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ORIGINAL PAPER

COPPER NANOPARTICLES ENHANCE VASCULAR CONTRACTION INDUCED BY PROSTAGLANDIN F_2-ALPHA AND DECREASE THE BLOOD PLASMA Cu-Zn RATIO IN WISTAR RATS*

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Abstract

Both copper (Cu) deficiency and excessive dietary exposure can induce alterations within the endothelium and vascular smooth muscles. This study examined the dietary effect of Cu nanoparticles (NPs) on the blood plasma ratio of Cu to zinc (Zn) and Cu to ceruloplasmin (Cp) as well as the vascular contraction of thoracic rings to potassium chloride (KCl), noradrenaline (NA), endothelin-1 (ET-1) and prostaglandin (PG) F_2-alpha in *in vitro* conditions. Male Wistar rats, divided randomly into three groups, were supplemented for 8 weeks with two forms of Cu (6.5 mg Cu kg^{-1} of a diet): either as nanoparticles (CuNPs group) or $CuCO_3$ (control group). Experimental rats not supplemented with Cu were defined as a negative control (Cu deficiency – CuD group). Significant decrease in body weight was observed only in CuD rats compared to rats supplemented with CuNPs. Dietary replacement of $CuCO_3$ with CuNPs resulted in decreased blood plasma Cu and Cp, while the Zn content remained unchanged. Moreover, the blood plasma Cu-Zn ratio decreased, while Cu-Cp was not modified. Supplementation with CuNPs increased the PGF_2-alpha-induced contraction, but did not modify the contractile response to KCl, NA or ET-1. CuD diet resulted in decreased Cu-Zn and increased Cu-Cp ratios. Contractile response to NA was not altered, although the vascular response to ET-1 and PGF_2-alpha was increased when compared to Cu supplemented groups. The results indicate that 8 weeks of supplementation with 6.5 mg Cu kg^{-1} of diet in the form of CuNPs may favor the onset of a pro-inflammatory environment. Given the significant role of PGF_2-alpha in the vascular tone regulation, it is likely that alterations in specific channels as well as PG receptors function and/or expression represent an important mechanism in the blood flow regulation during exposure to CuNPs.

Keywords: endothelin-1, noradrenaline, potassium chloride, prostaglandin F_2-alpha.

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INTRODUCTION

Vascular regulation is mediated by both the endothelium and the smooth muscle cells. The smooth muscle cells directly drive the contraction of the vascular wall, hence regulate the size of the blood vessel lumen (Brozovich et al. 2016), which can be measured by isolated blood vessels technique (Majewski et al. 2019*a*). However, in response to reactive oxygen species (ROS), diet or other factors (Majewski et al. 2018, 2019*b*), atherosclerosis or vessel remodeling may occur. There are a number of mechanisms proposed for transient vasoconstriction of the vessels, and there is some evidence supporting the role for hyperactivity of the adrenergic nervous system, endothelin (ET) receptors, oxidative stress and cyclooxygenase (COX) products during (patho)physiological conditions (Brozovich et al. 2016).

In the human body, copper (Cu) is involved in mitochondrial respiration, synthesis of melanin and cross-linking of collagen and elastin. On the kynurenine pathway of tryptophan metabolism, Cu regulates the activity of enzymes (Majewski et al. 2016), which can generate toxic products when dysregulated (Majewski et al. 2018). Cu ions in the free state promote formation of ROS, and thus are bound to the Cu carrier, ceruloplasmin (Cp), to prevent oxidative damage. Cu is able to modulate systemic inflammation by inducing arachidonic acid conversion and regulating prostaglandin (PG) synthesis (Wang et al. 1992, Majewski et al. 2019b). Endothelial dysfunction is usually associated with Cu deficiency, although Cu accumulation can also induce a variety of cardio-vascular alterations (Majewski et al. 2017, 2019*b*).

Cu bioavailability in the body can be increased by administration of this trace element in the form of Cu nanoparticles, CuNPs (Ognik et al. 2019). However, due to the catalytic properties of dietary CuNPs, they can induce toxic effects on an organism as a result of elevated production of ROS and modified activity of antioxidant enzymes (Ognik et al. 2019). This is contrary to the previously described beneficial anti-diabetic and cardioprotective role of CuNPs (Sharma et al. 2016, 2018) with decreased production of inflammatory mediators (Cholewińska et al. 2018) and increased blood catalase and plasma antioxidant capacity, measured as the ferric reducing ability of a sample, FRAP (Majewski et al. 2019b).

Dietary CuNPs have been recently studied for vascular regulations with a great focus on the vasodilatory mechanism(s); and the nitric oxide (NO) synthase and COX have been described as the target of dietary CuNPs (Majewski et al. 2017, 2019*b*). However, the mechanism(s) involved in the vascular contraction induced by supplementation with CuNPs have not been studied yet.

First, we aimed to examine whether the blood plasma Cu-Zn and Cu-Cp ratios are modified after dietary replacement of $CuCO_3$ with CuNPs. Next, we studied receptor dependent and/or independent mechanism(s) of vascular contraction to potassium chloride (KCl), noradrenaline (NA), endothelin-1

(ET-1) and prostaglandin F_2-alpha (PGF_2-alpha). Last but not least, we aimed to compare the effects of Cu supplemented diets with a Cu deficient diet.

MATERIALS AND METHODS

All procedures were approved by the Local Ethics Committee for Animal Experiments (65/2017) according to the European Union guidelines (Directive 2010/63/EU for animal experiments) and conform 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).

Animals and experimental protocol

Healthy male albino Wistar rats (Han IGS rat [Crl: WI(Han)]) were housed individually in stainless steel cages under a stable temp. of 21-22°C, a ventilation rate of 20 air changes per hour and a relative humidity of 50 ± 10%.

At 4 weeks of age, rats were randomly divided into three groups of 10 animals each. Rats were fed for 8 weeks a diet supplemented with a standard dose of Cu (6.5 mg Cu kg^{-1} of diet), either as CuNPs of 40-60 nm diameter or $CuCO_3$ – the control diet. Experimental rats not supplemented with Cu were defined as a negative control – CuD. The accepted dose of Cu in a daily diet of rats has been set in the range of 5-6.5 mg Cu kg^{-1} of diet (Majewski et al. 2019*b*, Ognik et al. 2019), and that was 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 an equivalent oil content.

During the experiment, 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 (Table 1).

Blood plasma Cu, Zn and Cp

At 12 weeks of age, rats were anaesthetized and 5 cm^3 of blood was taken from the *vena cava* and kept in tubes containing heparin + EDTA as an anticoagulant. Samples were centrifuged at 3000 x g for 10 min and blood plasma was separated and stored at –80°C until further analysis.

The concentrations of Cu and Zn (μmol dm^{-3}) were determined by the inductively coupled plasma optical emission spectrometry method (ICP-OES) with the certified reference material NIST1577C (bovine liver) used for quality control. The plasma ceruloplasmin (Cp) concentration (U dm^{-3}) was deter-

Table 1

Composition of basal experimental diet fed to rats

Item	Content (%)
Unchangeable ingredients	
casein[1]	14.8
DL-methionine	0.2
cellulose[2]	8.0
choline chloride	0.2
rapeseed oil	8.0
cholesterol	0.3
vitamin mix[3]	1.0
maize starch[4]	64.0
Changeable ingredient	
mineral mix[5,6,7]	3.5
Calculated content	
crude protein	13.5

Explanation:
[1] Casein preparation: crude protein 89.7%, crude fat 0.3%, ash 2.0% and water 8.0%.
[2] α-cellulose (SIGMA, Poznan, Poland), main source of dietary fiber.
[3] AIN-93G-VM (Reeves, 1997), g kg^{-1} mix: 3.0 nicotinic acid, 1.6 Ca pantothenate, 0.7 pyridoxine-HCl, 0.6 thiamin-HCl, 0.6 riboflavin, 0.2 folic acid, 0.02 biotin, 2.5 vitamin B-12 (cyanocobalamin, 0.1% in mannitol), 15.0 vitamin E (all-rac-α-tocopheryl acetate, 500 IU g^{-1}), 0.8 vitamin A (all-trans-retinyl palmitate, 500000 IU g^{-1}), 0.25 vitamin D-3 (cholecalciferol, 400000 IU g^{-1}), 0.075 vitamin K-1 (phylloquinone), 974.655 powdered sucrose.
[4] Maize starch preparation: crude protein 0.6%, crude fat 0.9%, ash 0.2%, total dietary fiber 0% and water 8.8%.
[5] (g kg^{-1}) mix: 357 – calcium carbonate anhydrous $CaCO_3$, 196 – dipotassium phosphate K_2HPO_4, 70.78 – potassium citrate $C_6H_5K_3O_7$, 74 – sodium chloride NaCl, 46.6 – potassium sulfate K_2SO_4, 24 – magnesium oxide MgO, 18 – microelement mixture[6], starch to 1 kg = 213.62.
[6] Microelements mixture (g kg^{-1} mix): 31 – iron (III) citrate (16.7% Fe), 4.5 – zinc carbonate $ZnCO_3$ (56% Zn), 23.4 – manganese (II) carbonate $MnCO_3$ (44.4% Mn), copper carbonate $CuCO_3$ (55.5% Cu)[7], 0.04 – potassium iodide KI, citric acid $C_6H_8O_7$ to 100 g.
[7] Changeable dietary ingredients in relation to copper level and citric acid: mineral mixture (the base according to NRC, 1995) with two copper sources (standard source and experimental nano-copper of 40 nm diameter): (i) Cu deficient diet 0 – $CuCO_3$, 40.7 – citric acid, (ii) Cu as carbonate $CuCO_3$ 1.85, citric acid 39.21, (iii) copper-nanoparticles 0 $CuCO_3$, 40.7 citric acid.

mined 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

Rats were sacrificed by decapitation and a thoracic aorta isolated from each animals was cleaned of adherent tissue, and cut into 6-8 aortic rings of 3 to 4-mm length. Aortic rings were suspended horizontally under a resting tension of 1 g (determined during preliminary experiments) in 5-cm^3 tissue baths (stagnant Graz Tissue Bath System) containing Krebs-Henseleit solution (KHS) in mmol dm^{-3}: NaCl – 115; $CaCl_2$ – 2.5; KCl – 4.6; KH_2PO_4 – 1.2; $MgSO_4$ – 1.2; $NaHCO_3$ – 25; glucose – 11.1. 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 HSE) to measure and analyze (LabChart 8 software) the isometric force.

After the initial equilibration period of 60 min, a contractile response was elicited by a single depolarizing concentration of KCl (75 mmol dm^{-3}) to check the functional integrity.

The rings were rinsed 3 times with KHS for 60 min, and then cumulative concentration-response curves to NA (0.0001-10 μmol dm^{-3}), ET-1 (0.001-1 μmol dm^{-3}) and PGF_2-alpha (0.001-1 μmol dm^{-3}) were plotted. Only one cumulative concentration-response curve was performed on each aortic ring.

Drugs and reagents

The drugs used were: NA as hydrochloride, ET-1, KCl (Sigma-Aldrich) and PGF_2-alpha (Cayman chemical). Stock solutions (10 mmol dm^{-3}) of these drugs were prepared in distilled water, except for NA which was dissolved in NaCl (0.9%) + ascorbic acid (0.01% w v^{-1}) solution. These solutions were maintained at –20°C and appropriate dilutions were made in KHS on the day of the experiment.

The bovine liver (NIST1577C) was sourced from Sigma-Aldrich and $CuCO_3$ (purity ≥ 99%) was obtained from Poch (Gliwice, Poland). The CuNPs (40-60 nm size metal nanopowder, 12 $m^2 g^{-1}$) were purchased from Sky Spring Nanomaterials, Inc. (Houston, TX, US). The nanoparticles had 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.

Data analysis and statistics

The calculations and graphs were supported by the software GraphPad Prism 7. Vasoconstriction induced by KCl, NA, ET-1 and PGF_2-alpha was represented in mg of developed tension.

To determine the maximal response (E_{max}) and the potency of the drug (pD_2 calculated as the negative logarithm of the concentration causing a half-maximum effect), the values were obtained from individual concentration-response curves. The results thus obtained were compared by analysis of variance (ANOVA) with the Tukey's multiple comparisons test. Results were expressed as means ± SEM of n = 6-10 rats. A value of $P \leq 0.05$ was considered to be significant.

RESULTS

The final body weight of Cu supplemented rats did not differ between the groups (in g, CuNPs: 414.9 ± 8.043 *vs.* $CuCO_3$: 396.6 ± 5.534, $P = 0.15$). However, significant decrease in body weight was observed in CuD rats compared to rats supplemented with CuNPs (in g, CuD: 386.2 ± 5.885 *vs.* CuNPs: 414.9 ± 8.043, $P = 0.02$).

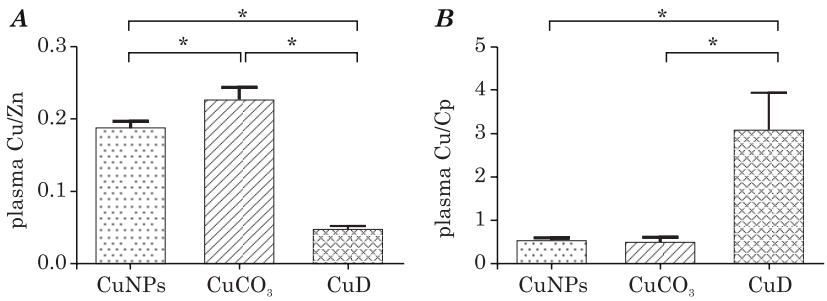

Fig.1. Effect of 8-week experimental treatment on the Cu-Zn (*A*) and Cu-Cp (*B*) ratio in the blood plasma of Wistar rats. Rats were fed with Cu (6.5 mg kg^{-1} diet) as 40 nm particles (CuNPs) and Cu carbonate ($CuCO_3$). The rats not supplemented with Cu were defined as the negative control (CuD). Results are expressed as means ± SEM, of $n = 8$ rats, $*P \leq 0.05$ (ANOVA with the Tukey's multiple comparisons test)

Blood plasma Cu-Zn and Cu-Cp ratio

Experimental treatment with CuNPs markedly reduced the blood plasma Cu-Zn ratio to 0.83-fold, compared to animals treated with $CuCO_3$ ($P < 0.0001$). In addition, the plasma Cu-Zn ratio was increased in rats supplemented with CuNPs and $CuCO_3$ by 3.83- and 4.60-fold, respectively, compared to CuD group (Figure 1*a*).

The blood plasma Cu-Cp ratio was not modified in CuNPs treated rats ($P = 0.25$). However, supplementation with CuNPs and $CuCO_3$ resulted in the Cu-Cp ratio being decreased by 0.18- and 0.17-fold, respectively, compared to the CuD diet (Figure 1*b*).

Dietary replacement of $CuCO_3$ with CuNPs resulted in decreased blood plasma Cu and Cp, while the Zn content remained unchanged (Table 2).

Table 2

Blood plasma Cu, Zn and Cp content in rats fed with experimental diets

Treatment	Cu	Zn	Cp
CuNPs	$15.79 \pm 0.409^{a,b}$	83.1 ± 0.8735^{b}	$28.01 \pm 0.731^{a,b}$
$CuCO_3$	18.35 ± 0.379^{b}	80.52 ± 1.132	36.17 ± 3.128^{b}
CuD	3.764 ± 0.371	75.72 ± 2.786	1.54 ± 0.327
Value of *P*	≤0.001	≤0.019	≤0.012

Data are expressed as means ± SEM of $n = 6$-10 rats; ^{a} *vs.* $CuCO_3$, ^{b} *vs.* Cu deficient ($P \leq 0.05$; one-way ANOVA with the Tukey's multiple comparisons test). CuNPs – Cu as nanoparticles, $CuCO_3$ – Cu as carbonate, CuD – Cu deficient diet

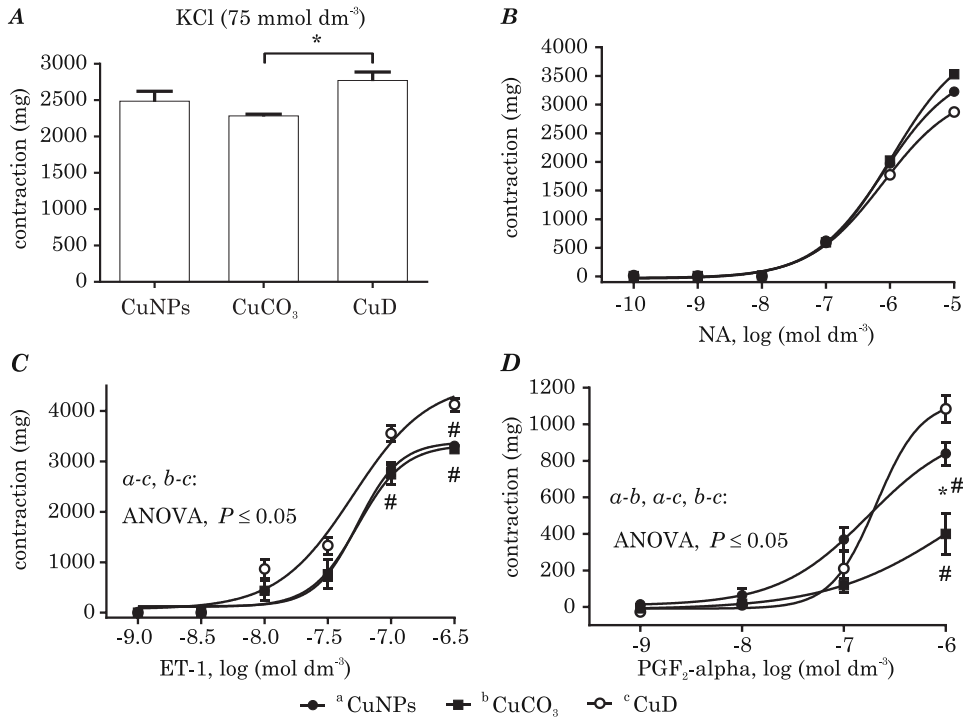

Fig. 2. Effect of 8-week experimental dietary treatment on the concentration-dependent contraction to potassium chloride (*A*), noradrenaline (*B*), endothelin-1 (*C*) and prostaglandin F_2-alpha (*D*) in aortic rings from Wistar rats. Rats were supplemented with Cu (6.5 mg kg^{-1} diet) either as CuNPs of 40 nm or as $CuCO_3$. The rats not supplemented with Cu were defined as the negative control – CuD. Results are expressed as means ± SEM, of $n = 8$ rats, **vs.* $CuCO_3$, #*vs.* CuD ($P \leq 0.05$, ANOVA with the Tukey's multiple comparisons test)

Vascular reactivity studies

Aortic rings obtained from Cu supplemented rats contracted in a similar way subjected to either a single dose of KCl (75 mmol dm^{-3}) or cumulative concentrations of NA and ET-1 (Figures 2*A-C*). In contrast, supplementation with CuNPs resulted in a marked increase in the PGF_2-alpha-induced contraction compared to the $CuCO_3$ group (Figure 2*D*).

The CuD diet resulted in a marked, 1.21-fold increase in the KCl-induced contraction of aortic rings compared to $CuCO_3$ treated rats, an effect which was unobserved in CuNPs treated rats ($P \leq 0.05$) – Figure 2*A*.

Moreover, the CuD diet did not modify the contractile response to NA, while increasing the response to ET-1 and PGF_2-alpha, compared to Cu supplemented rats (Figures 2*C-D*).

The E_{max} and pD_2 parameters are presented in Table 3.

Table 3

Changes in the maximal responses (E_{max}, expressed as a mg of contraction) and pD_2 parameters to noradrenaline, endothelin-1 and prostaglandin F_2-alpha in isolated aortic rings from rats fed with experimental diets

Treatment	CuNPs		$CuCO_3$		CuD	
	E_{max} (mg)	pD_2	E_{max} (mg)	pD_2	E_{max} (mg)	pD_2
Noradrenaline	3683 ± 260.4	6.099 ± 0.109	4157 ± 422.9	5.988 ± 0.153	3288 ± 508.4	6.111 ± 0.244
Endothelin-1	3387 ± 232.3^b	7.265 ± 0.051	3327 ± 325.8^b	7.265 ± 0.079	4575 ± 448.5	7.302 ± 0.085
Prostaglandin F_2-alpha	990.7 ± 336.8^{a,b}	6.759 ± 0.397^a	903.4 ± 65.55^b	5.876 ± 8.461	1133 ± 77.82	6.683 ± 0.163

Data are expressed as means ± SEM of n = 6-10 rats; ^{a} *vs.* $CuCO_3$, ^{b} *vs.* Cu deficient ($P \leq 0.05$; one-way ANOVA with the Tukey's multiple comparisons test). CuNPs – Cu as nanoparticles, $CuCO_3$ – Cu as carbonate, CuD – Cu deficient diet

DISCUSSION

We found that the blood plasma Cu-Zn ratio was decreased in rats supplemented with CuNPs compared to $CuCO_3$ treated rats. This was due to decreased blood plasma Cu content, and is consistent with the results obtained previously by our research team (Cholewińska et al. 2018, Majewski et al. 2019*b*). We also described previously that CuNPs were more effective than $CuCO_3$ in inhibiting oxidation and nitration of DNA and proteins, although a dietary CuNPs treatment enhanced lipid oxidation processes (Ognik et al. 2019). In addition, antioxidant mechanism(s) of blood, reflected as elevated catalase and plasma FRAP, became more intensive during CuNPs supplementation (Majewski et al. 2019*b*).

In the presence of inflammatory factors, intrinsic mechanism(s) may increase the blood concentration of Cu and decrease Zn, thus a high blood Cu-Zn ratio constitute an important marker describing (patho)physiological conditions. While aging, there is a higher incidence of oxidative stress and inflammation, which are associated with an increased risk of cardiovascular disorders, hence the Cu-Zn ratio could be measured to recognize ongoing processes and to allow timely intervention (Malavolta et al. 2015).

Cp is routinely measured in clinical chemistry as an immunoreactive protein, and variations in Cp levels indicate Cu toxicity as well as serious disorders (Connemann et al. 2010). In other study blood Cp concentration has been increased during exposure to cytokines (Malavolta et al. 2015). In addition, increased oxidative stress observed during aging may promote an increase in the blood Cu-Cp levels (Massie et al. 1979). In our study, we did not observe any changes to the plasma Cu-Cp ratio between Cu supplemented rats, although the blood plasma Cu and Cp decreased in rats supplemented with CuNPs. Moreover, the CuD diet resulted in an increased Cu-Cp ratio, which was due to the decreased blood plasma Cu and Cp content, and this is consistent with our previous results (Ognik et al. 2019, Majewski et al. 2019*b*).

In this paper, we are reporting, for the first time, that dietary replacement of $CuCO_3$ with CuNPs resulted in an enhanced maximum tension generated by PGF_2-alpha in aortic rings from Wistar rats. This adds to our previous results, where a nonselective inhibitor of COX, indomethacin, did not modify the ACh-induced response, suggesting that the net vasodilator effect had been lost (Majewski et al. 2019*b*). These data provide evidence that CuNPs may favor the onset of a pro-inflammatory environment within the vasculature. Surprisingly, the contractile-response to KCl, NA and ET-1 in our study was not modified in rats supplemented with CuNPs. This indicates that the intrinsic ability of smooth muscles to contract was intact and the alpha-adrenergic receptor and ET receptor dependent mechanisms for vasoconstriction were not altered.

Moreover, we also observed, unreported ever before, an increased response to PGF_2-alpha in CuD group compared to Cu supplemented rats, which points to the greater role of this prostanoid during Cu deprivation. Another important finding of the present study is that the submaximal contraction induced by direct depolarization of the vascular smooth muscle to KCl was enhanced compared to $CuCO_3$ treated rats but not CuNPs ones. The KCl-induced contraction increases calcium entry *via* voltage-operated calcium channels (VOCC) in smooth muscles, and Cu deficiency interferes with this mechanism. In addition, we examined the role of the alpha-adrenergic receptor agonist, NA which involves Ca^{2+} influx through VOCC and voltage-independent Ca^{2+} channels – SOCC (Miwa et al. 2005). However, we observed no difference in NA-induced contraction, which is opposite to the previously described augmented contraction to NA in female Wistar rats (Kitano 1980). The endothelium produces ET-1, a major vasoconstrictor peptide which binds to its receptors (typically ETA receptor) and activates Ca^{2+} influx through voltage-independent Ca^{2+} channels (NSCC-1, NSCC-2 and SOCC) (Miwa et al. 2005), therefore aortic contraction to ET-1 was used to assess the Ca^{2+} influx in CuD diet. We observed, for the first time, an increased contraction to ET-1 in CuD rats. The above results provide evidence that a different Ca^{2+} channel influx is involved in the Cu dependent vascular response. PGF_2-alpha elevates blood pressure and promotes atherosclerosis (Yu et al. 2009). PGF_2-alpha is formed by reduction of PGH_2 but it can also be formed from other PGs such as PGE_2 and PGD_2, thus the participation of other PGs and other PG receptors cannot be ruled out. Endothelium-derived PGF_2-alpha can influence vascular contraction either directly or through potentiation of the response to other endogenous vasoconstrictors. PGF_2-alpha dose-dependently elevates blood pressure *via* direct activation of the PGF (FP) receptors despite the absence of detectable FP receptor expression site in the rat aorta (Yu et al. 2009). Under certain conditions, PGs can act through other receptors in the aorta, and this is likely to be the thromboxane (TP) receptor (Kang et al. 1996). Moreover, in arteries, PGF_2-alpha increases ROS formation and induces vascular smooth muscle cell hypertrophy (Rice at al. 2008). These have further impact on NO synthase activity as well as on

the basal release of NO (Perez Martinez et al. 1998). The biologically active isomers, F_2 isoprostanes, are produced both enzymatically and by lipid peroxidation induced by ROS (Jiang et al. 2003). Enhanced lipid peroxidation, as the response to dietary supplementation with CuNPs, has been previously described in Wistar rats (Ognik et al. 2019).

Little is known about the ionic mechanisms that mediate the contractile response to PGF_2-alpha. However, Cl^- channel activation is an important mechanism mediating vascular smooth muscle contraction in response to PGF_2-alpha (Jiang et al. 2003).

CONCLUSIONS

The results indicate that 8 weeks of CuNPs supplementation with 6.5 mg Cu kg^{-1} in a diet may favor the onset of a pro-inflammatory environment. Given the significant role of PGF_2-alpha in the vascular tone regulation, it is likely that alterations in Cl^- channels as well as TP receptors function and/or expression represent an important mechanism in the blood flow regulation during supplementation with CuNPs.

Conflict of interest

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

Author contribution

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

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