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Determination of *Trans*-resveratrol Action on Two Different Types of Neuronal Cells, Neuroblastoma and Hippocampal Cells

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Abstract

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The effects of resveratrol on a cancer cell line derived from the nervous system, neuroblastoma and a cell line derived from hippocampal neurons under hydrogen peroxide-induced oxidative stress were evaluated and compared. Our studies indicate that resveratrol exerts different effects on tumour and normal cells. Interestingly, in the presence of an oxidising factor resveratrol can sensitise cancer cells and decrease their viability substantially. Under conditions of oxidative stress similar to those found in the tumour environment, resveratrol also intensified apoptosis in Neuro-2a cells. Our data indicate that resveratrol does not protect neuronal, hippocampal cells from oxidative damage.

Keywords: cancer; oxidative stress; polyphenol; stillbenoid

Resveratrol is one of the most intensively researched polyphenols. It is produced by some plants in response to multiple harmful factors such as attack by pathogens, UV radiation or increased oxidative stress. Dark grapes (*Vitis vinifera*) are the richest natural source of resveratrol. In larger quantities it is also present in plants such as plums, peanut root, strawberries, mulberries, and raspberries (Nakata *et al.* 2012). Resveratrol exists in *trans* and *cis* isomeric forms; however, only the *trans* isomer is bio-active (Vian *et al.* 2005). This polyphenolic compound is considered to be one of the strongest exogenous antioxidants with numerous beneficial properties. Many extensive investigations have proven that this extraordinary polyphenol slows the progression of or even prevents chronic diseases. It exerts these therapeutic effects mainly due to its potent free-radical scavenging and antioxidant activity (Cordova-Gomez *et al.* 2013). It plays a critical role in cardiovascular protection (*via* increased nitric oxide production) (Smidrkal *et al.* 2010; Harmatha *et al.* 2011). It also possesses beneficial properties such as anti-inflammatory and blood sugar-lowering ones (Delmas *et al.* 2011; Vang *et al.* 2011). Many experiments suggest that it triggers mechanisms that counteract aging-related effects and plays a role in insulin resistance as well (Catalgol *et al.* 2012; Sirerol *et al.* 2016). Recently several studies have revealed that it may also exhibit both neuroprotective and anti-tumour actions (Sun *et al.* 2008; Li *et al.* 2012).

Resveratrol exerts anticancer effects in different systems based on its ability to inhibit diverse cellular events associated with effective inhibition of the three stages of carcinogenesis: tumour initiation, promotion, and progression. Many pathways of direct or indirect action of resveratrol have been suggested but three major mechanisms are considered important. First of all, resveratrol demonstrates antioxidant effects (Iuga *et al.* 2012). It seems to be an effective free radical scavenger. Resveratrol's chemical structure (the presence of hydroxyl groups in the 3, 5, and 4' positions) ensures its antioxidant activity. Numerous studies have revealed that especially the 4'-OH group in the phenol ring of resveratrol dominates in the radical-scavenging efficiency and it is responsible for its biological activity (Stojanović *et al.* 2001). The free-radical scavenging mechanism utilized by resveratrol involves the generation of 4'-phenoxy radicals followed by semiquinone and quinone structures (Rodacka *et al.* 2015). It also improves endogenous

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cellular antioxidant systems – superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), and the content of glutathione (GSH). It also increases mRNA expression of glutathione S-transferase (GST), NAD(P)H:quinone oxidoreductase-1 (NOQ1), cardiac thioredoxin-1 (Trx-1), cardiac thioredoxin-2 (Trx-2), and heme oxygenase-1 (HO-1). This polyphenol has been shown to modulate cellular processes mainly by activating targets such as sirtuin 1 (SIRT1), peroxisome proliferator-activated receptor γ co-activator-1α (PGC-1α) and AMPK (Khan *et al.* 2012, Gerszon *et al.* 2014). Resveratrol may possess anticancer effects due to SIRT1's activation, which leads to deacetylation *inter alia* via the tumour suppressor retinoblastoma (Rb), β-catenin, and caspase 3. Resveratrol exerts diverse influences on tumour cells, leading to their apoptosis. More recent studies have revealed steps that play a role in this process. The low pH environment of tumours intensifies resveratrol's therapeutic, chemopreventive effects, while their acidic environment enhances growth inhibition, DNA damage and apoptosis induction by resveratrol in tumour cells (Shamim *et al.* 2012). Lin et al. (2009) examined the cellular events associated with *inter alia* resveratrol-induced platelet apoptosis and suggested that resveratrol causes caspase-8 activation, followed by cleavage of Bid into tBid, which leads to Bax translocation into mitochondria and induction of Cyt c release from mitochondria (Lin *et al.* 2009). The same apoptotic mechanism was proposed, specifically in a neuroblastoma model.

To date, many beneficial properties of resveratrol and many possible direct or indirect molecular targets have been proposed. Despite substantial knowledge in this field its precise mechanism of action remains to be defined. There is an urgent necessity to conduct more studies that will allow scientists to determine whether resveratrol is worthy of further recommendation for its chronic administration to human beings.

In these studies we evaluate the sustained action of resveratrol on mouse neuroblastoma (Neuro-2a) and hippocampal (mHippoE-18) cells under oxidative stress conditions. It is the first work to compare the influence of resveratrol on normal neuronal cells and tumour cells derived from the nervous system under identical experimental conditions (oxidative stress conditions). We examined several cell processes after treatment with resveratrol and after combined treatment with resveratrol and an oxidising factor (hydrogen peroxide). It included induction of apoptosis/necrosis and loss of activity of NAD(P)H-dependent cellular oxidoreductase enzymes. These studies prove that resveratrol exerts various effects on different cell types. Resveratrol may modulate the cytotoxicity of hydrogen peroxide, depending on the compound's concentration.

MATERIAL AND METHODS

Chemicals. Resveratrol (RSV), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), Dulbecco's modified Eagle's medium (DMEM), foetal bovine serum (FBS), dimethylsulphoxide (DMSO), hydrogen peroxide (H_2O_2), ethidium bromide, and acridine orange were obtained from Sigma (St. Louise, USA). PE Annexin V Apoptosis Detection Kit I was obtained from BD Pharmingen™ (San Diego, USA).

Cell cultures. An embryonic mouse hippocampal cell line (mHippoE-18) was purchased from Cederlane (CLU199). The mouse neuroblastoma cell line (Neuro-2a) was purchased from the American Type Culture Collection ATCC (CCL-131). Both cells line were cultured in Dulbecco's modified Eagle's essential medium (DMEM) containing 10% fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 µg/ml antimycin A at 37°C and 5% CO_2.

For an MTT assay, cells were seeded in flat bottom clear 96-well plates at the following densities: Neuro-2a – 1×10^4 cells/well and mHippoE-18 – 1.5×10^4 cells/well in 200 µl of DMEM medium. For apoptosis assays, cells were seeded in 12-well plates at the following densities: Neuro-2a – 1.5×10^5 cells/well and mHippoE-18 – 2.5×10^5 cells/well in 1 ml of DMEM medium. Cells were cultured about 20 h under growth conditions for cell attachment and then treated with resveratrol.

Compound treatments. Resveratrol was dissolved in dimethylsulphoxide (DMSO; Sigma) and diluted with culture medium to the working concentrations just before use. DMSO, at the concentrations used, did not influence the viability of either cell line.

In the first part of the experiments, cells were treated with different concentrations of RSV (0–50 µM) (Figure 1A), or with different concentrations of H_2O_2 (0–50 µM) (Figure 1B). Data from these experiments let us choose an appropriate range of RSV and oxidising factor (H_2O_2) concentrations for further studies. In the second part of our studies, we preincubated cells with the chosen concentrations of resveratrol for 3 or 6 h (Figure 2) and next the cells were exposed to H_2O_2 for 24 hours. The concentration of

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hydrogen peroxide was 30 and 20 μM for Neuro-2a and mHippoE-18 cells, respectively.

Cell viability (MTT assay). To examine the cytotoxicity of resveratrol, hydrogen peroxide, and resveratrol and hydrogen peroxide together on Neuro-2a and mHippoE-18 cells we conducted MTT assays. In living cells, NAD(P)H-dependent cellular oxidoreductase enzymes reduce the tetrazolium dye MTT to its insoluble form, purple formazan. The absorbance of dissolved formazan can be measured spectrophotometrically. After exposure of mHippoE-18 and Neuro-2a cells to: RSV, H_2O_2 or RSV and H_2O_2 simultaneously, 20 μl of MTT (5 mg/ml) was added to each well and incubated for 2 h under growth conditions. Next, the medium was discarded, 100 μl of DMSO was added to each well to dissolve the formazan crystals, and the absorbance was measured at 570 nm using a microplate spectrophotometer (BioTek). Cell viability was calculated as a percentage. The absorbance of untreated cells was taken as a control, at 100% viability.

Annexin V staining and flow cytometry for analysis of apoptosis. Apoptotic processes were investigated by using the Annexin V detection kit. The applied fluorescent annexin V conjugates provide detection of early stages of apoptosis by binding with phosphatidylserine. Propidium iodide binds to double stranded DNA by intercalating between base pairs and it stains only dead cells.

The cells were harvested and washed twice with cold PBS. The supernatant was discarded and the cells were resuspended with annexin-binding buffer. To each 100 μl of cell suspension 5 μl of annexin V and 5 μl of propidium iodide were added and the cells were incubated in the dark at room temperature for 15 minutes. After the incubation period, 400 μl of annexin-binding buffer was added and the stained cells were analysed by flow cytometry.

Visualisation of cell morphology, apoptosis, and necrosis. Double staining with fluorescent dyes – acridine orange (AO) and ethidium bromide (EB) – was performed to distinguish viable cells from apoptotic and necrotic ones. Acridine orange enters all cells and stains the nucleus green. Ethidium bromide stains only cells with damaged cell membranes and stains the nucleus orange/red. Cells were identified as follows: viable cells – normal morphology and green nucleus; early apoptotic cells – green nucleus with condensed or fragmented chromatin; late apoptotic cells – condensed or fragmented orange/red chromatin; necrotic cells – morphologically normal orange/red nucleus.

Both dyes were added to each well at a concentration 2 μg/ml for 2 minutes. The cells were washed with PBS and visualised under a fluorescence/phase contrast microscope (Olympus CKX41) at 400× magnification.

Statistical evaluations. Results are presented as mean ± SD. In order to determine the statistical significance of the results for the operation of the oxidising agents and resveratrol we used the t-test. The statistical significance of the impact of resveratrol and incubation time was rated by two-way analysis of variance. STATISTICA, v. 10.0 was used for the calculations.

RESULTS

Cell viability. The MTT assay provides information about the metabolic activity of living cells. It is commonly used for assessments of cell viability. Therefore, first we tested the effect of RSV on the viability of neuroblastoma and hippocampal cells. Cells of both lines were incubated with RSV (2.5–50 μM) for 24 hours. As shown in Figure 1A, RSV reduced the viability of Neuro-2a cells to a greater extent than in mHippoE-18 cells. In the case of hippocampal cells, the lowest concentrations of resveratrol (2.5–10 μM) even slightly increased cell viability (up to 10%). RSV at a concentration of 25 μM did not influence mHippoE-18 cell viability. At the final concentration of RSV (50 μM) cell viability was reduced to 88% for hippocampal cells and to 60% for neuroblastoma cells. The resveratrol-induced loss of cell viability was dependent on concentration; however, cancer cells were much more sensitive to resveratrol action than hippocampal neurons (Sun *et al.* 2008).

Then, we examined cell viability under the influence of hydrogen peroxide. Figure 1B shows that H_2O_2 was toxic to both cell lines in a concentration-dependent manner. For the Neuro-2a cell line, the IC_{50} value was 24.6 μM, while for the mHippoE-18 cell line the IC_{50} value amounted to 36.6 μM. The results indicate that neuroblastoma cells are more susceptible to hydrogen peroxide exposure compared to the hippocampal cell line. Similar data were reported by Yoshizaki *et al.* (2009). They proved that hydrogen peroxide exerts a pro-senescent effect on cancer cells (Yoshizaki *et al*. 2009). Additionally, in cancer cells, low levels of antioxidant enzymes such as catalase and glutathione peroxide act to increase the concentration of hydrogen peroxide (Punnonen *et al.* 1994). This added dose

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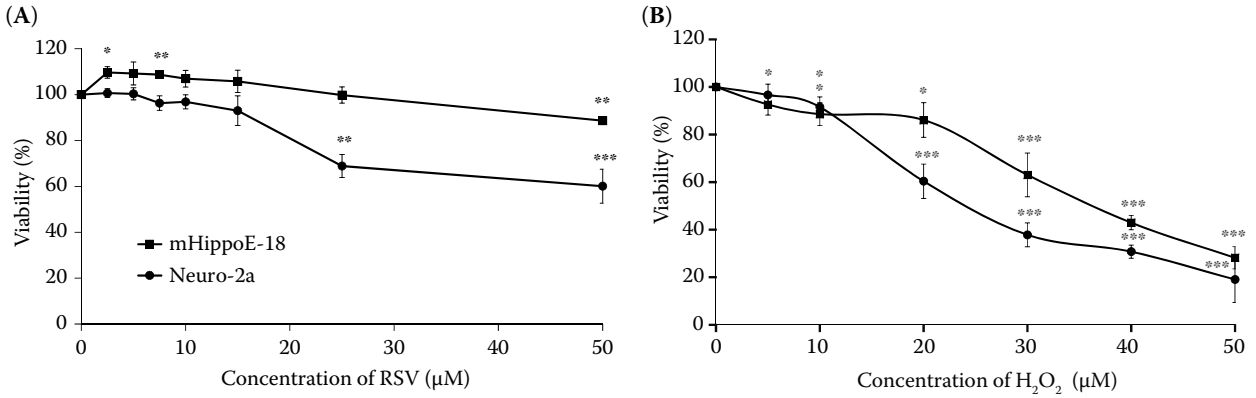

Figure 1. Viability of Neuro-2a and mHippoE-18 cells after 24 h of incubation with: (**A**) resveratrol and (**B**) hydrogen peroxide (n = 4–10, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. cells not treated with RSV or H_2O_2)

of hydrogen peroxide which accumulates in cancer cells might act as a prodrug (Wlassoff *et al.* 2007).

Data from these experiments let us choose appropriate ranges of RSV and oxidising factor concentrations for the following studies.

In the next stage we conducted experiments with combined treatment of cells – resveratrol and hydrogen peroxide – in order to verify the influence of resveratrol on mHippoE-18 and Neuro-2a cells under oxidative stress conditions. We pretreated cells with the chosen concentrations of resveratrol for 3 or 6 h, then added H_2O_2 and incubated for 24 hours. Based on the previous MTT assays we chose concentrations of oxidising factor that caused reductions in cell viability by about 50–60%. We applied hydrogen peroxide at a concentration of 30 and 20 μM for mHippoE-18 and Neuro-2a, respectively.

In the case of hippocampal cells we did not observe any statistically significant differences in cell viability between cultures preincubated for 3 and 6 h with RSV in the concentration range of 2.5–20 μM and afterwards treated with hydrogen peroxide compared to cells exposed to H_2O_2 only (Figure 2A). Resveratrol did not protect the cells from oxidative stress damage but neither did it enhance the effect of H_2O_2.

Quite different results were obtained for Neuro-2a cells. We observed considerable viability reduction in cells preincubated with RSV and treated with hydrogen peroxide in comparison with cells treated with hydrogen peroxide alone. The highest cytotoxicity occurred after 3 hours of the cell pretreatment with RSV (Figure 2B). When the preincubation time (6 h) of the cells with polyphenol was extended, cell viability increased and the metabolic ability of the cells improved.

The major impact on cells is probably due to various metabolites of polyphenol such as resveratrol glucuronides and resveratrol sulphates. Autophagy and senescence in human cancer cells are probably induced mainly by sulphate metabolites. The latest

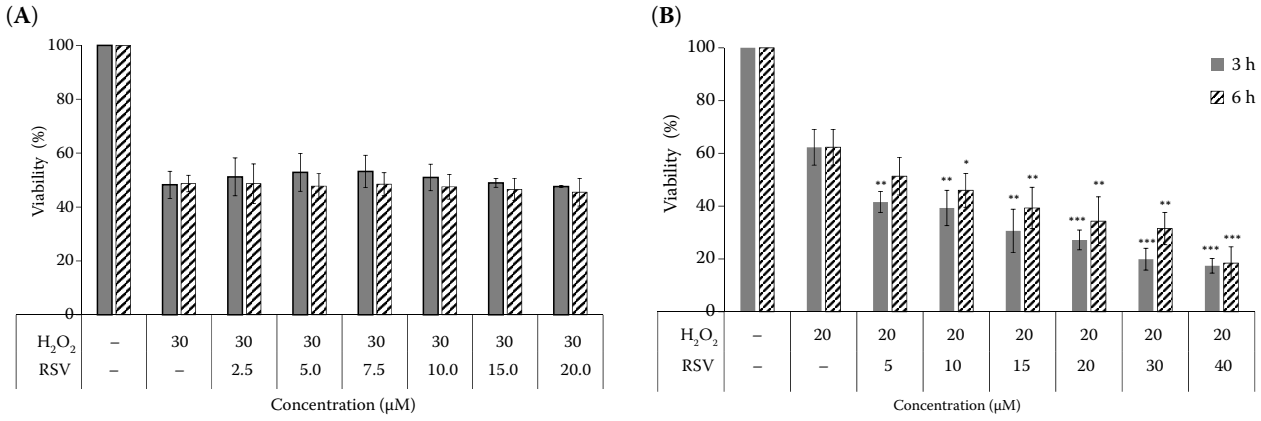

Figure 2. Viability of mHippoE-18 (**A**) and Neuro 2a (**B**) cells after combined treatment with resveratrol and hydrogen peroxide (n = 4–10, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. cells treated only with H_2O_2

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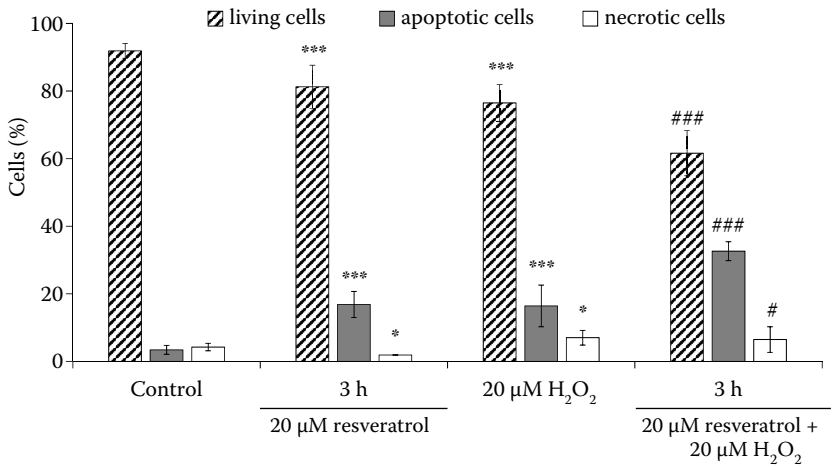

Figure 3. The percentage of living, dead, early, and late apoptotic mHippoE-18 cells after 27 and 36 h of incubation with resveratrol alone and after 3 h of pretreatment with resveratrol (20 μM) and afterwards 24 h of incubation with hydrogen peroxide (n = 4–8, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. cells not treated with RSV or H_2O_2, $\#P < 0.05$, $\#\#\#P < 0.001$ vs. cells treated only with H_2O_2)

data suggest that resveratrol is delivered to target tissues in a stable sulphate-conjugated form (Patel *et al.* 2013). These metabolites are able to penetrate into the living cells where they are gradually regenerated into the parent compound. The addition of hydrogen peroxide (after 3 h) to cultures with resveratrol metabolites intensified its prooxidative effect in cancer cells. "Transformed resveratrol" acts as a prodrug (Wlassoff *et al.* 2007). However, a longer period of preincubation with RSV increased cell viability. This is probably linked with the absence or diminished amounts of resveratrol metabolites in the cells. More and more literature data have indicated that polyphenols (*inter alia* resveratrol) induce apoptotic processes in cancer cell lines (Cai *et al.* 2015; Zielińska-Przyjemska *et al.* 2015).

Cell death studies. To investigate whether or not the loss of viability in response to resveratrol, hydrogen peroxide and combined treatment with RSV and hydrogen peroxide correlates with apoptotic changes, we carried out a flow cytometric analysis after staining with annexin V and propidium iodide as well as microscope observations after staining with acridine orange (AO) and ethidium bromide.

To determine the level of apoptosis induced by resveratrol, hydrogen peroxide and combined treatment with resveratrol and hydrogen peroxide we chose an RSV concentration of 20 μM (a concentration which did not cause any substantial changes in mHippoE-18 cell viability and produced a decrease in Neuro-2a cell viability). The concentration of H_2O_2 was selected by analogy with previous experiments (20 μM for Neuro-2a and 30 μM for mHippoE-18).

Incubation of hippocampal cells with resveratrol (20 μM) increased the percentage of apoptotic cells (Figure 3). This increase was higher than it would be expected from the results of the viability assays. Resveratrol at a concentration of 20 μM did not affect the cell survival but increased the cell apoptosis (increase up to 10% compared to the control). The MTT assay evaluates mitochondrial function and metabolic activity. If cells are in the early phase of apoptosis or apoptosis takes place through an extrinsic pathway, the mitochondrial function in cells can still be maintained

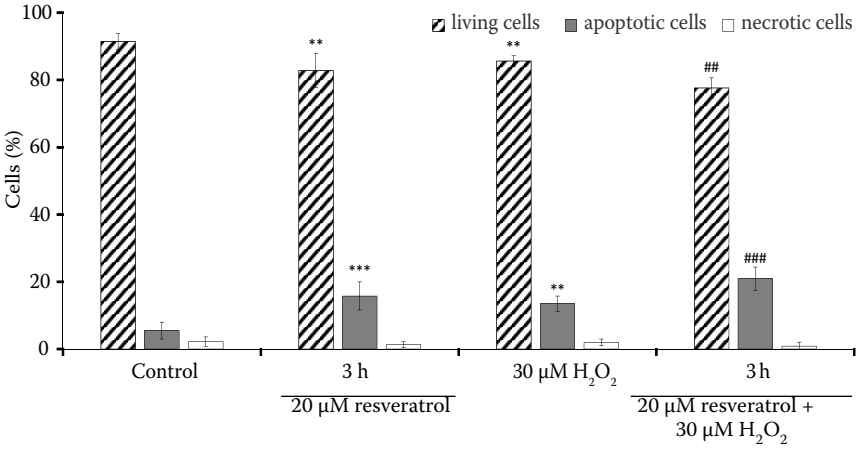

Figure 4. The percentage of living, dead, early, and late apoptotic Neuro-2a cells after 27 and 36 h of incubation with resveratrol alone and after 3 hours of pretreatment with resveratrol (20 μM) and afterwards 24 hours of incubation with hydrogen peroxide (n = 4–8, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. cells not treated with RSV or H_2O_2, $\#\#P < 0.01$, $\#\#\#P < 0.001$ vs. cells treated only with H_2O_2)

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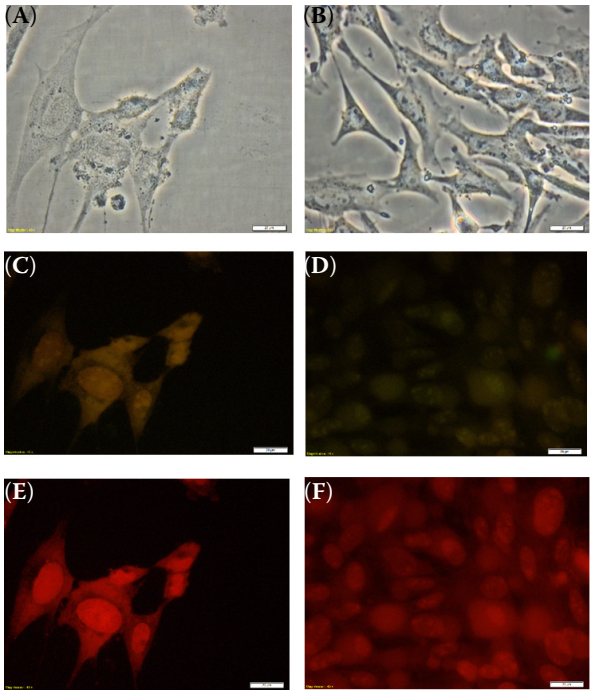

Figure 5. mHippoE-18 cells. A, C, E – control. B, D, F – cells treated with resveratrol and hydrogen peroxide (30 μM)

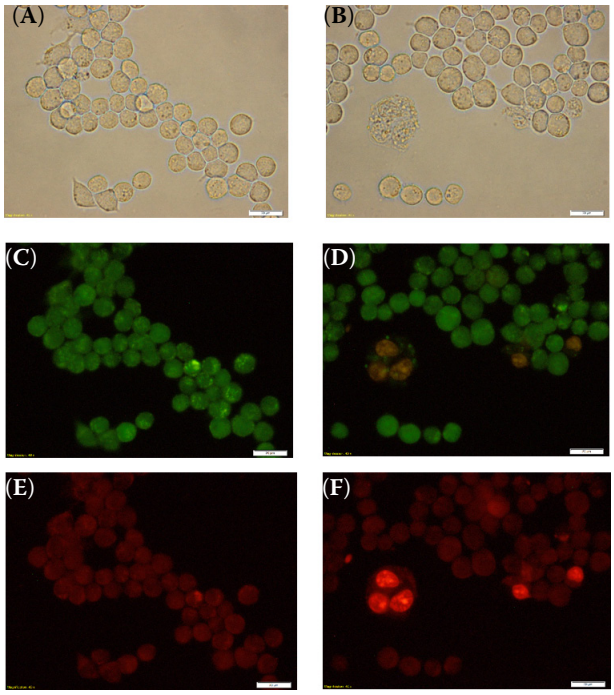

Figure 6. Neuro-2a cells. A, C, E – control. B, D, F – cells treated with resveratrol and hydrogen peroxide (20 μM)

(Fulda & Debatin 2006). This may be the reason why the results for apoptosis did not correlate with the results for survival.

Incubation of cancer cells with resveratrol also resulted in an increase in the percentage of apoptotic cells (approx. 15% compared to the control) (Figure 4). Other studies have also demonstrated that RSV affects the activation of apoptosis in neuroblastoma cells and suppresses the cell division (Rahman *et al.* 2012; Ren *et al.* 2015).

Incubation of both tested cell lines with hydrogen peroxide (20 μM for Neuro-2a and 30 μM for mHippoE-18 cells, respectively) caused a decline in the percentage of viable cells to 76 and 86%, respectively, for Neuro-2a and mHippoE-18 cells (Figures 3 and 4). In Neuro-2a cells, hydrogen peroxide also increased necrotic processes.

Preincubation of hippocampal and neuroblastoma cells with resveratrol for 3 h and then treatment with H_2O_2 and incubation for 24 h caused an increase in the percentage of apoptotic cells compared to cells treated with RSV only. If hydrogen peroxide and resveratrol acted synergistically, we would expect apoptosis in approx. 30%. In the combined treatment of hippocampal cells with resveratrol and hydrogen peroxide we saw smaller effects than they should result from their combined action. In this respect, resveratrol has an insignificant protective effect. However, combined treatment of Neuro-2a (H_2O_2 + RSV) resulted in a decrease in the percentage of living cells (approx. 27% compared to cells treated with hydrogen peroxide alone). The percentage of apoptotic cells was higher than what would be expected from the combined action of the two factors – hydrogen peroxide and resveratrol. Intensification of apoptotic processes in cancer cells may be the result of, among other influences, oxidative damage to different biomolecules caused by the formation of phenoxyl radicals of resveratrol, efflux of cytochrome C, and the influence of RSV on mitochondrial membrane proteins, which may result in a decrease in the mitochondrial potential (van Ginkel *et al.* 2007, Li *et al.* 2012; Xi *et al.* 2012; Rodacka *et al.* 2014). The greater sensitivity of tumour cells to the action of resveratrol or its metabolites in combination with hydrogen peroxide may also be due to conditions in the tumour microenvironment – low pH, reduced activity of antioxidant enzymes, increased oxidative stress (Shamim *et al.* 2012).

Light and fluorescent microscopy have also identified various morphological changes that occurred after treatment of mHippoE-18 and Neuro-2a cells with resveratrol, and after combined treatment with resveratrol and hydrogen peroxide. In the latter

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case (after treatment of cells with resveratrol and an oxidising factor) we observed extensive plasma membrane blebbing and more cells in early or late stages of apoptosis as well as more necrotic cells (Figures 5 and 6).

DISCUSSION

Recently resveratrol has received a lot of attention mainly because of its potential anti-cancer properties and the possibility it can be applied in chemotherapy as a supporting agent. It also became popular as a result of its reputed anti-aging effects (Lekli *et al.* 2010; Jayasena *et al.* 2013). The greatest advantage of resveratrol is that it is ubiquitous in a wide range of commonly consumed food plants such as blueberries, peanuts, chocolate. However, grapes and red wine are the best-known sources of resveratrol. The amount of resveratrol in wine depends both on the type of grapes and on the climate where they grow – the colder the climate, the higher the level of resveratrol in grapes. The most popular wines include Malbec, Petite Sirah, St. Laurent, and Pinot Noir. The low coronary heart disease death rates despite high intake of saturated fat and dietary cholesterol in France have been associated with drinking larger volumes of red wine. After the discovery and study of this so-called "French Paradox" even the FDA (Saunders 1998) stated that balanced consumption of red wine could be beneficial. Also the antioxidant properties of this extraordinary polyphenol are well known and are believed to contribute to its beneficial effects. A large effort has been made to evaluate how resveratrol influences both normal and tumour cells. Especially interesting seem to be neuronal cells. However, the exact mechanisms that could be responsible for its effects on cells have not been defined yet. We therefore decided to evaluate the sustained action of resveratrol on mouse malignant neuroblastoma and mouse hippocampal cell lines under oxidative stress conditions.

Our study demonstrates that neuroblastoma tumour cells are approximately 1.5 fold more sensitive to the action of resveratrol compared to normal hippocampal cells. Our data also indicate that under oxidative stress conditions resveratrol reduces the cell viability to a greater extent in cancer cells (Yoshizaki *et al.* 2009). Furthermore, in the present study, we confirmed that resveratrol administration efficiently induced apoptosis in a cancer cell line, which might be the result of many factors (Piotrowska *et al.* 2012). Most probably administrated resveratrol is converted into more stable metabolites that act as prodrugs in tumour cells (Biasutto *et al.* 2014).

The opposite trend in hippocampal cells is evidence that the cell type and specific metabolic abilities determine the impact of various factors. The metabolic properties of cancer cells are remarkably different from those of normal cells (Zhao *et al.* 2013). Cells with higher rates of metabolism are more sensitive to the actions of polyphenols and oxidising factors as well (Peltz *et al.* 2012). Furthermore, a point that must always be borne in mind is that effects may change a "sign" depending on the concentration. In this regard it is worth citing the results which Peltz's research group obtained. They claimed that resveratrol's functional discrepancies appear to have resulted from different cells being exposed to it at different concentrations in different experimental settings. Low concentrations of this polyphenol stimulate pro-survival effects in stem cells; however, higher concentrations initiate pro-death effects in cancer cell lines (Peltz *et al.* 2012).

Needless to say that new therapies and safer drugs are essential. We conclude that resveratrol seems to be a promising future target as a natural chemotherapeutic agent, supporting traditional therapies; however, still more evidences and further investigations of its action and its molecular targets are needed in order to use it wisely as a drug.

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