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RESEARCH

Neither resveratrol nor metformin protects human cardiomyocytes against toxicity of epirubicin and radiation *in-vitro*

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ABSTRACT

Oncological treatment is frequently followed by malfunction of cardiovascular system. Because of the high risk of developing cardiotoxicity after anticancer therapy, there is need for effective prevention. Promising method is the use of cardioprotective agents, which can attenuate toxic effects of therapy to the heart. In this study we analyzed the potential of resveratrol and metformin to minimize cytotoxic effect of ionizing radiation and/or epirubicin on human cardiomyocytes. Cultured *in vitro* human cardiomyocytes were treated with epirubicin and/or ionizing radiation. Simultaneously, the cells were treated with metformin or resveratrol. Survival of cells after different doses of tested agents was assessed by MTT and clonogenic assay. Changes in cell cycle phases distribution, apoptosis intensity, and in ROS concentration were measured as well. Exposure of cardiomyocytes to epirubicin had a negative effect on cell survival. Resveratrol and metformin alone had no significant effect on cardiac cells. When combined with epirubicin and/or ionizing radiation, resveratrol had no impact on cardiomyocytes condition, while metformin enhanced radiation and epirubicin toxicity. In general the results of this study do not provide a clear answer on a possible use of resveratrol or metformin as substances with the potential to reduce the cardiotoxicity of epirubicin and ionizing radiation.

Key words: anthacyclines; cardiotoxicity, cardioprotection, resveratrol, metformin, ionizing radiation, cancer therapy

Abbreviations: AMPK - 5' adenosine monophosphate-activated protein kinase; DNA - deoxyribonucleic acid; HER2 - human epidermal growth factor receptor 2; MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ROS - reactive oxygen species; TUNEL - terminal deoxynucleotidyl transferase dUTP nick end labeling.

1. INTRODUCTION

Improved efficacy of anticancer treatment and prolonged survival of cancer patients is frequently connected with increased long-term risk of side effects, which could markedly reduce quality of patients life. Almost every type of anticancer treatment is associated with some risk of developing cardiac toxicity, yet different mechanisms of cardiotoxicity could be involved depending on nature of therapeutic factor (Monsuez et al, 2010). Among anticancer factors with known potential to cause cardiotoxicity are anthracyclines and ionizing radiation, frequently used in combination that markedly increases their deleterious effects (Rahman et al, 2007; Zeglinski et al, 2011). Underlying mechanism of damage to the cardiovascular system induced by anthracyclines and/or radiation exposure is oxidative stress associated with increased formation of free radicals and disruption of antioxidant mechanisms (Monsuez et al, 2010; Zeglinski et al, 2011; Mokni et al, 2012; Senkus-Konefka et al, 2007). Varying degrees of cardiotoxicity associated with these factors could be observed in about 25% of treated patients, and development of such toxicity is observed even after 30 years of successful completion of cancer treatment (Simůnek et al, 2009; Shaikh et al, 2012). Because of the risk of cardiotoxicity following anticancer treatment, monitoring of cardiac functions during and after treatment is frequently implemented in clinical practice. However, such approach only allows for early detection but not for prevention of heart damage (Cardinale et al, 2010; Fiuza et al, 2012). Reduction of cumulative dose, modification of schemes of administration and/or using less toxic drug analogs could be considered to minimize the risk of cardiotoxicity. Unfortunately these options are not always possible without reducing efficacy of primary treatment (Cardinale et al, 2010; Fiuza et al, 2012; Bird et al, 2008). Preferred approach to minimize or completely eliminate toxic effects of cancer therapy to the heart would be an administration of cytoprotective agents. Although several substances have been tested as potential cardioprotectants, only one – dexrazoxane – has been approved for clinical use, yet its applicability is still rather limited (Simůnek et al, 2009; Kik et al, 2006). Hence, there is a constant need to develop and test novel compounds with potential applicability for chemical cardioprotection.

Resveratrol and metformin are among substances with hypothetical cytoprotective activity, which could be potentially used for cardioprotection against factors used in cancer treatment. Resveratrol is a natural plant-derived phenol (stilbenoid) possessing some antioxidant, anti-inflammatory, anti-angiogenic, and anti-tumor activities (Mokni et al, 2012; Rezk et al, 2006; Zhang et al, 2013). The published reports suggest that resveratrol is able to reduce heart damage induced by doxorubicin, which is manifested primarily by lowering intensity of necrosis in cardiomyocytes and reduction of myocardium fibrosis (Arafa et al, 2014; Osman et al, 2013). Metformin is a synthetic biguanide used as an antidiabetic drug. Beside lowering a concentration of glucose in the blood (An et al, 2006; Viollet et al, 2012) metformin could affect blood level of cholesterol and triglycerides, and reduce risk of cardiovascular and cancer complications of diabetes (El Messaoudie et al, 2011). Several reports indicate that metformin could increase the effectiveness of anticancer treatment (Barrière et al, 2013; Asensio-López et al, 2013; Kourelis et al, 2011; Isakovic et al, 2007). Moreover, a few reports suggested that metformin could reduce myocardial injury caused by HER2 inhibitors (Barrière et al, 2013; Kourelis et al, 2011) and doxorubicin (Asensio-López et al, 2013; Trobo et al, 2014). Two anthacyclines - doxorubicin and epirubicin, are anticancer drugs intercalating into DNA, disrupting the activity of topoisomerase II, and generating free radicals (Simůnek et al, 2009; Osman et al, 2013; Gewirtz, 1999). In some chemotherapy regiments epirubicin is favored over doxorubicin because of lower level of side effects, hence it is frequently used in (neo)adjuvant treatment of breast cancer, either alone or in combination with radiotherapy (Adamowicz et al, 2008; Arriola et al, 2007). Mechanisms of cardiotoxic activity of epirubicin are less studied than such activity of doxorubicin, and there are only a few reports on chemoprotection against epirubicin-related cardiotoxicity (Lopez et al, 1998; Jakobsen et al, 1994; Seymour et al, 1999), none of them concerning resveratrol or metformin. Similarly, there are no published report regarding hypothetical cytoprotection activity of resveratrol or metformin against radiotherapy-induced cardiotoxicity. The aim of this study was to assess potential effect of resveratrol and metformin on toxicity of epirubicin and ionizing radiation to human cardiomyocytes cultured *in vitro*.

2. MATERIALS AND METHODS

Cell line and materials. Human cardiomyocytes were obtained from Celprogen (1311001-09) and cultured in flasks covered with fibronectin. Cells were cultured in DMEM/F12 medium supplemented with 10% FBS, 2 mM L-glutamine and antibiotics, in 37°C humidified atmosphere containing 5% CO_2. Epirubicin, metformin and resveratrol were purchased from Sigma. Irradiation of cells was performed using CLINAC 600 linear accelerator with 6MV photons and 1 Gy/min dose rate.

Cell viability assay. MTT assay was used to determine cell viability. In brief, cells were cultured and exposed to analyzed factors in 96-well plates, to which the MTT reagent (Biotium) was added. After 3 hours of incubation in 37°C medium was removed and formazan crystals formed in cells were dissolved by adding isopropanol with HCl. The absorbance was measured on a plate reader at a 570 nm wavelength.

Clonogenic assay. Cells were seeded into 6-well plates at a density of 1000 cells per well and treated subsequently with analyzed factors, then cultured in fresh medium for 7-14 days. The colonies were stained with a solution containing 0.5% crystal violet and 50% methanol, rinsed with water to remove an excess of dye, and then the number of colonies was counted.

Cell cycle analysis. To characterize a distribution of the cell cycle phases cells treated with analyzed factors were harvested by trypsinization and fixed with 70% ethanol. DNA was stained in collected cells with a solution containing 1 ng/ml DAPI and 0.1 µg/ml RNase in PBS, and then analyzed by flow cytometry using FACSCanto cytometer (Becton Dickinson).

Assessment of apoptosis. Cells with fragmented chromatin were detected by the TUNEL assay. Briefly, cells were harvested by trypsinization, fixed with 1% formaldehyde in PBS, washed in PBS and stored in 70% ethanol in -20°C, and then stained with the APO-BRDU™ Kit (BD Pharmingen) according to manufacturer's protocol and quantitated using flow cytometry. Cells expressing CD95 (Fas) death receptor were harvested and fixed as described above, then stained with FITC-conjugated anti-Fas antibody (Exbio Praha) for 30 minutes and quantitated by flow cytometry.

Assessment of reactive oxygen species. Intracellular level of reactive oxygen species was determined using Total ROS/Superoxide Detection Kit (Enzo Life Sciences) according to manufacturer's protocol. Briefly, cells were cultured and exposed to analyzed factors in 96-well plates, then treated with ROS/Superoxide Detection Solution and stained for 60 minutes at 37°C in the dark. Intensity of green and orange fluorescence (at 488 nm and 550 nm) corresponding to levels of total ROS and superoxides, respectively, was measured by microplate reader.
Statistical analysis. All experiments were performed in triplicate at least. Significance of differences between compared groups was assessed by the two sided student's t-test. P value <0.05 was considered significant.

3. RESULTS

In this work we assessed hypothetical effect of resveratrol or metformin on toxicity of epirubicin and/or ionizing radiation for human cardiomyocytes cultured *in vitro*. In pilot experiments we tested several doses of epirubicin (up to 50 µg/ml) and ionizing radiation (up to 8 Gy) to select doses having measurable effects in different cellular and molecular tests. Moreover, different doses of resveratrol (up to 20 µM) and metformin (up to 15 mM) were tested to select doses neutral to cardiomyocytes (i.e., not revealing toxicity or reducing viability). Importantly, doses of all factors selected for further experiments were clinically relevant and could be reached in blood after systemic treatment (Isakovic et al, 2007; Eksborg, 1990; Scott et al, 2012). Cells were either incubated with epirubicin (for 24 hours) or irradiated alone, or treatment with epirubicin started 20 hours after irradiation (to address combined effect of both factors). Continuous treatment with resveratrol or metformin started 4 hours before irradiation and/or 24 hours before start of exposure to epirubicin, and lasted for 48 hours (i.e. to the end of exposure to epirubicin), which constituted the start of observation time after all treatments (see Figure 1). Such experimental settings corresponded to hypothetical prophylactic/preventive usage of both compounds.

Viability and metabolic activity of cardiomyocytes treated with cytotoxic factors (epirubicin and/or ionizing radiation) and/or potential cytoprotective compounds (resveratrol or metformin) was assessed using the MTT assay after the end of combined treatments. Visible reduction of cell viability (~50%) was observed after treatment with epirubicin at 5 µg/ml, and this concentration was selected for further experiments. On the other hand, about 50% reduction of cell viability was observed only at the highest radiation dose (8 Gy), yet more clinically relevant dose (4 Gy) was selected for further experiments. Resveratrol had no significant effect on cell viability with concentrations up to 20 mM, while metformin in concentrations higher than 1 mM reduced viability of cells. Consequently, resveratrol at concentrations 1 and 10 µM and metformin at concentration 1 and 5 mM were used in further experiments. Figure 2 shows results of experiments where cardiomyocytes pre-conditioned with either resveratrol or metformin were treated with epirubicin (5 µg/ml) and/or exposed to radiation (4 Gy). We observed reduction of cell viability after exposure to epirubicin, either alone or in combination with ionizing radiation. However, no statistically significant effect on drug-induced toxicity was observed in cells conditioned with resveratrol. Incubation of cells with metformin also did not prevent reduction of viability induced by epirubicin and radiation. Moreover, metformin further reduced cell viability in all experimental settings, which was particularly visible at 5 mM concentration. We concluded that conditioning of cardiomyocytes with resveratrol or metformin does not protect against reduction of metabolic activity and cell viability induced by epirubicin and radiation.

The effect of resveratrol and metformin on clonogenic survival of cardiomyocytes treated with epirubicin and ionizing radiation was assessed by the clonogenic assay, where number of viable clones of surviving cells was analyzed 1-2 weeks after the treatment. Clinically-relevant 2 Gy dose of radiation and 0.1 µg/ml of epirubicin were selected for further analyses (practically no proliferating clones were detected after exposure with higher concentrations of epirubicin, see Figure 3A). Neither resveratrol (at concentration 1 and 10 µM) nor metformin (at concentrations 0.01 to 1 mM) itself affected clonogenic survival of cardiomyocytes (see Figure 3A). Next, we analyzed cardiomyocytes conditioned with either resveratrol (2 µM) or metformin (0.1 and 1 mM), and then treated with epirubicin (0.1 µg/ml) and/or exposed to radiation (2 Gy). We observed reduction in the number of surviving clones after exposure to epirubicin, radiation, and combined treatment: to about 5%, 90%, and 5% of control, respectively. Importantly, conditioning with resveratrol or metformin did not result in increase of the surviving clones number (Figure 3B). We concluded that treatment with neither resveratrol nor metformin does not enhance clonogenic survival of cardiomyocytes exposed to epirubicin and radiation.

Potential effect of resveratrol and metformin on drug- and radiation-induced blockade of cell cycle progression was assessed by flow cytometry-based analysis of the cell cycle phases distribution. The analysis revealed that both epirubicin (0.1 µg/ml) and radiation (2 Gy)

induced prolonged block in the G2/M phase, which was still detectable 72 hours after the end of treatment; the ratios of G2/G1 in control and epirubicin-, radiation- or radiation/epirubicin-treated cell population were 0.23, 0.55, 0.40 and 0.76, respectively. The effect of epirubicin was dose-dependent and higher dose of the drug (1 μg/ml), either alone or in combination with radiation, totally changed distribution of the cell cycle phases (the G2/G1 ratio was about 20) and increased number of cells representing sub-G1 subpopulation. Neither resveratrol (2 μM) nor metformin at lower concentration (0.1 mM) affected distribution of the cell cycle phases, while higher concentration of metformin (1 mM) increased number of cells in S and G2/M phases (the G2/G1 ratios were 0.30, 0.30 and 0.70, respectively). Conditioning of cells with resveratrol and metformin at lower concentration slightly affected distribution of the cell cycle phases after treatment with epirubicin and/or radiation. In contrast, treatment with higher concentration of metformin (1 mM) caused major dysregulation of the cell cycle and deepened clastogenic effects observed in cells exposed to both genotoxic factors (Figure 4). We concluded that conditioning with resveratrol or metformin does not protect against the cell cycle block induced in cardiomyocytes by epirubicin and radiation.

Potential effect of resveratrol and metformin on drug- and radiation-induced apoptosis was further analyzed by assessment of cells with chromatin fragmentation (by the TUNEL assay) and cells with expression of Fas/CD95 death receptor 72 hours after the end of combined treatment. We found that epirubicin (0.1 μg/ml) induced significant increase in the number of TUNEL-positive (Figure 5A) and Fas-positive (Figure 5B) cells. This was further increased after combined treatment with the drug and 2 Gy dose of radiation, while effect of radiation alone was relatively weaker. Neither resveratrol (2 μM) nor metformin (0.1 mM) alone affected the number of TUNEL-positive or Fas-positive cells. We observed that conditioning with either resveratrol or metformin reduced the number of TUNEL-positive cells induced by epirubicin (and epirubicin combined with radiation). Some reduction in the number of Fas-positive cells was also observed in such experimental setting, yet differences between cardiomyocytes conditioned and not conditioned with resveratrol or metformin did not reach the level of statistical significance. We concluded that conditioning of cardiomyocytes with either resveratrol or metformin could only slightly reduce number of cells undergoing apoptosis induced by epirubicin.

Finally, we assessed potential effect of analyzed factors on intercellular levels of reactive oxygen species (ROS) by colorimetric assay directly after the end of combined treatment. We found that exposure to neither epirubicin (0.1 μg/ml) nor ionizing radiation (2 Gy) nor their combination affected total level of ROS (Figure 6A) or level of superoxides (Figure 6B) at the analyzed time point. Furthermore, levels of ROS and superoxides were affected by conditioning with neither resveratrol (2 μM) nor metformin (0.1 mM). We concluded, that analyzed factors, both cytotoxic and hypothetical cytoprotectants, do not affect markedly production of reactive oxygen species in human cardiomyocytes cultured *in vitro*.

4. DISCUSSION

Potential influence of metformin on toxic effects of epirubicin (as well as ionizing radiation) to human cardiac cells has not been studied yet. However, there are a few reports indicating the ability of metformin to reduce toxicity of doxorubicin (Asensio-López et al, 2013; Trobo et al, 2014; Kobashigawa et al, 2014). Metformin at concentration 4 mM reduced doxorubicin-induced damage and oxidative stress in mouse cardiomyocytes (Asensio-López et al, 2011). Moreover, it was shown that metformin in concentration of 1-2 mM could limit production of ROS and reduce apoptosis in rat cardiomyocytes (An et al, 2006). More recently it was shown that 0.1 mM metformin could protect cardiomyocytes against doxorubicin-induced toxicity through the AMPK-mediated pathway (Kobashigawa et al, 2014). In this study we showed that metformin used in 0.1 or 1 mM concentration did not reduce toxicity of epirubicin to human cardiomyocytes cultured *in vitro*. The concentration of metformin in plasma of diabetic patients could reach 6-30 μM, yet the drug accumulates in different tissues at higher levels (Isakovic et al, 2007; Alimova et al, 2009). Nevertheless, concentration of metformin used in current study was similar to that reported in the available reports on *in vitro* studies, which was typically 1-4 mM (An et al, 2006; Asensio-López et al, 2013; Isakovic et al, 2007; Alimova et al, 2009). Importantly, we showed here that metformin used at concentrations higher than 1 mM affected metabolic activity and the cell cycle of human cardiomyocytes cultured *in vitro*. As a consequence, harmful effects of epirubicin were enhanced in cells pre-conditioned with metformin instead of expected protection (which was clearly visible in results of the MTT test or distribution of the cell cycle). Hence, one should assume that in some circumstances combined treatment with metformin and epirubicin should be avoided.

In the existing literature there are also a few reports demonstrating a protective activity of resveratrol to myocardium of mice treated with doxorubicin (Arafa et al, 2014; Osman et al, 2013), yet no data regarding effects of resveratrol on irradiated cardiomyocytes is available. Cytoprotective activity of resveratrol used in biologically-relevant concentration against toxic effects of epirubicin and ionizing radiation were not observed when human cadiomyocytes cultured *in vitro* were used here as an experimental model. This is noteworthy, that analyzed cells were relatively more resistant to ionizing radiation than to epirubicin when either factor was used in clinically relevant dose, which apparently confirmed high radioresistance of cardiomyocytes reported in previous studies (Boerma et al, 2002).

General results of this study indicated that neither resveratrol nor metformin markedly reduced toxicity of epirubicin and/or ionizing radiation to human cardiomyocytes cultured *in vitro*. The only exception was slightly reduced level of epirubicin-induced TUNEL-positive cells after pretreatment with either resveratrol or metformin, yet such protection was not confirmed in other tests. Lack of protection against toxic effect of epirubicin and ionizing radiation could be attributed to specific features of experimental model. It is generally postulated that cardiotoxic effects of anthracyclines and ionizing radiation are related with damage induced by oxidative stress (Monsuez et al, 2010; Zeglinski et al, 2011; Mokni et al, 2012; Senkus-Konefka et al, 2007), and that cytoprotective effect of resveratrol and metformin could be related to their antioxidant activity. In analyzed cardiomyocytes neither epirubicin nor ionizing radiation induced enhanced production of reactive oxygen species. Hence, lack of protection by hypothetical antioxidants could be associated with lack of oxidative stress induced by toxic factors, which phenomenon could be specific for a given cellular model. Moreover, cultured cardiomyocytes were characterized by fast growth rate, while

corresponding heart cells have rather limited mitotic activity. Hence, hypothetical protective effects of resveratrol or metformin should be further analyzed using other experimental models more closely resembling *in vivo* conditions. Nevertheless, presented data did not reveal potential of resveratrol or metformin to reduce harmful effects induced by epirubicin and ionizing radiation in human cardiomyocytes *in vitro*.

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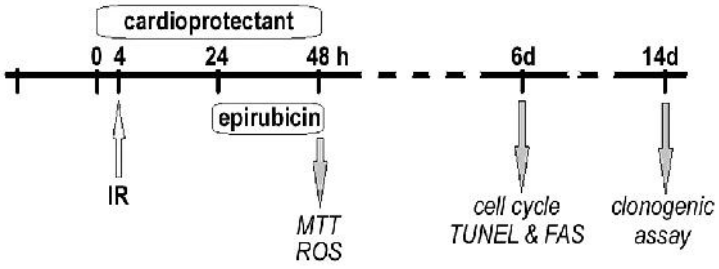

Figure 1

Experimental model

Cardiomyocytes were conditioned with resveratrol or metformin before and during treatment with epirubicin and/or ionizing radiation. All tests were performed after the end of combined treatment with all factors.

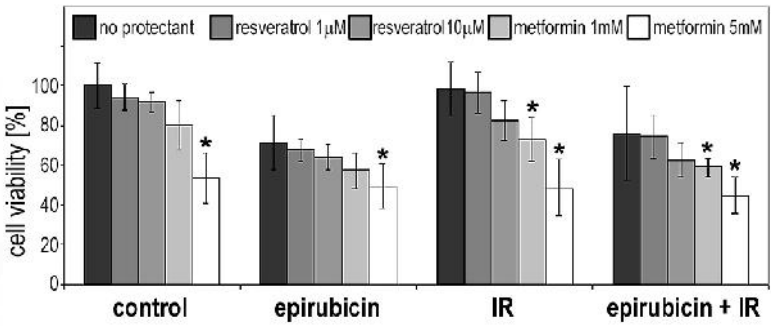

Figure 2

The influence of analyzed factors on viability of cardiomyocytes

Metabolic activity of cells exposed to epirubicin and ionizing radiation, either alone or in different combinations with resveratrol or metformin, was assessed by the MTT assay. Cell were analyzed directly after the end of treatment; 5 μg/ml epirubicin, 4 Gy ionizing radiation (IR), 1 and 10 μM resveratrol, and 1 and 5 mM metformin were used. Metabolic activity of cells and presented in relation to untreated controls; shown are the mean values ±S.D. for 10 replicas, statistically significant differences (in relation to cells not conditioned with protectants) are marked with asterisks.

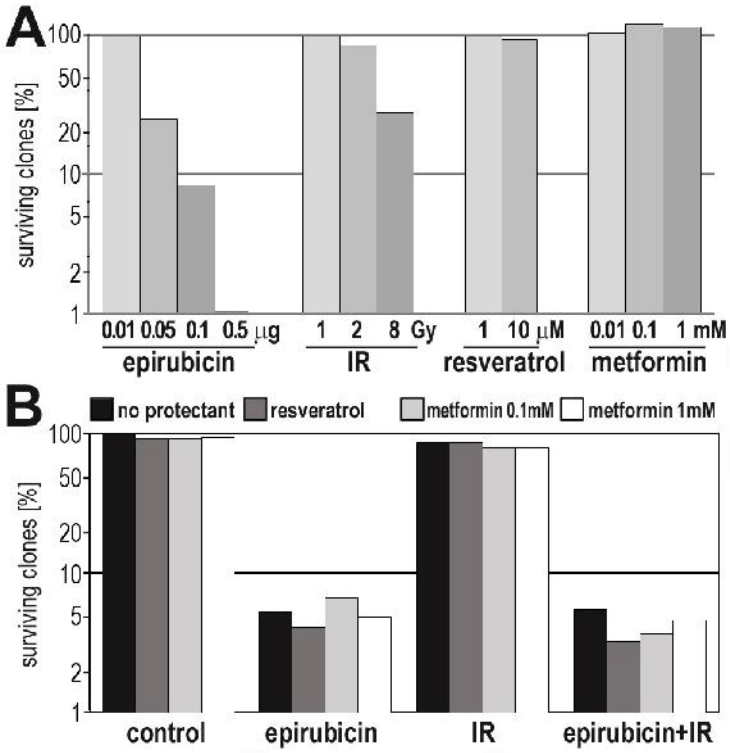

Figure 3

The influence of analyzed factors on clonogenic survival of cardiomyocytes

Number of clonogenic cells was assessed after treatment with epirubicin, ionizing radiation, resveratrol and metformin alone (A). Furthermore, surviving clones were quantitated for cells conditioned with resveratrol (2 µM) or metformin (0.1 and 1 mM), and then exposed to epirubicin (0.1 µg/ml) and/or radiation (IR; 2 Gy) (B). Numbers of surviving clones are presented in relation to untreated controls (100%).

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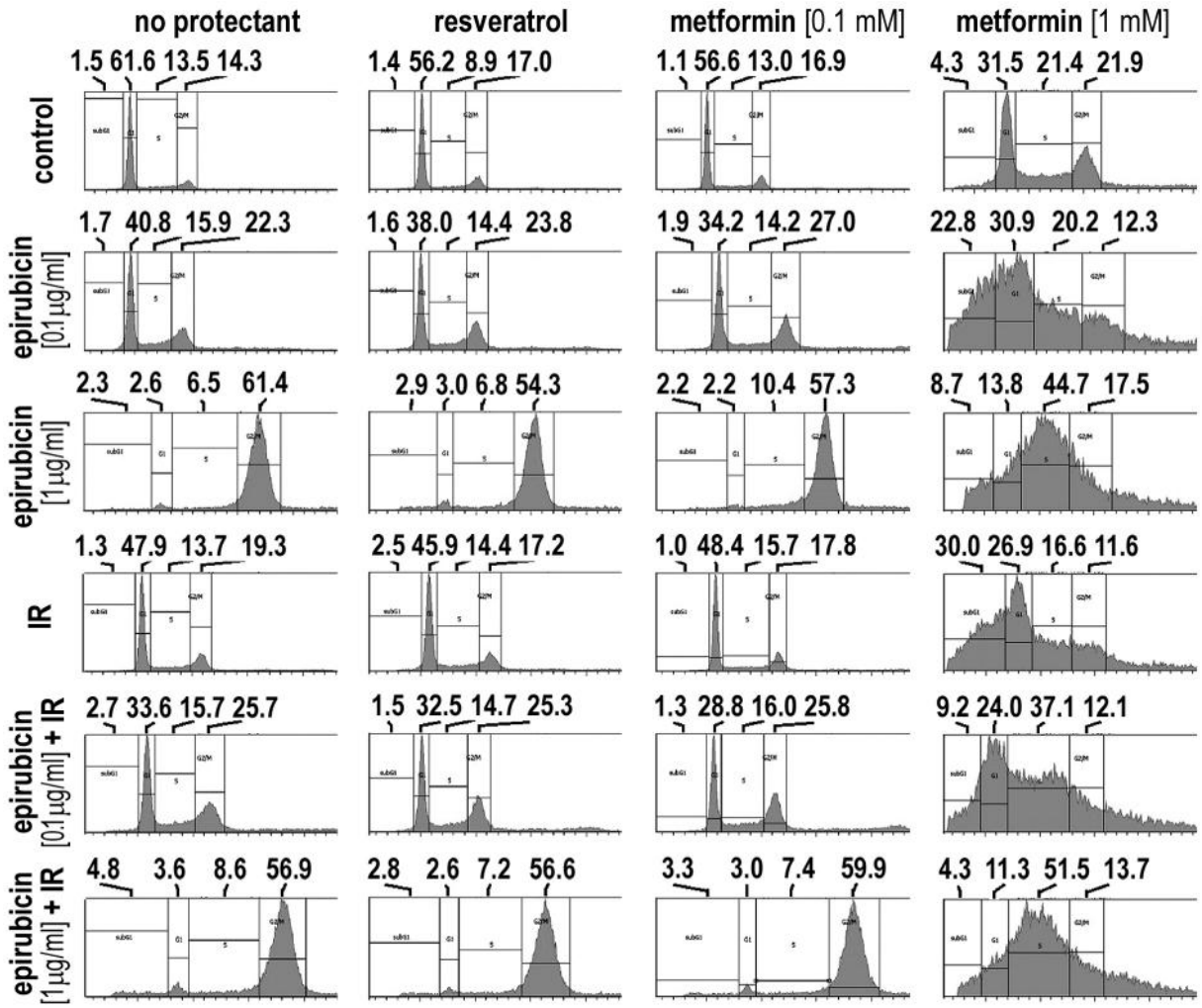

Figure 4

The influence of analyzed factors on distribution of the cell cycle phases

Numbers of cells in different phases were counted by flow cytometry 72 hours after the end of combined treatment and expressed as a percentage of all cells counted in a given sample. Average percentage of cells in subG1, G1, S and G2/M phases is showed above each diagram (from left to right). 2 Gy radiation dose (IR) and 2 µM resveratrol concentration was used in each case.

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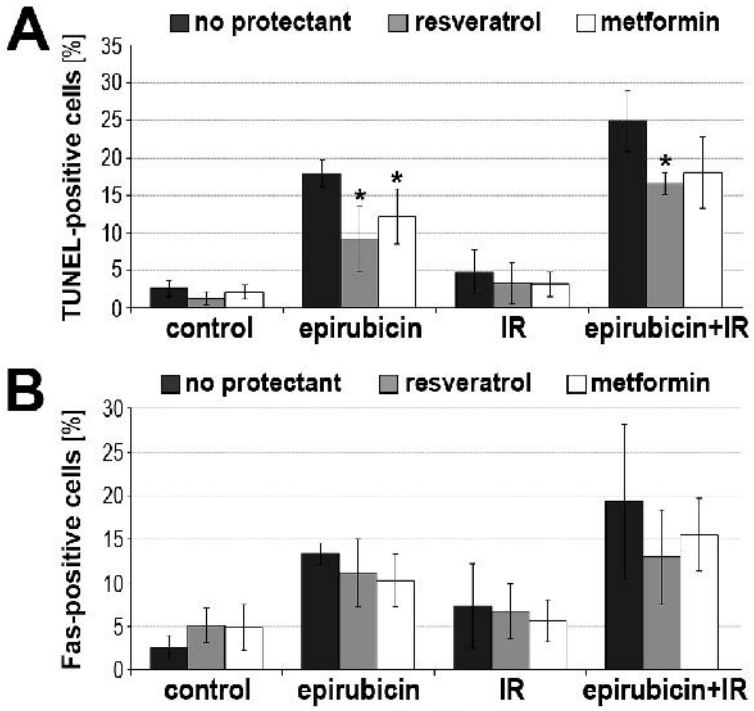

Figure 5

The influence of analyzed factors on induction of apoptotic cell death

Numbers of TUNEL-positive cells (A) and Fas-positive cells (B) was assessed by flow cytometry in population of cardiomyocytes 72 hours after the end of combined treatment and expressed as a percentage of all cells counted in a given sample. Cell were treated with epirubicin (0.1 µg/ml), radiation (IR; 2 Gy), resveratrol (2 µM) and/or metformin (0.1 mM); shown are the mean values ±S.D. for 4 replicas, statistically significant differences (in relation to cells not conditioned with protectants) are marked with asterisks.

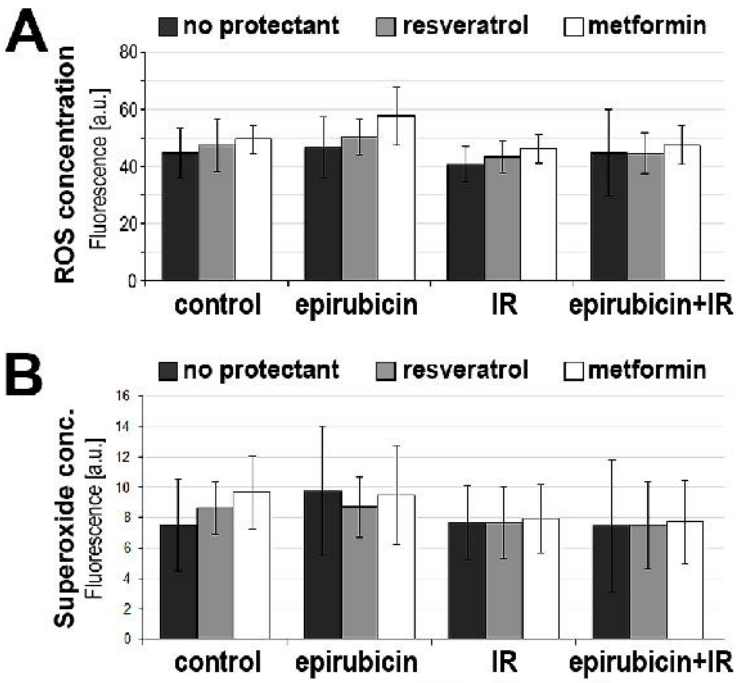

Figure 6

The influence of analyzed factors on intracellular level of reactive oxygen species

Amounts of total ROSs (A) and superoxides (B) were assessed colorimetrically directly after the end of combined treatment and expressed as intensity of fluorescence. Cells were treated with epirubicin (0.1 μg/ml), radiation (IR; 2 Gy), resveratrol (2 μM) and/or metformin (0.1 mM); shown are the mean values ±S.D. for 3 replicas.

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