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Interaction of citrinin and resveratrol and their effect on Caco-2 cell growth

Vzájomné pôsobenie citrinínu a resveratrolu a ich vplyv na rast Caco-2 buniek

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Abstract

The aim of this study was to use Caco-2 cells as an *in vitro* model of human intestinal barrier. This model was employed to investigate interaction of citrinin and resveratrol and to determine the doses that affect cell number. Citrinin (CTN) is a toxic secondary metabolite produced by fungi of the genera *Penicillium*, *Aspergillus* and *Monascus.* It is contaminant of cereals, grains, food and feed products. Previous studies have shown that CTN has teratogenic, nephrotoxic, hepatotoxic and embryotoxic effects. Resveratrol (RES) is polyphenol belongs to the group of stilbenes. At lower doses it has positive effects on human health and higher doses may induce apoptosis. The results of study indicate a significant ($P < 0.05$) decrease in cell number in all conditions: A (RES 20 μM), B (CTN 100 $\mu g*ml^{-1}$), C (CTN 250 $\mu g*ml^{-1}$), D (CTN 100 $\mu g*ml^{-1}$ + RES 20 μM), E (CTN 250 $\mu g*ml^{-1}$ + RES 20 μM) in comparison with the control group of non-treated cells (NT) and control group with ethanol (Ce). A significant ($P <0.05$) decrease in the cells number was observed between groups A - E, B - C and D - E. Citrinin effect seems to be dose-dependent.

Keywords: Caco-2 cell line, citrinin, mycotoxin, polyphenol, resveratrol

Abstrakt

Cieľom práce bolo použitie Caco-2 buniek ako *in vitro* model črevnej bariéry na zistenie vzájomného pôsobenia citrinínu a resveratrolu a stanovenie dávok, ktoré majú vplyv na počet buniek. Citrinín (CTN) je toxický sekundárny metabolit, ktorý produkujú mikroskopické huby patriace do rodov *Aspergillus, Penicillium* a

Monascus. Kontaminuje cereálie, obilniny, potraviny a krmivá. Predchádzajúce štúdie ukázali, že CTN má teratogénne, nefrotoxické, hepatotoxické a embryotoxické účinky. Resveratrol (RES) je polyfenol, ktorý patrí do skupiny stilbénov. Pri nižších dávkach má pozitívne účinky na ľudské zdravie a pri vyšších spôsobuje apoptózu buniek. Výsledky štúdie poukazujú na signifikantný ($P < 0.05$) pokles počtu buniek vo všetkých experimentálnych skupinách A (RES 20 μM), B (CTN 100 μg*ml^{-1}), C (CTN 250 μg*ml^{-1}), D (CTN 100 μg*ml^{-1} + RES 20 μM), E (CTN 250 μg*ml^{-1} + RES 20 μM) v porovnaní s kontrolnou skupinou s neošetrenými bunkami (NT) a kontrolnou skupinou s prídavkom etanolu (Ce). Významný ($P < 0.05$) pokles buniek sa zaznamenal medzi skupinami A - E, B - C a D - E. Pôsobenie citrinínu je dávkovo závislé.

Kľúčové slová: Caco-2 bunková línia, citrinín, mykotoxín, polyfenol, resveratrol

Introduction

The human Caco-2 cell line is one of the most widely used cell model to study absorption, metabolism and bioavailability of drugs and xenobiotics (Artursson et al., 2001). Although originated from colonocytes, Caco-2 cells have many properties of enterocytes, including microvilli, intercellular junctions and many of enzymes, nutrient transporters and efflux transporters that render them similar to small intestinal mucosa (Ward et al*.,* 2000).

Mycotoxins are toxic compounds, produced by the secondary metabolism of toxigenic moulds in the *Aspergillus*, *Alternaria*, *Claviceps*, *Fusarium*, *Penicillium* and *Stachybotrys* genera occurring in food and feed commodities both pre- and post-harvest (Milićević et al., 2010). Mycotoxins have various acute and chronic effects on humans and animals (especially monogastrics) depending on species and susceptibility of an animal within a species (Zain, 2011). Contamination of cereals and grains and related products with mycotoxins causes food and feed-borne intoxications (mycotoxicoses) in man and livestock (Mellor, 2001). Citrinin (CTN) is produced in foodstuffs by *Monascus* species (*Monascus purpureus, M. ruber*), *Penicillium* species (*Penicillium citrinum, P. expansum, P. radicicola, P. verrucosum*) (Ostry et al., 2013). Though *A. niger* is reported to be the highest producer of citrinin among the *Aspergillus* species*,* other citrinin producers of this genus include *A. awentil, A. ostianus, A. fumigatus, A. niveus, A.awamori and A. parasiticus* (Li et al., 2010)*.* The toxic effects of citrinin include severe kidney damage, hepatic damage and immunosuppression as a consequence of damage to the immune system (Quinn et al., 2011). In animals and humans the toxin accumulates in the kidneys and can cause severe renal failure. Physiological investigations identified different adverse effects on the kidneys, liver and the gastrointestinal tract (Krejci et al., 1996). The cytotoxic effects of several mycotoxins including CTN on target tissues and cultured cells are thought to correlate with their apoptosis-inducing ability (Chan, 2007; Yu et al., 2006). Ostry et al. (2013) reported that CTN was found in foodstuffs of vegetable origin (e.g., cereals, pomaceous fruits, black olive, roasted nuts, spices), food supplements based on rice fermented with red microfungi *Monascus purpureus* and

in foodstuffs of animal origin (e.g., cheese). Jeswal and Kumar (2015) determined high concentration of citrinin in red chilli, black pepper and dry ginger.

Polyphenols are a specific group of plant products and may be classified into flavonoids, lignans, phenolic acids and stilbenes (Biesalski, 2007). Flavonoids belong to a group of natural substances with variable phenolic structures and are found in fruit, vegetables, grains, bark, roots, stems, flowers, tea and wine (Middleton, 1998). The chemical nature of flavonoids depends on their structural class, degree of hydroxylation, other substitutions and conjugations, and degree of polymerization (Heim et al., 2002). Resveratrol (RES) (3,5,4´ -trihydroxystilbene) is a natural polyphenol with a stilbene structure (Takaoka, 1940). The main source of RES is grape skin and is also present in a large variety of fruits (e.g., cranberry, mulberry, lingberry, bilberry, partridgeberry, sparkleberry, deerberry, blueberry, jackfruit), peanut and also in a wide variety of flowers and leaves including gnetum, butterfly orchid tree, white hellebore, scots pine, corn lily, eucalyptus, spruce etc. Resveratrol is also synthesized in response to environmental stressors that include water deprivation, UV irradiation and especially fungal infection (Das and Maulik, 2006). Red grapes and wines contain a considerable amount of resveratrol, 5 - 40 μM (Sato et al.,1997; Frémont, 2000). Resveratrol affects all three discrete stages of carcinogenesis (initiation, promotion and progression) by modulating signal transduction pathways that control cell division and growth, apoptosis, inflammation, angiogenesis and metastasis, and hence is considered by some to be a promising anticancer therapy (Kraft et al., 2009). At high doses, resveratrol not only hinders tumor growth but also inhibits the synthesis of RNA, DNA and proteins, causes structural chromosome aberrations, chromatin breaks, base exchanges, weak aneuploidy, higher S-phase arrest, blocks cell proliferation, decreases wound healing, endothelial cell growth by fibroblast growth factor-2 (FGF-2) and vascular endothelial growth factor, and angiogenesis in healthy tissue cells leading to cell death (Mukherjee et al., 2010).

The aim of this study was to investigate interaction of citrinin and resveratrol and their effect on the number of Caco-2 cells.

Materials and methods

Caco-2 cell line maintenance and differentiation

The human colon cancer Caco-2 cells were obtained from European Collection of Cell Cultures (no. 86010202). The cells were maintained in DMEM (Dulbecco's modified Eagles medium, Corning, Amsterdam, Netherlands) with high content of glucose (4.5 $g*l^{-1}$), supplemented with FBS (heat inactivated fetal bovine serum, 10 % v/v), 2 mM L- glutamine and mixture penicillin (100 $U*ml^{-1}$) streptomycin (100 $\mu g*ml^{-1}$) and amphotericin B (250 $ng*ml^{-1}$) (all from PAN-Biotech, Aidenbach, Germany). The cells were cultured in 100 x 20 mm Falcon ® standard dishes for tissue culture at 37 °C, in a humified atmosphere of 5 % CO_2. Cells were passaged with 0.25 % trypsin/ 0.02 % EDTA (PAN-Biotech, Aidenbach, Germany) and washed in PBS (phosphate buffered saline) without Ca and Mg ions (Corning, Amsterdam, Netherlands). The medium was changed two times a week. Cells were seeded in Falcon ® 24- well plates (approximately 10 000 cells per well) and incubated with

mycotoxin citrinin and stilbene resveratrol alone or in combination at different dilutions for 48 h.

Preparation of citrinin and resveratrol

Citrinin [C13H14O5, IUPAC: (3R,4S)-4,6-dihydro-8-hydroxy-3,4,5-trimethyl-6-oxo-3H-2-benzopyran-7- carboxylic acid] and resveratrol [3,4′,5-Trihydroxy-*trans*-stilbene, 5-[(1*E*)-2-(4-Hydroxyphenyl)ethenyl]-1,3-benzenediol] was obtained from Sigma Aldrich (Munich, Germany). Citrinin was dissolved in DMSO (Sigma Aldrich, Munich, Germany) to prepare a stock solution of 5 mg*ml^{-1} (200 µl of sterile DMSO (PAN-Biotech, Aidenbach, Germany) and the rest was filled with sterile water). Resveratrol was dissolved in ethanol (POCh, Gliwice, Poland) to prepare 10 mM stock solution. Stock solution was subsequently diluted with sterile water and certain concentrations were prepared and applied to the cell cultures according to the experimental design (Table 1).

Table 1. Design of experiment.

Tabuľka 1. Návrh experimentu.

Groups	Concentration of applied substances
NT	Non-treated cells
Ce	Control group with ethanol
A	RES 20 µM
B	CTN 100 µg*ml^{-1}
C	CTN 250 µg*ml^{-1}
D	CTN 100 µg*ml^{-1} + RES 20 µM
E	CTN 250 µg*ml^{-1} + RES 20 µM

Staining cells with crystal violet

After cultivation the medium was aspirated from wells and cells were washed with PBS. Caco-2 cells were fixed with 3.7 % buffered formaldehyde (formaldehyde diluted in 150 mM sodium phosphate buffer, pH 7.4), stained with a 0.5 % crystal violet solution (crystal violet dissolved in methanol-water (1:5 v/v)) and destained with destaining solution (68.8 mM citric acid and 42.2 mM tri-sodium citrate dissolved in distilled water with addition of methanol) (all chemicals were obtained from Sigma Aldrich (Munich, Germany)). After finished procedure, 100 µl of destaining solution was transferred to 96- well microtitration plate and absorbance was measured on Microplate reader (Bio-Rad, Model 680) at a wavelength of 540 nm. The calculation

of cell numbers in samples was performed on the basis of a standard curve prepared from suspensions of known cell number.

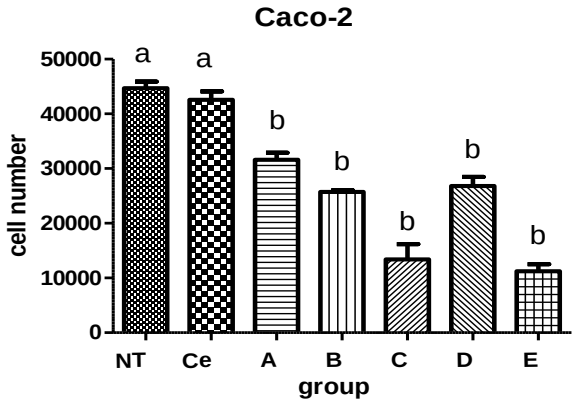
Statistical analysis

The data are expressed as mean ± standard deviation (SD) and analysed by one-way analysis of variance (ANOVA) using Tukey's multiple comparison test and the statistical analysis tool in GraphPad Prism 5 software (GraphPad Software, Inc, La Jolla, CA, USA). Differences were compared for statistical significance at the level $P < 0.05$.

Results

Different concentrations of citrinin and resveratrol were applied alone and in combination, as is shown in Table 1, and the impact of these agents was monitored on Caco-2 cell proliferation.

As shown in the Figure 1, the Caco-2 cell numbers were significantly ($P < 0.05$) lower in all of experimental groups (A, B, C, D, E), as compared to the non- treated control group (NT) and ethanol treated control group (Ce).



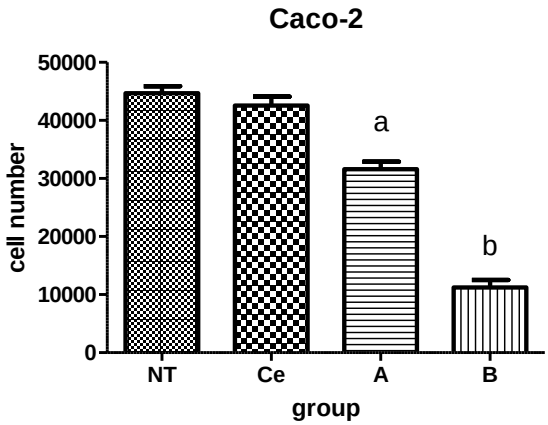
a, b - means significant difference ($P < 0.05$)

a, b- znamená signifikantný rozdiel ($P < 0.05$)

Figure 1. Effect of citrinin and resveratrol alone and in combination on the Caco-2 cell number in comparison to NT and Ce groups. NT- non treated cells, Ce- control group with ethanol, A (RES 20 μM), B (CTN 100 $\mu g*ml^{-1}$), C (CTN 250 $\mu g*ml^{-1}$), D (CTN 100 $\mu g*ml^{-1}$+ RES 20 μM), E (CTN 250 $\mu g*ml^{-1}$+ RES 20 μM)- groups with different addition of following substances, CTN- citrinin, RES- resveratrol.

Obrázok 1. Vplyv citrinínu a resveratrolu jednotlivo a v kombinácii na počet Caco-2 buniek v porovnaní s NT a Ce skupinami. NT- neošetrené bunky, Ce- kontrolná skupina s etanolom, A (RES 20 μM), B (CTN 100 $\mu g*ml^{-1}$), C (CTN 250 $\mu g*ml^{-1}$), D (CTN 100 $\mu g*ml^{-1}$ + RES 20 μM), E (CTN 250 $\mu g*ml^{-1}$ + RES 20 μM)- skupiny s rozličnými prídavkami daných látok, CTN- citrinín, RES- resveratrol.

In experimental group B (CTN 250 μg*ml^{-1} + RES 20 μM) cell count of Caco-2 was lower than in the experimental group A (RES 20 μM) without addition of mycotoxin. The statistically significant ($P < 0.05$) difference between the groups A and E is presented in the Figure 2 and the lower cell number observed in the group treated with mixture of resveratol and citrinin.



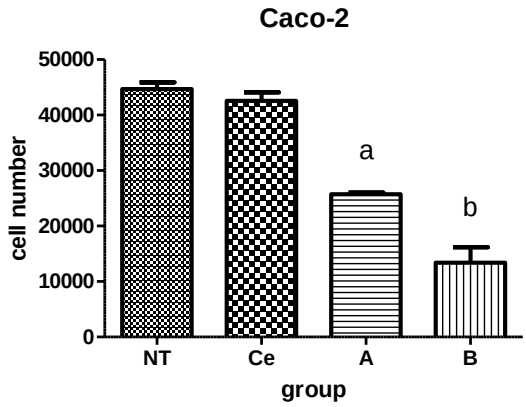
a, b - means significant difference ($P < 0.05$)

a, b- znamená signifikantný rozdiel ($P < 0.05$)

Figure 2. Decrease number of Caco-2 cells in group B (CTN 250 μg*ml^{-1} + RES 20 μM) in comparision to group A (RES 20 μM) without citrinin addition.

Obrázok 2. Zníženie počtu Caco-2 buniek v skupine B (CTN 250 μg*ml^{-1} + RES 20 μM) v porovnaní so skupinou A (RES 20 μM) bez prídavku citrinínu.

The anti-proliferative effects of citrinin are dose-dependent. Higher concentration of citrinin caused a more pronounced decrease of Caco-2 cell number (Figure 3). Differences between group A (CTN 100 μg*ml^{-1}) and group B (CTN 250 μg*ml^{-1}) are statistically significant ($P < 0.05$).



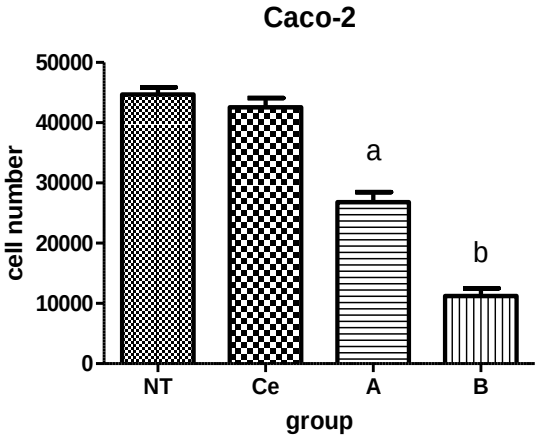
a, b - means significant difference ($P < 0.05$)

a, b- znamená signifikantný rozdiel ($P < 0.05$)

Figure 3. Decrease number of Caco-2 cells in group B (CTN 250 μg*ml^{-1}) in comparison to group A (CTN 100 μg*ml^{-1}).

Obrázok 3. Zníženie počtu Caco-2 buniek v skupine B (CTN 250 μg*ml^{-1}) v porovnaní so skupinou A (CTN 100 μg*ml^{-1}).

Significant reduction ($P < 0.05$) in cell numbers occurred in group E (CTN 250 μg*ml^{-1} + RES 20 μM) in comparison to group D (CTN 100 μg*ml^{-1} + RES 20 μM) with different concentration of citrinin (Figure 4).



a, b - means significant difference ($P < 0.05$)

a, b- znamená signifikantný rozdiel ($P < 0.05$)

Figure 4. Decrease number of Caco-2 cells in group B (CTN 250 μg*ml^{-1} + RES 20 μM) in comparison to group A (CTN 100 μg*ml^{-1} + RES 20 μM).

Obrázok 4. Zníženie počtu Caco-2 buniek v skupine B (CTN 250 μg*ml^{-1} + RES 20 μM) v porovnaní so skupinou A (CTN 100 μg*ml^{-1} + RES 20 μM).

Discussion

In the present study was used an assay that allows to determine total number of viable (adherent) cells in each condition. The number of adherent cells depends on the proliferation rate and possible cell death (dead cells detach and are not stained). In this experiment was detected neither cytotoxicity (massive cell detachment) nor any signs of apoptosis (cell shrinking or blebbing). The decrease in cell numbers from the groups treated with citrinin or resveratrol or combination of both may be caused by the slow down or block of proliferation.

This phenomenon might be considered beneficial in case of premalignant or malignant in situ cancer lesions, but in case of healthy mature intestinal mucosa, built of non-proliferating cells, should not have a severe adverse effects. Only the proliferating epithelial cell progenitors and stem cells located in the crypts might be affected, which could be a health concern as it potentially impairs intestinal mucosa renewal or regeneration rate.

The Caco-2 cell numbers were significantly ($P < 0.05$) lower in all of experimental groups A (RES 20 µM), B (CTN 100 µg*ml^{-1}), C (CTN 250 µg*ml^{-1}), D (CTN 100 µg*ml^{-1} + RES 20 µM), E (CTN 250 µg*ml^{-1} + RES 20 µM), as compared to the non-treated control group (NT) and ethanol treated control group (Ce). Study of Chang et al. (2010) suggested that CTN-activated G2/M arrest primarily arises from the inhibition of tubulin polymerization and associated mitotic spindle formation. It suggests that cell division is adversely affected. In experimental group E (CTN 250 µg*ml^{-1} + RES 20 µM) cell count of Caco-2 was significantly ($P < 0.05$) lower than in the experimental group A (RES 20 µM). At lower dose, resveratrol can be very useful in maintaining the human health whereas at higher dose, resveratrol has pro-apoptotic actions on healthy cells, but can kill tumor cells (Mukherjee et al., 2010). Higher concentration of citrinin in group C (CTN 250 µg*ml^{-1}), in comparison to group B (CTN 100 µg*ml^{-1}), caused significant ($P < 0.05$) reduction of Caco-2 cells, which means that anti-proliferative effects of CTN are dose-dependent. In the study of authors Pascual-Ahuir et al. (2014) citrinin triggers a fast and dose dependent activation of stress responsive promoters. It has been reported that CTN stimulates ROS generation and c-Jun N-terminal kinase (JNK) activation for mitochondria-dependent apoptotic signaling in human hepatoma G2 cells, and these apoptotic biochemical events are blocked by pretreatment with resveratrol, which exerts antioxidant effects (Chen and Chan, 2009). In the present study was observed a significant ($P < 0.05$) decrease number of cells in group E (CTN 250 µg*ml^{-1} + RES 20 µM) in comparison to group D (CTN 100 µg*ml^{-1} + RES 20 µM). Previous study has shown that polyphenol quercetin has no cytoprotective effect against alternaria toxins when they were exposed simultaneously in Caco-2 cells (Fernández-Blanco et al., 2016). However, the results from the group C (CTN 250 µg*ml^{-1}) and the group E (CTN 250 µg*ml^{-1} + RES 20 µM) are not significantly different, so it suggests the lack of additivity or synergism in anti-proliferative effect of both compounds. Similarily, there is no diference between cell numbers from the groups B (CTN 100 µg*ml^{-1}) and D (CTN 100 µg*ml^{-1} + RES 20 µM), which further supports this conclusion.

The action of citrinin is not strenghtened by resveratrol, nevertheless, it cannot be excluded that the simultaneous treatment with citrinin with mixture of various

biologically active dietary componds, such as resveratol or other phytochemicals may modify the cellular response to mycotoxins.

Conclusions

Citrinin exerts a strong dose-dependent inhibitory effect of the caco-2 cell proliferation. Resveratrol mildly reduces the Caco-2 cell number, comparing to control. These two compound likely act through the different biological mechanisms and influence separate intracellular targets, because their overall effects on proliferation are not additive, nor synergistic.

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