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Učinak nanokristala resveratrola na angiogenezu i rast Ehrlichova ascitesnoga i solidnoga tumora u miša

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Effects of resveratrol nanocrystals on angiogenesis and proliferation of Ehrlich ascites and solid tumour in mice

DOCTORAL DISSERTATION

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SAŽETAK

Resveratrol (3,5,4′-trihidroksi-stilben) jest potencijalan protutumorski spoj zbog svoje antioksidativne, protuupalne, proapoptotičke, antiangiogene i antiproliferativne aktivnosti. Međutim, terapijska primjena resveratrola ograničena je njegovim fizikalno-kemijskim (slaba topljivost, foto-nestabilnost) i farmakokinetičkim (brzi metabolizam i eliminacija, niska bioraspoloživost) svojstvima. Temeljem navedenog, cilj rada bio je primijeniti resveratrol u obliku nanokristala te istražiti njegov učinak na rast angiogenetski ovisnog, brzo rastućeg Ehrlichovog ascitesnog i solidnog tumora (EAT) te interakciju nanokristala s biološkim tkivom kroz biokemijske i histološke promjene stanica bubrega, jetre i EAT. Rezultati pokazuju da primjena otopine resveratrola (RSV) i suspenzije nanokristala resveratrola (NANO-RSV) dovodi do značajnog smanjenja proliferacije tumorskih stanica u trbušnoj šupljini, te smanjenja broja krvnih žila u potrbušnici, uz nisku sistemsku toksičnost. Prooksidativni učinak resveratrola primijenjenog *ip* vodi kroničnoj upali, polarizaciji makrofaga od M1 prema M2 fenotipu te skraćenom životnom vijeku nositelja tumora. U histopatološkim ispitivanjima, vidljiva je veća hepatocelularna nekroza i apoptoza, jetrena fibroza oko središnje vene i degeneracija s manjim masnim promjenama u miševa nakon obrade s RSV nego u miševa nakon obrade s NANO-RSV. Primjena RSV i NANO-RSV vodi upali s proksimalnom tubularnom nekrozom i oticanjem bubrežnog glomerula, zajedno s blagim povećanjem biokemijskih parametara kao pokazatelja narušenog funkcionalnog kapaciteta bubrega. Intratumoralna primjena resveratrola i njegovih nanokristala u solidni EAT ne utječe na biodostupnost u tkivu tumora; duža nazočnost nanokristala unutar tumorskog tkiva posljedica je učinkovitijeg antiangiogenog i imunomodulatornog doza-ovisnog učinka nanokristala u odnosu na resveratrol te značajno većeg preživljenja nositelja tumora. Sažimajući navedeno, te uzimajući u obzir dobrobit ljudi i ekološku sigurnost, mehanizam toksičnosti resveratrola i njegovih nanokristala potrebno je detaljnije proučiti u cilju boljeg iskorištavanja pozitivnih učinaka nanokristala resveratrola te smanjenja potencijalnih nuspojava njihove terapijske primjene.

Ključne riječi: resveratrol, nanokristali, Ehrlichov ascitesni tumor u miša, angiogeneza, protutumorski učinak, toksičnost nanokristala.

SUMMARY

Introduction

According to the WHO, cancer-mediated deaths are on the second place globally. Cancer-related deaths are predominantly attributed to either metastasis or cancer-mediated co-morbidity. In 2020 cancer was responsible for an estimated 10 million deaths. Traditional cancer therapy involves chemotherapy, radiotherapy, immunotherapy and surgical procedures which can be applied as stand-alone treatment, successively or combined depending on a cancer type and a patient. Moreover, majority of available cancer therapies are expensive, often non-selective and with numerous side effects. There is an urgent need for alternative therapies with optimal effects, lower toxicity and resulting in a better quality of life. Due to low toxicity and multiple mechanisms, natural compounds could play a significant role in the treatment of different diseases, including cancers. Natural polyphenol and phytoalexin - resveratrol (3,5,4′ - trihydroxystilbene) could be a good candidate due to its proven anti-oxidative, cardioprotective, anti-inflammatory and anti-tumour properties. It has been shown that resveratrol affects all three levels of carcinogenesis, i.e., inhibits the processes of initiation, promotion and progression. Resveratrol inhibits glucose uptake in different tumour cell lines, such as human leukemic cell lines (U-937 and HL-60) and human ovarian cancer cells due to its structural similarities with tyrosine kinases, as known inhibitors of GLUT1. Moreover, resveratrol induces apoptosis or autophagy and prevents the proliferation of ovarian cancer cells by interfering with the AMPK/mTOR signalling pathway at different levels. One of target molecules for resveratrol is also serine/threonine-protein kinase (TBK1), which is involved in many chronic inflammatory processes that can lead to the development of tumours. Studies have shown that resveratrol is capable of modulating HIF-1 related signalling pathways in cancer cells under hypoxia, resulting in induction of apoptosis and suppression of metastatic potential. The antitumor activity of resveratrol is based on its ability to regulate the level of reactive oxygen species, modulate signalling pathways, activate apoptosis and inhibit the cell cycle. Despite the numerous positive effects of resveratrol, many studies have indicated that RSV has a biphasic, concentration-dependent effect. Hence, low concentrations of RSV show antioxidant effects that protect against DNA damage and oxidative stress, while high concentrations of RSV can cause pro-oxidant effects promoting DNA damage with increased oxidative stress both *in vitro* and *in vivo*. More specifically, RSV acts hermetically in different types of human cell lines and can be considered a double-edged sword, i.e., with opposite effects at low and high doses in both healthy and cancerous cells. Nevertheless, his therapeutic use is limited by its physico-

chemical (poor solubility, photo instability) and pharmacokinetic (rapid metabolism and elimination, low bioavailability) properties. To overcome these traits and increase solubility, stability and bioavailability, different approaches have been investigated such as chemical modifications, synthesis of prodrugs or incorporation in lipid and polymeric carriers, including nanoparticles, micelles and vesicles. Among these strategies, nanocrystals may be a promising approach, as several formulations of flavonoid nanocrystals have shown enhanced pharmacological effects *in vitro* and *in vivo.*

Swiss albino mice with an Ehrlich ascites tumour (EAT) were selected as a suitable tumour model to investigate the antitumor and antiangiogenic properties of resveratrol and its nanocrystals.

The aim of this study was to investigate the potential use of resveratrol (RSV) and its nanocrystals (NANO-RSV) as antitumor agents in Ehrlich ascites tumour (EAT)-bearing mice in ascites and solid form and the interaction of nanocrystals with biological tissue through biochemical and histological changes of kidney, liver and EAT cells.

Materials and Methods

The RSV nanocrystal suspension was prepared by the wet media milling technique. Biocompatible polymer Pluronic® F127 was used as a stabilizer of the RSV nanocrystal suspension. Of the eight experimental groups, four groups received a suspension of resveratrol nanocrystals intraperitoneally (*ip*) or intratumorally (*it*) (at a dose of either 50 mg/kg bw or 25 mg/kg bw; NANO-RSV50 and NANO-RSV25) and four groups received resveratrol dissolved with DMSO and then diluted with distilled water to adjust to 1% DMSO for the final concentration for injection (at a dose of either 50 mg/kg bw or 25 mg/kg bw; RSV50 and RSV25). DMSO was selected as the most commonly used dissolving agent for resveratrol.

In the study, we used Swiss albino inbred mice obtained from the Department of Animal Physiology, Faculty of Science, University of Zagreb. A total of 200 mice were included in the study and were inoculated intraperitoneally (*ip*) with 2.5×10^6 or subcutaneally (*sc*) with 1×10^6 viable EAT cells (Day 0. of the study). Over the course of 14 days, animals were treated every other day with water for injection (control EAT groups; *ip* with 0.5 mL or intratumoral (*it*) with 0.2 mL depending on the type of the tumor) or with resveratrol nanocrystals or resveratrol solution at dose of either 25 or 50 mg/kg bw (experimental groups). On the 15th day, 10 animals in each group were sacrificed. From the EAT ascites tumor bearing mice, ascites fluid, serum, liver, kidney and spleen tissue samples were collected for further analyses.

From the EAT solid tumor bearing mice, serum, liver, kidney, spleen as well as tumor samples were collected. The remaining animals in each group were kept alive to monitor animal survival after treatment.

Ascites fluid was used for the evaluation of tumour growth, total cell number in the peritoneal cavity, differential count of cells present in the peritoneal cavity. Serum and ascites fluid were used for the biochemical analysis: VEGF, matrix metalloproteinase (MMP-2 and MMP-9), Th1, Th2, Th17 cytokines. We measured arginase and nitric oxide synthase activity, as well as parameter of oxidative stress. The peritoneum was opened and the inner lining of the peritoneal cavity examined for angiogenesis and for histological analysis of the peritoneum. Ascites fluid, liver and kidney were used for the histological examinations.

Mice with solid tumor were used for the analyses of the tumor growth. Tumor angiogenesis was determined via count of microvesssel density. Serum was collected for biochemical analyses. Liver, kidney and spleen were used for measurments of parameter of oxidative stress.

Results

A wet media milling technique in a miniature scale was used for the production of resveratrol nanocrystals suspension (NANO-RSV). We were able to produce stabile suspension of resveratrol nanocrystals suitable for further analyses. Mean particle size of the RSV nanocrystals was 270 ± 7.2 nm, giving a polydispersity index (PDI) of 0.310±0.005.

We investigated the *in vivo* antitumor effects of resveratrol and its nanocrystals on EAT-bearing mice in ascites form. The treatment of EAT-bearing mice with NANO-RSV showed a significantly reduced total number of cells in the abdominal cavity compared to the EAT control group. If calculated as the % inhibition of tumour growth in relation to the control, the growth inhibition for NANO-RSV (50 mg/kg and 25 mg/kg) was 73.43% and 60.52%, while for RSV (50 mg/kg and 25 mg/kg) it was 29.34% and 47.30%, respectively. The ascites fluid volume was reduced by 51.64% and 56.85% for animals treated with NANO-RSV50 and NANO-RSV25, respectively, and 0.41% and 37.94% for animals treated with RSV50 and RSV25, respectively in relation to the control. Despite a significant reduction in the number of cells in the abdominal cavity, overall survival of animals was not statistically improved compared to the control, except NANO-RSV25. Diferential cell analyses of the ascites fluid showed increased levels of the active macrophages for NANO-RSV groups in both doses. The percentage of active macrophages in the peritoneal fluid after treatment with NANO-RSV50 and NANO-RSV25 was about 8.0 times higher than then control, while treatment with RSV50

and RSV25 showed percentages of macrophages 5.10 and 2.4 times higher compared to the control. Total amount of VEGF, MMP-2 and MMP-9 secreted into the ascites fluid and serum of mice was quantified using an ELISA assay. NANO-RSV suppressed the secretion of VEGF (dose: 50 $P < 0.05$; 25 $P < 0.01$) and MMP-2 in ascites fluid but had no effect on level of MMP-9 compared to the control. RSV50 suppressed the secretion of VEGF by 64.46% ($P < 0.01$) compared to the control but had no effect on MMP-2 and MMP-9. There was a statistically significant difference ($P < 0.001$) between NANO-RSV and RSV at both doses for MMP-2. Moreover, totoal amount of VEGF was also reduced in the serum of NANO-RSV50 group. We examined the microvessel density (MVD) of the peritoneum. When compared to 53.79 ± 6.79 microvessels present in the peritoneum of the control group EAT bearing mice on the 14th day, the peritoneum of NANO-RSV and RSV treated mice showed only 31–38 microvessels; the tested component reduced the number of microvessels by 59–72% when compared to 100% microvessels in control mice. MVD analyses of kidney and liver NANO-RSV treated groups showed decreased number of vessels for both organs compared to the control group, although not statisticly significant, while MVD was significantelly lover for RSV treated groups. Analysis of enzymes and proteins in serum showed increased activity of ALP, as a parameters of liver function, in NANO-RSV groups (dose: 50 $P < 0.001$; 25 $P < 0.05$) while ALT was increased in the RSV groups (dose: 50 $P < 0.01$; 25 $P < 0.05$) compared to the control. Significantly lower amylase levels were observed in the RSV50 group ($P < 0.01$) compared to the NANO-RSV50 group. Total protein and albumin, known markers of liver function, did not differ from the control group. Analyses of metabolites and substrates in serum of NANO-RSV groups showed a decreased level of phosphorus (dose: 50 $P < 0.05$; 25 $P < 0.01$) and increased the level of creatinine as an indicator of kidney function though not significantly, while RSV at both doses showed increased K+ levels compared to the NANO-RSV50 groups (dose: 50 $P < 0.001$; 25 $P < 0.01$). Levels of nitric oxide (NO) in the spleen were eleveted for NANO-RSV treated groups, as well as levels of arginase (Arg1). Analysis of NO in ascites fluid for NANO-RSVs groups showed a dose-dependent decrease (dose: 50 $P < 0.01$; 25 $P < 0.001$), while RSV in both doses showed a decrease in NO though without statistical significance. Arg1 levels in the ascites fluid were significantly reduced after treatment with NANO-RSV50 ($P < 0.05$), while RSV25 showed an increase in arginase levels in ascites fluid compared to the control. Cytokine analysis in ascites fluid showed an increase in Th1 (IL-2, IL-10, IL-12, IFN-γ and TNFβ), Th2 (IL-2, IL4, IL-5, IL-10, and IL13), and Th17 (IL-6, IL17A, IL23, TNFα and TGFβ) cytokines in NANO-RSV at both doses and after treatment with RSV50. The activity of TGF-β was significantly increased in the NANO-RSV50 group compared to the control ($P < 0.01$)

and RSV25 ($P < 0.01$), as were TNFα (NANO-RSV50 *vs* Contr. $P < 0.05$ and NANO-RSV50 *vs* RSV25 $P < 0.05$) and IL-12 (NANORSV50 *vs* RSV25 $P < 0.05$). The activity of IFN-γ was significantly increased with RSV50 compared to the control ($P < 0.05$) and RSV25 ($P < 0.05$), as were IL17A (RSV50 *vs* RSV25 $P < 0.05$), IL- 13 (RSV50 *vs* Contr. $P < 0.05$ and RSV50 *vs* RSV25 $P < 0.01$), IL-12, IL-10 and IL-5 (RSV50 *vs* Contr. $P < 0.05$ and RSV50 *vs* RSV25 $P < 0.05$), IL-4 (RSV50 *vs* RSV25 $P < 0.05$) and IL-2 (RSV50 *vs* RSV25 $P < 0.01$). Measurements of the oxidative stress parameters in the ascites fluid showed direct antioxidative effect of NANO-RSV, especially for NANO-RSV25 since activity of SOD, as well as concetration of MDA and GSH were elevated. Histopatological examinations showed signifficantelly decreased number of EAT cells in the proliferation phase as well as elevated number of cells in the necrosis and apoptosis after treatment with NANO-RSV and RSV. Liver tissue showed hepatocellular necrosis and apoptosis, hepatic fibrosis around the central vein and degeneration with minor fatty change after treatment with RSV, while less liver damage was seen in the NANO-RSV group. Damaged and dilated blood vessels were also visible in the liver in all NANO-RSV and RSV groups. Normal histological architecture of the kidney was observed in kidney sections of control mice, while experimental groups treated with RSV or NANO-RSV showed renal proximal tubule necrosis involving most of the nephrons. Furthermore, cellular fragments were found in the tubule lumen and renal glomerulus swelling as well as active inflammation, and endothelial dysfunction and destruction, especially in RSV treated groups.

We investigated the *in vivo* antitumor effects of resveratrol and its nanocrystals on EAT-bearing mice in solid form. From the obtained data it was clear that tumor volume in NANO-RSV and RSV groups had significantelly smaller volume whwn compared to the control group. Moreover, we calculated te percentage of the growth inhibition (%TVI) and data showed that RSV25 group had 93,78 % TVI and NANO-RSV25 group 78,81 %, while NANO-RSV 50 and RSV50 had 73,48 % and 69,31 % TVI in comparison to the control group, respectively. Data obtained from the survival study showed that all treated animals, both NANO-RSV and RSV groups, had improved overall survival compared to the control group, especially RSV50 group (20,27 %). We examined the microvessel density (MVD) of the solid EAT tumor. From the obtained data, it was clear that NANO-RSV50 ($P < 0,05$) had a statistically lower MVD when compared to microvessels in control group. In addition, all other treated groups had lower MVD than the control group, but not with statistical significance. Analysis of enzymes and proteins in serum showed decreased levels of ALT in RSV50 group and decreased levels of GLOB in RSV25 when compared to the control group. Total proteins and ALP do not differ among

treated groups of mice and control group. Analyses of metabolites and substrates in serum showed decreased levels of BUN in RSV25 group ($P < 0,01$) when compared to the control group. Increased levels of PHOS were observed in all NANO-RSV and RSV groups, esspecially NANO-RSV50 ($P < 0,05$), when compared to the control. Levels of K^+ were elevated in NANO-RSV50 and RSV25 ($P < 0,05$) when compared to the control. In addition, levels of TBIL, Na^+ and Ca do not differ among NANO-RSV and RSV groups and control group. Measurements of the oxidative stress parameters in kidney showed elevated levels of MDA together with decerased levels of GSH, SOD and CAT. Antioxidative capacity of the liver was increased in treated groups, while spleen of the treated mice did not show significat changes.

Discussion

The results show that the administration of resveratrol and its nanocrystals lead to significant reductions in the proliferation of tumour cells in the abdominal cavity, and a reduction of the number of blood vessels in the peritoneum, with low systemic toxicity. Destruction of tumour cells and their disappearance was apparent, and we can confirm that nanocrystals caused a dose-dependent effect, wherebthe higher dose showed a stronger antitumour effect. Moreover, our research shows that the new nanoformulation of resveratrol, i.e., resveratrol nanocrystals in suspension form, displayed a better antitumour effect than resveratrol itself. Our data showed rapid tumour growth and an increase in ascites fluid volume in the control EAT group, while administration of RSV or its nanocrystals significantly reduced ascites fluid volume and the total number of cells. Furthermore, a positive effect of RSV and NANO-RSV was also seen in the significant reduction of blood vessel density and VEGF levels as a potent mediator of peritoneal fluid accumulation and tumour progression. Due to pro-oxidative effects of resveratrol after *ip* administration, chronic inflammation, macrophage polarisation from M1 to M2 phenotype as well as shorter lifespan of resveratrol-treated mice was observed. In histopathological examinations, greater hepatocellular necrosis and apoptosis, hepatic fibrosis around the central vein and degeneration with minor fatty change were observed with RSV than with NANO-RSV. Inflammation with proximal tubular necrosis and renal glomerulus swelling were also observed, together with slight elevation of several biochemical parameters in both the RSV and NANO-RSV groups.

Intra-tumoral administration does not effects bioavailability of the resveratrol, but longer presence of nanocrystals in the tumor tissue improves anti-angiogenic and immunomodulatory dose- depending effects of resveratrol, including overall survival of the mice.

With respect for human health and environmental safety, the mechanisms of toxicity require further clarification. In order to increase the beneficial effects and reduce the possible risks of therapeutic application of RSV nanocrystals, parameters such as dose, genetic factors, health status and nature of target cells (normal *vs* tumour) should be considered. In order to improve the quality of life and survival of the host, further research efforts are needed, primarily regarding the therapeutic dose that would provide significant clinical benefits, including improved efficacy, tumour targeting, and reduced systemic toxicity.

Key words: resveratrol, nanocrystals, Ehrlich ascites tumor in mice, angiogenesis, anti-tumor effect. toxicity of nanocrystals