Magnesium nitrate attenuates blood pressure rise in SHR rats

Reinis Vilskersts\textsuperscript{1,2}, Janis Kuka\textsuperscript{1}, Edgars Liepinsh\textsuperscript{1}, Helena Cirule\textsuperscript{1}, Anita Gulbe\textsuperscript{1}, Ivars Kalvinsh\textsuperscript{1}, Maija Dambrova\textsuperscript{1,2}

\textsuperscript{1}Latvian Institute of Organic Synthesis, Aizkraukles Str. 21, Riga, LV-1006, Latvia; \textsuperscript{2}Rigas Stradins University, Dzirciema Str. 16, Riga, LV-1007, Latvia

Correspondence: R Vilskersts. Latvian Institute of Organic Synthesis, Aizkraukles Str. 21, Riga LV-1006, Latvia
<Reinis.Vilskersts@biomed.lu.lv>

Abstract. The administration of magnesium supplements and nitrates/nitrites decreases arterial blood pressure and attenuates the development of hypertension-induced complications. This study was performed to examine the effects of treatment with magnesium nitrate on the development of hypertension and its complications in spontaneously hypertensive (SHR) rats. Male SHR rats with persistent hypertension at the age of 12-13 weeks were allocated to two groups according to their arterial blood pressure. Rats from the control group received purified water, while the experimental animals from the second group received magnesium nitrate dissolved in purified water at a dose of 50 mg/kg. After four weeks of treatment, blood pressure was measured, the anatomical and functional parameters of the heart were recorded using an ultrasonograph, vascular reactivity was assayed in organ bath experiments and the cardioprotective effects of magnesium nitrate administration was assayed in an \textit{ex vivo} experimental heart infarction model. Treatment with magnesium nitrate significantly increased the nitrate concentration in the plasma (from 62 ± 8 \textmu mol/l to 111 ± 8 \textmu mol/L), and attenuated the increase in the arterial blood pressure. In the control and magnesium nitrate groups, the blood pressure rose by 21 ± 3 mmHg and 6 ± 4 mmHg, respectively. The administration of magnesium nitrate had no effect on the altered vasoreactivity, heart function or the size of the heart infarction. In conclusion, our results demonstrate that magnesium nitrate effectively attenuates the rise in arterial blood pressure. However, a longer period of administration or earlier onset of treatment might be needed to delay the development of complications due to hypertension.

Key words: magnesium nitrate, hypertension, spontaneously hypertensive rat, heart infarction, vascular reactivity

Essential hypertension is a disease that affects more than 1.5 billion people worldwide \cite{1}. Elevated blood pressure is considered to be a main risk factor for the development of various diseases involving more than just the cardiovascular system \cite{2}. Thus, the discovery of new antihypertensive drugs remains a challenging field of research. Although new drug candidates and novel surgical approaches to treat hypertension are still being developed, it has been shown that elevated blood pressure can be lowered with lifestyle changes and dietary supplementation with relatively inexpensive macro-elements \cite{3} and inorganic ions such as nitrate and nitrite \cite{4, 5}.

Nitrite and nitrate are naturally occurring anions found in vegetables and meats. For a long time, it was thought that the ingestion of both of these nitrogen-containing anions increased the incidence of oncological diseases. However, recent data from toxicological studies do not support this statement \cite{6}. Instead, it has been concluded that dietary nitrates and nitrites may play a significant role in cardiovascular health \cite{4, 5}.
Magnesium nitrate attenuates blood pressure rise

In previous studies, treatment with nitrates or nitrites has lowered blood pressure in experimental animals [7, 8] as well as in humans [9]. Bryan and colleagues demonstrated that the administration of nitrite and nitrate protected the heart against ischemia/reperfusion injury [10]. Moreover, treatment with nitrate and nitrite has demonstrated vasoprotective effects [9, 11, 12]. The pharmacological effects of nitrates and nitrites have mainly been attributed to their ability to be converted in vivo to nitric oxide (NO) [4, 5].

Magnesium supplementation has shown similar cardiovascular effects as nitrate and nitrite administration [13]. As with nitrate administration, magnesium supplementation has been shown to reduce blood pressure in retrospective clinical studies [3] as well as in an experimental animal model [14]. The administration of additional magnesium in an experimental model of atherosclerosis in cholesterol-fed rabbits attenuated the development of atherosclerotic lesions [15]. Moreover, the vasoprotective effects of magnesium supplementation were observed in an experimental model of autosomal recessive, ectopic mineralising disorder [13], and in a case of balloon injury-induced endothelial dysfunction [16]. The pharmacological effects of magnesium ions have been attributed to their ability to act as an antagonist of calcium channels. In addition, magnesium ions stimulate the production of NO and vasodilator prostacyclins, and alter the vascular responses to vasoconstrictive agents [17].

In this study, we evaluated the effects of concomitant treatment with both magnesium ions and nitrate in the form of magnesium nitrate on the development of hypertension and its complications. In previous studies, it has been shown that in cases of the simultaneous administration of two or more different blood pressure lowering drugs, the hypotensive effect is potentiated [18, 19]. Thus, we hypothesised that treatment with both ions might have synergistic, hypotensive effects.

The effect of the administration of magnesium nitrate was studied in an experimental model of hypertension, spontaneously hypertensive (SHR) rats, which is a widely used experimental model for the study of blood pressure lowering agents [20]. The dose of magnesium nitrate was chosen to be equimolar to the dose of nitrates used in a previous study [10] with the corresponding amount of magnesium.

Materials and methods

Chemicals

Sodium pentobarbital (Narkodorm) solution was purchased from Alfasan (Woerden, The Netherlands). Heparin sodium was purchased from Panpharma (Fougeres, France). Magnesium nitrate hexahydrate, sodium chloride, potassium chloride, calcium chloride dihydrate, magnesium chloride hexahydrate, sodium hydrogen carbonate, potassium dihydrogenphosphate, glucose, ethylenediaminetetra-acetic acid (EDTA), L-phenylephrine hydrochloride, sodium nitroprusside (SNP) and acetylcholine chloride were purchased from Sigma (Schnelldorf, Germany).

Experimental animals

Male, 8- to 9-week-old SHR rats (n=18), weighing 140 to 150 g were obtained from the Harlan Laboratories (Great Britain) and housed under standard conditions (21-23°C, 12-hour light/dark cycle, relative humidity 45%-65%), with unlimited access to food (R70 diet, Lactamin AB, Kimstad, Sweden) and water. The R70 diet contained 0.25% w/w magnesium. The rats acclimatised to their new conditions for four weeks before the beginning of the study.

The experimental procedures were carried out in accordance with the guidelines of the European Community, and local laws and policies and were approved by the Latvian Animal Protection Ethical Committee of the Food and Veterinary Service, Riga, Latvia.

All experimental procedures and analyses were performed by a person blinded to the treatment groups.

Treatment and experimental protocol

Systolic blood pressure was measured in conscious SHR rats at the beginning of the study using an ML125 Non-Invasive Blood Pressure Controller connected to a PowerLab8/30 system (ADInstruments, Chalgrove, UK) and a PC. Afterwards, the experimental animals were divided into two groups so that the average systolic blood pressure in both groups was similar. The animals in the first experimental group received magnesium nitrate for four weeks at a dose of 50 mg/kg together with
purified water, while the rats in the second group (control group) received purified water alone. The dosage of magnesium nitrate was confirmed by measuring the consumption of drinking water every two days and adjusting the concentration of the supplemented substance. The average concentration of magnesium nitrate in the purified water during the study was 0.5 mg/mL. After four weeks of treatment, the systolic blood pressure was measured in conscious rats from both groups. Twenty-four hours after the measurement of the systolic pressure, the experimental animals were anesthetised with sodium pentobarbital at a dose of 60 mg/kg. After the onset of anaesthesia, the animals were put in a decubitus position and the chest was shaved. During the echocardiographic procedure, the rats were allowed to breathe spontaneously. M-mode tracings of the left ventricle were recorded at the papillary muscle level using an iE33 ultrasonograph equipped with a linear L15-7io transducer (Philips Healthcare, Andover, USA). After the echocardiographic parameters were recorded, heparin (1 IU/g i.p.) was administered. Afterwards, the chest was opened, the heart was isolated to perform the experimental heart infarction ex vivo, and the aorta was excised to test the vascular reactivity. Blood (∼5 mL) was collected in heparin-containing test tubes and centrifuged at 3000 rpm for 10 min at 4°C. The plasma obtained was used for the quantification of nitrates and nitrites.

Organic chamber experiments

Vascular reactivity in aortic rings was examined in organ bath experiments as described previously [21]. In brief, the excised thoracic aorta was immersed in ice-cold Krebs-Henseleit (K-H) buffer solution and cleaned of fatty and connective tissue. The aorta was cut into 3- to 4-mm-long aortic rings that were suspended between two stainless steel hooks in a 20 mL organ bath filled with the K-H buffer solution. The bathing solution was continuously bubbled with 95% O₂ and 5% CO₂ and maintained at 37°C (pH of 7.3-7.5). The passive tension was set to 20 mN. After a period of equilibration (1 h), the maximal contractile function was assessed by the application of 60 mM KCl. The aortic rings were then precontracted to 60-80% of their maximum with phenylephrine, and the cumulative response to acetylcholine (10⁻¹⁰-10⁻⁵ M) was assessed. In addition, to test vascular smooth muscle function, the aortic rings were precontracted to the same level as in the previous experimental setup, and the cumulative response to SNP (10⁻¹⁰-10⁻⁵ M) was assessed. The relaxation of the aortic rings in response to acetylcholine or SNP was expressed as a percentage of the phenylephrine-induced constriction.

Experimental heart infarction ex vivo

The infarction study was performed according to the Langendorff technique as described previously with slight modifications [22]. The hearts were perfused in retrograde with an oxygenated K-H buffer solution via the aorta at a constant pressure of 60 mmHg. A water-ethanol mixture (1:1)-filled balloon, connected to a physiological pressure transducer (ADInstruments, Chalgrove, UK) was inserted into the left ventricle, and the baseline end-diastolic pressure was set between 5 and 10 mmHg. The heart rate, left ventricle-developed pressure (LVDP), contractility, and relaxation were recorded continuously using the PowerLab8/30 system from ADInstruments. Coronary flow was measured using an ultrasound flow detector system connected to a PowerLab8/30. A 4-0 coated, braided silk suture (Sofsilk; Syneture, Dublin, Ireland) was passed under the left anterior descending coronary artery and threaded through a small plastic tube to permit reversible occlusion of the coronary artery. The hearts were allowed to acclimatise for 20 minutes, and the occlusion was performed for 30 minutes by constricting the threads through the plastic tube. The reperfusion was achieved by releasing the threads. At the end of the 120-minute reperfusion, the amount of necrotic myocardium was quantified as described previously [23]. The infarct size was expressed as the percentage of necrosis in the risk zone.

Quantification of magnesium, nitrate and nitrite ions in blood plasma

The concentration of magnesium ions in plasma samples was measured in an accredited diagnostic laboratory (http://www.egl.lv), using automated diagnostic equipment.
Before the nitrates and nitrites were measured in the plasma, it was filtered through Amicon Ultra 10 kDa centrifugal filter devices to separate out the macromolecules. The nitrates and nitrites were quantified in the filtered plasma using commercially available kits for the measurement of nitrates and nitrites (Nitrate/Nitrite Fluorometric Assay Kit, Cayman Chemical Company, Ann Arbor, USA), according to the manufacturer’s instructions.

Statistical analysis
The results were expressed as the mean ± SEM. The Kolmogorov–Smirnov test was used to test the distribution of the results obtained. Because the data were normally distributed, the significance of the differences between the mean values was evaluated using parametric tests. Thus, the differences between the means were evaluated using a two-tailed t-test, and p values of less than 0.05 were considered significant.

Results

Excluded animals
During the administration period, two experimental animals from the magnesium nitrate receiving group died for unknown reasons. The lethality in magnesium group was apparently not due to the administration of magnesium nitrate, as weight increase in both groups was similar and rats from magnesium nitrate group did not show any sign of sickness or suffering.

Plasma levels of magnesium, nitrate and nitrite ions
Administration of magnesium nitrate at the dose of 50 mg/kg did not change the concentration of magnesium ions in plasma. The magnesium ion concentrations in control and treated group animals were 0.76 ± 0.05 mmol/L and 0.72 ± 0.01 mmol/L, respectively.

As shown in figure 1, the basal concentration of nitrates in the blood plasma from the control group animals was 62 ± 8 µmol/L. The administration of magnesium nitrate at a dose of 50 mg daily for four weeks increased the level of nitrate in the blood plasma to 111 ± 11 µmol/L (p<0.01). The concentration of nitrite in the plasma was below the detection level of the kit (<30 nM).

Effect of magnesium nitrate supplementation on blood pressure
Before the beginning of the treatment, the average blood pressure in the control group rats was 168 ± 5 mmHg, and in the magnesium nitrate-treated group animals the average blood pressure was 172 ± 3 mmHg. After the four-week treatment period, the average blood pressure in the control group was 188 ± 6 mmHg, but in the magnesium nitrate-treated group, the average blood pressure was 177 ± 3 mmHg (figure 2A). We did not observe any statistically significant difference between the mean values for the systolic blood pressure of both groups after the four-week treatment; even so, the increase in blood pressure in the magnesium nitrate-treated group was significantly (p = 0.02)
**Figure 2.** Systolic blood pressure in the SHR male rats before and after the four-week treatment with magnesium nitrate (A); alterations in systolic blood pressure (B). The administration of magnesium nitrate for four weeks did not induce statistically significant changes in the systolic pressure. However, the treatment with magnesium nitrate significantly delayed the increase in the systolic blood pressure. The data are shown as the mean ± SEM from seven to nine animals; *p<0.05, Student’s t-test.

Effect of magnesium nitrate supplementation on echocardiographic parameters

The treatment with magnesium nitrate at a dose of 50 mg/kg did not influence the echocardiographic parameters of the heart, and there were no statistically significant differences between the echocardiographic parameters, including the interventricular septal thickness at end-diastole, left ventricular posterior wall thickness at end-diastole, left ventricular internal dimension at end-diastole, left ventricular ejection fraction, left ventricular fractional shortening, and the heart to body mass index of the rats from both groups.

Effect of magnesium nitrate supplementation on vascular reactivity

As can be observed in **figure 3A**, the four-week treatment with magnesium nitrate had no influence on the endothelium-dependent vasodilation in the isolated thoracic aortic rings. The maximal relaxation observed in the isolated aortic rings was ~50-60% of the Phe-induced contraction. In addition, the treatment with magnesium nitrate did not influence the reactivity of the vascular rings to SNP (**figure 3B**).

**Effect of magnesium nitrate supplementation on infarct size**

The cardioprotective effect of the administration of magnesium nitrate was studied in the experimental heart infarction model *ex vivo*. As can be observed in **figure 4A**, there was no difference between the mean values of the area-at-risk between the groups. The average area-at-risk was from 55 to 60% of the left ventricle. The analysis of the stained heart slices revealed that there was no statistically significant difference between the sizes of the heart infarct. The average infarction size was 33 ± 5 in the control group and 40 ± 5% in the magnesium nitrate group (**figure 4B**). The analyses of the heart rate, LVDP, contractility, relaxation and coronary flow...
Magnesium nitrate attenuates blood pressure rise

Figure 3. Effects of magnesium administration on endothelium-dependent (A) and endothelium-independent (B) vasorelaxation. The treatment with magnesium nitrate for four weeks did not influence endothelium-dependent or endothelium-independent relaxation in the SHR rats. The data are shown as the mean ± SEM from seven to nine animals.

Figure 4. Effects of magnesium nitrate treatment on infarct size in an experimental heart infarction model ex vivo. The size of the area at risk (A) and the size of the infarction (B) in the experimental model of heart infarction ex vivo. The administration of magnesium nitrate at a dose of 50 mg/kg for four weeks had no effect on the infarct size. The data are shown as the mean ± SEM from seven to nine animals.
during the experiment revealed that there were no differences between the experimental and control groups.

Discussion

The results of the present study demonstrate that the administration of magnesium nitrate at a dose of 50 mg/kg for four weeks increased the nitrate concentration in the plasma and, at the same time, significantly attenuated the increase in the systolic blood pressure in hypertensive SHR rats. In addition, the four-week treatment with magnesium nitrate had no effect on the altered vasoconstriction, heart function or size of the heart infarction.

The effects of the treatment with magnesium and nitrate/nitrite separately on the development of hypertension in SHR rats have been studied before [8, 14]. In comparison with previously published data, in which treatment with magnesium ions alone was used [14], we used a much shorter treatment period (only 4 weeks), and SHR rats with more severe hypertension (figure 2A), which has been shown to limit the hypotensive action of the magnesium administration [14]. Nevertheless, a statistically significant attenuation of the increase in systolic blood pressure was observed (figure 2B). Thus, peroral administration of magnesium nitrate could be an effective approach to attenuate the rise in blood pressure, even in cases where elevated blood pressure has been persistent for longer time periods.

Recent data have demonstrated that an intravenous bolus administration of nitrite, dose-dependently lowered the blood pressure in SHR rats [8], and the peroral administration of nitrates to rats with unilateral nephrectomy, which were fed on a high salt diet, dose-dependently lowered the blood pressure and simultaneously increased the concentration of nitrates in plasma [7]. The analysis of the plasma samples from our experimental animals revealed that rats receiving peroral magnesium nitrate had increased plasma nitrate levels (figure 1); however, there was no significant correlation between the plasma concentration of nitrates and the changes in the systolic blood pressure (data not shown). Moreover, it has been shown that supplementation with magnesium ions also decreases elevated blood pressure [13, 14]. Analysis of plasma samples revealed that there was no difference between magnesium ion concentrations in plasma from animals from both groups. Magnesium ions are mainly found intracellularly and only ~0.3% of the total magnesium pool is present in the serum, the serum concentration usually does not reflect the real magnesium pool in the tissues [13], and additional supplementation will not alter the concentration of magnesium ions in the plasma.

Nitrate/nitrite administration, and thus increased levels of both anions, is associated with vasoprotective effects [9, 11]. In the SHR rat model, endothelial dysfunction is characterised by unaltered nitric oxide synthesis, and the acetylcholine-induced endothelial synthesis of prostacyclines and prostaglandins induces vasoconstriction by overpowering nitric oxide relaxation [24]. Thus, the administration of additional nitrates or nitrites should not attenuate the development of endothelial dysfunction by providing additional nitric oxide. However, a long-term blood pressure decrease may inhibit the development of endothelial dysfunction, as it has been shown that the level of vasoconstriction correlates with the age of the SHR rats and the severity of hypertension [24]. There was no difference in the reactivity of the aortic rings to acetylcholine or SNP between the treated and untreated animals, which is most likely due to the relatively short treatment period. However, in an experimental model with a different mechanism for the development of endothelial dysfunction, longer administration or earlier onset of the treatment with magnesium nitrate could also attenuate the development of endothelial dysfunction.

The results from a previous study have shown that the administration of nitrates protects the myocardium from ischemia/reperfusion induced damage in healthy animals [10]. Recently Fantinelli et al. demonstrated that the heart tissues of SHR rats at the hypertensive stage are more susceptible to ischemia-reperfusion injury than the heart tissues of their healthy counterparts [25]. In our experimental setup, we used experimental animals with severe hypertension (figure 2A); the treatment with magnesium nitrate did not reduce the infarct size compared with the untreated rat group. Although the substances that demonstrate cardioprotective effects in healthy animals could be cardioprotective in unhealthy counterparts [25, 26], it has been shown that the hypertension in SHR rats can blunt the effects of post-conditioning [27] as well as pre-conditioning.
in older SHR rats [28]. Thus, the hypertension alters the activity and/or expression of proteins that are involved in cardioprotection, and if these proteins are involved in the cardioprotective action of the nitrates and nitrates, then the cardioprotective activity of both anions could be blunted.

In addition, a study published by Bryan et al., [10] showed that administration of nitrates protected heart against ischemia/reperfusion injury and increased the concentration of nitrates in plasma. It has been demonstrated that nitrates, via different molecular pathways, induce protective effects in tissues [29, 30]. In our experimental set-up, no increase in nitrates in the plasma of treated animals was observed; consequently, mean infarct size in both groups was similar.

In conclusion, the results from the present study demonstrate that the administration of magnesium nitrate attenuates the increase in systolic blood pressure after a relatively short administration period. However, a longer period of administration or an earlier onset of treatment might be needed to delay the development of complications due to hypertension such as hypertrophy of the left ventricle and altered vaso-reactivity.

Disclosure

Financial support: this study was supported by the Latvian State Research Program BIOMEDICINE and Rigas Stradins University research project No. RSU ZP 09/2013. Conflict of interest: none.

References


