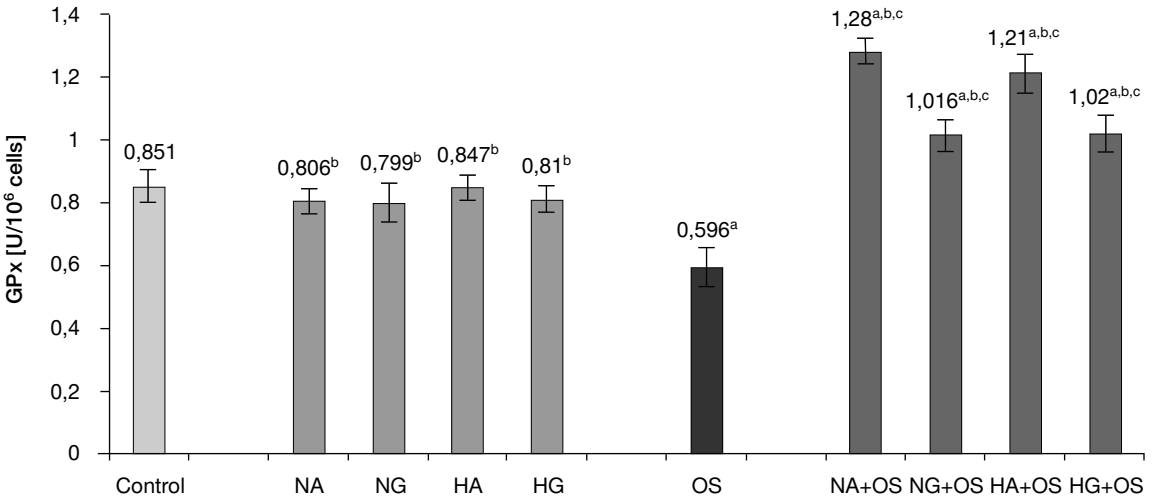
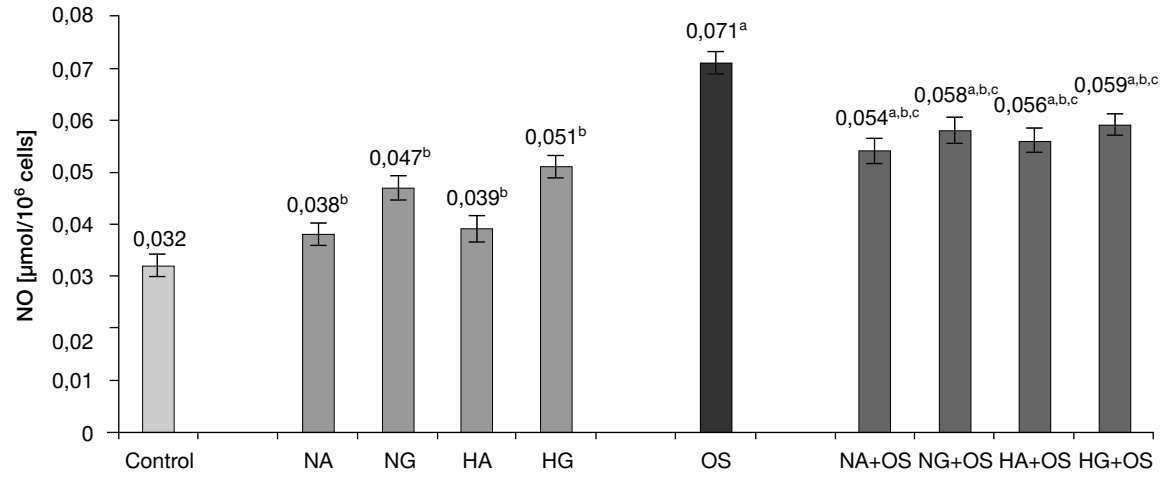
Fig. 2. Flavanones effect on SOD activity in fibroblasts exposed to oxidative stress
Ryc. 2. Wpływ flawanonów na aktywność SOD w fibroblastach narażonych na stres oksydacyjny



Experimental conditions as per that described in Figure 4.
Warunki eksperymentu takie jak opisano przy rycinie 4.

Fig. 3. Flavanones effect on GPx activity in fibroblasts exposed to oxidative stress
Ryc. 3. Wpływ flawanonów na aktywność GPx w fibroblastach narażonych na stres oksydacyjny



Each value represents the mean ±SD (n=5);
[a] p<0,05 vs. control
[b] p<0,05 vs. oxidative stress
[c] p<0,05 vs. flavanones control (NA, NG, HA, HG)
OS – oxidative stress (Fe^{+2}/ascorbic acid)
NA – culture with naringenin (10^{-5} mol/L)
NA+OS – culture with naringenin (10^{-5} mol/L) exposed to oxidative stress
NG – culture with naringin (10^{-5} mol/L)
NG+OS - culture with naringin (10^{-5} mol/L) exposed to oxidative stress
HA – culture with hesperetin (10^{-5} mol/L)
HA+OS – cultures with hesperetin (10^{-5} mol/L) exposed to oxidative stress
HG – cultures with hesperidin (10^{-5} mol/L)
HG+OS – cultures with hesperidin (10^{-5} mol/L) exposed to oxidative stress

Wyniki są prezentowane jako wartość średnia ±SD (n=5)
[a] p<0,05 vs. kontrola
[b] p<0,05 vs stress oksydacyjny
[c] p<0,05 vs. kontrola flawanonów (NA, NG, HA, HG)
OS – stres oksydacyjny (Fe^{+2}/kwas askorbinowy)
NA – hodowle z narynginą (10^{-5} mol/L)
NA+OS – hodowle z naryngenina (10^{-5} mol/L) narażone na stres oksydacyjny
NG – hodowle z naringiną (10^{-5} mol/L)
NG+OS – hodowle z naringiną (10^{-5} mol/L) narażone na stres oksydacyjny
HA – hodowle z hesperetyną (10^{-5} mol/L)
HA+OS – hodowle z hesperetyną (10-5 mol/L) narażone na stres oksydacyjny
HG – hodowle z hesperydyną (10^{-5} mol/L)
HG+OS – hodowle z hesperydyną (10^{-5} mol/L) narażone na stres oksydacyjny

Fig. 4. Flavanones effect on NO concentration in fibroblasts exposed to oxidative stress
Ryc. 4. Wpływ flawanonów na stężenie NO w fibroblastach narażonych na stres oksydacyjny

parison with the control culture (Fig 2–3). However, following the introduction of flavanones, nitric oxide concentration increased by 19–22% for aglycone forms and by 47–59% for glycosidic forms (Fig. 4).

The effects of flavanones and oxidative stress on defence parameters in fibroblasts

The generation of oxidative stress in fibroblast cultures with flavanones caused the increase in SOD activity for naringenin (NA+Fe/ASC) and hesperetin (HA+ Fe/ASC) by 20% and 12%, respectively. For naringin (NG+ Fe/ASC) and hesperidin (HG+ Fe/ASC), there was no significant difference in comparison with the culture exposed to oxidative stress (OS) (Fig. 2).

The activity of GPx in cultures with flavanones also increased in the presence of oxidative stress. In the case of GPx, a very high increase in the enzyme activity was determined: 120% for NA+ Fe/ASC, 70% for NG+ Fe/ASC, 105% for HA+ Fe/ASC and 71% for HG+ Fe/ASC, in comparison with the culture exposed to oxidative stress (OS) (Fig. 3). In the case of the culture subjected to oxidative stress, a decrease in the concentration of NO was determined, as a result of pre-incubation of cells with flavanones. The changes amounted to: 16,9% for HG+ Fe/ASC and 18,3% for NG+ Fe/ASC and 21,1% for HA+ Fe/ASC i 23,9% for NA+ Fe/ASC (Fig. 4).

In all cases, the activity or concentration of the parameters measured (SOD, GPx, NO) in fibroblast cultures with flavanones in the presence of oxidative stress were higher ($p<0.05$) than in cultures with flavanones only (Fig. 2–4).

The effects of structure of flavanones on defence parameters in fibroblasts

It was determined that in the cultures subjected to oxidative stress the extent of changes in the SOD, GPx and NO concentrations depends on the chemical form of flavanone. In the case of glycoside forms (NG+ Fe/ASC and HG+ Fe/ASC), the activities of the measured SOD and GPx enzymes were lower than in the case of aglycone forms (NA+ Fe/ASC and HA+Fe/ASC). Higher concentrations of nitric oxide were determined for glycoside forms. The differences between them were statistically significant ($p<0.05$) (Fig. 2–4).

DISCUSSION

Oxidative stress appears when there is a disturbance in the pro-oxidative-antioxidative balance. It may result from an increased ROS generation and from reduction in their removal by antioxidative cell defence system. The increase in SOD activity in fibroblasts incubated with Fe/ASC observed in our study was accompanied by a decrease in the activity of GPx, an enzyme for which hydrogen peroxide is a substrate. An increase in SOD activity (catalyzing the generation of H_2O_2), which is not accompanied by a sufficient increase in the activity of enzymes removing H_2O_2, may cause oxidative stress, as shown by Kalender et al. [15]. Other studies demonstrated a decrease in the activity of both enzymes - SOD and GPx – under the influence of oxidative stress [16].

In our study, the activity of SOD and GPx, measured in cells incubated only with flavanones, did not differ from the one measured in control fibroblasts. This means that in the tested concentrations both compounds used separately do not influence the oxidative-reductive balance of fibroblasts. Yen et al. [17] demonstrated in their study that flavanones (naringenin and hesperetin) in the concentrations of 0–200 µM did not cause any increase in H_2O_2 in human lymphocytes. They also demonstrated that, among the flavonoids tested, hesperetin and naringenin have the least pro-oxidative effect. Wilms et al. [18] determined that the level of oxidative damage to human leukocyte DNA depended on quercetine concentration. Low concentrations of quercetine (1, 10 i 50 µM) caused a decrease in the damage to DNA caused by hydroxy radical. However, the concentration of 100 µM caused an increase in the damage. Therefore, the concentration of flavanones of 10 µM should not have caused oxidative-reductive changes.

As research indicates, flavonoid compounds may display both antioxidative and prooxidative activity. Antioxidative activity consists in the possibility of neutralizing the effects of free radical activity through various mechanisms, among others: direct reactions with ROS, activating antioxidative enzymes [19] or increasing the antioxidative activity of small molecule antioxidants [20]. In our study, we determined an increase in both SOD and GPx activity in cultures preincubated with flavanones subjected to oxidative stress. Jagetia et al. [21] observed in their studies *in vitro* a decrease in the activity of SOD and GPx under stress caused by Fe^{+3} and an increase in the activity of these enzymes in cultures with naringenin and Fe^{+3}.

Apart from generating reactive forms of oxygen (ROS), a disturbance in cell homeostasis may take place as a result of generating reactive forms of nitrogen (RNS). The introduction of exogenous substances – which flavanones are – into cell cultures

caused a disturbance in fibroblast homeostasis, expressed by the production of nitric oxide. The production of NO in fibroblasts incubated only with flavanones, without any changes in SOD and GPx activity, could be caused by their antioxidative effect through increasing the concentrations of intracellular glutathione. Moellering et al. [22] demonstrated that nictric oxide in physiological and low concentrations may regulate the expression of genes controlling glutathione synthesis (GSH), which results in the increase of its intracellular concentration and additionally protects cells against oxidative stress. Nitric oxide may also inactivate antioxidative enzymes, including superoxide dismutases, glutathione peroxidase and catalase [23]. Therefore, one of the reasons for a significant decrease in GPx activity measured in our study as a result of oxidative stress could be the production of large amounts of NO in fibroblasts. The intensification of nitric oxide synthesis in cells, through chemical stabilization of tetrahydrobiopterine (natural cofactor of NO synthase), as a result of treating the cells with ascorbic acid, has been determined by Heller et al. [24]. Walker et al. [25] demonstrated that the cytotoxicity initiated by NO depended on the total amount of glutathione in the cells. On the other hand, the generation of excessive amounts of oxidized glutathione (GSSG) in the cells and a deficiency in reduced glutathione (GSH) may inhibit GPx activity.

So far, the ability of flavonoids, to scavenge reactive forms of nitrogen has been studied only to a modest extent. Sueishi et al. [26] measured the antioxidative potentials of 6 natural antioxidants. They established that NO reacts with hydrophilic antioxidants depending on their redox potential and that the essential chemical process of the reaction of an antioxidant with NO may be determined based on the transfer of an electron from NO to the antioxidant. In order to measure the rate of free radical scavenging by water-insoluble lipophilic antioxidants, Sueishi and Hori [27] used a solubiliser. The researchers proved that the tested antioxidants showed lower NO binding rates than hydrophilic antioxidants (uric and caffeic acid). Research showed that the mechanism of NO scavenging by lipophylic antioxidants is not of a free radical nature.

The properties of flavanones are connected with their structure, especially the location of chemical substituents. The value of the change in the antioxidative enzymes activity depends on the chemical form of a flavanone. Flavanones in the form of aglycones – hesperetin and naringenin – in cultures under oxidative stress caused greater SOD and GPx increase and a greater decrease in NO concentration than the glycosidic forms – hesperidin and naringin. Lesser changes in the tested parameters in glycosidic forms result from the fact that, having also a sugar residue in their structure, they are less absorbed than the aglycone forms. In their studies, Nakamura et al. and Rice-Evans et al. [28, 29] determined that the combination of aglycone with a sugar residue in a glycosidic form decreased the antioxidative properties. Di Majo et al. [30] measured the antioxidative properties of nine different flavanones. They determined that the configuration and the total number of hydroxyl groups, as well as the presence of a methyl and glycoside group, influence antioxidative properties. The presence of these groups impacts the speed of free radical scavenging, which may result from a steric effect caused by these substituents, impairing the ability to carry electrons.

CONCLUSION

Our results demonstrate that the addition of naringenin, hesperetin, naringin and hesperidin has a protective effect on cells subjected to oxidative stress through the activation of antioxidative enzymes and a decrease in nitric oxide release. The changes observed are particularly visible in the case of aglycone forms of both compounds.

Despite the protective properties against oxidative stress which flavanones display, we determined distrubances in redox homeostasis in comparison to the control culture, manifested by an increase in the superoxide dismutase and glutathione peroxidase activity, as well as in the concentration of nitric oxide. The impact of the interactions taking place between particular antioxidants and the influence of their interrelationships on living organisms is still unknown. Therefore, research needs to be conducted on the influence of flavanones intake with food and dietary supplements on cell and living organisms homeostasis.

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