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Original article

Copper nanoparticles modify the blood plasma antioxidant status and modulate the vascular mechanisms with nitric oxide and prostanoids involved in Wistar rats

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ABSTRACT

Background: We aimed to analyze whether a diet supplemented with a standard dose of copper (Cu) in the form of nanoparticles, as an alternative to carbonate, exerts beneficial effects within the vasculature and improves the blood antioxidant status.
Methods: Male Wistar rats were fed for 8 weeks with a diet supplemented with Cu (6.5 mg Cu/kg in the diet) either as nanoparticles (40 nm diameter) or carbonate – the control group. Moreover, a negative control was not supplemented with Cu. At 12 weeks of age, blood samples, internal organs and thoracic aorta were taken for further analysis. Blood antioxidant mechanism was measured together with Cu and Zn.
Results: Diet with Cu as nanoparticles resulted in an elevated catalase activity and ferric reducing ability of plasma, however decreased Cu (plasma), and ceruloplasmin (Cp) compared to carbonate. The participation of vasoconstrictor prostanoid was increased, as indomethacin did not modify the acetylcholine (ACh)-induced response. Arteries from Cu nanoparticle and carbonate rats exhibited a reduced maximal contraction to potassium chloride and an increased response to noradrenaline. The endothelium-dependent vasodilation to ACh was enhanced while exogenous NO donor, sodium nitroprusside, did not modify the vascular response. Down-regulation of BKCa channels influenced hyperpolarizing mechanism. The superoxide dismutase and HDL-cholesterol were decreased opposite to an increased lipid hydroperoxides, malondialdehyde, Cu (plasma and liver) and Cp.
Conclusion: Despite the increased antioxidant capacity in blood of Cu nanoparticle fed rats, vasoconstrictor prostanoids and NO are involved in vascular regulation.

Introduction

Cu is one of the most important microelements involved in energy metabolism (cytochrome c oxidase), antioxidant defense (Cu/Zn-SOD), the synthesis of neurotransmitters (dopamine β-monooxygenase), neuropeptides (peptidylglycine α-amidating enzyme) and tryptophan metabolism on the kynurenine pathway [1,2]. Cu is able to inhibit systemic inflammation by inducing arachidonic acid conversion and prostanoid synthesis [3]. Cu in its inorganic form (sulfate and/or carbonate) has been well studied for its anti-inflammatory, anti-proliferative and anti-oxidant properties [4–7].

However, some studies have questioned the safety of the standard dose (5–6.5 mg/kg diet) of Cu looking for an alternative which would neither induce inflammation nor cause harmful effects to the intestinal epithelium and liver. This is why Cu

Abbreviations: ACh, acetylcholine; ANOVA, analysis of variance; CAT, catalase; CCRC, cumulative concentration-response curve; COX, cyclooxygenase; Cp, ceruloplasmin; Cu, copper; CuD, copper deficient; CuNPs, copper nanoparticles; E_{max}, maximal response values; FRAP, ferric reducing antioxidant power; HDL, high density lipoprotein; K^+, potassium; K_{ATP}, ATP-dependent potassium channels; K_{Ca}, calcium-dependent potassium channels; KHS, Krebs-Henseleit solution; LDL, low density lipoprotein; L-NAME, Nω-Nitro-L arginine methyl ester; LOOH, lipid hydroperoxides; MDA, malondialdehyde; NA, noradrenaline; NO, nitric oxide; NOS, nitric oxide synthase; pD_2, drug concentration exhibiting 50% of the E_{max} expressed as negative log molar; ROS, reactive oxygen species; SEM, standard error of the mean; SNP, sodium nitroprusside; SOD, superoxide dismutase; TG, triglycerides; Zn, zinc.

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nanoparticles have been studied as an alternative to ionic Cu [4,17,20,21]. Recent studies have reported a significant anti-diabetic and cardioprotective role of Cu as nanoparticles with decreased production of inflammatory mediators [4,8–10]. Treatment with low dose of Cu nanoparticles (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 has increased the serum bioavailability of NO [9].

There are some reports that question the safety of nanoparticles, which tend to exhibit quite different properties, when compared to larger particles of the same element [11]. The enhanced reactivity of nanoparticles has its origin in the increased surface-to-volume ratio, or might result from preferential uptake in specific cells and tissues [12,13]. Toxicities to the kidney, liver and spleen have been reported, with exposure to dietary 23.5 nm Cu particles in experimental mice [14]. Liu et al. have reported the toxic effects of nasally inhaled 23.5 nm Cu particles to the liver and kidneys of mice after administering high doses (40 mg/kg body weight) at three times per week [15]. Body weight retardation has been also reported in the same series of experiments. The toxicity of Cu nanoparticles has been found to be gender-dependent and male mice were more prone to these effects at the same dosage when compared to females [14]. In addition, increased prostaglandin E_2 release and enhanced TNF-α and IL-1 production in cerebral microvessels [16], as well as high-grade hydropic degeneration and necrotic lesions in the liver [17] have been described as the mechanism of Cu nanoparticles.

On the other side, Cu-deficiency inactivates Cu/Zn-SOD and promotes accumulation of ROS. This process impairs NO-mediated vasodilation and triggers vascular inflammation in which COX-2 is upregulated [7]. Altered production of those factors could induce vascular dysfunction [18], which may contribute to the increase in blood pressure.

We aimed to investigate the possible therapeutic potential of supplementation with Cu nanoparticles on the vasculature, as an alternative to ionic Cu, together with the exploration of the involved mechanism(s).

In the presented study, we analyzed the blood plasma glucose, total lipid profile, Cu and Zn concentration together with the antioxidant status of the blood, reflected as SOD, CAT, FRAP, MDA, LOOH and Cp. The endothelium dependent and independent vasodilatory mechanisms were studied with ACh and SNP. The participation of prostanoid in the ACh-induced vasodilation was studied with the nonspecific COX inhibitor – indomethacin. The vasodilator response to the BK_{Ca} and K_{ATP} channel openers was analyzed.

Materials and methods

Drugs and reagents

Cu as carbonate (purity ≥ 99%) was sourced from Poch (Gliwice, Poland). The nanoparticles of Cu (40–60 nm size nanopowder, 12 m^2/g) were purchased from Sky Spring Nanomaterials, Inc. (Houston, TX, US), 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.

The drugs used were: ACh as chloride, NA as hydrochloride, L-NAME as hydrochloride, indomethacin, KCl, SNP (Sigma-Aldrich) and NS1619, pinacidil (Cayman chemical).

Stock solutions (10 mM) of these drugs were prepared in distilled water, except for NS1619, indomethacin and pinacidil which were dissolved in ethanol, and NA which was dissolved in a NaCl (0.9%) – ascorbic acid (0.01% w/v) solution. These solutions were maintained at −20 °C and appropriate dilutions were made in KHS of the following composition (mM): NaCl 115; $CaCl_2$ 2.5; KCl 4.6; KH_2PO_4 1.2; $MgSO_4$ 1.2; $NaHCO_3$ 25; glucose 11.1, on the day of the experiment.

Animals

This 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).

Experimental protocol

Healthy male albino Wistar rats (Han IGS rat [Crl: WI(Han)]) at 4 weeks of age, weighing between 152.9 and 192.3 g, were randomly divided into three groups of eight animals each. During the 8 weeks of experimental feeding, 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%. The rats had free access to tap water and experimental diets, which were prepared weekly and then stored at 4 °C in hermetic containers. The experimental diets were modifications of a casein diet for laboratory rodents recommended by the American Institute of Nutrition (see supplementary materials, Table S.1).

Experimental diets were supplemented with standard dose of Cu (6.5 mg Cu/kg of diet) (i) as nanoparticles of 40–60 nm diameter and (ii) Cu as carbonate – control diet. Moreover, a negative control was not supplemented with Cu – Cu deficient diet (iii). The accepted dose of Cu in the daily diet of rats has been set in the range of 5–6.5 mg Cu/kg of diet [4,17,19,20] and that is the dose we used in our 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.

Biochemical assay and organ examinations

Rats were anaesthetized and killed by decapitation. The organs such as liver, kidneys, heart, spleen and brain were then carefully removed and weighed accurately.

Blood samples were kept in tubes containing heparin + EDTA as an anticoagulant. Samples were centrifuged at 3000 x *g* for 10 min and blood plasma was separated for analysis. Blood plasma was stored at −80 °C until further analysis.

The concentrations of Cu and Zn (μmol/l) were determined by the inductively coupled plasma optical emission spectrometry method (ICP-OES) with the certified reference material NIST1577C. Bovine liver was used for quality control.

The content of glucose, total cholesterol, HDL-cholesterol, LDL-cholesterol and triglycerides (mmol/l) was measured using a biochemical auto-analyzer (Plasma Diagnostic Instruments, Horiba, Kyoto, Japan).

The activity of SOD (U/ml) was determined in erythrocytes using Ransod and Ransel diagnostic kits (Randox), and CAT (U/ml) was determined according to Aebi [22]. Total antioxidant potency expressed as FRAP, the main primary products of lipid peroxidation – LOOH, and the secondary product during lipid peroxidation MDA, were analyzed (μmol/l) in blood plasma according to the method described previously [23].

Cp (U/l) was determined 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

Briefly, the thoracic aorta was isolated and placed in an ice-cold KHS at pH 7.35-7.45. Isolated aorta was cleaned of adherent tissue, and cut into 6–8 aortic rings of 3 to 4-mm length, which were 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 mixture of 95% oxygen and 5% carbon dioxide, and maintained at 37 °C. Each ring was connected to a transducer (F-30) to measure isometric force.

After the initial equilibration period of 60 min, contractile response elicited by a single depolarizing concentration of KCl (30 mM) was assessed. After 30 min, aortic ring were washed 3 times in KHS. After the equilibration period (60 min), aortic rings were contracted for 4–5 min with NA (0.1 μM) that induced approximately 1 g of contraction and then ACh (0.0001–10 μM) was added in 30–60 sec intervals to assess the endothelium-dependent vasodilation.

In addition, the CCRCs were built in respective intervals for SNP (1 min, 0.0001–10 μM), NS1619 (10 min, 0.001–10 μM) and pinacidil (3–5 min, 0.1–10 μM).

In other set of experiment, CCRCs to ACh and SNP were built on precontracted aortic rings with KCl (30 mM).

Vasodilation induced by ACh was also analyzed in the absence and presence of the NO synthase (NOS) inhibitor – L-NAME (100 μM), and the nonspecific COX inhibitor – indomethacin (10 μM). These drugs were added 30 min before contraction with NA. Only one CCRC was performed in each aortic ring.

Data analysis and statistics

The calculations and graphs were done and analyzed in GraphPad Prism 7. Contractions were expressed in mg of developed tension for both KCl and NA. Vasodilation was represented as a percentage of the maximal response to 0.1 μM NA or 30 mM KCl. To determine the maximal response (E_{max}) and the potency, the pD_2 values (the negative logarithm of the concentration causing a half-maximum effect) were determined from the individual CCRC by non-linear regression analysis. Data were expressed as means ± SEM from n = 8 rats, and were compared by either one- or by two-way ANOVA with Tukey's multiple comparisons test, where appropriate. The model assumption of normality and homogeneity of variance was tested for all data. A value of $p \leq 0.05$ was considered to be significant.

Results

The characteristics of the rats

The food consumption and the body weight of the animals were not significantly different between experimental groups (Table 1). The liver, spleen, kidneys and brain mass-to-body weight ratio didn't differ between groups ($p > 0.05$), see Table 2. However, an increase in the heart-to-body mass ratio was observed in Cu deficient animals ($p \leq 0.03$).

Cp, Cu, Zn and the antioxidant status

Nanoparticle fed rats had 0.86- and 0.76-fold lower Cu and Cp content, respectively compared to carbonate ($p \geq 0.002$).

However, animals which received Cu as nanoparticles and carbonate in their daily diet had a higher blood plasma Cu content ($p = 0.001$) and Cp ($p = 0.006$) compared to Cu deficient animals. In addition, the Zn level was increased in Cu nanoparticle but not in carbonate rats, compared to Cu deficient animals ($p = 0.015$) (Table 3).

There was no difference in the activity of SOD and the blood plasma MDA and LOOH values between Cu nanoparticle and carbonate rats ($p \geq 0.8$). However, animals which received Cu as nanoparticles and carbonate had increased values of MDA and LOOH opposite to decreased SOD activity, compared to Cu deficient animals ($p \leq 0.05$).

The activity of CAT and blood plasma FRAP were increased in nanoparticle rats by 1.28- and 1.14-fold, respectively compared to carbonate ($p = 0.01$). In addition, CAT activity was increased in Cu nanoparticles exclusively ($p = 0.0017$), whereas FRAP was decreased in carbonate ($p = 0.39$) rats, compared to Cu deficient animals, see Table 3.

Glucose and the lipid profile

The blood plasma glucose and the total lipid profile were not different between Cu nanoparticle and carbonate rats ($p > 0.05$), see Table 3. However, Cu nanoparticle and carbonate rats had decreased HDL-cholesterol compared to Cu deficient animals ($p \leq 0.05$). Moreover, Cu carbonate, but not nanoparticle rats had increased TG and decreased glucose, compared to Cu deficient animals ($p \leq 0.02$).

Vascular reactivity studies

There was no difference in the KCl- and NA-induced vasoconstriction and ACh-induced vasodilation between Cu nanoparticle and carbonate aortic rings ($p \geq 0.8$), see Figs. 1 and 2. However, the aortic rings isolated from these two studied groups, exhibited decreased contractile response to 30 mM KCl and an increased response to 0.1 μM NA ($p \leq 0.05$) when compared to Cu deficient animals (Fig. 1).

Moreover, the vasodilation induced by ACh was increased in Cu nanoparticle and carbonate aortic rings compared to Cu deficient aortas ($p \leq 0.05$, Fig. 2A). In nanoparticle aortic rings, preincubation with the nonselective COX inhibitor, indomethacin (10 μM, 30 min), increased the maximal response to ACh compared to carbonate, and shifted the CCRC to the right when compared to Cu deficient aortic rings (Fig. 2B).

The vasodilator response induced by the exogenous NO donor, SNP, was not significant different between experimental groups (Fig. 3).

Table 1
Effect of experimental diet on food consumption and body weight of Wistar rats.[a].

Experimental group	Food consumption[b]	Initial body weight	Final body weight
Cu carbonate	1069 ± 29.79	180.37 ± 2.18	388.93 ± 6.69
Cu nanoparticles	1101 ± 41.18	177.64 ± 3.67	403.24 ± 9.89
Cu deficient	1091 ± 52.69	177.80 ± 3.46	392.41 ± 6.09

Data are expressed as means ± SEM of n = 8 experiments ($p > 0.05$; one-way ANOVA with Tukey's multiple comparisons test).
[a] In grams.
[b] For diet composition, see supplementary materials (Table S.1).

Table 2
The effect of experimental diet on the mass of internal organs (in g/100 g of final body weight) in Wistar rats.

Experimental group	Heart	Liver	Spleen	Kidneys	Brain
Cu carbonate	0.231 ± 0.007*	3.674 ± 0.123	0.219 ± 0.0146	0.589 ± 0.016	0.488 ± 0.007
Cu nanoparticles	0.230 ± 0.008*	3.518 ± 0.102	0.207 ± 0.013	0.586 ± 0.016	0.473 ± 0.008
Cu deficient	0.271 ± 0.015	3.713 ± 0.166	0.197 ± 0.009	0.592 ± 0.013	0.480 ± 0.008

Data are expressed as means ± SEM of n = 8 experiments; *vs.* Cu deficient diet ($p \leq 0.05$; one-way ANOVA with Tukey's multiple comparisons test).

Table 3
Blood analysis and copper content in the liver of rats fed with experimental diets.

	Cu in liver (mg/kg of tissue)	Cu in plasma (μmol/l)	Zn (μmol/l)	Cp (U/l)	SOD (U/ml)	CAT (U/ml)	FRAP (μmol/l)	LOOH (μmol/l)	MDA (μmol/l)	TC (mmol/l)	LDL (mmol/l)	HDL (mmol/l)	TG (mmol/l)	GLU (mmol/l)
Cu carbonate	0.22 ± 0.006#	18.31 ± 0.476#	80.11 ± 0.971	37.51 ± 2.71#	36.53 ± 0.367#	19.12 ± 0.606	160.8 ± 5.724#	14.43 ± 0.901#	8.543 ± 0.239#	2.784 ± 0.229	0.544 ± 0.069	0.461 ± 0.031#	2.579 ± 0.217#	17.9 ± 0.721#
Cu nanoparticles	0.20 ± 0.003#	15.67 ± 0.373*#	83.14 ± 0.757#	28.41 ± 0.748*#	37.16 ± 0.879#	24.45 ± 1.781*#	183.3 ± 5.521*	14.12 ± 0.807#	7.755 ±0.643#	2.366 ± 0.136	0.478 ± 0.044	0.482 ± 0.032#	2.001 ± 0.221	20.56 ± 0.622
Cu deficient	0.04 ± 0.005	3.441 ± 0.372	75.65 ± 2.715	1.335 ± 0.274	58.27 ± 1.677	17.67 ± 0.791	198.2 ± 7.398	10.26 ± 0.302	6.51 ± 0.289	2.579 ± 0.099	0.488 ± 0.048	0.63 ± 0.061	1.69 ± 0.216	22.89 ± 1.898

Data are expressed as means ± SEM of n = 8 experiments; *vs.* Cu as carbonate, #*vs.* Cu deficient diet ($p \leq 0.05$; one-way ANOVA with Tukey's multiple comparisons test).

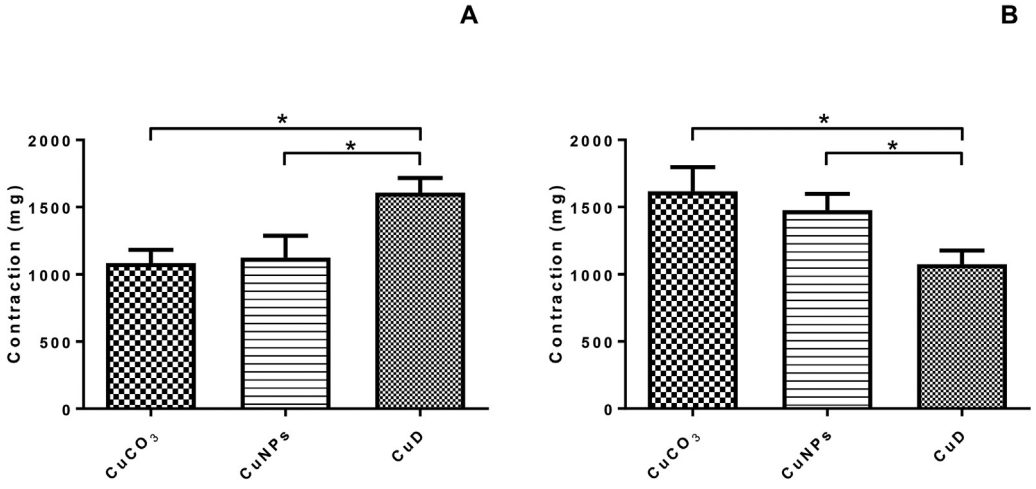

Fig. 1. Contractile response to potassium chloride (KCl 30 mM) (A) and noradrenaline (NA 0.1 μM) (B) in the isolated thoracic rings from rats fed with experimental diets. Values are expressed as means ± SEM, n = 8, *$p \leq 0.05$ (one-way ANOVA with Tukey's multiple comparisons test).

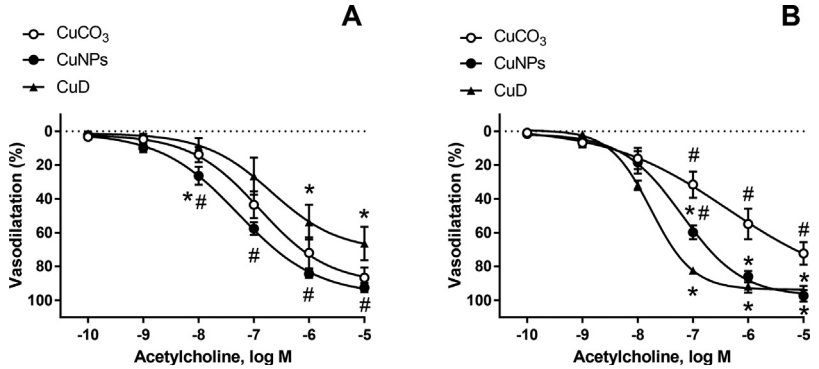

Fig. 2. The cumulative concentration-response curves to acetylcholine in the isolated thoracic rings from rats fed with experimental diets. Control conditions (A). The aortic rings were incubated with indomethacin (10 μM, 30 min) (B). Results (means ± SEM, n = 8) are expressed as a percentage of inhibition of the contraction induced by 0.1 μM noradrenaline, *vs.* Cu as carbonate, #*vs.* Cu deficient diet ($p \leq 0.05$, two-way ANOVA with Tukey's multiple comparisons test).

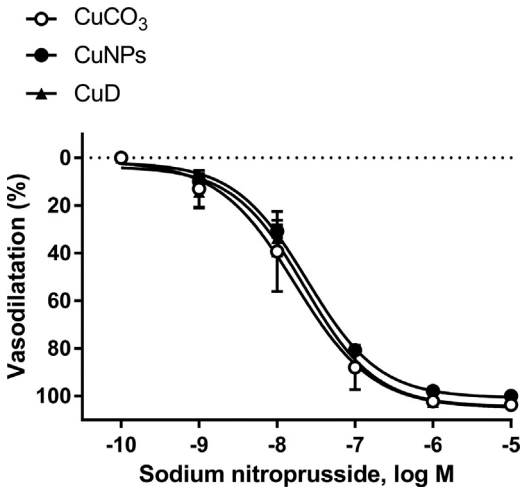

Fig. 3. The cumulative concentration-response curves to sodium nitroprusside in the isolated thoracic rings from rats fed with experimental diets. Results (means ± SEM, n = 8) are expressed as a percentage of inhibition of the contraction induced by 0.1 μM noradrenaline, $p > 0.05$ (two-way ANOVA with Tukey's multiple comparisons test).

In the presence of the NOS inhibitor (L-NAME, 100 μM, 30 min), the ACh-induced vasodilation was completely inhibited in the aortic rings from all studied groups (Fig. 4). Incubation with indomethacin (10 μM, 30 min), did not modify the ACh-induced response in aortic rings from nanoparticle group. In contrast, in aortic rings from Cu carbonate rats, the maximal response was attenuated whereas in Cu deficient – enhanced (Fig. 4). The aortic rings precontracted with 30 mM KCl responded in the similar same way subjected to ACh (Fig. 4).

In Cu nanoparticle aortic rings, the vasodilation induced by SNP was not modified when compared to the control conditions (Fig. 5). However, in Cu carbonate and Cu deficient rats, SNP induced vasodilation of the KCl-precontracted aortic rings was reduced when compared to the control conditions.

There was no difference in the vascular response to NS1619 between Cu nanoparticle and carbonate aortic rings. However, the NS1619-induced vasodilation was attenuated in Cu nanoparticle and carbonate aortic rings compared to Cu deficient arteries ($p \leq 0.05$). The vasodilation induced by the K_{ATP} channel opener, pinacidil, was similar in all three studied groups (Fig. 6).

The E_{max} and pD_2 parameters are presented in Table 4.

Discussion

The supplementation with Cu nanoparticles, used in our study, did not cause any loss of appetite or changes in the body weight of rats. We observed heart enlargement in Cu deficient animals, which is in agreement with our previous results [17,19] and other studies [24,25], and together with decreased blood plasma Cu concentration confirm Cu deficiency.

Cu fluctuations during the period of intensive development and growth of the body adversely affect the metabolism and absorption of other micronutrients, such as iron, calcium and zinc [4], as was also observed in our study (Table 3), which in turn can have a direct impact on metabolic processes. Elevated levels of Cu, as was observed in nanoparticle and carbonate rats, may increase oxidative stress, inflammation [26] and lead to anxiety, which can increase blood pressure when persistent [27].

One of the adverse effects of oxidative stress is ROS generation and the induction of lipid peroxidation which has damaging effects on cells and tissues. We found increased primary products of lipid peroxidation (LOOH and MDA levels) in the blood plasma of Cu nanoparticle and carbonate rats, which points toward an increased oxidative stress in tissues of these rats compared to Cu deficient animals.

Surprisingly, we noticed increased values of FRAP in animals fed with Cu nanoparticles. This may suggest an increased antioxidant capacity of blood plasma of Cu nanoparticle animals opposite to carbonate fed rats.

Another interesting finding, is the increased CAT activity. The increased CAT, which is involved in hydrogen peroxide detoxification, points to the influence of nanoparticles on the redox status. The increased activity of CAT, accompanied by stable activity of SOD, points toward the activation of enzymatic defense mechanisms, as the response to increased oxidative stress.

Cu is also involved in the biosynthesis of fatty acids, as well as carbohydrates, which suggests that lipid profile and glucose are highly sensitive to changes in Cu ion fluctuations [17]. Abnormal Cu metabolism can contribute to the development of diabetes, obesity and finally to cardiovascular disorders [28]. Low Cu content in the blood plasma of rats has been associated with impaired glucose tolerance characterized by increased blood glucose [29]. However, in our study, decreased blood plasma Cu and Cp, observed in Cu nanoparticle rats, did not influence the blood glucose. In addition, the total lipid profile was not different between Cu nanoparticle and carbonate rats.

Surprisingly, after 8 weeks of Cu nanoparticle feed diet, the KCl- and NA-induced contraction and endothelium–dependent vasodilation to ACh remained unchanged compared to carbonate group.

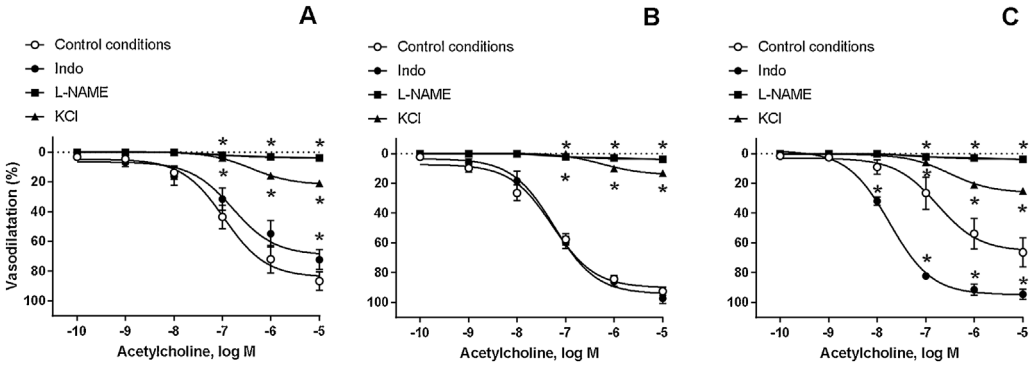

Fig. 4. The effect of preincubation with indomethacin (10 μM, 30 min) and L-NAME (100 μM, 30 min) on the cumulative concentration-response curves to acetylcholine in the isolated thoracic rings. Rats were fed with Cu as carbonate (A), nanoparticle (B) and Cu deficient (C) diet. Results (means ± SEM, n = 8) are expressed as a percentage of inhibition of the contraction induced by 0.1 μM NA or 30 mM KCl, $^{*}p \leq 0.05$ *vs.* control conditions (two-way ANOVA with Tukey's multiple comparisons test).

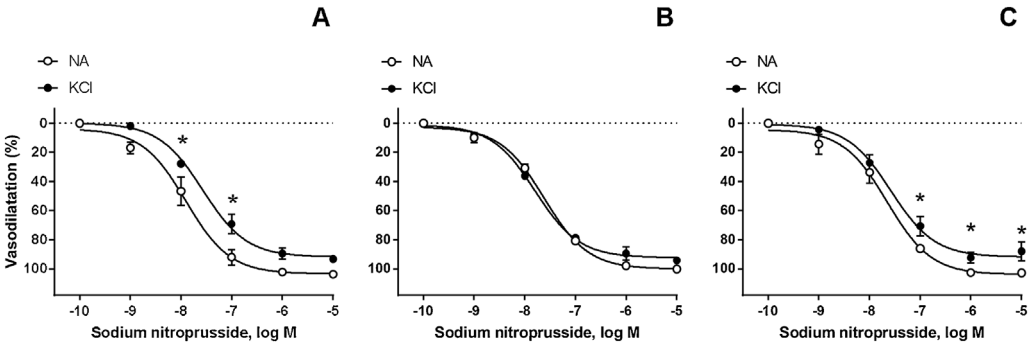

Fig. 5. Cumulative concentration-response curves to sodium nitroprusside in the thoracic rings of rats fed with experimental diets. Rats were fed with Cu as carbonate (A), nanoparticle (B) and Cu deficient (C) diet. Results (means ± SEM, n = 8) are expressed as a percentage of inhibition of the contraction induced by 0.1 μM NA or 30 mM KCl, $^{*}p \leq 0.05$ (two-way ANOVA with Tukey's multiple comparisons test).

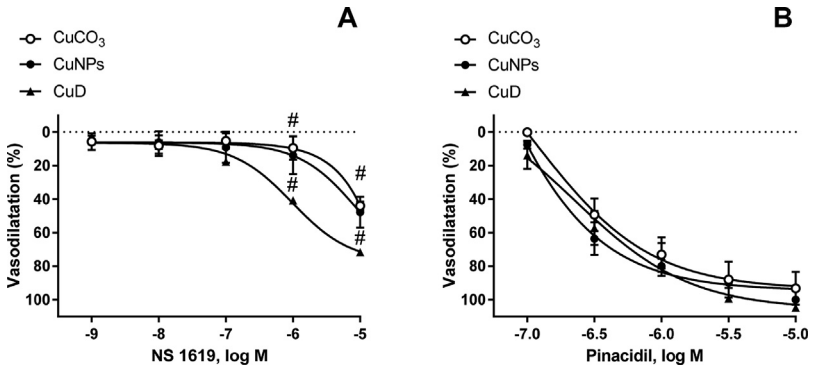

Fig. 6. Cumulative concentration-response curves to the BK_{Ca} (A) and K_{ATP} (B) channel openers. Results (means ± SEM, n = 8) are expressed as a percentage of inhibition of the contraction induced by 0.1 μM noradrenaline, *vs. Cu as carbonate, #vs. Cu deficient diet ($p \leq 0.05$, two-way ANOVA with Tukey's multiple comparisons test).

Table 4
Changes in the maximal responses (E_{max}, expressed as a percentage of dilation) and pD_2 parameters to acetylcholine, sodium nitroprusside, NS1619 and pinacidil in isolated aortic rings from rats fed with experimental diets.

Treatment	Cu carbonate		Cu nanoparticles		Cu deficient	
	E_{max} (%)	pD_2	E_{max} (%)	pD_2	E_{max} (%)	pD_2
ACh	89.7 ± 5.56[b]	7.02 ± 0.19[b]	90.16 ± 4.58[b]	7.27 ± 0.14[b]	73.63 ± 6.14	6.79 ± 0.27
+ INDO	79.92 ± 5.98*[b]	6.77 ± 0.12[b]	94.33 ± 3.28[a,b]	7.24 ± 0.12[a,b]	94.85 ± 1.07*	7.75 ± 0.03*
+ L-NAME	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
ACh ^{KCl}	21.98 ± 1.39	6.41 ± 0.32	14.22 ± 1.72	6.25 ± 0.21	26.29 ± 2.14	6.51 ± 0.64
SNP ^{NA}	103.6 ± 3.18	7.89 ± 0.10	100.1 ± 0.98	7.62 ± 0.03	103.9 ± 1.95	7.65 ± 0.06
SNP ^{KCl}	91.78 ± 5.27*[b]	7.57 ± 0.16	92.24 ± 1.53	7.89 ± 0.05	89.63 ± 2.57*	7.39 ± 0.08
NS1619	43.95 ± 2.4[b]	N.D.	47.78 ± 9.18[b]	N.D.	71.7 ± 0.58	N.D.
Pinacidil	94.77 ± 3.92	6.53 ± 0.08	95.38 ± 3.27	6.63 ± 0.07	102.4 ± 4.18	6.6 ± 0.06

Data are expressed as means ± SEM of n = 6–8 experiments; *vs. control conditions, [a]vs. Cu carbonate, [b]vs. Cu deficient ($p \leq 0.05$; one-way ANOVA with Tukey's multiple comparisons test). *Abbreviations:* ACh, acetylcholine; E_{max}, maximal response values; INDO, indomethacin; KCl, potassium chloride; L-NAME, N(ω)-nitro-L-arginine methyl ester; N.D., not determined; pD_2, drug concentration exhibiting 50% of the E_{max} expressed as negative log molar; SNP, sodium nitroprusside.

This is contrary to our previous report, which had revealed an increased vasoconstriction to the selective α-1 receptor agonist phenylephrine during 4 weeks of dietary experiment [19]. This may in part be explained by the increased duration of experimentation to 8 weeks, and to the development of some compensatory mechanism(s), as well as to the choice of the non-selective α-1/2 receptor agonist – noradrenaline, instead of phenylephrine.

Even though we did not currently report any changes to ACh-induced vasodilation between the Cu nanoparticle and carbonate aortic rings, the contribution of other factors such as NO, prostanoid and hyperpolarizing mechanism(s) cannot be ruled out.

We observed that NOS inhibition with L-NAME blocked the ACh-induced response in aortas from all three studied groups, indicating that the vasodilatory response to ACh is mainly due to the NO, as was described for large elastic arteries [30].

Considering that the sensitivity of the smooth muscles to NO between the studied groups was not altered (confirmed with SNP), prostanoid may also be responsible for the dilation of large arteries, as has been suggested by Trickler et al. [16]. So far, the proposed mechanisms of the vasoactive effect of Cu nanoparticles have been due to an increased bioavailability of NO in the arteries of diabetic rats [9] and increased prostaglandin E_2 formation in endothelial cells of microvessels in rat brain [16]. Preincubation with the nonselective COX inhibitor, indomethacin, decreased the response to ACh in Cu carbonate aortic rings, indicating the involvement of a vasodilatory net effect of prostanoids. However, in Cu nanoparticle

arteries, the response to ACh was not altered, indicating reduced sensitivity of the smooth muscles to prostanoids. Furthermore, when the prostanoids are altered, the formation of NO is also modified, indicating that NO is involved. The increased response to ACh in Cu nanoparticle aortic rings might be explained by the increased bioavailability of NO. This is supported by the increased production of NO which has been described by Sharma et al. after the exposure to nanoparticles [9].

In view of these results, the hyperpolarizing effect of NO was also investigated. Our results show that after blocking hyperpolarization with KCl, the response to ACh was not altered in arteries from Cu nanoparticle aortic rings. This indicate that the nanoparticles negatively affect the participation of hyperpolarizing mechanisms in the vasodilator responses.

BK_{Ca} and K_{ATP} channels have an important role in vascular tone regulation. Taking into account that nanoparticles decreased the participation of hyperpolarizing mechanisms induced by SNP, the vasodilator response to the BK_{Ca} and K_{ATP} channel openers NS1619 and pinacidil, were analyzed respectively. Surprisingly, we observed that Cu nanoparticles and carbonate decreased the NS1619-induced response, suggesting that BK_{Ca} channels rather than K_{ATP} are involved in the vasodilator response induced by Cu but not nanoparticles.

Conclusion

Our findings show that Cu in form of nanoparticles reduce the sensitivity of the smooth muscles to prostanoids (i) and potentiate NO-dependent ACh-induced vasodilation (ii), as well as decrease the participation of hyperpolarizing mechanisms in the vasodilator response (iii), in which BK_{Ca} channels might be involved (iv). These together with the increased CAT activity and FRAP (v) and reduced Cu and Cp (vi) is the mechanism involved for the role of Cu as nanoparticles.

Conflict of interest

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

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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 the responsibility for the integrity of the data analysis.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.pharep.2019.02.007.

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