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Original article

The interaction between resveratrol and two forms of copper as carbonate and nanoparticles on antioxidant mechanisms and vascular function in Wistar rats

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A B S T R A C T

Background: Experimental studies have emphasized that cardiovascular alterations can be improved by the long-term use of resveratrol (*trans*-3,5,4′-trihydroxystilbene; RSV) as well as dietary copper (Cu) intake.
Methods: Male Wistar rats were supplemented for 8 weeks with Cu (6.5 mg/kg diet) as either nanoparticles (40 nm, CuNPs) or carbonate ($CuCO_3$). Half of the studied animals were supplemented with RSV (500 mg/kg diet). Vascular function and blood plasma antioxidant status, expressed as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), lipid hydroperoxides (LOOH) and malondialdehyde (MDA) were analyzed. The activity of ceruloplasmin (Cp), lipid profile, fasting glucose, and concentrations of Cu and zinc (Zn) were analyzed.
Results: RSV supplementation resulted in the elevated activity of SOD and decreased CAT, GPx and LDL-cholesterol in both groups. RSV supplementation on CuNPs increased the participation of vasoconstrictor prostanoids and decreased ACh-induced vasodilation, while the participation of hyperpolarizing mechanism(s) was restored by activating K_{ATP} channels. Blood plasma glucose was decreased. RSV supplementation on $CuCO_3$ enhanced ACh- and SNP-induced vasodilation and decreased NA-induced vasoconstriction. The lipid profile was improved, as well as Zn concentration. Meanwhile, Cu and Cp, and the markers of lipid peroxidation, reflected as LOOH and MDA, were decreased.
Conclusion: The use of RSV during $CuCO_3$ intake improves vascular responses, the lipid profile and the antioxidant mechanism(s). The beneficial role of RSV was not observed in the CuNP group and decreased ACh-induced vasodilation and increased participation of vasoconstrictor prostanoids in the vascular regulation were noticed.

Introduction

Copper (Cu) is an essential micronutrient, with either anti- or pro-oxidant properties, that is able to control the Cu dependent enzymes: superoxide dismutase (SOD), lysyl oxidase and monoamine oxidase [1]. Cu is involved in tryptophan metabolism by regulating the activity of enzymes in the kynurenine pathway [2], which can generate toxic products when dysregulated [3]. Cu is also involved in proper vascular functioning [4,5]. Because of its role as a metal co-factor, Cu possesses the ability to generate reactive oxygen species (ROS) and form the Cu-peroxide complexes with the ability to cause DNA damage [6,7].

Recent studies have reported a beneficial role for Cu as a nanoparticle (CuNP) with decreased production of inflammatory mediators [8–10]. Treatment with low doses of CuNPs (1 mg/kg/day, 4 weeks, *po*) has shown a significant increase in the phosphorylation of both protein kinase B and glycogen synthase kinase 3β, against ischemia/reperfusion induced myocardial injury in rats. These have further diminished oxidative stress, inflammatory cytokines, apoptosis and increased the serum bioavailability of nitric oxide (NO) [9].

However, there are some reports that question the safety of nanoparticles. Currently, the toxicity to CuNPs has been reported as being due to prostaglandin E_2 release and enhanced tumor necrosis factor (TNF)-α; as well as interleukin (IL)-1 production

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in cerebral microvessels [11]. During 4 weeks of feeding with CuNPs the ceruloplasmin (Cp) activity, antioxidant enzymes and lipid peroxidation markers have been influenced in Wistar rats [12]. In another study, increased blood catalase (CAT) and plasma antioxidant capacity, measured as FRAP have been observed after 8 weeks of experimental feeding with CuNPs [4].

Resveratrol (trans-3,5,4′-trihydroxystilbene; RSV), a polyphenolic compound of plant origin, found in the skin of red grapes, berries, peanuts, dried roots and rhizomes of *Polygonum cuspidatum*, has been of much interest to researchers due to its cardiovascular, antioxidant, anti-inflammatory and anticarcinogenic properties [13–16].

New RSV formulations, with potential therapeutic properties have been recently developed to improve its biological activity, which is otherwise limited by low water solubility and chemical instability [17,18].

Under physiological conditions, the action of RSV is mediated through its intrinsic anti-oxidant properties and the ability to activate sirtuin 1, endothelial NO synthase (eNOS) and AMP-activated protein kinase [19,20], as well as *via* hydroxyl-radical ($^{\bullet}$OH) scavenging and glutathione-sparing mechanisms [21].

RSV has been recognized as a naturally occurring antioxidant; however it can also act as a pro-oxidant agent, especially in the presence of Cu ions with the ability to promote DNA fragmentation [5,13,22–24]. RSV interacts with Cu (II), leading to its reduction to Cu (I) [25] with the concomitant production of oxidative RSV radicals [26,27]. The mechanism also involves the mobilization of endogenous Cu and its consequent pro-oxidant action [24,28].

In view of the above considerations, this study was undertaken to clarify the hypotheses that RSV supplementation can act as either an anti- or pro-oxidant agent, and the effect is dependent on the type of Cu particles.

Materials and methods

Animal protocol and dietary treatment

The study was approved by the Local Ethics Committee for Animal Experiments according to European Union guidelines (Directive 2010/63/EU for animal experiments) and conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publications No. 86–26, revised 2014).

Healthy male albino Wistar rats (Han IGS rat [Crl:WI(Han)]) at 4 weeks of age were randomly divided into 4 groups of 9 animals each. Rats were supplemented for 8 weeks with Cu as $CuCO_3$ (group 1), $CuCO_3$ + RSV (group 2), CuNPs (group 3) and CuNPs + RSV (group 4). Experimental diets were composed of Cu 6.5 mg/kg (groups 1–4) and RSV 500 mg/kg (group 2 and 4).

Rats were housed individually in stainless steel cages under a stable temperature of 21–22 °C, a ventilation rate of 20 air changes per hour and a relative humidity of 50 ± 10%. During the experimental period, the rats had free access to tap water and 20 g/day of experimental diet. Diets were prepared every 7 days and then stored at 4 °C in hermetic containers until the end of the experiment. Cu as a nano-suspension was prepared in rapeseed oil, and the same amount of pure rapeseed oil was added to the other two experimental diets to have equivalent oil content, as described elsewhere [4]. The experimental diets were modifications of a casein diet for laboratory rodents recommended by the American Institute of Nutrition.

Drugs and reagents

The drugs used were: acetylcholine (ACh) chloride, noradrenaline (NA) hydrochloride, N(ω)-nitro-L-arginine methyl ester (L-NAME) hydrochloride, indomethacin, potassium chloride (KCl), sodium nitroprusside (SNP) (Sigma-Aldrich), NS1619, pinacidil (Cayman chemical). Stock solutions (10 mmol/l) of these drugs were prepared in distilled water; except for NA, which was dissolved in a NaCl (0.9%) + ascorbic acid (0.01% w/v) solution; indomethacin, NS-1619 and pinacidil were dissolved in ethanol and administered from a prepared stock in such a way that the maximal ethanol concentration of the medium was less than 0.001% (vol/vol). These solutions were maintained at −20 °C and appropriate dilutions were made in Krebs-Henseleit solution (KHS) on the day of the experiment.

Cu carbonate (purity ≥ 99%, $CuCO_3$) was sourced from Poch (Gliwice, Poland), NIST1577C from Sigma-Aldrich and trans-resveratrol from Cayman chemical. The CuNPs particles (40–60 nm size nano powder) were purchased from Sky Spring Nanomaterials, Inc. (Houston, TX, USA), with a purity of 99.9% on a trace metals basis, with a spherical morphology of 0.19 g/cm^3 bulk density, and an 8.9 g/cm^3 true density.

Blood biochemical assay

Rats were anaesthetized and killed by decapitation. Immediately after blood collection, samples were kept in tubes containing heparin + EDTA as an anticoagulant. Samples were centrifuged at 3000 g for 10 min and blood plasma was separated and stored at −20 °C until further analysis.

The concentration of Cu and zinc (Zn) was determined by the inductively coupled plasma optical emission spectrometry method (ICP-OES) with the certified reference material NIST1577C (bovine liver) for quality control. The units were expressed as μmol/l.

The content of glucose (GLU), total cholesterol (TC), high density lipoprotein (HDL)-cholesterol, low density lipoprotein (LDL)-cholesterol and triglycerides (TG) was measured using a biochemical auto analyzer (Plasma Diagnostic Instruments, Horiba, Kyoto, Japan). The units were expressed as mmol/l.

The activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx) were determined in erythrocytes using Ransod and Ransel diagnostic kits (Randox). Catalase (CAT, U/ml) was determined according to Aebi [29]. The main primary products of lipid peroxidation, lipid hydroperoxides (LOOH) and the secondary product during lipid peroxidation, malondialdehyde (MDA) were analyzed in blood plasma according to the method described previously [4]. The units were expressed as (μmol/l).

Plasma ceruloplasmin (Cp, U/l) concentration was determined according to the method of Broderius et al. [30], based on the fact that Cp catalyzes the oxidation of p-phenylenediamine, forming a colored product that can be directly determined by spectrophotometry.

Vascular reactivity studies

The thoracic aorta was isolated and placed in ice-cold KHS of the following composition (mmol/l): NaCl 115; $CaCl_2$ 2.5; KCl 4.6; KH_2PO_4 1.2; $MgSO_4$ 1.2; $NaHCO_3$ 25; and glucose 11.1; at pH 7.4. Next, the aorta was cleaned of adherent tissue, cut into 6–7 rings of 3- to 4-mm length, and was suspended horizontally under a resting tension of 1 g (determined during preliminary experiments) in 5-ml tissue baths (stagnant Graz Tissue Bath System) containing KHS. The solution had been aerated with a carbogen (95% oxygen and 5% carbon dioxide), and maintained at 37 °C. Each ring was connected to a transducer (F-30 HSE) to measure isometric force.

After the initial equilibration period of 60 min, contractile response elicited by a single depolarizing concentration to potassium chloride (KCl, 30 mmol/l) was assessed. The cumulative

Table 1
Biochemical parameters in the blood of rats fed with experimental diets.

	Cu (μmol/l)	Zn (μmol/l)	Cp (U/l)	SOD (U/ml)	CAT (U/ml)	GPx (U/ml)	MDA (μmol/l)	LOOH (μmol/l)	GLU (mmol/l)	TC (mmol/l)	LDL (mmol/l)	HDL (mmol/l)	TG (mmol/l)
$CuCO_3$	18.14 ± 0.63	80.14 ± 0.78	37.91 ± 2.24	36.90 ± 0.38	19.08 ± 0.79	11.42 ± 0.65	8.30 ± 0.23	13.61 ± 1.19	17.66 ± 0.77	2.88 ± 0.24	0.56 ± 0.08	0.45 ± 0.03	2.78 ± 0.18
$CuCO_3$ + RSV	**15.34 ± 0.75***	**85.58 ± 0.91***	**22.98 ± 0.69***	**44.17 ± 1.72***	**13.82 ± 0.90***	**8.61 ± 0.96***	**7.10 ± 0.38***	**11.20 ± 0.28***	17.89 ± 0.97	**2.19 ± 0.11***	**0.34 ± 0.05***	**0.58 ± 0.03***	**2.00 ± 0.20***
CuNPs	15.66 ± 0.42#	82.56 ± 0.56	28.01 ± 0.73#	37.28 ± 1.19	26.07 ± 1.96#	25.51 ± 1.24#	7.69 ± 0.69	13.94 ± 1.09	20.37 ± 0.67	2.43 ± 0.14#	0.49 ± 0.05	0.49 ± 0.04	2.06 ± 0.24
CuNPs + RSV	16.24 ± 0.25	84.29 ± 2.07	25.46 ± 1.07	**42.75 ± 1.04***	**13.98 ± 0.95***	**8.89 ± 0.86***	7.91 ± 0.35	13.27 ± 1.02	**16.96 ± 1.00***	2.26 ± 0.10	**0.38 ± 0.03***	0.52 ± 0.03	2.19 ± 0.19

Values are expressed as means ± SEM, *n* = 8, *vs. not supplemented, #vs. respective $CuCO_3$ control ($p \le 0.05$, two-way ANOVA, Tukey's).

concentration-response curves (CCRCs) of ACh (0.0001–10 μmol/l), sodium nitroprusside (SNP, 0.0001–10 μmol/l), NS-1619 (0.001–10 μmol/l) and pinacidil (0.1–10 μmol/l) were analyzed to determine the dilator responses on endothelium-intact rings that had been precontracted with submaximal concentrations (determined during preliminary experiments) of NA (0.1 μmol/l).

In another set of experiments, ACh-induced vasodilation was analyzed in the absence and presence of the NO synthase (NOS) inhibitor – L-NAME (100 μmol/l), and the nonspecific cyclooxygenase (COX) inhibitor – indomethacin (10 μmol/l).

Only one CCRC was performed on each aortic ring.

Data analysis and statistics

The calculations and graphs were done and analyzed in GraphPad Prism 7. Contraction was expressed in mg of developed tension for both KCl and NA. Vasodilation was represented as a percentage of the maximal response to 0.1 μmol/l NA. In addition, the maximal response (E_{max}) and the potency (pD_2, the negative logarithm of the concentration causing a half-maximum effect) were determined. Data are expressed as means ± SEM (Standard Error of the Mean) from *n* = 9 rats, and were compared by two-way analysis of variance (ANOVA) followed by a *post-hoc* Tukey's test, where appropriate. The model assumption of normality and homogeneity of variance was tested for all data. A value of $p \le 0.05$ was considered to be significant.

Results

Plasma Cu, Cp and Zn

Supplementation with RSV markedly reduced the blood plasma Cu content and Cp activity to 0.85- and 0.62-fold, respectively meanwhile increased the blood Zn level by 1.07-fold in the $CuCO_3$ group exclusively (all values of $p \le 0.05$), see Table 1.

The antioxidant mechanisms and the lipid peroxidation markers

RSV markedly increased activity of SOD to 1.20- and 1.15-fold, in the $CuCO_3$ and CuNP groups. RSV decreased activities of CAT to 0.72- and 0.54-fold; and GPx to 0.75- and 0.35-fold, respectively. In addition, in the $CuCO_3$ group, RSV markedly decreased MDA to 0.85-fold and LOOH to 0.82-fold ($p \le 0.05$). RSV did not modify MDA and LOOH values in the CuNP group ($p > 0.05$), see Table 1.

Plasma glycaemia and the lipid profile

RSV markedly reduced the fasting glucose level in the blood plasma of the CuNP fed rats to 0.83-fold ($p \le 0.05$), however not in the $CuCO_3$ fed animals ($p = 0.85$), see Table 1.

RSV markedly decreased LDL-cholesterol in rats fed with $CuCO_3$ and CuNPs to 0.60- and 0.76-fold, respectively. Meanwhile, TC, TG and HDL-cholesterol were modified only in the $CuCO_3$ group by 0.76-, 0.72- and 1.29- fold, respectively ($p \le 0.05$), see Table 1.

Vascular reactivity studies

All studied aortic rings contracted in a similar way subjected to KCl (30 mmol/l), see Fig. 1A. However, RSV induced a marked 0.74-fold decrease in the NA (0.1 μmol/l) induced contraction of aortic rings from the $CuCO_3$ group of rats ($p \le 0.05$), but not in CuNPs ($p = 0.99$), see Fig. 1B.

RSV markedly shifted to the left the ACh-induced vasodilation in the $CuCO_3$ group and reduced the maximal response in CuNPs (Fig. 2A). Preincubation with non-selective COX inhibitor – indomethacin (10 μmol/l, 30 min) resulted in an enhanced

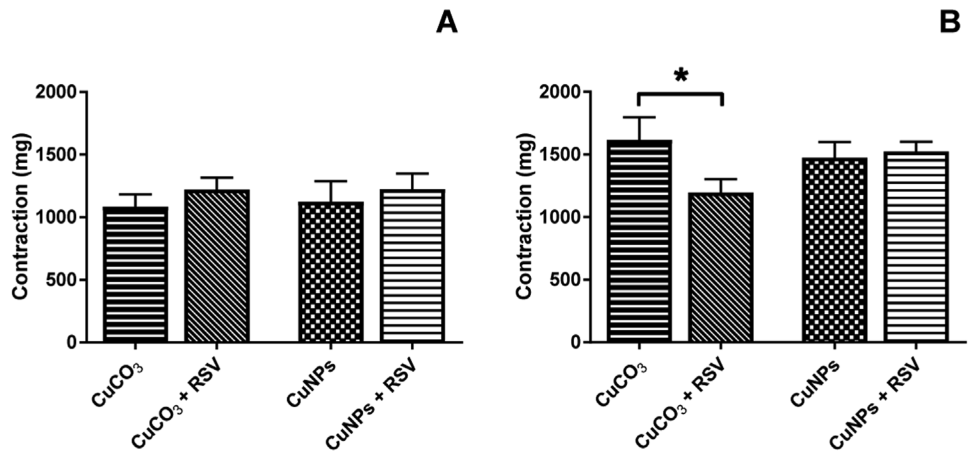

Fig. 1. Contractile responses to potassium chloride (KCl, 30 mmol/l) (A) and noradrenaline (NA, 0.1 μmol/l) (B) in the thoracic rings of rats fed with experimental diets. Values are expressed as mean ± SEM, $n = 8$, $^{*}p \leq 0.05$ *vs.* respective control (two-way ANOVA with Tukey's multiple comparisons test).

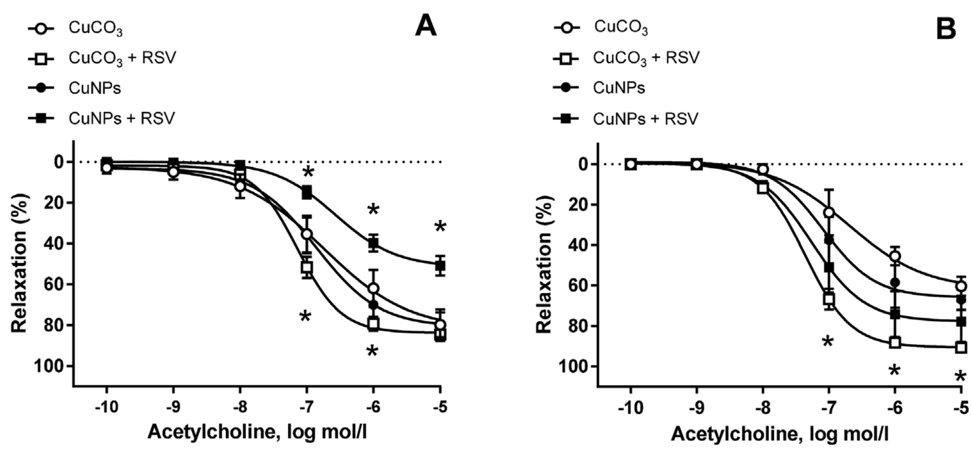

Fig. 2. The cumulative concentration-response curves to acetylcholine (ACh) in the thoracic rings of rats fed with experimental diets (A). The aortic rings were incubated with indomethacin (10 μmol/l, 30 min) (B). Results (means ± SEM, $n = 8$) are expressed as a percentage of inhibition of the contraction induced by noradrenaline (0.1 μmol/l), $^{*}p \leq 0.05$ *vs.* respective control (two-way ANOVA with Tukey's multiple comparisons test).

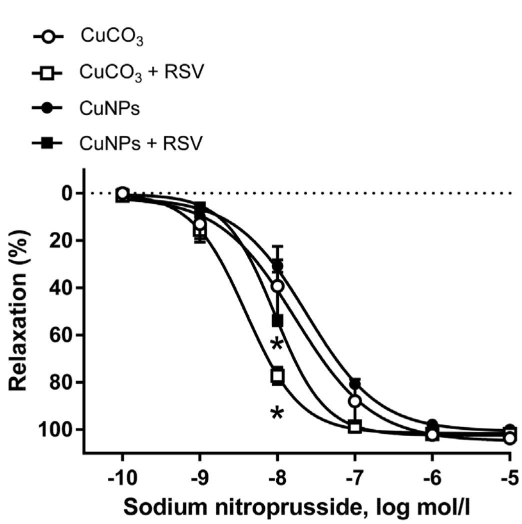

Fig. 3. The cumulative concentration-response curves to sodium nitroprusside in the thoracic rings of rats fed with experimental diets. Results (means ± SEM, $n = 8$) are expressed as a percentage of inhibition of the contraction induced by noradrenaline (0.1 μmol/l), $^{*}p \leq 0.05$ *vs.* respective control (two-way ANOVA with Tukey's multiple comparisons test).

vasodilation to ACh in the $CuCO_3$ group, and shifted the CCRCs to the left. RSV did not modify the above described response in the CuNP group (Fig. 2B).

RSV shifted to the left the vasodilator response induced by the exogenous NO donor, SNP, in the $CuCO_3$ group ($p = 0.003$), see Fig. 3.

In the presence of NOS inhibitor, L-NAME, the ACh-induced vasodilation was attenuated in aortic rings from all studied groups (Fig. 4A–D). In arteries from the $CuCO_3$ group, pre-incubation with COX inhibitor, indomethacin, decreased the dilatory response induced by ACh (Fig. 4A), while it did not modify the response in RSV supplemented rats (Fig. 4C). However, in the CuNP group, RSV in the presence of indomethacin, enhanced the impaired ACh-induced vasodilation ($p \leq 0.001$) (Fig. 4B and D).

The participation of hyperpolarizing mechanisms, in the SNP-induced response, was analyzed by precontraction of aortic rings with KCl (30 mmol/l) to block the membrane hyperpolarization (Fig. 5A–D). Under these conditions, RSV shifted to the right the SNP-induced vasodilation in the $CuCO_3$ group (Fig. 5A and C), and decreased the vasodilation in the CuNP group (Fig. 5B and D).

The participation of two potassium channels, BK_{Ca} and K_{ATP}, was analyzed with channel openers NS-1619 and pinacidil, respectively. RSV didn't modify the response to NS-1619 in any of group

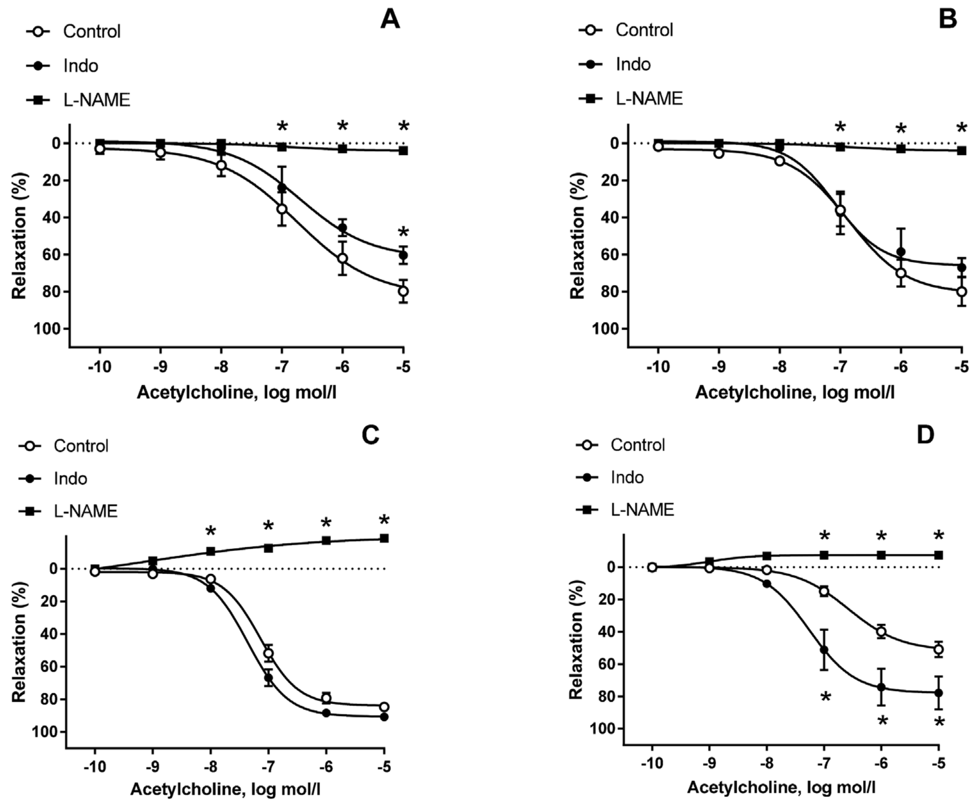

Fig. 4. The effect of preincubation with COX inhibitor, indomethacin (10 μmol/l, 30 min) and NOS inhibitor, L-NAME (100 μmol/l, 30 min) on the cumulative concentration-response curves to acetylcholine (ACh) in isolated thoracic rings. Rats were fed for 8 weeks with Cu as carbonate (A), nanoparticles (B), carbonate + RSV (C), and nanoparticles + RSV (D). Results (mean ± SEM, $n = 8$) are expressed as a percentage of inhibition of the contraction induced by noradrenaline (NA, 0.1 μmol/l), $^{*}p \leq 0.05$ *vs*. control conditions (two-way ANOVA with Tukey's multiple comparisons test).

(Fig. 6A); however, markedly shifted to the left the vasodilator response induced by pinacidil in rats treated with CuNPs (Fig. 6B).

The E_{max} and pD_2 parameters are presented in Table 2.

Discussion

This study was designed to investigate the effects of RSV supplementation on vascular reactivity, oxidative stress and lipid peroxidation induced by the dietary intake of two different forms of Cu as a CuNPs and a $CuCO_3$. The most significant findings from the RSV supplemented CuNP group were as follows: 1) a significant decrease in ACh-induced vasodilation was due to increased participation of vasoconstrictor prostanoids; 2) an increased participation of K_{ATP} channels as the compensatory mechanism to improve the vasodilation; and 3) the markers of lipid peroxidation, reflected as LOOH and MDA, were not influenced by RSV. We have previously reported that dietary replacement of $CuCO_3$ with CuNPs decreased blood plasma Cu and Cp, and enhanced CAT and FRAP [4], meanwhile we now present that supplementation with RSV made this insignificant.

Normally, the nanoparticle absorption rate is higher than the non-nanoparticle compounds. However, we observed decreased Cu content and Cp activity in the CuNP group when compared to $CuCO_3$, which confirm our previous results [4].

Surprisingly, RSV supplementation decreased Cu content and Cp activity and increased plasma Zn, only in the $CuCO_3$ group. Our results are in accordance with Asadi et al. [31], who have also found decreased Cu content in the blood of type 2 diabetic rats supplemented with RSV.

In our study, RSV improved blood plasma glucose only in the CuNP group. Asadi et al. [31] have also reported improved blood glucose by RSV.

After 8 weeks of experimental supplementation with RSV, the antioxidant defense system was stimulated in the $CuCO_3$ group, which was reflected as an increased activity of SOD and decreased activities of CAT and GPx, as well as in decreased markers of lipid peroxidation: MDA and LOOH. In contrast, RSV did not modify MDA and LOOH in the CuNP group, however in a similar way to $CuCO_3$; it enhanced SOD and attenuated CAT and GPx activities.

The increased activity of SOD indicates the effective means of scavenging superoxide anion ($^{\bullet}O_2^-$) that might be generated in excess. This stays in agreement with Asadi et al. [31], who have also reported increased SOD activity in type 2 diabetic rats supplemented with RSV. When SOD activity is increased, $^{\bullet}O_2^-$ and peroxynitrite ($ONOO^-$) are scavenged, and more hydrogen peroxide (H_2O_2) is generated, hence we observed decreased CAT and GPx activities, as the response to increased H_2O_2.

In this place, it is important to highlight, that in the presence of Cu ions, H_2O_2 is able to generate the highly destructive hydroxyl free radicals, $^{\bullet}OH$ as well as more $^{\bullet}O_2^-$ [32], which in turn would further augment the above vicious circle. Cu ions also inhibit CAT activity, which can lead to H_2O_2 accumulation. This seems to be reflected in the $CuCO_3$ group exclusively, as the blood plasma Cu content was decreased as well as MDA and LOOH levels. In the vascular wall, $^{\bullet}O_2^-$, compromises vasomotor function, by not only scavenging endothelium-derived NO but also inhibiting prostacyclin synthesis due to the formation of $ONOO^-$ [33]. This suggests that COX products could also be involved in the vascular regulation of RSV fed rats. In our study, an increase in the ACh-induced vasodilation in the presence of a COX inhibitor, indomethacin, was observed in the CuNP group supplemented with RSV, indicating the prevalence of a net effect of vasoconstrictor prostanoids. More surprisingly, was the effect of RSV in $CuCO_3$ rats, in which indomethacin did not modify the vasodilation. This might be

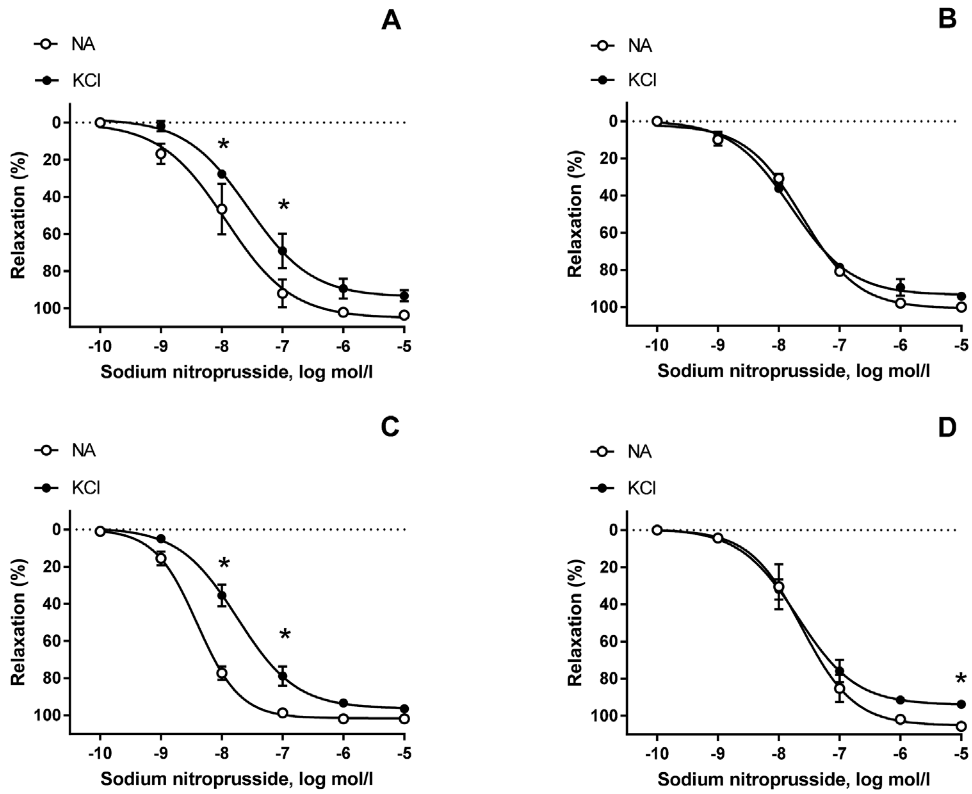

Fig. 5. Cumulative concentration-response curves to sodium nitroprusside in noradrenaline (NA) and potassium chloride (KCl) precontracted isolated thoracic rings. Rats were fed for 8 weeks with Cu as carbonate (A), nanoparticles (B), carbonate + RSV (C), and nanoparticles + RSV (D). Results (mean ± SEM, $n = 8$) are expressed as a percentage of inhibition of the contraction induced by NA (0.1 μmol/l) or KCl (30 mmol/l), $^{*}p \leq 0.05$ (two-way ANOVA with Tukey's multiple comparisons test).

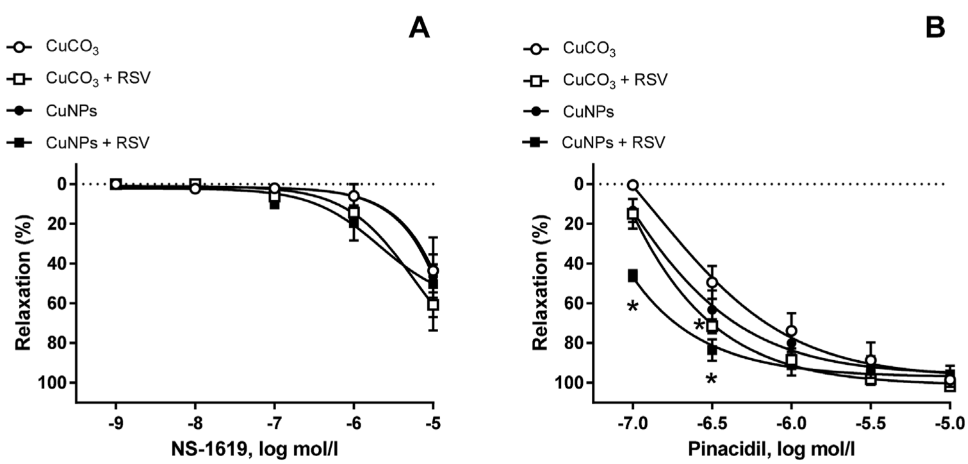

Fig. 6. Cumulative concentration-response curves to the BK_{Ca} (A) and K_{ATP} (B) channel openers. Results (mean ± SEM, $n = 8$) are expressed as a percentage of inhibition of the contraction induced by noradrenaline (NA, 0.1 μmol/l), $^{*}p \leq 0.05$ *vs.* respective control (two-way ANOVA with Tukey's multiple comparisons test).

attributed to the reduced sensitivity of the smooth muscles to prostanoids due to prolonged Cu intake in the form of $CuCO_3$.

In our study, decreased TC, TG, LDL-cholesterol and increased HDL-cholesterol levels corresponded with RSV supplementation in the $CuCO_3$ treated group. However, in CuNPs the effect of RSV on the lipid profile was less pronounced and only LDL-cholesterol was decreased.

The above described observations stay in agreement with the beneficial effect of RSV against strenuous exercise-induced oxidative damage and lipid peroxidation [34], and on improved oxidative status in RSV fed type 2 diabetic rats [31]. Since Cu enhances the formation of free radicals, the hypolipidemic potential of RSV might be altered, to some extent, due to Cu toxicity so we conclude, that RSV might not be effective in reducing the cholesterol levels during Cu intoxication.

After the 8 weeks of RSV supplementation, the vasoconstriction to NA and vasodilation to ACh and SNP were improved in the $CuCO_3$ group. RSV has been previously found to possess vaso-protective properties in mouse [35] and rat [36] models. Soylemez et al. [37] and Han et al. [38] have reported that RSV improved ACh-induced vasodilation in 3 month old male Wistar rats. However, Soylemez et al. [37] have also reported, that RSV treatment (3 weeks, 50 mg/l

Table 2
Vasodilatory effects (in %) of acetylcholine (ACh), sodium nitroprusside (SNP) and pinacidil in the aortic rings from rats fed with resveratrol and two forms of copper either as nanoparticles or carbonate.

	$CuCO_3$		$CuCO_3$ + RSV		CuNPs		CuNPs + RSV	
	Emax (%)	pD_2	Emax (%)	pD_2	Emax (%)	pD_2	Emax (%)	pD_2
ACh	87.09 ± 8.56	6.66 ± 0.34	83.15 ± 2.36	7.14 ± 0.07*	81.88 ± 9.58	6.84 ± 0.18	52.75 ± 4.24*	6.55 ± 0.17
+ Indo	64.00 ± 9.76#	6.64 ± 0.35	90.39 ± 2.30*	7.36 ± 0.07*	64.67 ± 8.00	7.10 ± 0.19	77.73 ± 5.95#	7.26 ± 0.18#
+ L-NAME	3.75 ± 0.16	6.99 ± 0.11	−16.72 ± 1.50*	8.24 ± 0.35*	3.75 ± 0.16	6.98 ± 0.11	−7.66 ± 0.05*	9.05 ± 0.03*
SNP ^{NA}	104.90 ± 6.52	7.77 ± 0.18	101.5 ± 6.59	8.40 ± 0.06*	100.8 ± 4.49	7.62 ± 0.25	102.4 ± 3.94	7.98 ± 0.36
SNP ^{KCl}	93.88 ± 6.2$	7.56 ± 0.16	96.33 ± 5.23	7.74 ± 0.07$	93.49 ± 4.53	7.79 ± 0.05	94.1 ± 2.31$	7.71 ± 0.08
Pinacidil	102.9 ± 6.99	6.42 ± 0.15	107 ± 4.40	6.65 ± 0.10	100.6 ± 4.73	6.62 ± 0.11	99.25 ± 3.10	7.01 ± 0.11*

Values are expressed as means ± SEM, *n* = 8, **vs.* not supplemented, #*vs.* ACh control conditions, $*vs.* SNP control conditions ($p \leq 0.05$, two-way ANOVA, Tukey's).

in drinking water) did not change SNP-induced vasodilation in the aortas of Wistar rats. We conclude, that the dose and duration of RSV intake may be responsible for the above-described discrepancies, which is in agreement with Fogacci et al. [15].

Surprisingly, in our study the beneficial effects of RSV on vascular function were not documented in the CuNP group, as we observed a decreased ACh-induced vascular response. Similar to our results, Han et al. [38] have also reported a decreased ACh-induced response of thoracic arteries, however in old Wistar rats. Thus, we conclude, that the obtained results could be interpreted as a variable Cu-dependent effect of RSV on endothelial function, which is also dependent on the pathophysiological conditions of organism.

Now, we present, for the first time, that the consumption of RSV decreases the ACh-induced vascular response in rats supplemented with CuNPs.

Since the endothelium is able to synthesize EDHF and/or substances that hyperpolarize the cell membrane, such as H_2O_2, the participation of hyperpolarizing mechanism(s) of exogenous NO donor, SNP, was analyzed. Our results indicate that NO partially hyperpolarizes the cell membrane and that CuNPs decrease the degree of SNP-induced hyperpolarization, which was partially restored by the RSV-supplementation. It is well known that BK_{Ca} and K_{ATP} channels have an important role in vascular tone regulation [39] and that their function has been modulated *in vitro* by RSV [40,41]. We observed that RSV increased the pinacidil-induced responses in arteries from the CuNP group. This vasodilator response elicited by the K_{ATP} channel opener was much more significant to the effect found in arteries from $CuCO_3$ rats, and would constitute the compensatory effect to the decreased ACh-induced vasodilation.

It is important to note the methodological limitations of this study, as species differences between rodents and humans should be considered. We used young Wistar rats instead of aged, whereas most health effects are found in the elderly. Male rats are commonly used in such experimentations, thus testing of both sexes is not mandatory. Finally, we used one single dose of RSV, however different dosages should be implemented.

The potential health benefits of RSV are limited by instability [42], and rapid metabolism, which require higher or more frequent doses [43], thus other formulations of RSV should be considered in future studies. In this framework, the incorporation of RSV in PEG-modified liposomes have been proven to safely deliver RSV, ensuring the preservation of stability and antioxidant efficacy of this polyphenol against induced oxidative stress [18]. This would allow to distinguish whether the observed results are due to the oxidation of RSV or to other mechanism(s).

Conclusion

In conclusion, our data point to the beneficial supplementation with RSV during $CuCO_3$ intake reflected as an improved vasoconstriction and vasodilation, as well as in decreased markers of lipid peroxidation. This role of RSV was not observed in CuNP rats, and decreased ACh-induced vasodilation and increased participation of vasoconstrictor prostanoids in vascular response was observed.

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Author contributions

Study design: MM. Study conduct: MM. Data collection: MM, KO, JJ. Data analysis: MM. Funding acquisition: MM. Drafting manuscript: MM. Wrote the manuscript: MM. Approving final version of manuscript: all the authors. MM take responsibility for the integrity of the data analysis.

Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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