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Research Article

Trimethylamine N-oxide does not impact viability, ROS production and mitochondrial membrane potential of adult rat cardiomyocytes

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Abstract: Trimethylamine N-oxide (TMAO) is an organic compound derived from dietary choline and L-carnitine. It behaves as an osmolyte, a protein stabilizer and an electron acceptor, showing different biological functions in different animals. Recent works point out that in humans high circulating levels of TMAO are related with the progression of atherosclerosis and other cardiovascular diseases. Nevertheless, studies on a direct role of TMAO on cardiomyocytes parameters are still limited. This work focuses on the effects of TMAO on isolated adult rat cardiomyocytes. TMAO both 100µM and 10mM, from 1 to 24 h of treatment, does not affect cell viability, sarcomere length, intracellular ROS and mitochondrial membrane potential. Furthermore, the simultaneous treatment with TMAO and known cardiac insults, H_2O_2 or Doxorubicin, does not affect the effect of the treatment. In conclusion, TMAO cannot be considered a direct cause or an exacerbating risk factor of cardiac damage at the cellular level in acute conditions.

Keywords: Trimethylamine N-oxide; cardiomyocytes; cardiotoxicity; ROS; mitochondrial membrane potential

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1. Introduction

Trimethylamine N-oxide (TMAO) is an amine oxide directly introduced through the diet or synthetized from its precursors, primarily L-carnitine and choline, that are transformed into trimethylamine (TMA) by the gut microbiota. Once absorbed, TMA in most mammals is oxidized by hepatic FMOs to form TMAO which enters the systemic circulation. Several studies illustrates different biological functions of TMAO in other animals. In elasmobranchs and deep sea fishes it acts as an osmolyte able to counteract either osmotic or hydrostatic pressure. It is a protein stabilizer preserving protein folding and it also acts as an electron acceptor balancing oxidative stress [1,2]. TMAO is also reduced to TMA in the anaerobic metabolism of a number of bacteria. Although TMAO is involved in several reactions within cells, recent studies highlight its detrimental role when present in high plasmatic concentrations in some mammals. In fact, TMAO seems to be involved in accelerating endothelial cell senescence, enhancing vascular inflammation and oxidative stress [3,4]; it also could be involved in the stimulation of platelets hyperreactivity and in the onset of thrombosis, exacerbating atherosclerotic lesions [5]. Several studies also underline the role of TMAO in the pathogenesis of type 2 diabetes mellitus [6]. There are limited data on its function in mediating direct cardiac injuries, and they are mainly focused on its role in the impairment of mitochondrial metabolism [7] and calcium handling [8]. Instead, recent papers revalue the role of TMAO, underlining the emerging debate on its direct effect in causing or exacerbating cardiovascular diseases (CVD) [9,10]. First criticisms point out that populations that have diets with high concentrations of TMAO, like those rich in fish products, when compared to Western diets rich in its precursors, have reduced risks of CVD or diseases assumed to be related to high TMAO plasma levels [11]. Another study demonstrate that TMAO does not affect macrophage foam cells formation and lesion progression in ApoE$^{-/-}$ mice expressing human cholesteryl ester transfer protein, suggesting that the molecule does not worsen atherosclerosis [12]. Furthermore, administration of TMAO seems to ameliorate symptoms related to streptozotocin induced diabetes in rats and mice, highlighting no direct contribution of the molecule in exacerbating this condition [13]. Finally, data about TMAO plasma concentrations in health and pathological subjects are not clear: lack of plasma concentration ranges of the molecule highlights the difficulties in referring to TMAO as a protective or a damaging factor in CVD. Starting from these conflicting considerations, aim of this work is to evaluate for the first time the effect of TMAO in an *in vitro* model of adult rat cardiomyocytes exposed to different concentrations of the compound from 1 h to 24 h of treatment. To show whether TMAO exacerbates or reduces induced cell stress, cardiomyocytes are simultaneously treated with TMAO and H_2O_2 or Doxorubicin (DOX). Investigations have been focused to cell viability after TMAO or TMAO and stressors co-treatment, assessing cell morphology and functionality with α-actinin staining and specific probes that measure oxidative stress status and mitochondrial membrane potential.

2. Results

2.1 TMAO and cell viability

In order to investigate the effect of TMAO on cell viability, cardiomyocytes are treated with TMAO 100μM, TMAO 10mM, H_2O_2 50μM and H_2O_2 50μM+TMAO 100μM. After 1 h or 24 h of treatment, cardiomyocytes are labeled with PI and marked nuclei of suffering cells are detected by confocal microscopy at 568nm. Concentrations used have been taken from literature: TMAO 100μM is recognized as a marker of cardiovascular risk, TMAO 10mM is over the physiological range and here tested to detect any effect induced by high concentrations of the compound [14]. As shown in Figure 1a, there is no effect of TMAO 100μM nor TMAO 10mM at either time of treatment, while H_2O_2, here used as positive control, has effects only after 24 h. Simultaneous treatment with H_2O_2 and TMAO does not ameliorate nor worsen the effect of the stressor on cell viability (1 h: CTRL: 83.95±6.59, n=3, 52 cells; TMAO 100μM: 85.52±7.01, n=5, 81 cells; TMAO 10mM: 84.08±5.84, n=5, 92 cells; H_2O_2: 52.92±16.46, n=3, 56 cells; H_2O_2+TMAO: 50.93±18.50, n=3, 58 cells. 24 h: CTRL: 83.42±2.29, n=3, 101 cells; TMAO 100μM: 85.66±6.48, n=3, 91 cells; TMAO 10mM: 62.22±10.47 n=3, 119 cells; H_2O_2: 2.38±2.38, n=3, 82 cells(***$P<0.001$); H_2O_2+TMAO: 1.33±1.33, n=3, 41 cells(***$P<0.001$)). Figure 1b displays confocal images of cardiomyocytes from a representative experiment of PI staining after 24 h of treatment. White arrows point out PI-stained, damaged cardiomyocytes.

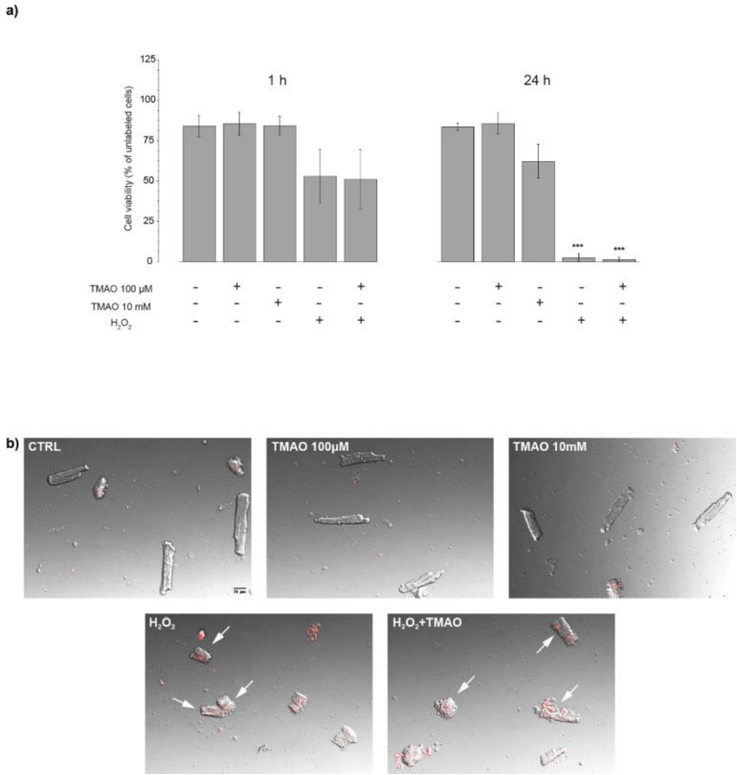

Figure 1. Cell viability after TMAO exposure. a) Bar graph of cell viability after 1 h and 24 h of treatment. Cells viability results reduced only after H_2O_2 treatment for 24 h, condition not ameliorated nor worsened by the simultaneous treatment with TMAO (refer to the main text for numerical values). b) Merged images in bright field and fluorescence of cells treated for 24 h with TMAO 100μM and TMAO 10mM, H_2O_2 and H_2O_2 + TMAO and labeled with propidium iodide (PI) (20X magnification). White arrows point out PI-stained, damaged cardiomyocytes.

2.2 TMAO and sarcomere length

To evaluate if TMAO is able to alter sarcomere structures after 24 h of treatment, sarcomere length is measured in α-actinin stained cardiomyocytes. As shown in Figure 2, no changes in sarcomere length are observed in cells treated with TMAO, while H_2O_2 50μM used as a positive control, cause cardiomyocytes shrinkage, condition that is not ameliorated nor worsened by the simultaneous treatment with TMAO. In cardiomyocytes treated with DOX 1μM for 24 h we do not observe sarcomere length variations, as in our model DOX treatment is designed to induce a mild damage preceding cell shortening. Even so, 100μM TMAO does not modify DOX-treated cardiomyocytes (sarcomere length in μm is: CTRL: 1.69±0.01, n=7, 42 cells; TMAO 100μM: 1.69±0.01, n=6, 34 cells; TMAO 10mM: 1.67±0.02, n=3, 19 cells; H_2O_2: 1.22±0.04, n=5, 33 cells (***$P<0.001$); H_2O_2+TMAO: 1.28±0.03, n=3, 24 cells (***$P<0.001$); DOX: 1.62±0.02, n=3, 16 cells; DOX+TMAO: 1.65±0.01, n=3, 15 cells).

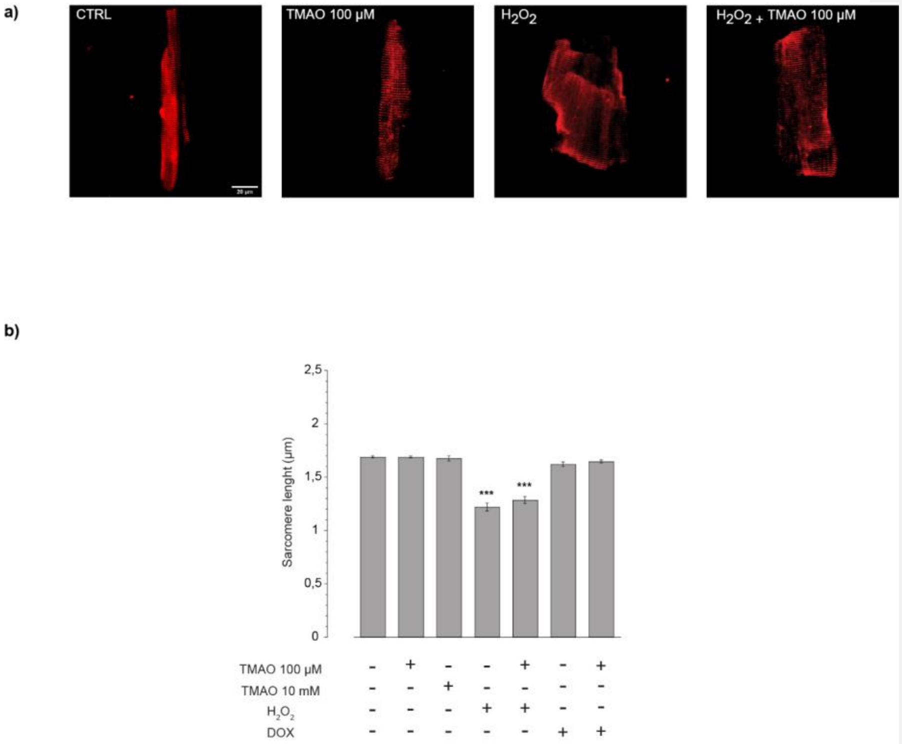

Figure 2. Sarcomere length after TMAO treatment. a) Confocal microscopy images of fixed cells labeled for α-actinin protein (40X magnification). b) Bar graph showing sarcomere length after 24 h of treatment with TMAO and other stressors: no cell shrinkage is measured when cells are exposed to different TMAO concentrations (refer to the main text for numerical values).

2.3 TMAO and intracellular Reactive Oxygen Species (ROS)

In order to determine a variation in total ROS produced after treatment with TMAO for 1 h, 3 h or 24 h, cells are labeled with DCF-DA probe and its fluorescence is quantified and related to control. As shown in Figures 3 (1 h, 3 h) and 4 (24 h) no fluorescence variations after TMAO treatment is detected at any concentration and time used. As a positive control we employ DOX 1μM for 24 h [15]; this drug caused a significant variation in ROS production referred to control condition. TMAO 100μM does not modify ROS production in DOX treated cardiomyocytes (Fig. 4). (1h: TMAO 100μM: 1.30±0.21, n=3, 34 cells; TMAO 10mM: 1.32±0.23, n=3, 40 cells, vs CTRL; 3h: TMAO 100μM: 0.96±0.05, n=3, 45 cells; TMAO 10mM: 1.15±0.09, n=3, 52 cells, vs CTRL; 24h: TMAO 100μM: 1.18±0.04, n=5, 40 cells; TMAO 10mM: 1.24±0.16, n=3, 52 cells; DOX: 1.33±0.11, n=4, 21 cells (**P<0.01); DOX+TMAO: 1.27±0.01, n=6, 31 cells (***P<0.001) vs CTRL).

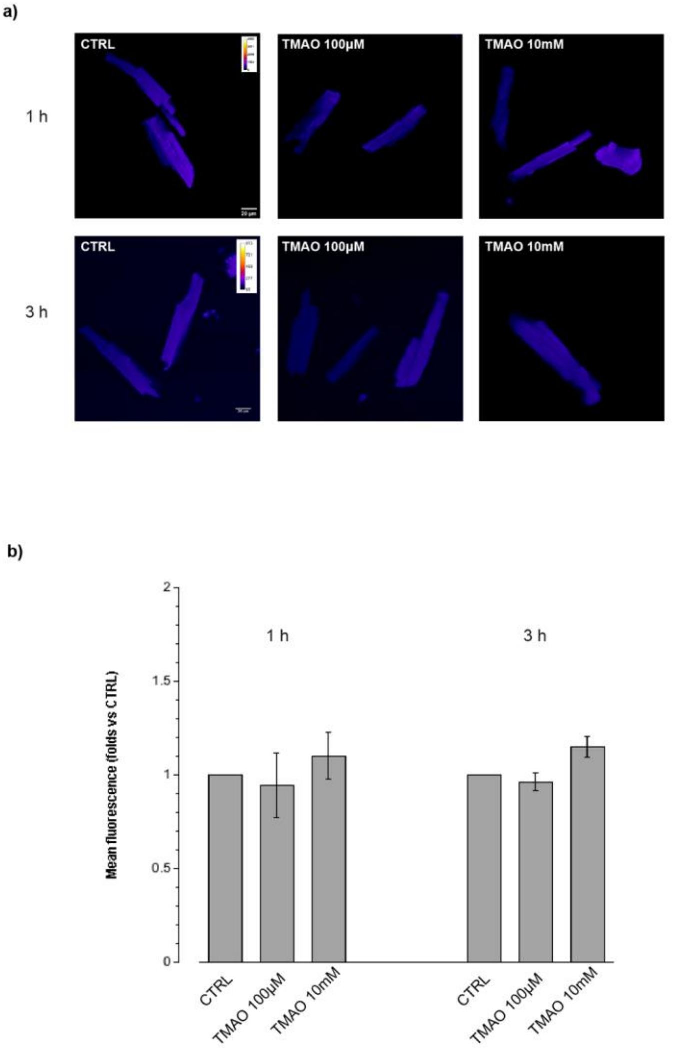

Figure 3. ROS production after 1 h and 3 h of treatment. a) Confocal microscopy images of cells treated with TMAO 100μM and TMAO 10mM for 1 h and 3 h and labeled with DCF-DA probe (60X magnification). b) Bar graph showing mean fluorescence after 1 h and 3 h of treatment, no variations or ROS produced are detectable (refer to the main text for numerical values).

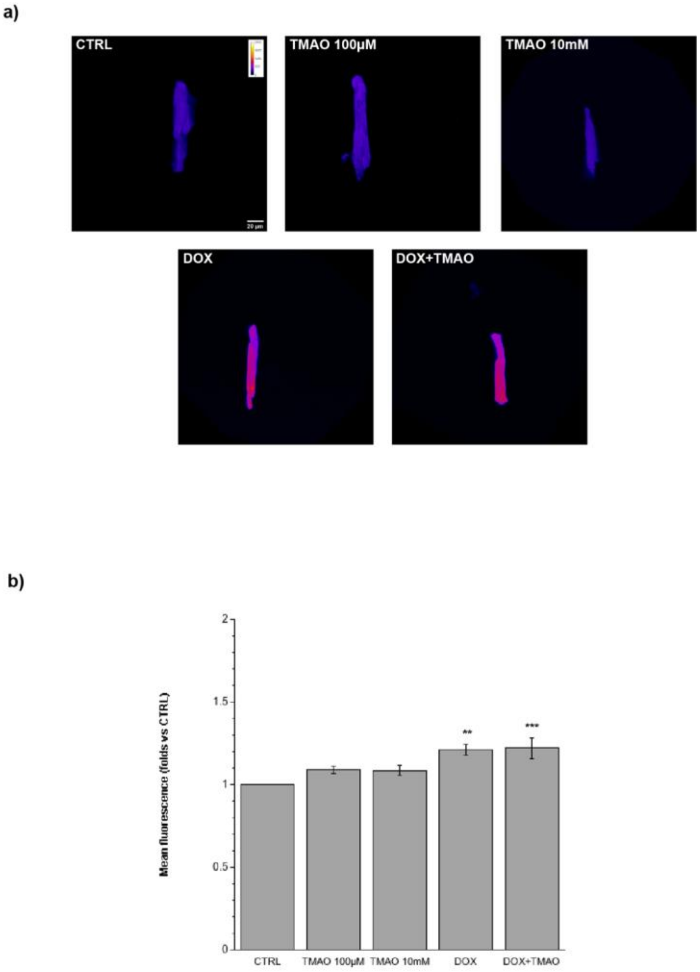

Figure 4. ROS production after 24 h of treatment. a) Confocal microscopy images of cells treated with TMAO 100μM and TMAO 10mM for 24 h and labeled with DCF-DA probe. In these experiments Doxorubicin (DOX) is used as positive control (60X magnification). b) Bar graph showing mean fluorescence after 24 h of treatment. No variations of ROS produced are detectable after TMAO treatment, and a small but significant increase is visible after DOX treatment (here used as positive control), this increase is not changed by a simultaneous treatment with TMAO (refer to the main text for numerical values).

2.4 TMAO and mitochondrial membrane potential

To investigate the potential metabolic damage induced by TMAO, cardiomyocytes treated with TMAO 100μM and 1mM for 1 h, 3 h or 24 h are labeled with the JC-1 probe. Figures 5 (1 h, 3 h) and 6 (24 h) show variations in mitochondrial membrane potential (red/green fluorescence ratio) detected by confocal microscopy in living cells. TMAO treatment from 1 to 24 h does not cause any difference towards control, indicating no mitochondrial effect of the molecule, while, as expected, DOX cause a depolarization of mitochondrial membrane potential after 24 h of treatment. TMAO 100μM does not modify mitochondrial membrane potential in DOX treated cardiomyocytes (Fig. 6) (1 h: TMAO 100μM: 1.09±0.08, n=5, 65 cells; TMAO 10mM: 1.11±0.11, n=3, 49 cells, vs CTRL. 3 h: TMAO 100μM: 1.02±0.06, n=3, 26 cells; TMAO 10mM: 0.88±0.05, n=4, 39 cells, vs CTRL; 24 h: TMAO 100μM: 1.08±0.11, n=3, 21 cells; TMAO 10mM: 0.95±0.15, n=3, 54 cells; DOX: 0.73±0.02, n=3, 24 cells (**P<0.01); DOX+TMAO: 0.69±0.05, n=3, 22 cells (**P<0.01), vs CTRL).

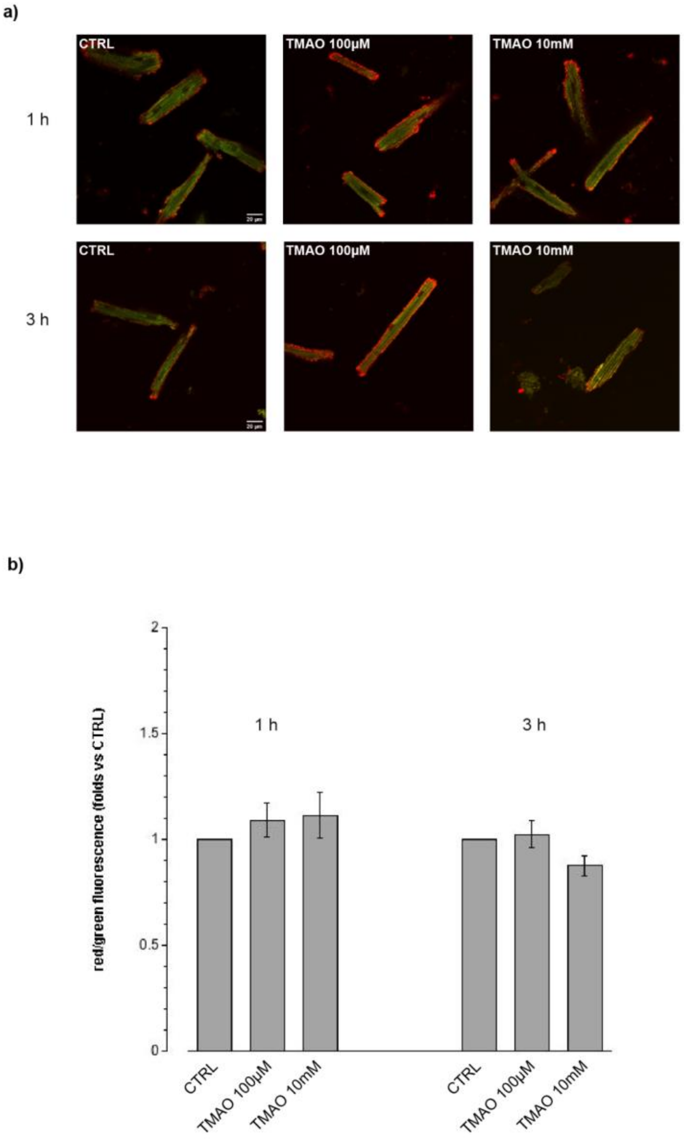

Figure 5. Mitochondrial membrane potential variation following 1 h and 3 h of treatment. a) Confocal microscopy images of cells treated with TMAO 100μM and TMAO 10mM for 1 h and 3 h and labeled with JC-1 probe (60X magnification). b) Bar graph showing red/green fluorescence after 1 h and 3 h of treatment, no variations of mitochondrial membrane potential is detected (refer to the main text for numerical values).

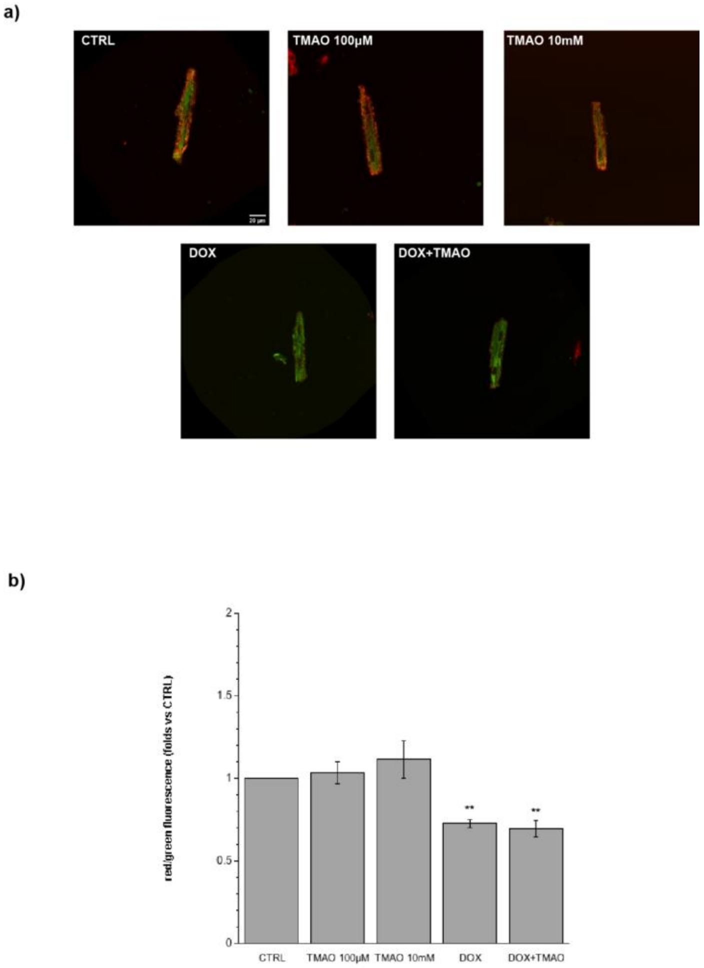

Figure 6. Mitochondrial membrane potential variation following 24 h treatment. a) Confocal microscopy images of cells treated with TMAO 100μM and TMAO 10mM for 24 h and labeled with JC-1 probe. In these experiments Doxorubicin (DOX) is used as positive control (60X magnification). b) Bar graph showing red/green fluorescence after 24 h of treatment, no variations of mitochondrial membrane potential is detected, a little but significant reduction of the ratio is visible after DOX treatment here used as positive control, condition not changed in a simultaneous treatment with TMAO (refer to the main text for numerical values).

3. Discussion

This study provides novel insights into the physiological role of TMAO on isolated adult rat cardiomyocytes. Our findings do not show effects of TMAO on cell viability, sarcomere length, ROS production and mitochondrial membrane potential within the range of concentration used. Moreover, we demonstrate that TMAO does not exacerbate or counteract the effect of known insults such as H_2O_2 or Doxorubicin, tested for up to 24 h of treatment. Taken together these results suggest that TMAO should not be considered a primary cause of acute cardiac damage and that the molecule could not revert or worsen existing risk factors of cardiac damage.

In the last few years, many studies suggest a strong relationship among diet, gut microbiota and cardiovascular diseases [16]. In particular, some attention has been pointed to either TMAO directly coming from diet (fish), or produced from L-carnitine and choline conversion by gut microbiota into TMA and oxidized in the liver by FMO3 enzymes [17, 14].

Experiments have mainly now focused on endothelial cells damaging role of TMAO. It has been described to upregulate cellular senescence reducing cell proliferation, increasing the expression of senescence markers, as p53 and p21, and impairing cell migration [3]. TMAO also increases endothelial cells oxidative stress through a down-regulation of SIRT-1 and impairs NO production that causes endothelial dysfunction [4]. ~~Prolonged hypertensive~~Hypertensive effects of TMAO ~~angiotensin II~~ have been evaluated by Ufnal and colleagues who demonstrate that TMAO has a role in stabilizing the action of Ang II and in prolonging its hypertensive effect, underlining the role of TMAO in stabilizing protein conformation and no direct role of the molecule in mediating hypertension ~~promoting~~ ~~this condition~~ [18]. Koeth and colleagues underline the strong relationship among high consumption of TMAO precursors, high TMAO plasma concentrations and the development of atherosclerosis [19], while another study underlines the effect of the metabolite in enhancing platelet hyperreactivity and thrombosis risk in subjects with high TMAO plasma concentrations [5]. In relation with cardiovascular effects of TMAO, Dambrova and collaborators evidence that high plasma concentrations of the molecule are linked with increased body weight, insulin resistance and it directly correlates with an augmented risk of diabetes [20].

Only a few studies are centered on the direct effect of the molecule on cardiac cells; in particular they focus on the impairment of mitochondrial metabolism in the heart and underline TMAO as an agent that increases the severity of cardiovascular events or that enhances the progression of cardiovascular diseases [7]. Savi et colleagues show a damaging effect of TMAO in cardiomyocytes because it worsens intracellular calcium handling with a reduced efficiency in the intracellular calcium removal and consequent loss in functionality of cardiac cells, furthermore TMAO seems to alter energetic metabolism and to facilitate protein oxidative damage [8].

This scenario presents TMAO as either a marker or a direct agent involved in vascular and cardiac outcomes, but recent papers seem to oppose this point of view, highlighting uncertainty about the causative relation between TMAO and CVD [9]. It is still debated the function of TMAO that is, for example, controversial in fish-rich diets, because of the higher bioavailability of the compound in seafood products and their well-known role in lowering risk of CVD. Also, TMAO does not enhance atherosclerosis development because it seems not to be involved in foam cell formation even at higher concentrations than physiological ones [12], and, there is no direct correlation between high plasma TMAO concentrations and coronary heart diseases [21, 22]. Last findings by Huc et al., underline also a protective role of TMAO in reducing diastolic dysfunction and fibrosis in pressure-overloaded heart [23].

The present study fits into this debate and the results presented agree with other works supporting TMAO as a non-damaging factor. In fact, it is well known that loss of vital cardiac cells is a damaging condition that hampers primarily the functionality of the heart and has several aggravating responses also in peripheral tissues. Our first investigations underline no toxic effect in cardiomyocytes exposed to high concentrations of TMAO highlighting that the molecule is not involved in inducing cardiac tissue cells loss ~~(Fig. 1)~~, and, no alterations of cardiac structure emerge from the evaluation of sarcomere length and cytoskeletal organization ~~(Fig. 2)~~. Oxidative stress could be considered one of the causative factors of senescence in cells, and one of the promoters of cardiometabolic reorganization in response to injury. Considering TMAO as a possible inducer of ROS rising, both in cytoplasmic and mitochondrial environment, we show no variation in ROS production even after 24 h of treatment ~~(Fig. 3 and 4)~~ and we detect no depolarization of

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mitochondrial membrane potential underling no direct influence of the molecule in inducing cardiac cell senescence ~~(Fig. 5 and 6)~~.

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4. Materials and Methods

4.1 Animal care and sacrifice

Experiments are performed on female adult rats which are allowed *ad libitum* access to tap water and standard rodent diet. The animals receive human care in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), and in accordance with Italian law (DL-116, Jan. 27, 1992). The scientific project is supervised and approved by the Italian Ministry of Health, Rome, and by the ethical committee of the University of Torino. Rats are anaesthetized by i.p. injection of Pentobarbital (Nembutal, 100 mg/Kg) and killed by stunning and cervical dislocation.

4.2 Solutions and drugs

Tyrode standard solution ~~contain~~containing (in mM): 154 NaCl, 4 KCl, 1 $MgCl_2$, 5.5 D-glucose, 5 HEPES, 2 $CaCl_2$, pH adjusted to 7.34 with NaOH. Ca^{2+} free Tyrode solution contain (in mM): 154 NaCl, 4 KCl, 1 $MgCl_2$, 5.5 D-glucose, 5 HEPES, 10 2,3-Butanedione monoxime, 5 taurine, pH 7.34. All drug-containing solutions are prepared fresh before the experiments and Tyrode solutions are oxygenated (O_2 100%) before each experiment. Unless otherwise specified, all reagents for cells isolation and experiments are purchased from Sigma-Aldrich.

4.3 Adult rat ventricular cells isolation

Isolated cardiomyocytes are obtained from the hearts of adult rats (200–300 g body wt) according to the previously described method [~~23~~24]. Briefly, after sacrifice, the rat hearts are explanted, washed in Ca^{2+} free Tyrode solution and cannulated via the aorta. All the following operations are carried on under a laminar flow hood. The heart is perfused at a constant flow rate of 10 ml/min with Ca^{2+} free Tyrode solution (37°C) with a peristaltic pump for approximately 5 min to wash away the blood and then with 10 ml of Ca^{2+} free Tyrode supplemented with collagenase (0.3mg/ml) and protease (0.02mg/ml). Hearts are then perfused and enzymatically dissociated with 20ml of Ca^{2+} free Tyrode containing 50μM $CaCl_2$ and the same enzymatic concentration as before. Atria and ventricles are then separated and the ventricles are cut in small pieces and shaken for 10 minutes in 20ml of Ca^{2+} free Tyrode solution in presence of 50μM $CaCl_2$, collagenase and protease. Calcium ions concentration is slowly increased to 0.8mM. Cardiomyocytes are then plated on glass cover slips or glass bottom dishes (Ibidi, Germany), both treated with laminin to allow cell adhesion.

4.4 Cell viability

Cell viability is evaluated by propidium iodide (PI) staining on glass bottom dishes adherent cells. At the end of the treatments cells are incubated with PI (10μg/ml, Invitrogen) for 5 min in the dark. Nuclei of suffering cells are detected with confocal microscopy using Olympus Fluoview 200 microscope at 568nm (magnification 20X). Merged images are created with ImageJ (Rasband, W. S., ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/, 1997-2017) and cells viability is calculated as percentage of (total cells-labeled cells)/total cells.

4.5 Evaluation of sarcomere length

Cardiomyocytes on glass coverslips are stimulated with TMAO and H_2O_2, as a positive control. Cells are treated for 24 h with TMAO, at 100μM and 10mM, then the sarcomere protein α-actinin, localized in the Z lines, has been detected using confocal microscopy. Then cells are fixed in 4% PFA for 40 min. After two washes with PBS, cells are incubated for 20 min with 0.3% Triton and 1% bovine serum albumin (BSA) in PBS and stained for 24 h at +4°C with a mouse monoclonal anti-α-actinin primary antibody (Sigma, 1:800). Cover slides are washed twice with PBS and incubated 1 h at room temperature with the secondary antibody (1:2000, anti-mouse Alexa Fluor 568, Thermo Fisher). After two washes in PBS, coverslips are mounted on standard slides with DABCO and observed after 24 h under confocal microscope. Confocal fluorimetric measurements are acquired using a Leica SP2 laser scanning confocal system, equipped with a 40X water-immersion objective. Image processing and analysis are performed with ImageJ software. Sarcomere length is evaluated measuring the distance between Z lanes in n=10 sarcomeres/cell.

4.6 Intracellular Reactive Oxygen Species (ROS) measurement

Production of ROS is evaluated by fluorescence microscopy using the 2'-7'-Dichlorofluorescein diacetate probe (DCF-DA). After adhesion on glass bottom dishes, DCF-DA solution (5μg/ml) is added to each dish 30min prior to the end of the treatment, then cells are washed with standard Tyrode solution. Fluorescence images at 488nm have been acquired with Olympus Fluoview 200 microscope (magnification 60X). Fluorescence variations are calculated with the definition and measurement of Regions Of Interest (ROIs) using the software ImageJ and expressed as relative Medium Fluorescence Index (MFI) compared to control, fixed at 1.

4.7 Mitochondrial membrane potential measurement

Mitochondrial membrane potential is evaluated by staining cardiomyocytes with the dye 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide (JC-1)**.** JC-1 solution (10μM) is added to each dish 30 min prior to the end of the treatment, then cells are washed with Tyrode standard. Fluorescence images at 488nm and 568nm have been acquired with Olympus Fluoview 200 microscope (magnification 60X). Amounts of the monomeric form of the dye are quantified using the red/green fluorescence ratio in the ROIs using the software ImageJ and expressed as folds towards control, fixed at 1.

4.8 ~~Statitical~~Statistical analysis

All data are expressed as mean±Standard Errors of the mean. For differences between mean values Bonferroni's multiple comparisons test has been performed. Differences with P<0.05 are regarded as statistically significant.

5. Conclusions

In summary, this study demonstrates that TMAO is not directly involved in causing or exacerbating cardiac damage in an acute stress model (Fig. 7). However, there are some limitation of this study: a very wide range of plasmatic TMAO concentrations is presented in literature, ~~and even~~ within different mammals, and also between different sexes of the same species, several orders of magnitude can be considered physiological [~~24,~~ 25, 26], so, to test direct effect of the molecule, it has been used high concentrations, even over human physiological ones. Another weakness of the study could be linked to the time of treatment, because evaluations are no longer than 24 h and they could only represent an acute exposure to TMAO. ~~In~~Moreover, in order to evaluate more deeply the mechanism involved in TMAO-mediated responses it could be necessary to treat cells for longer time to assess a chronic stress compatible with the development of CVD. Furthermore we only study TMAO effect on female ventricular cardiomyocytes and it could be interesting to extend the analysis also to male cardiomyocytes as gender differences have been observed in cardioprotective mechanisms [27] and TMAO induced intracellular calcium imbalance has been described in male cardiomyocytes [8]. Finally, our findings provide new insights into cardiac effect of TMAO, exploring the direct treatment of isolated cardiomyocytes.

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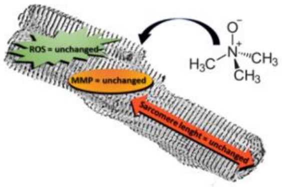

Figure 7. No effects of TMAO is detectable on isolated adult rat cardiomyocytes viability, sarcomere length, ROS production and mitochondrial membrane potential (MMP).

Author Contributions: MG and RL conceived the study, assisted its design and revised the manuscript for important intellectual content. GQ and SA carried out all the experiments, statistical analysis, all the authors interpreted the results. GQ wrote the manuscript. All authors read and approved the final manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

TMAO	Trimethylamine N-oxide
PI	Propidium iodide
ROS	Reactive Oxygen Species
DCF-DA	2′-7′-Dichlorofluorescein diacetate
JC-1	5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide

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