TITLE: CHRONIC, LOW-DOSE TMAO TREATMENT REDUCES DIASTOLIC DYSFUNCTION AND HEART FIBROSIS IN HYPERTENSIVE RATS.

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Running title: TMAO improves cardiac parameters in hypertensive rats.

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**AUTHOR’S CONTRIBUTION**

Conception and design of the work: TH, AD, MU. Acquisition, analysis, and interpretation of hemodynamic data: TH, AD, MG, KB and MU. Acquisition, analysis, and interpretation of spectrometry data: ES, MD and MU. Acquisition, analysis, and interpretation of PCR data: MG, AWT, LP and MU. Acquisition, analysis, and interpretation of ELISA data: MK, EZ and MU. Acquisition, analysis, and interpretation of histopathology data: TH and MU. Drafting the work: MU. All authors reviewed the manuscript and approved the final version.

**ABSTRACT**

Several studies suggest negative effects of trimethylamine oxide (TMAO) on the circulatory system. However, a number of studies showed protective functions of TMAO, a piezolyte and osmolyte, in animals exposed to high hydrostatic and/or osmotic stress. We evaluated the effects of TMAO treatment on the development of hypertension and its complications in male, Spontaneously Hypertensive Rats maintained on water (SHR-WATER) and SHR drinking TMAO water solution from weaning (SHR-TMAO). Wistar-Kyoto rats (WKY) were used as normotensive controls to discriminate between age-dependent and hypertension-dependent changes. Telemetry measurements of blood pressure (BP) were performed in rats between 7th and 16th week of life. Anaesthetized rats underwent echocardiographic, electrocardiographic and direct left ventricular end-diastolic pressure (LVEDP) measurements. HE and van Gieson staining for histopathological evaluation were performed. Plasma TMAO measured by chromatography coupled with mass spectrometry was significantly higher in SHR-WATER than in WKY (≈20%). TMAO treatment increased plasma TMAO by 4-5-fold, and did not affect the development of hypertension in SHR. 16-week-old SHR-WATER and SHR-TMAO (12-week-TMAO-treatment) showed similar BP, angiopathy and cardiac hypertrophy. However, SHR-TMAO had lower plasma NT-proBNP, LVEDP, and cardiac fibrosis. In contrast to age-matched WKY, 60-week-old SHR showed hypertensive angiopathy and heart failure with preserved ejection fraction. In comparison to SHR-WATER, SHR-TMAO (56-week-TMAO-treatment) showed significantly lower plasma NT-proBNP and vasopressin, significantly lower LVEDP and cardiac fibrosis. In conclusion, 4-5-fold increase in plasma TMAO does not exert negative effects on the circulatory system. In contrast, increased dietary TMAO seems to reduce diastolic dysfunction in pressure-overloaded heart in rats.

**Keywords:** hypertension, cardiac fibrosis, heart failure, trimethylamine, trimethylamine N-oxide

**NEW AND NOTEWORTHY**

Hypertensive rats show higher plasma TMAO level than age-matched normotensive rats. Furthermore, chronic, low-dose TMAO treatment that increases plasma TMAO by 4-5 fold, reduces plasma NT-pro-BNP and vasopressin, left ventricle end-diastolic pressure and cardiac fibrosis in pressure-overloaded heart in hypertensive rats. Our study provides evidence that a moderate increase in plasma TMAO does not have a negative effect on the circulatory system. In contrast, increased dietary TMAO seems to reduce diastolic dysfunction in pressure-overloaded heart.

**INTRODUCTION**

Hypertension is a major risk factor of cardiovascular events. Recently it has been found that high plasma level of TMAO (trimethylamine N-oxide), a liver metabolite of gut bacteria-produced TMA (trimethylamine), is associated with an increased cardiovascular risk (18, 30, 35, 36, 42, 44). However, the mechanism of plasma TMAO increase as well as the effect of TMAO on the circulatory system is not clear. On the one hand, several experimental studies suggest that TMAO may produce harmful changes in the circulatory system including platelet hyperreactivity (56), decreased beta-oxidation of fatty acids in myocardiocytes (21), alterations in cholesterol and sterol metabolism (42), exacerbated inflammatory reactions of vascular wall and increased reactive oxygen species production (41). In addition, previously we found that a 2-week 100-fold increase in blood TMAO in normotensive rats prolonged the hypertensive effect of concomitantly given low-dose angiotensin II, however, it did not produce significant effects if given alone (45). On the other hand, several-fold higher TMAO plasma levels were observed in humans after ingestion of fish and vegetarian diet than after ingestion of red meat and eggs (4). Therefore, it seems that fish-rich and vegetarian diet, which is beneficial or at least neutral for cardiovascular risk, is associated with a significantly higher plasma TMAO than red-meat and eggs-rich diets which are considered to increase the cardiovascular risk (1, 16). Moreover, TMAO plays a protective role in deep-sea animals that are exposed to high hydrostatic pressure. Namely, deep-sea fishes use TMAO as a piezolyte, a molecule that counteracts the protein-destabilizing effects of hydrostatic pressures (47, 50). Finally, TMAO has been found to protect proteins from the destabilizing effects of urea, high temperature and NaCl (46) and as a protein stabilizer, it has been tested to treat protein-folding diseases (3, 40, 43, 53).

In this study we evaluated the effect of chronic 12-week and 56-week-long treatments with TMAO on the development of hypertension and its complications in spontaneously hypertensive rats.

**GLOSSARY**

**TMAO -** Trimethylamine N-oxide

**SHR -** Spontaneously Hypertensive Rats

**WKY -** Wistar-Kyoto rats

**SHR-WATER -** SHR drinking tap water

**SHR-TMAO -** SHR drinking TMAO-water solution

**BP -** Blood Pressure

**LVEDP -** Left Ventricular End-Diastolic Pressure

**HE -** Haematoxylin and Eosin stain

**NT-proBNP -** N-terminal pro B-type Natriuretic Peptide

**HR -** Heart Rate

**ABP -** Arterial Blood Pressure

**ECG -** Electrocardiography

**TMA -** Trimethylamine

**AT1a -** Angiotensin II receptors 1a

**AT2 -** Rngiotensin II receptors 2

**qRT-PCR -** quantitative Real-Time Polymerase Chain Reaction

**SBP -** Systolic blood pressure

**DBP -** Dystolic blood pressure

**HF -** Heart Failure

**GFR -** Glomerular Filtration Rate

**LVEDV** - left ventricle end diastolic volume

**LVESV** - left ventricle end systolic volume

**SV** – stroke volume

**EF** - ejection fraction

**IVSs** - interventricular septum diameter during systole

**METHODS**

**Animals**

The study was performed according to Directive 2010/63 EU on the protection of animals used for scientific purposes and approved by the I Local Bioethical Committee in Warsaw (permission:100/2016). Male, spontaneously Hypertensive rats (SHR) and normotensive Wistar-Kyoto rats (WKY) were obtained from the Central Laboratory for Experimental Animals, Medical University of Warsaw, Poland. Rats were housed in groups of 2-3 animals, in polypropylene cages with environmental enrichment, 12h light/12h dark cycle, temperature 22-23oC, humidity 45-55%, food and water ad libitum.

**Experimental protocol**

After weaning, 4-5 week-old SHR were maintained on tap water (SHR-WATER group) or water containing TMAO (abcr GmbH - Karlsruhe, Germany 333 mg/l, SHR-TMAO group). WKY rats (WKY group) were used as normotensive controls to discriminate between age-dependent and hypertension-dependent histopathological changes in SHR.

To choose a dose of TMAO, we did pilot studies with SHR receiving TMAO for 4 weeks at a dose of 1g/l (n=3), 0.333 g/l (n=3) and 0.1 g/l (n=3). We have chosen a dose that increased blood TMAO concentration 3-5 times to mimic possible physiological concentrations and to avoid suprapharmacological blood TMAO concentration.

**The effect of TMAO on the development of hypertension and its complications in 16-week-old rats**

6-week-old WKY (n=6), SHR-WATER (n=7) and SHR-TMAO (n=7) were implanted with telemetry transmitters (HD-S10, Data Sciences International, St. Paul, USA) under general anesthesia with ketamine (Bioketan 100 mg/ml, Vetoquinol Biowet, Poland) 100 mg/kg bw and xylazine (20mg/ml, Xylapan, Vetoquinol Biowet, Poland) 10 mg/kg bw i.p., as previously described (38). Continuous recordings of heart rate (HR) and mean arterial blood pressure (MABP) were started with ART software (Data Sciences International, St. Paul, USA) one week after the surgery and were performed continuously for 9 weeks. Afterwards the rats were maintained for two days in metabolism cages to evaluate 24hr water and food balance and to collect urine for biochemical analysis.

Next day, the rats were anaesthetized with urethane (1.5 g/kg bw i.p., Sigma-Aldrich, Poland) and the left femoral artery was cannulated with polyurethane catheter for ABP and HR recording. The recordings were started 40 minutes after the induction of anaesthesia and 15 minutes after inserting the arterial catheter. After 10 min of ABP recordings, Millar Mikro-Tip SPR-320 (2F) pressure catheter was inserted via the right common carotid artery and simultaneous left ventricle end-diastolic-pressure (LVEDP) and ABP recordings were performed. The catheter was connected to Millar Transducer PCU-2000 Dual Channel Pressure Control Unit (Millar, USA) and Biopac MP 150 (Biopac Systems, USA). For electrocardiogram (ECG) recordings, standard needle electrodes (Biopac Systems, USA) were used. After hemodynamic recordings, blood from the right ventricle of the heart was taken and rats were killed by decapitation. The heart, the kidneys and arteries were harvested for further analysis.

**Plasma and urine TMA, TMAO and general biochemistry evaluation**

Plasma and urine TMA and TMAO were evaluated using Waters Acquity Ultra Performance Liquid Chromatograph coupled with Waters TQ-S triple-quadrupole mass spectrometer. The mass spectrometer operated in the multiple-reaction monitoring (MRM) - positive electrospray ionization (ESI) mode, as previously described (13). Plasma and urine sodium, potassium, creatinine and urea were analyzed using Cobas 6000 analyzer (Roche Diagnostics, Indianapolis, USA).

**ELISA tests**

The following EIAab Kits (Wuhan EIAab Science (Co. Ltd., China) were used for the evaluation: NT-proBNP (cat. no. E0485r), Angiotensin II (cat. no. E0005Ge), Aldosterone (cat. no. E0911Ge), Vasopressin (cat. no. E1139Ge), TNF-α (cat. no. E0133r) and IL-10 (cat. no. E0056r). All procedure were performed according to the standard protocol by ELISA Kit Operating Instruction. The absorbance intensity was measured at 450 nm with the Multiskan microplate reader (Termo Fisher Scientific). All experiments were performed in doubles.

**Histopathological evaluation**

Tissues sections fixed in 10% buffered formalin were dehydrated using graded ethanol and xylene baths and embedded in paraffin wax. Sections of 3–4 μm were stained with haematoxylin and eosin (HE) and van Gieson stain (for connective tissue fibres). General histopathological examination was evaluated at magnification of 10x, 40x and 100x (objective lens) and 10x (eyepiece) and photographic documentation was made. Additionally, we performed morphometric measurements: width of cardiomyocytes, wall thickness of the aorta and carotid artery at magnification of 40x (objective lens). Level of fibrosis was calculated as a percentage of the connective tissue in the general surface of the myocardium. Micrographs were taken at the magnification of 40x (objective lens) and 10x (eyepiece), using a standard light microscope Olympus BX41 and CellSens software (Olympus Corporation, Tokyo, Japan). For each sample, 10 fields of view of myocardium in histological slides were evaluated. The percentage of fibrosis was calculated using Open Source software ImageJ (Fiji).

**qRT-PCR for cardiac expression of angiotensin system genes**

Approximately 25 mg of left ventricle was homogenized in lysis buffer (Qiagen, Germany) in a TissueLyser bead mixer (Qiagen, Germany) at 25 Hz in two 5-min repetitions.

Total RNA from tissue was extracted using EZ1 RNA Tissue Mini Kit and Biorobot EZ1 (Qiagen, Germany) according to the manufacturer's protocol. RNA was quantified by measuring λ=260nm absorbance using a spectrophotometer NanoDrop 1000. RNA samples were frozen and stored at −80°C for subsequent analysis.

Gene expression levels were assessed using real-time PCR (real-time polymerase chain reaction) and were measured with the ABI-Prism 7500 Sequence Detection System (Applied Biosystems, USA). Specific probes and primers of TaqMan Gene Expression Assays (Thermo Fisher Scientific; USA) for rat angiotensin II receptor type 1a, angiotensin II receptor type 2, angiotensinogen, as well as glyceraldehyde 3-phosphate dehydrogenase, were applied to each well of the 96-Well Optical Reaction Plate (Applied Biosystems, USA). All reactions were run in duplicate, NTC for tested genes in triplicate, and the threshold cycles (Ct) were determined.

**The effect of TMAO on hypertension-induced complications in 60-week-old rats**

60-week-old WKY (n=6), SHR-WATER (n=8) and SHR-TMAO (n=8) were maintained for two days in metabolism cages to evaluate 24hr water and food balance and to collect urine for biochemical analysis. Next day, the rats were anaesthetized with urethane (1.5 g/kg bw i.p., Sigma-Aldrich, Poland) and underwent echocardiography using a Samsung HM70 Ultrasound system equipped with a linear probe 5-13 mHz. The probe was placed on the shaved chest wall to obtain images from the right parasternal short-axis. After the echo examination, the left femoral artery and the right common carotid artery were cannulated for hemodynamic recordings. LVEDP, ABP, HR and histopathological and biochemistry tests were performed as described above for 16-week-old rats.

**Data analysis and statistics**

In chronic telemetry study, mean arterial blood pressure (MABP) and heart rate (HR) were calculated by ART software (Data Sciences International, St. Paul, USA). For the evaluation of MABP and HR changes in time, one-way analysis of variance (ANOVA) for repeated measures, followed by Tukey’s post hoc test was used. Differences between the groups were evaluated by multivariate ANOVA, followed by Tukey’s post hoc test.

In acute hemodynamic studies MABP, diastolic blood pressure (DBP), systolic blood pressure (SBP), heart rate (HR) and LVEDP were calculated on the BP tracing by AcqKnowledge 4.3.1 Biopac software (Biopac Systems, Goleta, USA). To evaluate ECG, the lead II wasused.

Differences between the groups were evaluated by one-way ANOVA, followed by Tukey’s post hoc test or by t-test, when appropriate. The Kolmogorov-Smirnov test was used to test normality of the distribution. A value of two-sided P<0.05 was considered significant. Analyses were conducted using Dell Statistica, version 13 (Dell Inc, Tulsa, USA).

**RESULTS**

**The effect of TMAO on the development of hypertension and its complications in 16-week-old rats**

**Chronic telemetry hemodynamic measurements**

Freely moving 7-week-old SHR showed a significantly higher MABP than WKY. There was no significant difference between 7-week-old SHR-WATER and SHR-TMAO in MABP and HR (Fig. 1). Treatment with TMAO did not affect the development of hypertension in SHR. Namely, SHR-TMAO and SHR-WATER showed a significant, gradual increase in MABP, which stabilized in 15/16-week-old rats. This was accompanied by a not significant decrease in HR. In contrast, WKY did not show any significant change in MABP and HR throughout the experiment (Fig. 1).

**Body mass and food intake**

There was no significant difference between 16-week-old WKY, SHR-WATER and SHR-TMAO in body mass. WKY showed a higher food intake than SHR. There were no significant differences in food intake between SHR-WATER and SHR-TMAO groups (Table 1).

**TMAO and TMA balance**

SHR-WATER showed a not significantly higher TMAO plasma level than WKY. SHR-TMAO showed a significantly higher plasma TMAO than SHR-WATER (4-fold increase). There was no significant difference in plasma TMA levels between groups. However, SHR-TMAO and SHR-WATER showed a significantly lower 24hr urine TMA excretion than WKY (Table 1).

**Water-electrolyte balance and kidney function**

SHR-WATER and SHR-TMAO showed a higher water intake than WKY. There was no significant difference in water intake and urine output between SHR-WATER and SHR-TMAO, however, SHR-TMAO showed moderately lower water intake and urine output than SHR-WATER (Table 1).

SHR-WATER showed a higher sodium plasma level and a lower 24hr sodium urine excretion than WKY. There was no significant difference between SHR-WATER and SHR-TMAO in those parameters (Table 1).

There was no significant difference between the groups in terms of plasma potassium, plasma urea, plasma creatinine levels and creatinine clearance (Table 1).

**Histopathology of the kidneys**

There were no evident pathological changes in 16-weeks-old rats in the kidney parenchyma (Fig. 2). However, SHR-WATER and SHR-TMAO exhibited thickening of the tunica media of the lobed and arcuate arteries, which were caused by hypertrophy and hyperplasia of smooth myocytes (Fig. 3 D,E,F).

**Hemodynamic and cardiac parameters**

Anaesthetized 16-week-old SHR showed a significantly higher MABP, SBP and a trend towards higher DBP (p=0.09) in comparison to WKY. There was no significant difference between SHR-WATER and SHR-TMAO in MABP, SBP and DBP (Table 1).

Directly measured LVEDP was significantly higher in SHR than in WKY. SHR-TMAO showed a moderately lower LVEDP than SHR-WATER (Table 1).

There was no significant difference between the groups in plasma NT-proBNP level, however, SHR-WATER showed a higher plasma NT-proBNP than SHR-TMAO and WKY (Table 1).

There was no significant difference between the groups in ECG morphology.

Heart mass was significantly higher in SHR than in WKY. SHR-TMAO showed a moderately lower heart mass than SHR-WATER (Table 1).

**Histopathology of the heart**

Morphometric analysis showed cardiomyocyte hypertrophy in SHR-TMAO and SHR-WATER. The increase in the width of cardiomyocytes in SHR-WATER was significantly higher than in SHR-TMAO (Fig. 4). Additionally, we found a connective tissue hyperplasia and perivascular fibrosis in the myocardium in SHR (Fig. 3 A,B,C and Fig. 4). Fibrosis was significantly greater in SHR-WATER than in SHR-TMAO (Fig. 4).

**Histopathology of arteries**

In SHR we found hypertrophy of smooth myocytes in the coronary arteries and carotid artery, which were more pronounced in SHR-WATER than in SHR-TMAO (Fig. 5). An increase in the wall thickness of aorta was comparable between SHR-WATER and SHR-TMAO (Fig. 3 G,H,I). In coronary and renal arteries we found hypertrophy of smooth myocytes and perivascular fibrosis, which were more pronounced in SHR-WATER than in SHR-TMAO (Fig. 3).

**The effect of TMAO on hypertension-induced complications in 60-week-old rats**

**Body mass and food intake**

There was no significant difference between 60-week-old WKY, SHR-WATER and SHR-TMAO in body mass and food intake (Table 2).

**TMAO and TMA balance**

SHR-WATER groups showed significantly higher TMAO plasma level than WKY. SHR-TMAO group showed a significantly higher plasma TMAO level than SHR-WATER group (5-fold increase). SHR-TMAO group showed a significantly lower plasma TMA level than SHR-WATER (Table 2).

**Water-electrolyte balance and kidney function**

There was no significant difference between the groups in water intake and urine output however, SHR showed a trend towards a higher water intake (P=0.2) and 24hr urine output (P=0.1) than WKY (Table 2).

There was no significant difference between the groups in plasma sodium level but SHR showed a significantly lower 24hr urine sodium excretion than WKY. SHR-TMAO showed a moderately higher 24hr urine output and 24hr urine sodium excretion than SHR-WATER (Table 2).

SHR-TMAO showed a significantly lower plasma creatinine and a significantly higher creatinine clearance than SHR-WATER (Table 2).

There was no significant difference in plasma Ang II and aldosterone level between groups whereas plasma vasopressin level was significantly lower in SHR-TMAO than in SHR-WATER (Table 2).

**Histopathology of the kidneys**

In contrast to WKY, SHR-WATER and SHR-TMAO showed arcuate and lobed arteries thickening caused by hyperplasia of smooth myocytes and moderate hyperplasia of the connective tissue accompanied by focal mononuclear cell infiltrates in the parenchyma, single casts in the renal tubules and weak to moderate glomerular collapse and glomerulosclerosis (Fig. 6 and Fig. 7). There were no evident differences between SHR-TMAO and SHR-WATER.

**Hemodynamic and cardiac parameters**

Anaesthetized 60-week-old SHR showed significantly higher MABP, SBP and DBP than WKY. There was no significant difference between SHR-WATER and SHR-TMAO in MABP and SBP whereas DBP was lower in SHR-TMAO (Table 2).

There were no significant difference between SHR and WKY in basic echocardiographic parameters including stroke volume and ejection fraction, however, SHR showed a trend towards an increased interventricular septum diameter (P=0.15) and an increased posterior wall diameter of the left ventricle (P=0.2) (Table 2), (Fig. 8).

LVEDP was significantly higher in SHR than in WKY. SHR-TMAO showed a significantly smaller (2-fold lower) LVEDP than SHR-WATER, (Fig. 9).

NT-proBNP level was significantly lower in SHR-TMAO than in SHR-WATER (Table 2).

There were no disturbances in heart rhythm in ECG recordings. SHR showed significantly wider QRS complexes than WKY. There was no significant difference between SHR-WATER and SHR-TMAO in ECG morphology (Table 2), (Fig. 10).

Heart mass was significantly higher in SHR than in WKY (Table 2).

**Histopathology of the heart**

There was an increase in the thickness of the left ventricular wall caused by significant hypertrophy of cardiomyocytes in SHR-TMAO and SHR-WATER. Morphometric analysis showed a significantly greater width of cardiomyocytes in SHR-WATER than in SHR-TMAO. Similarly, connective tissue hyperplasia in the myocardium was significantly greater in SHR-WATER than in SHR-TMAO (Fig. 11 and Fig. 7).

**Histopathology of arteries**

In contrast to WKY, SHR-WATER and SHR-TMAO showed hypertrophy of smooth myocytes, thickened connective tissue and endothelial damage (Fig.7 and 12). SHR-WATER and SHR-TMAO showed a significant increase in the thickness of the carotid artery wall (Fig. 12) and the aorta (Fig. 7). Additionally, in coronary and renal arteries we found hypertrophy of smooth myocytes and perivascular fibrosis (Fig. 7). Pathological changes in the coronary arteries were greater in SHR-WATER, whereas the changes in large arteries were similar between SHR-WATER and SHR-TMAO.

**Cardiac expression of genes of angiotensin system**

There was no significant difference in cardiac gene expression of angiotensinogen and angiotensin AT1a and AT2 receptors between SHR-WATER and SHR-TMAO groups (Fig. 13).

**DISCUSSION**

A new finding of our study is that 4-5-fold increase in plasma TMAO does not exert negative effects on the circulatory system. In contrast, a low-dose TMAO treatment is associated with reduced cardiac fibrosis and improved hemodynamic and biochemical parameters of failing heart in spontaneously hypertensive rats.

**The effect of TMAO on hypertension and pressure overloaded heart**

Essential hypertension is a major risk factor of cardiovascular events. Its etiology seems multifactorial and is poorly understood. Here, we did not find a significant effect of TMAO on the development of hypertension in SHR. Namely, both SHR-WATER and SHR-TMAO groups showed an increase in ABP between 7th and 15th week of life, and there was no difference in systolic, diastolic and mean ABP in 16-week-old rats.

Hypertension is known to produce a wide range of complications, such as hypertensive angiopathy, kidney failure and heart failure (HF). Accordingly, in SHR but not in age-matched WKY, we found multiorgan hypertensive angiopathy and water-electrolyte disturbances.

HF is a major complication of hypertension (6). Despite a significant progress in diagnosis and treatment, the mortality and cost of care due to HF remain very high (11). Patients with hypertension-induced heart disease usually present HF with preserved ejection fraction (diastolic HF) and an increased LVEDP which is associated with cardiomyocyte hypertrophy, cardiac hypertrophy and cardiac fibrosis (6, 14, 31). In the present study, 60-week-old SHR showed characteristic features of hypertension-induced diastolic HF including significantly increased heart mass, increased LVEDP, increased plasma NT-proBNP and vasopressin levels, mildly reduced stroke volume and substantial cardiac fibrosis. Interestingly, the hemodynamic and structural changes in the heart started to appear in 16-week-old SHR, i.e. 9-10 weeks after the onset of BP surge.

Strikingly, SHR treated with low-dose TMAO for 56 weeks showed significantly smaller hemodynamic, biochemical and histopathological indices of HF than untreated SHR. Namely, SHR-TMAO group had 2-fold lower LVEDP, significantly lower plasma NT-proBNP and significantly lower cardiac fibrosis. The positive effect of TMAO was also present in younger, 16-week-old SHR-TMAO group. Therefore, our findings strongly suggest that TMAO may have a beneficial effect on pressure overloaded-heart in hypertensive rats.

Several experimental studies suggest a negative effect of TMAO of the circulatory system (9, 21, 34, 54, 55). However, there are also some studies showing potentially beneficial effects of TMAO on the circulatory and the nervous system (5, 7, 20).

The discrepancy between our present findings and studies showing a negative effect of TMAO on the heart may result from several factors, such as tested doses of TMAO and experimental settings. For example, Yu et. al showed that TMAO increased the instability of atrial electrophysiology in normal canines, however, in contrast to our study, TMAO was injected locally i.e. atrial ganglionated plexi of the heart (54). Savi et al. showed that TMAO exposure worsened cardiomyocyte mechanics and intracellular calcium handling, however, the experiments were performed in vitro (34). Makrecka-Kuka et al. in in vivo experiments showed that increased plasma TMAO impairs pyruvate and fatty acid oxidation in cardiac mitochondria in mice. However, in the latter study, mice were treated with several times higher doses of TMAO than in our study, which was associated with a 22-23-fold increase in plasma TMAO level (21). Finally, Organ et al. found that heart failure severity is significantly enhanced in TAC mice fed TMAO-rich diet which increased plasma TMAO by 16-17-fold (27).

It seems that the design of our study may be more suitable for studying the effect of TMAO on the heart function, as data were gathered in the long-term in vivo experiments in which rats were treated with TMAO at a dose that may be achieved in real life conditions by consuming TMAO-rich food. Specifically, in our study, the concentration of TMAO in the plasma of TMAO-treated rats was only 4-5-fold higher than in untreated rats.

**Postulated mechanism of TMAO action on pressure-overloaded heart**

Research shows that pressure overload of the heart triggers transition of fibroblast to myofibroblast producing myocardial fibrosis due to accumulation of collagen (14), and that angiotensin and aldosterone independently of hypertension make a major contribution to myocardial fibrosis in hypertensive rats (24). In our study, there was no significant differences between SHR-TMAO and SHR-WATER in plasma angiotensin II, plasma aldosterone and cardiac gene expression of angiotensinogen and angiotensin receptors. This suggest a non-angiotensin dependent mechanism of TMAO effects.

We think that the beneficial effect of TMAO on the heart depends on the interaction of TMAO with cardiac proteins i.e. TMAO acts as a piezolyte. With this regard, a number of biophysical studies show that TMAO stabilizes structural proteins, enzymes, DNA and RNA in conditions of increased hydrostatic pressure, osmotic pressure, temperature or if exposed to denaturants such as urea (5, 8, 10, 19, 26, 28, 29, 39, 46). Furthermore, it is well-established that organisms exposed to high hydrostatic and/or osmotic pressures accumulate TMAO to protect their cells from osmotic and hydrostatic pressure stresses (32, 48-50, 52).

Certainly, cardiac cells, in particular cardiac cells of hypertensive subjects are exposed to substantial hydrostatic stress i.e. significant diastole-systole changes in intraventricular pressure (0 – 180 mmHg diastole-systole difference, or greater). We would hypothesize that cardiac cells of rats treated with TMAO were more resilient to hydrostatic stress caused by diastolic-systolic changes in hydrostatic blood pressure, which resulted in preserved biomechanical function of cardiomyocytes and lower fibrosis. Therefore, biophysical studies evaluating the effect of TMAO on pressure-dependent folding of structural proteins and enzymes of cardiomyocytes are needed.

**Increased plasma TMAO and cardiovascular risk**

The issue of a number of clinical studies showing a positive correlation between increased plasma TMAO and an increased cardiovascular risk (18, 30, 35, 36, 42, 44) needs to be addressed. However, some studies do not confirm such an association (7, 22, 51) or show that the correlation is dependent on race (37), or point to a high intra-individual variation of plasma TMAO level over time (17).

We found that 60-week-old hypertensive SHR-WATER had a significantly higher plasma TMAO level than normotensive WKY. However, altogether, our findings suggest that association of an increased plasma TMAO and cardiovascular disturbances may not be a causative relationship. Similarly, an increased plasma BNP is not a causative factor of HF but a compensatory response to failing heart. We would hypothesize that in cardiovascular diseases TMAO is accumulated in order to protect cells from hydrostatic and osmotic stresses i.e. pressure overload and water-electrolyte disturbances. Alternatively, an increased plasma TMAO may be a marker of other cardiovascular risk factors, such as low GFR (23), dietary habits e.g. high-salt intake (2) or disturbed gut-blood barrier. Specifically, research suggests that cardiovascular diseases, including HF and hypertension produce structural and hemodynamic disturbances in intestines (13, 15, 33), which increases permeability of the gut-blood barrier and facilitates the passage of gut bacterial metabolites including TMA, a TMAO precursor, to the portal blood (13).

**Limitations**

A limitation of our study is that it was performed on one animal model of hypertension and heart failure. However, the characteristics of HF in our study were similar to those found in other animal models of hypertension-induced heart failure, i.e. Dahl salt-sensitive rats (12) and deoxycorticosterone acetate-salt hypertensive rats (25). Finally, the model of pressure-overloaded heart that we used in the present study closely resembles a common type of hypertension-induced HF in humans in terms of structural, hemodynamic and biochemical characteristics (14, 31). This study would be enriched if we had evaluated the effects of chronic, low-dose TMAO treatment in healthy WKY rats. However, earlier we found that in healthy Sprague Dawley rats a 2-week 100-fold increase in plasma TMAO did not produce significant hemodynamic effects (45).

**Perspectives and Significance**

Our study provides new evidence for a potentially beneficial effect of a moderate increase in plasma TMAO on pressure-overloaded heart. This may imply that reported in clinical studies association of an increased plasma TMAO and an increased cardiovascular risk is not a causative relationship. In contrast, increased plasma TMAO may be a compensatory mechanism, similar to increased BNP level.

Therefore, we strongly believe that despite a significant workload and high cost of long-term in vivo experiments, further studies are needed to assess the effect of TMAO and TMA on the circulatory system.

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**DISCLOSURES**

None.

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**FIGURE LEGENDS:**

**Fig. 1**. (A) Mean arterial blood pressure (MABP, mmHg) and (B) heart rate (beats/min) in normotensive Wistar-Kyoto rats (WKY, n=6) and Spontaneously Hypertensive rats maintained on tap water (SHR-WATER, n=7) or water containing TMAO (SHR-TMAO, n=7). Values are means, ± SE. \* - p<0.05 - MABP changes in time in SHR, † - p<0.05 - MABP changes in time in SHR-TMAO (by one-way ANOVA for repeated measurements).

**Fig. 2.** Histopathological picture of kidneys in 16-week-old Wistar Kyoto rats (WKY), spontaneously hypertensive rats (SHR-WATER), and SHR treated with TMAO in drinking water (SHR-TMAO).

A, B, C – Parenchyma of renal cortex (Hematoxyline-Eosine stain); D, E, F – Parenchyma of renal cortex (van Gieson stain for connective tissue fibres).

**Fig. 3.** Histopathological picture of arteries in 16-weeks-old rats.

A, B, C – Coronary arteries in the heart (van Gieson stain for connective tissue fibres); D, E, F – Arcuate arteries in the kidney (Hematoxyline-Eosine stain); G, H, I – Wall of aorta, J - Wall thickness of the aorta (µm), K –The thickness of the periarterial connective tissue in heart (µm), \* - p<0.05 vs WKY, † - p<0.05 vs SHR-WATER [WKY (n=6) , SHR-WATER (n=6), SHR-TMAO (n=6), by one-way ANOVA followed by Tukey’s post-hoc test].

**Fig. 4**. Histopathological picture of heart in 16-week-old rats.

A, B, C – Myocardium (Hematoxyline-Eosine stain); D, E, F – Myocytes - cross-section (Hematoxyline-Eosine stain); G, H, I– Myocardium (van Gieson stain for connective tissue fibres); J-Width of cardiomyocytes (µm, group mean ± SE); K- Percentage of fibrosis (%, group mean ± SE). \* - p<0.05 vs WKY; † - p<0.05 vs SHR-WATER [WKY (n=6), SHR-WATER (n=6), SHR-TMAO (n=6), by one-way ANOVA followed by Tukey’s post-hoc test].

**Fig. 5.** Histopathological picture of arteries in 16-week-old rats.

A, B, C - Wall of carotid artery (Hematoxyline-Eosine stain); D, E, F - Coronary arteries in the heart (Hematoxyline-Eosine stain); G – Wall thickness of the carotid artery (µm). \* - p<0.05 vs WKY [WKY (n=6), SHR-WATER (n=6), SHR-TMAO (n=6), by one-way ANOVA followed by Tukey’s post-hoc test].

**Fig. 6.** Histopathological picture of kidneys in 60-week-old Wistar Kyoto rats (WKY), spontaneously hypertensive rats (SHR-WATER), and SHR treated with TMAO in drinking water (SHR-TMAO).

A, B, C – Parenchyma of renal cortex (Hematoxyline-Eosine stain); D, E, F – Parenchyma of renal cortex (van Gieson stain for connective tissue fibres).

**Fig. 7.** Histopathological picture of arteries in 60-week-oldrats.

A, B, C – Coronary arteries in the heart (van Gieson stain for connective tissue fibres); D, E, F – Arcuate arteries in the kidney (Hematoxyline-Eosine stain); G, H, I – Wall of aorta, J - Wall thickness of the aorta (µm), K –The thickness of the periarterial connective tissue in heart (µm), \* - p<0.05 vs WKY, † - p<0.05 vs SHR-WATER [WKY (n=6), SHR-WATER (n=7), SHR-TMAO (n=7), by one-way ANOVA followed by Tukey’s post-hoc test].

**Fig. 8.** Echocardiography in 60-week-old Wistar Kyoto rats (WKY), spontaneously hypertensive rats (SHR-WATER), and SHR treated with TMAO in drinking water (SHR-TMAO).

**Fig. 9.** Simultaneous recording of arterial blood pressure (ABP, mmHg) and left ventricular end-diastolic-pressure (LVEDP, mmHg) in 60-week-old Wistar Kyoto rats (WKY), spontaneously hypertensive rats (SHR-WATER), and SHR treated with TMAO in drinking water (SHR-TMAO).

A. Group means are presented with SE. LVEDP was calculated as the average of 10 consecutive LVEDP intervals for each rat. \* - P<0.05 vs. WKY, † - P<0.05 vs. SHR-WATER [WKY (n=6), SHR-WATER (n=7), SHR-TMAO (n=6), by one-way ANOVA followed by Tukey’s post-hoc test].

B, C, D panels present analog recordings of ABP and LVEDP of one rat from WKY, SHR-WATER, and SHR-TMAO groups respectively. Arrows show LVEDP interval.

**Fig. 10.** Electrocardiography in 60-week-old Wistar Kyoto rats (WKY), spontaneously hypertensive rats (SHR-WATER), and SHR treated with TMAO in drinking water (SHR-TMAO).

**Fig. 11.** Histopathological picture of heart in 60-week-old Wistar Kyoto rats.

A, B, C – Myocardium (Hematoxyline-Eosine stain); D, E, F – Myocytes - cross-section (Hematoxyline-Eosine stain); G, H, I – Myocardium (van Gieson stain for connective tissue fibres). J-Width of cardiomyocytes (µm, group mean ± SE); K- Percentage of fibrosis (%, group mean ± SE). \* - p<0.05 vs WKY; † - p<0.05 vs SHR-WATER [WKY (n=6), SHR-WATER (n=7), SHR-TMAO (n=7), by one-way ANOVA followed by Tukey’s post-hoc test].

**Fig. 12.** Histopathological picture of arteries in 60-week-oldrats

A, B, C - Wall of carotid artery (Hematoxyline-Eosine stain); D, E, F - Coronary arteries in the heart (Hematoxyline-Eosine stain); G - Wall thickness of the carotid artery (µm, group mean ± SE). \* - p<0.05 vs WKY [WKY (n=6), SHR-WATER (n=7), SHR-TMAO (n=7), by one-way ANOVA followed by Tukey’s post-hoc test).

**Fig. 13** Cardiac gene expression of angiotensinogen and angiotensin receptors in 16-week-old rats, SHR-WATER (n=5), SHR-TMAO and 60-week-old rats SHR-WATER (n=5), SHR-TMAO (n=5).

ΔCt (target gene threshold cycles (Ct) - reference gene threshold cycles). Mean ± SE are presented.

**TABLES:**

**Table 1.** Metabolic, kidney and cardiovascular parameters in 16-week-old normotensive Wistar Kyoto rats (WKY, n=6), spontaneously hypertensive rats (SHR-WATER, n=7), and SHR treated with TMAO in drinking water (SHR-TMAO, n=7).

Creatinine clearance calculated as urine creatinine x urine output (ml/min) / plasma creatinine.

LVEDP – pressure in the left ventricle during the end of diastole measured directly with a catheter. Values are means, ± SE. One-way ANOVA (WKY x SHR-WATER x SHR-TMAO) followed by Tukey’s post-hoc test. ns - not significant differences between the groups by ANOVA, \* - P<0.05 vs. WKY, † - P<0.05 vs. SHR by Tukey’s post-hoc test.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Group/****Parameter** | **WKY** | **SHR-WATER** | **SHR-TMAO** | **ANOVA** |
| **Body mass and food intake** |
| Body mass (g)  | 309.49±8.45 | 320.87±19.39 | 311.65±8.81 | ns |
| 24hr food intake (g) | 24.01±0.74 | 21.13±0.76\* | 21.52±0.83\* | P<0.05  |
| 24hr stool output (g) | 11.87±0.82 | 9.31±0.53\* | 11.62±0.83 † | P<0.05  |
| **TMA and TMAO balance** |
| Plasma TMAO (ng/ml)[µM] | 470.78±59.80[6.27±0.79] | 661.38±126.04[8.81±1.67] | 2801.73±610.05\*†[37.31±8.12] | P<0.05  |
| 24hr TMAO excretion (mg)[µmoles] | 0.68±0.08[9.05±1.06] | 0.56±0.08[7.45±1.06] | 6.01±1.45\*†[80.02±19.31] | P<0.05  |
| Plasma TMA (ng/ml)[µM] | 5441.62±269.5[92.06±4.56] | 5156.6±830.62[87.24±14.05] | 4309.29±286.4[72.90±4.84] | ns |
| 24hr TMA excretion (mg)[µmoles] | 0.53±0.05[8.97±0.84] | 0.36±0.04\*[6.09±0.67] | 0.21±0.03\*[3.55±0.51] | P<0.05  |
| **Water-electrolyte balance and kidney function** |
| 24hr water intake (ml) | 30.82±0.82 | 36.83±3.07\* | 34.71±2.98\* | P<0.05  |
| 24hr urine output (ml) | 10.14±0.57 | 15.14±1.36\* | 11.90±1.89 | P<0.05  |
| Plasma sodium (mg/dl) | 325.67±1.25 | 332.35±1.46\* | 331.03±2.01 | P<0.05  |
| 24hr sodium urine excretion (mg)  | 36.77±2.71 | 24.01±1.73\* | 18.96±2.18\* | P<0.05 |
| Plasma potassium (mg/dl) | 21.38±0.76 | 21.55±2.79 | 20.71±1.76 | ns |
| 24hr potassium excretion (mg)  | 170.77±14.12 | 155.19±8.07 | 137.53±13.51 | P=0.06 |
| Plasma creatinine (mg/dl) | 0.76±0.06 | 0.88±0.06 | 0.85±0.07 | ns |
| Plasma urea (mg/dl) | 69.00±2.33 | 73.00±8.87 | 65.83±3.83 | ns |
| Creatinine clearance (ml/min) | 0.37±0.02 | 0.34±0.03 | 0.33±0.02 | ns |
| **Hemodynamic and cardiac parameters in anaesthetized rats** |
| MABP (mmHg) | 83.73±4.46 | 101.60±4.34\* | 100.92±2.97\* | P<0.05 |
| Systolic blood pressure (mmHg) | 104.34±5.41 | 133.53±6.42\* | 129.13±5.77\* | P<0.05  |
| Diastolic blood pressure(mmHg) | 67.71±5.16 | 77.21±3.23 | 78.89±2.85 | P=0.09 |
| Heart Rate (based on RR-ECG) | 318.63±15.09 | 345.03±9.77 | 351.65±9.27 | P=0.17 |
| Heart mass (g) | 00.96±0.02 | 01.39±0.06\* | 01.26±0.04\* | P<0.05  |
| LVEDP (mmHg, direct measurements) | 03.90±0.18 | 07.16±0.51\* | 05.44±0.28 | P<0.05 |
| NT-proBNP (pg/ml) | 25.50±1.22 | 32.11±2.23 | 26.73±3.26 | ns |
| QRS width (ms, ECG) | 22.87±0.87 | 24.34±0.40 | 24.38±1.46 | ns |
| QRS amplitude (mV, ECG) | 3.25±0.48 | 2.80±0.24 | 3.19±0.55 | ns  |

**Table 2.** Metabolic, kidney and cardiovascular parameters in 60-week-old Wistar Kyoto rats (WKY, n=6), spontaneously hypertensive rats (SHR-WATER, n=8), and SHR treated with TMAO in drinking water (SHR-TMAO, n=8).

Creatinine clearance calculated as urine creatinine x urine output (ml/min) / plasma creatinine.

LVEDV - left ventricle end diastolic volume, LVESV - left ventricle end systolic volume, SV – stroke volume, EF - ejection fraction, IVSs - interventricular septum diameter during systole, posterior wall of the left ventricle during systole. LVEDP - pressure in the left ventricle during the end of diastole measured directly with a catheter.

Values are means, ± SE. One-way ANOVA (WKY x SHR-WATER x SHR-TMAO) followed by Tukey’s post-hoc test. ns- not significant differences between groups by ANOVA, \* - P<0.05 vs. WKY, † - P<0.05 vs. SHR by Tukey’s post-hoc test.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Group/****Parameter** | **WKY** | **SHR-WATER** | **SHR-TMAO** | **ANOVA** |
| **Body mass and food intake** |
| Body mass (g)  | 391.37±12.90 | 375.63±12.23 | 378.66±11.07 | ns |
| 24hr food intake (g) | 23.63±1.09 | 22.96±1.75 | 23.65±1.24 | ns |
| 24hr stool output (g) | 9.23±0.79 | 11.57±1.09 | 13.31±1.19\* | P<0.05 |
| **TMA and TMAO balance** |
| Plasma TMAO (ng/ml)[µM] | 391.83±49.63[5.22±0.66] | 608.52±80.59\*[8.10±1.07] | 3073.03±604.61\*†[40.92±8.05] | P<0.05 |
| 24hr TMAO excretion (mg)[µmoles] | 0.65±0.11[8.66±1.46] | 0.67±0.09[8.92±1.20] | 7.35±0.40\*†[97.88±5.33] | P<0.05 |
| Plasma TMA (ng/ml)[µM] | 5855.26±378.4[99.06±6.40] | 5879.99±469.7[99.47±1.19] | 3784.05±362.6\*†[64.02±6.13] | P<0.05 |
| 24hr TMA excretion (mg)[µmoles] | 0.61±0.06[10.32±1.02] | 0.75±0.07[12.69±1.18] | 0.75±0.1[12.69±1.69] | ns |
| **Water-electrolyte balance and kidney function** |
| 24hr water intake (ml) | 31.30±2.46 | 36.50±3.26 | 36.30±2.87 | P=0.2 |
| 24hr urine output (ml) | 15.87±2.33 | 16.16±1.66 | 19.78±1.50 | P=0.1 |
| Plasma sodium (mg/dl) | 316.86±2.49 | 313.41±2.48 | 319.82±2.18 | ns |
| 24hr sodium urine excretion (mg)  | 48.83±2.21 | 23.72±2.20\* | 29.37±3.64\* | P<0.05 |
| Plasma potassium (mg/dl) | 16.53±0.54 | 18.13±0.67 | 17.46±0.53 | ns |
| 24hr potassium excretion (mg)  | 79.38±6.27 | 95.59±7.83 | 104.89±7.11 | P=0.07 |
| Plasma creatinine (mg/dl) | 0.88±0.05 | 0.86±0.04 | 0.72±0.02\*† | P<0.05 |
| Plasma urea (mg/dl) | 53.16±2.31 | 57.33±2.74 | 51.12±1.89 | ns |
| Creatinine clearance (ml/min) | 0.26±0.02 | 0.28±0.03 | 0.36±0.03\* | P<0.05 |
| Plasma Ang II (pg/ml) | 1080.79±22.08 | 1193.40±21.09 | 1204.69±19.27 | ns |
| Plasma aldosterone (ng/ml) | 6.91±1.28 | 9.34±1.75 | 8.94±2.22 | ns |
| Plasma vasopressin (pg/ml) | 1304.7±122.6 | 1709.7±154.3\* | 1246.4±112.2† | P<0.05 |
| **Inflammatory markers in plasma** |
| TNF-α (pg/ml) | 20.21±4.26 | 41.28±12.71 | 35.46±8.78 | ns |
| IL-10 (pg/ml) | 18.02±1.75 | 15.91±1.90 | 18.02±1.42 | ns |
| **Hemodynamic and cardiac parameters in anaesthetized rats** |
| MABP (mmHg) | 81.79±2.19 | 120.98±5.03\* | 112.47±5.25\* | P<0.05 |
| Systolic blood pressure (mmHg) | 101.92±2.93 | 135.28±3.92\* | 139.32±5.89\* | P<0.05 |
| Diastolic blood pressure (mmHg) | 64.41±2.59 | 104.57±6.17\* | 92.24±5.29\*† | P<0.05 |
| Heart Rate (based on RR-ECG) | 305.90±22.4 | 343.62±6.75 | 335.66±12.85 | ns |
| Heart mass | 1.16±0.05 | 2.09±0.07\* | 1.98±0.07\* | P<0.05 |
| Wall thickness of the carotid artery (µm) | 67.65±5.97 | 106.08±12.45\* | 116.56±8.05\* | P<0.05 |
| LV EDV (ml, ECHO) | 0.48±0.09 | 0.46.±0.07 | 0.48±0.10 | ns |
| LV ESV (ml, ECHO) | 0.17±0.04 | 0.13±0.03 | 0.09±0.02 | ns |
| SV (ml, ECHO) | 0.37±0.07 | 0.33±0.05 | 0.39±0.09 | ns |
| EF (%, ECHO) | 78.25±3.30 | 72.40±3.25 | 81.60±3.34 | ns |
| IVSs (cm, ECHO) | 0.18±0.01 | 0.22±0.01 | 0.23±0.03 | P=0.15 |
| LVPWs (cm, ECHO) | 0.20±0.01 | 0.24±0.02 | 0.25±0.03 | P=0.2 |
| NT-proBNP (pg/ml) | 24.32±2.48 | 46.82±6.34\* | 30.34±1.11† | P<0.05 |
| QRS width (ms, ECG) | 18.54±1.09 | 29.54±1.62\* | 31.09±3.06\* | P<0.05 |
| QRS amplitude (mV, ECG) | 2.72±0.47 | 3.39±0.43 | 3.97±0.66 | ns |