

Resveratrol administration prevents radiation-related changes in metabolic profiles of hearts 20 weeks after irradiation of mice with a single 2 Gy dose

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We aimed to evaluate whether resveratrol affects radiation-induced changes in metabolite profiles of the mouse heart. Hearts were irradiated *in vivo* with a single 2 Gy dose during the resveratrol administration and metabolite profiles of heart tissue were analyzed by the untargeted HR-MAS NMR approach twenty weeks after irradiation. The administration of resveratrol mitigated the radiation-induced decline in the content of choline-containing compounds and unsaturated lipids, which might reflect the stabilization of cell membrane structure against radiation-related damage. Results obtained with this mouse model suggest that the resveratrol supplementation may prevent metabolic changes related to radiation-induced damage in the heart.

Key words: radiation; resveratrol; cardiotoxicity; cardioprotection; metabolomics

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Abbreviations: GPCho, glycerophosphocholine; HR-MAS NMR, High-Resolution Magic Angle Spinning Nuclear Magnetic Resonance; TUNEL, Terminal deoxynucleotidyl transferase dUTP Nick End Labeling

INTRODUCTION

Cardiotoxic effects of ionizing radiation represent a growing health problem because of the increased frequency of its medical usage and the increased life expectancy of exposed individuals, which allows for revealing of the long-term effects of radiation in a large population. Hence, finding an effective prevention against cardiotoxicity of ionizing radiation is a crucial issue, especially in the context of cancer therapy. The use of natural or synthetic cardioprotective compounds to attenuate or entirely neutralize the cardiotoxicity of anti-cancer treatment is an attractive concept (Fisher *et al.*, 2018). There are several substances, both natural and synthetic, which have been reported to show cardioprotective activity, however, most of them were not tested extensively in combination with radiation yet. Several compounds naturally occurring in plants, for example, flavonoids or compounds with phenolic structure, show high antioxidant potential and could hypothetically protect the heart against reactive oxygen species. Resveratrol is one of such compounds positively tested for its suitability in cardioprotection (deFreitas *et al.*, 2013; Zhang *et al.*, 2013; Arafa *et al.*, 2014; Ahmet *et al.*, 2017). However,

there are no *in vivo* studies available that have focused on resveratrol in the context of cardioprotection against ionizing radiation.

Several studies have shown that changes induced by ionizing radiation can be revealed at the metabolome level of irradiated tissue and that such molecular changes can follow macroscopic changes affecting the structure and function of exposed tissue (Schnackenberg *et al.*, 2016). Hence, aiming to evaluate the potential radioprotective effects of resveratrol *in vivo*, we analyzed the influence of resveratrol administration on the long-term effects of ionizing radiation on the metabolite profile of whole mouse heart tissue using the HR-MAS NMR spectroscopy.

MATERIALS AND METHODS

Animals. The C57Bl/6NCrl female mice housed in pathogen-free conditions were used in the study. The study protocol had been approved by the Local Ethics Committee (Silesian Medical University; approval no. 37/2018). The animals were randomly divided into six groups (5 animals in each group): (i) unexposed controls; (ii) mice consuming 5 mg/kg/d of resveratrol; (iii) mice consuming 25 mg/kg/d of resveratrol; (iv) mice irradiated with a single 2 Gy dose; (v) mice consuming 5 mg/kg/d of resveratrol and irradiated; (vi) mice consuming 25 mg/kg/d of resveratrol and irradiated. Resveratrol was administered in drinking water for six weeks in the period between 4 and 10 weeks of age (the amount was calculated based on the average water consumption and mice weight). Animals were irradiated at the age of 8 weeks. All animals were sacrificed at the age of 28 weeks (i.e., 20 weeks after irradiation). Irradiation was performed after heart positioning using Varian CLINAC 2300 linear accelerator, with 6 MV photons, the dose rate was set to 300 MU/min, as previously reported (Gramatyka *et al.*, 2020); mice were anesthetized with Avertin (0.5 mg/kg) before irradiation. Hearts were removed immediately after the animals' sacrifice and dissected in halves (vertically from the atrium to the apex of the heart). Halves of hearts were either snap-frozen in liquid nitrogen and stored at 80°C until further NMR analysis or fixed in formalin and embedded in paraffin for histological analysis (The Masson trichrome staining and the TUNEL test).

NMR measurement protocols. The samples of the heart tissue (15 µg) were packed into disposable 30 µl inserts with 5 µl of 75 mM sodium formate in D₂O and placed in HR-MAS rotors. ¹H NMR spectra were acquired on a 400 MHz Bruker Avance III spectrometer

(Bruker Biospin, Rheinstetten, Germany) at 4°C and a spinning rate of 4000 Hz. Registered HR-MAS NMR spectra included 2D J-resolved (pulse sequence: jresgp-prqf) and 1D Diffusion-edited (pulse sequence: ledb-pgp2s1dpr). The spectra were post-processed with a line broadening of 0.3 Hz, automatically phase-corrected (in Topspin software from Bruker Biospin), referenced to the formate peak at 8.46 ppm, and normalized to the total area of metabolites' spectral integrals to compensate for possible differences in sample concentration (in MNova software from Mestrelab Research).

Metabolite identification and quantification. The metabolites were identified by comparing their chemical shifts with standards from the Chenomx NMR Suite Professional database (Chenomx Inc., Edmonton, Canada). 1D projections of 2D J-resolved spectra onto the chemical shift axis (p-Jres) were chosen for the determination of signal integrals corresponding to low molecular weight metabolites. Lipid signals were evaluated in diffusion-weighted spectra. The peaks in the 1D spectra were integrated by area in MNova software (Mestrelab Research). The peak integrals were measured using individually adjusted spectral regions (i.e., for each metabolite separately). Signals from solvent (water) and impurities (ethanol), as well as those with very low signal to noise ratio, were excluded from the analyses. Metabolites were quantified based on normalization to the total area of metabolites' spectral integrals (no internal standard was used).

Statistical analyses. For each metabolite, the normality of distribution was assessed using the Shapiro-Wilk test to provide optimal tools for statistical analysis. Analysis of variances was performed using the ANOVA test or the Kruskal-Wallis test, when appropriate. Subsequent pairwise analyses were performed using the Tukey or Dunn post-hoc tests, respectively. Moreover, selected groups were compared pairwise using the t-test or the Mann-Whitney U test, depending on the normality of distribution. The data analysis was carried out using Statistica software (Statsoft v.12); *p*-values below 0.05 were considered as statistically significant.

RESULTS

Murine hearts were irradiated during supplementation with drinking water with resveratrol for four weeks be-

fore and two weeks after a single 2 Gy dose, then untargeted metabolome profiling was performed 20 weeks later using the HR-MAS NMR spectroscopy. In general, there were 17 metabolites identified based on the 1D projections of JRES spectra in the mouse heart tissue. Additionally, 7 lipid metabolites were identified based on the diffusion-weighted spectra. **Figure 1** illustrates representative NMR spectra with the components marked corresponding to the identified and quantified metabolites.

The overall analysis of variances revealed three types of compounds, which abundances were significantly different between experimental groups: glycerophosphocholine (GPCho; chemical shift 3.24 ppm), lipids with unsaturated double bonds (CH=CH; chemical shift 5.34 ppm), and glycine (chemical shift 3.57 ppm). Subsequent multiple comparison tests showed a significantly reduced level of GPCho in the group irradiated with 2 Gy when compared to control animals and animals with resveratrol supplementation (5 mg/kg). Moreover, the level of CH=CH was significantly reduced in irradiated animals when compared to animals co-treated with resveratrol. On the other hand, the supplementation with resveratrol (25 mg/kg) resulted in a significantly increased level of glycine when compared to untreated controls. Importantly, a significant difference was observed between animals supplemented with resveratrol alone and animals irradiated during such supplementation: irradiation resulted in reduced glycine level (**Fig. 2A**).

Furthermore, we looked specifically for pairwise differences between controls and irradiated not supplemented animals, between animals irradiated with and without resveratrol supplementation as well as between not irradiated mice kept on a normal diet and with resveratrol supplementation. This analysis additionally revealed differences in levels of GPCho between irradiated animals and animals irradiated during a high resveratrol supplementation (25 mg/kg). Moreover, reduced levels of total choline-containing compounds (including choline, phosphocholine, and GPCho; chemical shift 3.21–3.24 ppm) were observed in irradiated animals when compared to control animals and animals irradiated during resveratrol treatment (levels of choline compounds were comparable between the two later groups) (**Fig. 2B**). Hence, irradiation itself resulted in reduced levels of glycer-

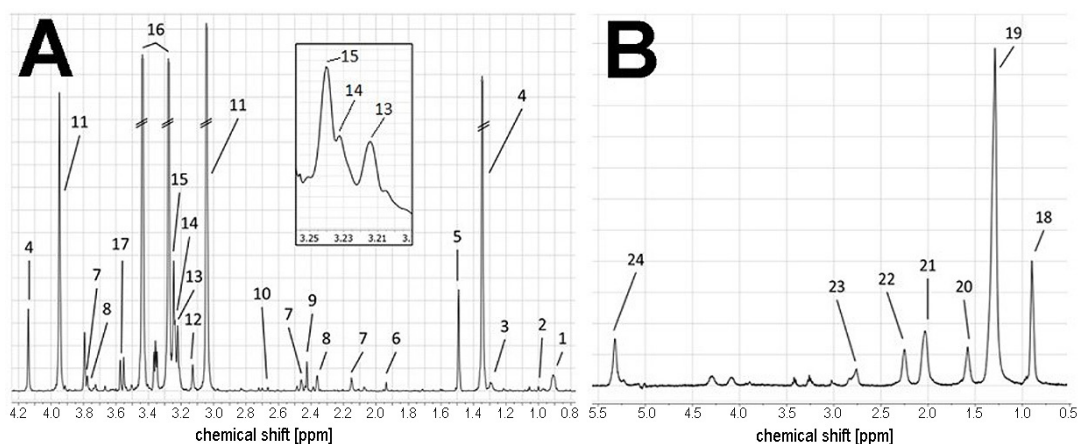


Figure 1. Representative NMR spectra.

(Panel A) 1D projection of 2D J-resolved spectrum with inserted region specific for choline-containing compounds. **(Panel B)** diffusion-weighted spectrum. Components identification: 1 – lipid's CH_2 ; 2 – BCAA (branched chain amino acids Val, Leu, Ile); lipid's $(\text{CH}_2)_n$; 4 – lactate; 5 – valine; 6 – acetate; 7 – glutamine; 8 – glutamate; 9 – succinate; 10 – hypotaurine; 11 – creatine; 12 – malonate; 13 – choline; 14 – phosphocholine; 15 – glycerophospholine; 16 – taurine; 17 – glycine; 18 – CH_3 ; 19 – $(\text{CH}_2)_n$; 20 – $\text{CH}_2\text{-CH}_2\text{-COO}$; 21 – $\text{CH}_2\text{-CH=CH}$; 22 – CH_2CO ; 23 – $\text{CH=CH-CH}_2\text{-CH=CH}$; 24 – CH=CH .

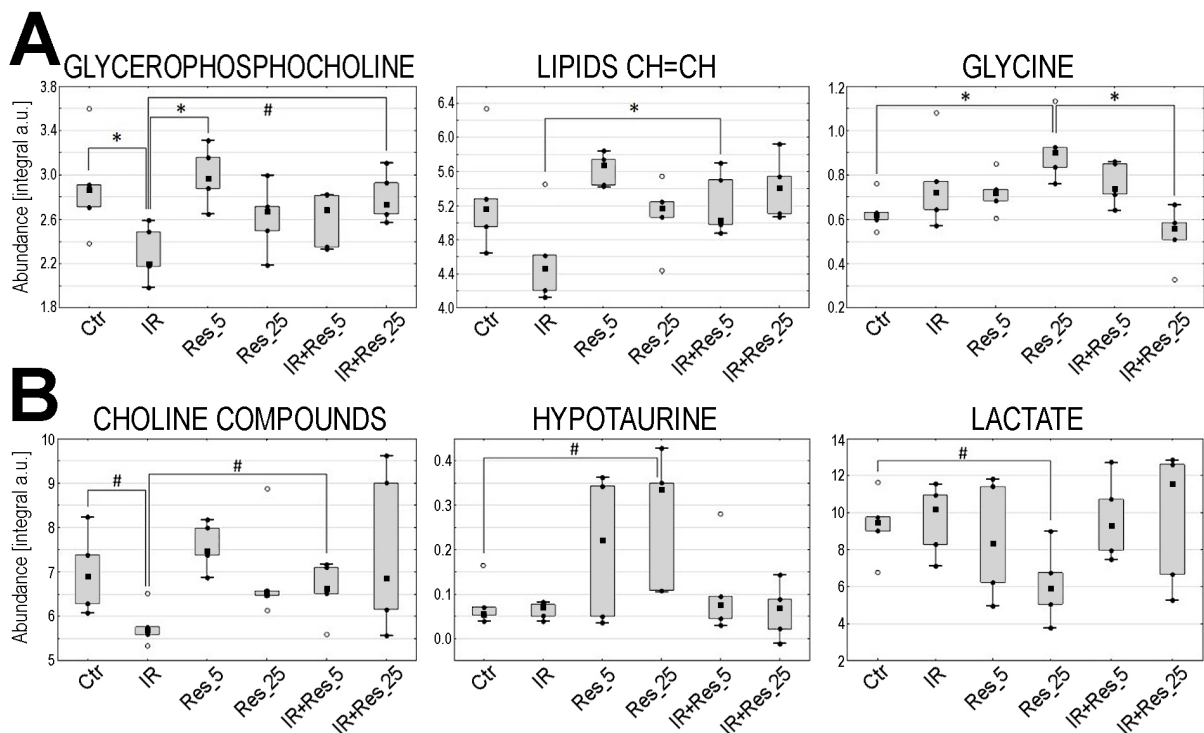


Figure 2. Levels of selected metabolites affected by radiation and resveratrol.

(Panel A) shows metabolites that generally discriminated compared groups in multiple comparison tests (asterisks mark the statistical significance; $p < 0.05$). (Panel B) shows metabolites that discriminated selected groups in pairwise tests (hash symbol mark the statistical significance; $p < 0.05$). Box plots show median, upper, and bottom quartiles, minimum, maximum; open dots represent statistical outliers. Group description: Ctr – unexposed control; IR – 2Gy irradiation alone; Res_5 – 5 mg/kg resveratrol supplementation; Res_25 – 25 mg/kg resveratrol supplementation; IR+Res_5 – 2Gy irradiation with 5 mg/kg resveratrol supplementation; IR+Res_25 – 2Gy irradiation with 25 mg/kg resveratrol supplementation.

ophosphocholines (and choline-containing compounds in general) as well as lipids with unsaturated CH=CH bonds. However, the administration of resveratrol influenced these radiation-related changes: in the groups irradiated in the presence of resveratrol the levels of lipid CH=CH signals, glycerophosphocholines, and choline-containing compounds were generally higher than in animals irradiated without resveratrol supplementation (they remained comparable to the ones in untreated not supplemented controls).

Moreover, some differences were noted in metabolic profiles of not irradiated animals that could be related to resveratrol action itself. In groups of mice supplemented with resveratrol, especially at the higher dose (25 mg/kg), the increased level of glycine and hypotaurine (chemical shift 2.66), as well as decreased level of lactate (chemical shift 1.34 ppm) was observed when compared to untreated control. However, these resveratrol-related changes were not observed in supplemented and irradiated animals, which could suggest more complex interactions between both factors (Fig. 2B).

Furthermore, no significant changes related to the exposure to radiation and resveratrol were observed at the level of histo-cytological features in corresponding tissues. The Masson trichrome staining revealed the presence of collagen fibers that were located mostly in the atria of the heart, yet the sizes of areas that were reached by the collagen fibers were comparable among all groups of animals, independent of radiation or resveratrol exposure. Moreover, only single apoptotic cells were detected in either tissue specimen (one TUNEL-positive nucleus per 10^3 nuclei) and no differences were detected between groups.

DISCUSSION

Several studies indicated the potential ability of resveratrol to reduce cardiotoxicity of anticancer therapy (Mokni *et al.*, 2007; Piasek *et al.*, 2009; Mitani *et al.*, 2014; Ko *et al.*, 2017), especially in the context of doxorubicin treatment (Arafa *et al.*, 2014; Gu *et al.*, 2018; Matsumura *et al.*, 2018). It has been also shown that resveratrol could reduce oxidative stress and damage to bone marrow cells in rats and mice exposed to total body irradiation (Carsten *et al.*, 2008; Zhang *et al.*, 2013). Moreover, it has been reported that the administration of black grape juice, a food product enriched in resveratrol, could reduce lipid peroxidation in the hearts of rats 24 hours after the total body irradiation with 6 Gy, which suggested that the administration of resveratrol could affect radiation-induced changes in the metabolism of heart tissue (deFreitas *et al.*, 2013). In the current report metabolite profiles were analyzed in mouse hearts irradiated *in vivo* with a single 2 Gy dose, i.e., typical fraction dose used during radiotherapy. Resveratrol was administered in drinking water four weeks before and two weeks after the irradiation to mimic its “dietary supplement” mode of action (doses of this compound were selected based on reports describing its protective effects; Zhang *et al.*, 2013; Ahmet *et al.*, 2017). Importantly, metabolomics analysis was performed 20 weeks after irradiation to address hypothetical features relevant to the long-term effects of radiation.

The study revealed molecular changes at the level of heart’s metabolome that could be associated with the hypothetical radioprotective activity of resveratrol. A decreased level of choline-containing metabolites and lipids with unsaturated fatty chains was observed in animals ir-

radiated with the dose of 2 Gy, which could be associated with a general influence of radiation on metabolism and stability of cell membranes. Glycerophosphocholine and phosphocholine are the major forms of choline storage and form in the breakdown of phosphatidylcholines, which are the main component of cell membranes. Changes in the level of choline-containing compounds indicate disturbances related to the synthesis and integrity of cell membranes and are frequently described in the literature as the effect of ionizing radiation (Jelonek *et al.*, 2017). Our previous study that implemented different analytical setting (profiling of tissue extracts by ¹H NMR spectroscopy) to analyze metabolite profiles in murine hearts 20 weeks after irradiation, revealed decreased levels of glutamine and acetylcarnitine, which are involved not only in energy metabolism but also with the stability of membrane structures (Gramatyka *et al.*, 2020). Hence, metabolic changes observed in the current study might reflect the influence of radiation-initiated mechanisms on the stability of cell membranes and disturbances in signaling pathways that involve choline-containing compounds. Notably, the administration of resveratrol seems to counteract the changes observed due to heart irradiation because the reduced level of choline-containing compounds and unsaturated lipids was not observed in co-treated animals.

Interestingly, in animals from the group supplemented with a higher dose of resveratrol, we have observed changes in the levels of metabolites associated with maintaining the balance of energy production. This indicated that resveratrol, although considered harmless, at higher concentrations may interfere with metabolic processes. Moreover, the interference between resveratrol and radiation was apparent since levels of resveratrol-affected compounds (i.e., glycine, hypotaurine, and lactate) were comparable in control animals and animals irradiated during the resveratrol supplementation. Hence, this aspect of metabolic changes related to the crosstalk between ionizing radiation and resveratrol also deserves further study. Nevertheless, the results obtained with a mice model that addressed long-term effects of exposure and included clinically relevant doses of radiation and resveratrol revealed the interference between both factors at the level of the heart metabolome. Although cardioprotective effects *per se* were not addressed with this simple model, obtained metabolomics data putatively reflect the cardioprotective role of the resveratrol supplementation against ionizing radiation.

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Conflicts of Interest

The authors declare no conflict of interest.

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