doi: 10.1111/fcp.12163

ORIGINAL ARTICLE

Immunohistochemical determination of the extracellular matrix modulation in a rat model of choline-deprived myocardium: the effects of carnitine

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Keywords
carnitine,
choline,
extracellular matrix,
heart,
immunohistochemistry,
rat

Received 25 March 2015;
revised 6 September 2015;
accepted 20 October 2015

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ABSTRACT

Choline has been identified as an essential nutrient with crucial role in many vital biological functions. Recent studies have demonstrated that heart dysfunction can develop in the setting of choline deprivation even in the absence of underlying heart disease. Matrix metalloproteinases (MMPs) are responsible for extracellular matrix degradation, and the dysregulation of MMP-2 and MMP-9 has been involved in the pathogenesis of various cardiovascular disorders. The aim of the study was to investigate the role of MMPs and their inhibitors (TIMPs), in the pathogenesis of choline deficiency-induced cardiomyopathy, and the way they are affected by carnitine supplementation. Male Wistar Albino adult rats were divided into four groups and received standard or choline-deficient diet with or without L-carnitine in drinking water (0.15% w/v) for 1 month. Heart tissue immunohistochemistry for MMP-2, MMP-9, TIMP-1, and TIMP-2 was performed. Choline deficiency was associated with suppressed immunohistochemical expression of MMP-2 and an increased expression of TIMP-2 compared to control, while it had no impact on TIMP-1. MMP-9 expression was decreased without, however, reaching statistical significance. Carnitine did not affect MMP-2, MMP-9, TIMP-1 or TIMP-2 expression. The pattern of TIMP and MMP modulation observed in a choline deficiency setting appears to promote fibrosis. Carnitine, although shown to suppress fibrosis, does not seem to affect MMP-2, MMP-9, TIMP-1 or TIMP-2 expression. Further studies will be required to identify the mechanism underlying the beneficial effects of carnitine.

INTRODUCTION

Choline belongs to the vitamin B_{12} complex and is a lipotropic agent crucial for many vital biological functions [1]. Choline deficiency has been associated with fatty liver disease, steatohepatitis and insulin resistance [1,2]. In the myocardium, choline deprivation has been shown to exert a constellation of manifestations, most notably fibrosis leading to significant diastolic dysfunction [3]. However, little is known about

the mechanisms underlying these effects. Matrix metalloproteinases (MMPs) are a family of latent zinc- and calcium-dependent proteolytic enzymes that mediate changes in the extracellular matrix (ECM) participating in many physiological processes such as morphogenesis, development and apoptosis [4]. Moreover, their role is crucial in various pathological processes as well, involved in the pathogenesis of different diseases such as chronic obstructive pulmonary disease [5], cancer [6], liver cirrhosis [7] and arthritis [8,9], while they are also engaged in cardiovascular diseases including arterial hypertension [10], pulmonary hypertension [11], coronary artery disease [12], stroke and cardiac hypertrophy [13]. They are gaining increasing clinical interest as important players in vascular remodeling, atherogenesis [14] and thrombosis [15,16].

There is compelling evidence that among all MMPs, MMP-2 and MMP-9 have been shown to be predominately responsible for cardiac matrix homeostasis [17]. Moreover, there is recent evidence that both of them have a multifactorial role, including, but not limited to, inflammation regulation [18], tissue remodeling and revascularization [19]. MMP-2 and MMP-9 upregulation has been implicated in the pathophysiology of atherosclerosis [20], congestive heart failure, viral myocarditis and nonischemic cardiomyopathy [21–25].

The activity of MMPs is tightly regulated by a family of tissue inhibitors, known as tissue inhibitors of metalloproteinases (TIMPs); when activated they can partially or completely neutralize MMP function. Homeostasis of the ECM depends on the balance between MMPs and TIMPs.

MMP-2 and MMP-9 activity is downregulated by TIMP-2 and TIMP-1, respectively. TIMP-2, the most abundantly expressed TIMP, is of particular interest, as it has the cardinal feature to suppress MMP-2 at high levels, whereas at low levels, it surprisingly upregulates MMP-2. TIMP-2 is upregulated in patients and animal models of heart failure [26].

Carnitine is a quaternary ammonium considered as a chemical analogue of choline [27]. It is biosynthesized in the liver and kidneys from the amino acids lysine and methionine [28]; cardiac tissue cannot synthesize it. Carnitine has been found to promote choline derivatives' biosynthesis [29], whereas choline can serve as a methyl donor in the synthesis of carnitine from methionine and lysine [30]. It is common knowledge that carnitine has significant antioxidant effects on the myocardium and the endothelium under stress conditions [28,31], while it is also indispensable for the mitochondrial oxidation of long-chain fatty acids. Moreover, carnitine has been recognized as a nutritional supplement in cardiovascular diseases [31] and it is currently used as an adjunctive therapy in various heart conditions [32].

Choline is a lipotropic agent whose deficiency has been associated with defects in fatty acid metabolism [33] and increased oxidative stress [34] leading to liver steatosis or even cirrhosis [2,35] that might interfere with the synthesis of carnitine as the last depends on proper liver function [36].

In our experimental model, we hypothesized that carnitine administration could potentially compensate for the extra energy demands that would arise from the increased fatty acid oxidation due to choline deficiency, as well as for the relative carnitine deficiency observed in the setting of choline deficiency [37–41].

We sought to determine whether MMPs' and/or TIMPs' dysregulation is implicated in the pathogenesis of choline deficiency-induced cardiomyopathy, as well as the effects of carnitine supplementation.

METHODS

Animals

Male Albino Wistar rats (n = 24) 10–12 weeks of age (350 ± 30 g body weight), obtained from the National Center of Scientific Research 'Demokritos', were used. On arrival, the animals were maintained on a rodent standard diet, consumed food and water ad libitum and were housed at a constant room temperature (22 ± 1 °C) under a 12-h light: 12-h dark (light 08:00–20:00 h) cycle. They were acclimatized to laboratory conditions for a minimum of 1 week before the beginning of the experiment. All animals procedures were carried out under the authority of the relevant project license obtained from the Prefecture of Athens, and were approved by the Institutional Animal Care and Use Committee of the University of Athens for Medical Sciences. The number and suffering of the animals were kept to the minimum.

Induction of choline deprivation and L-carnitine supplementation

After acclimatization for 1 week to constant environmental conditions, rats were randomly assigned in four groups of six animals, each fed with: (i) standard diet (Control-CA), (ii) choline-deficient diet (CDD), (iii) standard diet and L-carnitine in drinking water 0.15% w/v

(CARN), and (iv) choline-deficient diet and L-carnitine in drinking water 0.15%w/v (CDD + CARN). The mean daily dose of L-carnitine was approximately 200 mg/kg body weight. Diets were obtained from AnaLab Ltd, Athens, Greece; L-carnitine was obtained from Vianex SA, Athens, Greece. The analytical composition (g/kg) of the choline-deficient diet was as follows: sugar 413, starch 110, dextrin 110, hydrogenated vegetable oil 100, pea protein 90, soya protein isolate 60, corn oil 50, mineral mix 35, vitamin mix 10, cellulose 10, vitamin-free casein 10 and L-cystine 2. The standard diet was enriched by choline (1.1 g/kg) at the expense of sucrose. Rats were handled according to the above dietary pattern for 4 weeks, when they were sacrificed by decapitation.

Immunohistochemistry (IHC)

After decapitation of rats at the end of the first month, the hearts were fixed after incubation in 10% formalin solution and then embedded in paraffin. Six sequential tissue sections, 4 μm in thickness, were taken from each heart, at a distance approximately of 2 mm from each other. Immunohistochemical staining for MMP-2, MMP-9, TIMP-1 and TIMP-2 proteins was performed on the paraffin sections, after heating overnight at 37 °C and subsequent deparaffinization in xylene and rehydration through graded alcohols. To enhance antigen retrieval, sections were microwave-treated in 0.01 M citrate buffer pH 6.0 at 750 W for 15 min for TIMP-2 and in EDTA pH 8.0 (Trilogy; Cell Marque, Rocklin, CA, USA) at 750 W for 15 min for MMP-2, MMP-9 and TIMP-1. Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide in tris-buffered saline (TBS). After rinsing with TBS, a ready to use prediluted blocking reagent (SU-01; General Bioscience, Brisbane, CA, USA) was applied for 20 min, at room temperature, to block nonspecific antibody binding. Subsequently, sections were incubated overnight at 4 °C with the following antibodies: (i) a rabbit polyclonal antibody against MMP-2 (AP23331PU-N; Acris Antibodies GmbH, Herford, Germany) at a dilution of 1 : 80, (ii) a rabbit polyclonal antibody against MMP-9 (AR33090-100; Aviva Systems Biology, San Diego, CA, USA) at a dilution of 1 : 60, (iii) a rabbit polyclonal antibody against TIMP-1 (orb11483; Biorbyt, Cambridge, UK) at a dilution of 1 : 40 and (iv) a mouse monoclonal antibody against TIMP-2 (clone 67-4H11; Acris Antibodies GmbH, Herford, Germany) at a dilution of 1 : 100. A two-step polymer-conjugated peroxidase method [TruVisionTM Poly-HRP IHC Detection Kit (IHC-761-15; General Bioscience, Brisbane, CA, USA)] was used for visualization, with diaminobenzidine as a chromogen. The Poly-HRP Reagent (HRP-261) was prediluted and was applied for 30 min at room temperature. This kit is designed for rat tissue immunohistochemistry, while the studied antibodies react with the corresponding rat antigens. Finally, sections were counterstained with haematoxyline and mounted.

Evaluation of the immunohistochemical staining was performed independently by two pathologists through light microscopic observation and without knowledge of the clinical data for each animal. The cases of disagreement were only eight slides of a total of 112. After the necessary re-evaluation, consensus was finally achieved in all specimens.

The score was the average of 10 distinct high-power fields observed under ×400 magnification. Cytoplasmic immunoreactivity was observed for all proteins.

IHC scoring was interpreted using a semi-quantitative scoring system that has been widely accepted and described previously [42,43] taking into account the staining intensity and the number of positive cells (area extent). MMP and TIMP stained sections were graded in a blinded manner by two independent experienced pathologists as absent (0), weak (1), moderate (2) or intense (3) staining. The percentage of cells stained was, respectively, 0% (0), 1–10% (1), 11–50% (2) and 51–100% (3). As positive controls, we used a paraffin-embedded human breast carcinoma. Negative controls had the primary antibody omitted and replaced by nonimmune normal serum from the same species as the primary antibody.

In the present experimental setting, we have already published data concerning the degree of cardiac interstitial fibrosis [3], according to which the performed modified Masson's Trichrome staining [44] in the examined specimens revealed a significant fibrosis in the choline-deficient myocardial tissue [3]. The assessment of the extent of cardiac fibrosis was conducted in a way similar to the one that is usually used in such histopathologic studies [45]. For the comparative evaluation of myocardial fibrosis and the relevant alterations in the ECM composition (as were depicted by MMP-2, MMP-9, and their respective tissue inhibitors TIMP-1 and TIMP-2 immunostaining), the same specimens were used.

Additionally, we determined the immunohistochemical staining pattern of MMP/TIMP ratio, which reflects a crude index of the activation/inhibition balance of the collagenolytic system [46].

Statistical analysis

The statistical tests have been performed by the Statistical package SPSS 19 (Academic license). Prior to any statistical test the normality of the studied variables was evaluated by the Kolmogorov–Smirnov normality test, according to which the variables were found not to follow a normal distribution. For that reason, we performed the Kruskal–Wallis test for the comparison of the differences. Statistical significance was considered for P values of <0.05. Whatever the P value was lower than 0.05 the statistical significance between the groups was checked, one by one, by performing nonparametric Mann–Whitney test.

RESULTS

MMP-2, MMP-9, TIMP-1, and TIMP-2 were expressed in the cytoplasm of nearly all cells which comprise the heart, while MMP-9 and TIMP-2 were also localized occasionally at the nuclei of the vessel wall.

Relative tissue expression of the studied proteins is demonstrated in *Figures 1*–5.

The immunohistochemical expression of MMP-2 (*Figures 2* and *3*) was significantly suppressed in the CDD group compared to control ($P = 0.005$), as well as in the CDD + CARN group compared to control and CARN group. There was a trend toward higher MMP-2 expression in the CARN group compared to CDD without reaching statistical significance.

MMP-9 tissue expression was higher in the control group compared to CDD, CARN and CDD + CARN groups without, however, reaching statistical significance (*Figure 4*). Carnitine supplementation did not affect MMP-9 expression in the CDD group. A trend toward lower tissue expression of MMP-9 was noticed in the CDD + CARN group compared to CARN.

TIMP-1, although identified in the cardiac tissue of all groups, it presented a diffuse and weak immunostaining; therefore, we did not classify it as positive.

TIMP-2 tissue expression (*Figures 2* and 5) was significantly higher in the CDD and CDD + CARN groups compared to control and CARN groups ($P = 0.003$, $P = 0.005$, $P = 0.002$ and $P = 0.004$ respectively). Carnitine supplementation had no effect on immunostaining expression of TIMP-2 neither in the control nor in the CDD groups.

The examined ratio between the MMP-2 and TIMP-2 immunostaining expression suggested as well a shift toward a suppressed rate of the ECM degradation in the CDD group compared to control that the concurrent administration of carnitine could not prevent (*Figure 6*). Moreover, the performed Spearman rank correlation revealed that the ratio fluctuations correlate stronger to the MMP-2 than to TIMP-2 changes.

The MMP-9/TIMP-1 ratio did not reach statistical significance between the groups (data not shown).

DISCUSSION

In a previous study [3], we demonstrated that choline-deficient diet can lead in adult rats to impairment of heart mechanical properties and more specifically to a prominent early diastolic dysfunction, a condition that can be ameliorated by the supplementation of L-carnitine in drinking water. The aim of the present paper was to further investigate the mechanisms underlying this effect.

Studies have shown that the presence of a choline-deficient state for an experimental period similar to ours is associated with stimulation of the ECM turnover and deposition through increased expression of both MMPs and TIMPs [47–52]. Unfortunately, most of these studies concerned mainly the liver since choline deficiency is a well-established model of nonalcoholic fatty liver disease [53]. Moreover, MMP-2 has been shown to be upregulated in rat models of choline deficiency-mediated liver cirrhosis [54–57] and a similar pattern of expression has been observed for TIMP-1

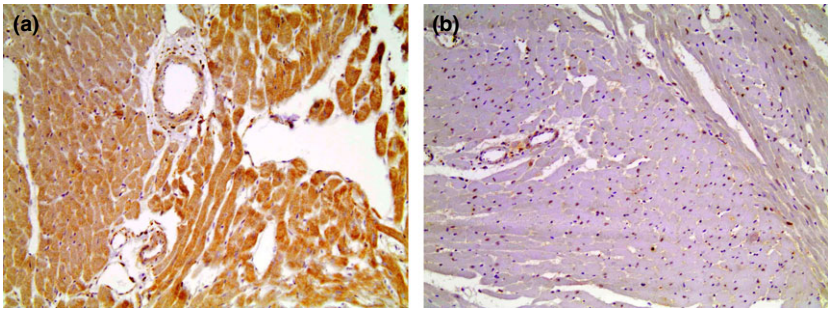

Figure 1 Representative photos indicating the expression of the studied proteins (by brown staining) in the: (a) myocardium, interstitium and vessels; (b) interstitium and vessels (×200).

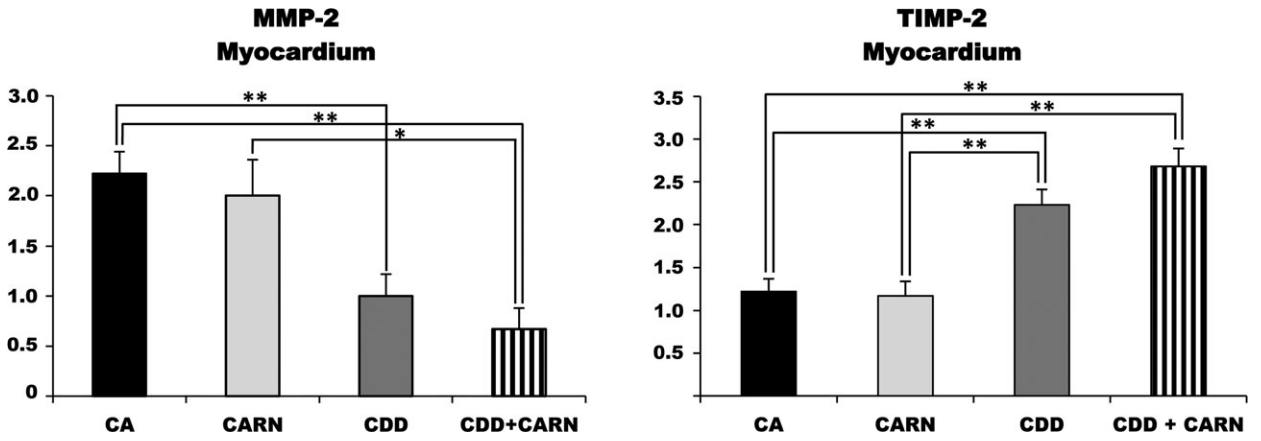

Figure 2 Effect of choline-deficient diet on cardiac MMP-2 and TIMP-2 immunohistochemical expression and modulation by carnitine co-administration after 1 month of intervention. Data are represented as mean ± SEM. The asterisk (*) represents the statistical significance (*P*) between the indicated groups and the number of asterisks corresponds to the level of the statistical significance (*$P < 0.05$, **$P < 0.01$). CA: rats receiving standard diet, CDD: rats fed with choline-deficient diet, CARN: rats receiving standard diet and carnitine in drinking water, CDD + CARN: rats receiving choline-deficient diet and carnitine in drinking water.

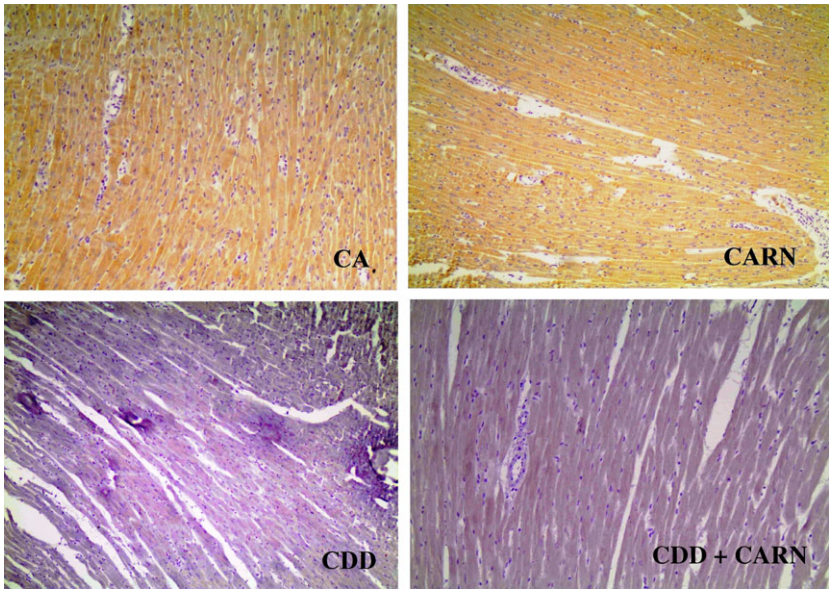

Figure 3 Moderate cytoplasmic expression (indicated by brown staining) of MMP-2 in the myocardium of control (CA) and carnitine-treated (CARN) rats. Suppressed expression of MMP-2 in the myocardium of choline-deficient rats without carnitine (CDD), or after carnitine supplementation (CDD + CARN), (×100).

[54,57,58], TIMP-2 [54] and MMP-9 [57]. On the contrary, the impact of inadequate levels of carnitine on MMPs modulation has been found to result in suppressed ECM degradation [59,60]. Except for their effect on the regulation of ECM homeostasis, MMPs interfere with cell survival, differentiation, inflammation and angiogenesis triggering the expression of vascular endothelial growth factor (VEGF) [61,62]. It is already known that choline deficiency has been associated with upregulation of VEGF [63–65], but the causative contribution of which in the pathogenesis of choline deficiency-mediated heart dysfunction [3] could be difficult to assume in this setting as on the other hand, the concurrent established carnitine deficiency [66] might induce downregulation of VEGF [67,68], (*Figure* 7).

Up to date, the patterns of expression of MMPs and TIMPs in the current experimental conditions, and especially as far as the heart tissue is concerned, have not been investigated. In addition, evidence from previous reports has demonstrated that diastolic heart dysfunction and fibrosis are associated with reduced MMPs activity that promotes collagen deposition [17,46]. This

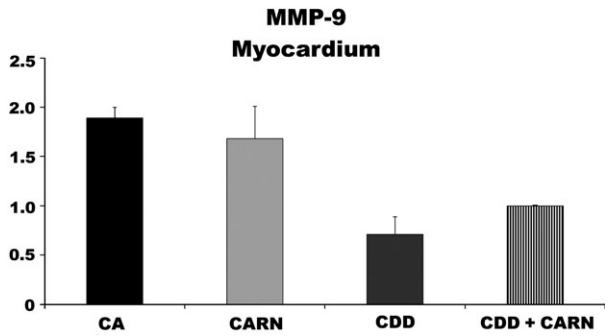

Figure 4 Cardiac MMP-9 immunohistochemical expression under choline deficiency conditions and modulation by carnitine co-administration after 1 month of intervention. Data are represented as mean ± SEM. There was no statistical significance between the groups. CA: rats receiving standard diet, CDD: rats fed with choline-deficient diet, CARN: rats receiving standard diet and carnitine in drinking water, CDD + CARN: rats receiving choline-deficient diet and carnitine in drinking water.

is interesting since such cardiac functional impairment has been observed in a choline-deficient state as well [3], where a mainly moderate cardiac fibrosis along with focal myocardial inflammation and cardiac interstitial edema was found [3].

MMPs, considered as key factors in the maintenance of the normal cardiac extracellular architecture, have also been implicated in changes of the vascularization process in various heart diseases. Although the contentious issue of the angiogenesis in cardiovascular diseases is out of the scope of the present study, it is important to notice that in the heart, MMP-2 increases the expression of VEGF [69] promoting vascular growth, whereas MMP-9 seems to down-regulate angiogenesis by promoting angiostatin expression [69], although this effect is of doubtful significance given its low activity. In general, the role of VEGF in the complex biology of cardiovascular diseases, although vital, has not yet been elucidated [70].

In our model, the pattern of MMP-2 tissue expression is in accordance with the changes observed in pressure overload models of heart failure [46] and in diabetic cardiomyopathy [71]. On the other hand, the pattern of MMP-9 tissue expression is different to that observed in other models of heart failure, like that induced by viral infection or ischemia/reperfusion injury [18,72]. This discrepancy could be attributed to the absence in our experimental setting of left ventricular (LV) dilatation which is mostly associated with upregulation of MMP-9 [25].

Nevertheless, the MMP-9 trend resembles to the pattern observed in diabetic cardiomyopathy [17,73]; this is not surprising as choline deficiency leads to mitochondrial dysfunction [2] which, in turn, is associated with insulin resistance [74].

In addition to MMPs, TIMPs, and especially TIMP-1 and TIMP-2, have been associated with the development of interstitial fibrosis of the heart, but in our model, we observed no change in the tissue expression

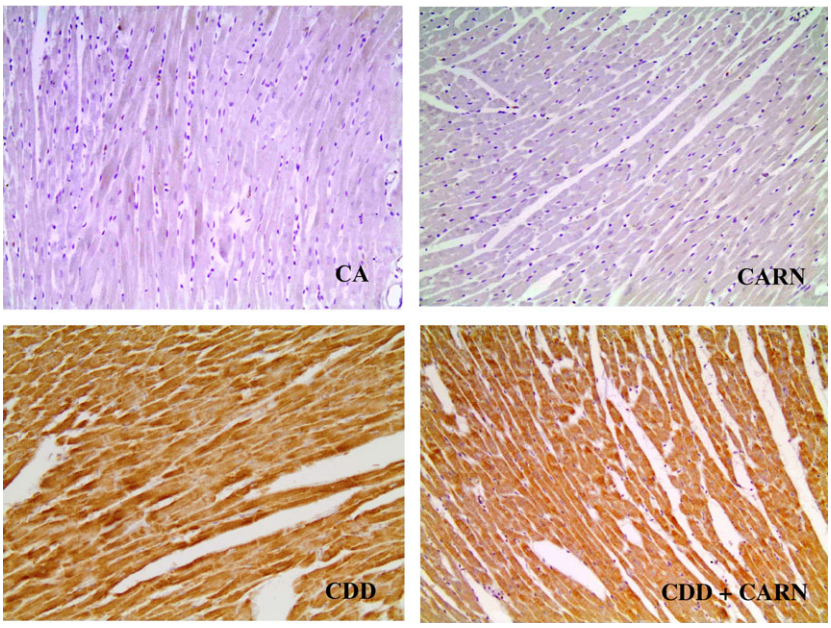

Figure 5 Faint expression of TIMP-2 in the myocardium of control (CA) and carnitine-treated (CARN) rats. Intense expression of TIMP-2 (indicated by brown staining) in the myocardium of choline-deficient rats without carnitine (CDD) and moderate TIMP-2 expression in choline-deficient rats with carnitine supplementation (CDD + CARN), (×100).

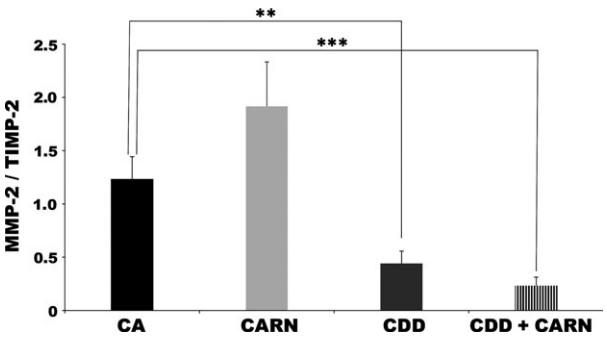

Figure 6 Graphical representation showing the modulation of MMP-2/TIMP-2 ratio in the myocardium (immunohistochemical expression) in a choline-deficient state with or without the concurrent carnitine administration after 1 month of intervention. Data are represented as mean ± SEM. The asterisk (*) represents the statistical significance (P) between the indicated groups and the number of asterisks corresponds to the level of the statistical significance (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). CA: rats receiving standard diet, CDD: rats fed with choline-deficient diet, CARN: rats receiving standard diet and carnitine in drinking water, CDD + CARN: rats receiving choline-deficient diet and carnitine in drinking water.

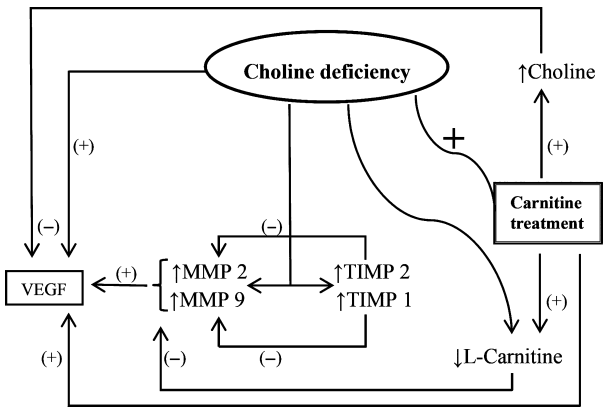

Figure 7 Diagrammatic representation of the known interactions between choline, carnitine, MMPs-TIMPs and VEGF.

of TIMP-1. This finding could be related to the short experimental period or/and the extent and severity of the myocardial damage in a choline-deficient setting. Moreover, it arises once again the question regarding the time- and space-dependent expression pattern of each TIMP family member during the various stages of cardiac remodeling, their multifactorial role and the possible MMP-independent TIMP-mediated processes during cardiac health and disease [75]. On the other hand, TIMP-2 has been found to decrease the expression of pro-angiogenic MMP-2, VEGF and increase the anti-angiogenic factors that result in detrimental LV remodeling [69,76]. Except for fibrosis itself, it is believed that aberrant TIMP-2 activation might promote heart dysfunction by inhibiting angiogenesis through its effects on MMP-2 activity [69,76]. TIMP-2 was significantly upregulated in the setting of choline deficiency.

The perturbed homeostasis of the ECM, as was indicated by the inhibition of the MMP-2 and increase of the TIMP-2 immunohistochemical expression under choline deficiency conditions, provides evidence of remodeling of the ECM, suggesting an aberrant ECM that could contribute to the myocardium stiffness [77] and progressive heart dysfunction observed in this case [3]. Moreover, the studied ratio of MMP-2/TIMP-2 reflecting the ECM dynamics that are indispensable during restructuring of tissue architecture confirmed that dietary deprivation of choline triggers collagen accumulation and deposition leading to cardiac interstitial fibrosis.

We also explored whether carnitine would have a measurable effect on tissue expression of MMPs and TIMPs in choline-deficiency conditions, as L-carnitine is a molecule with significant antioxidative, antiapoptotic and anti-inflammatory properties [78,79] and has been widely used as adjunct in cardiac diseases management [32]. In experimental studies, carnitine has been shown to ameliorate CCl_4-induced liver fibrosis [32,73,74,78,79] as well as cyclosporine and radiation-induced renal fibrosis [80], in part by modulating the TIMP-3 to MMP-9 activity ratio [32]. However, its potential favorable effect on ECM homeostasis is not always present, as shown in a model of bleomycin-induced lung fibrosis [81].

In the heart, L-carnitine has been shown to exert beneficial effects on interstitial remodeling in aged rabbits [82]. Moreover, it reduces myocardial fibrosis in hypertensive rats by modulating PPAR-γ expression and NOX2, NOX4, TGF-β1, and CTGF production, as well as prostacyclin release through the arachidonic acid pathway [83,84]. According to our data, L-carnitine has the capacity to significantly reduce choline-deficient diet-induced myocardial fibrosis and to maintain cardiac function [3]. The fact that L-carnitine has no significant effect on MMP-9, MMP-2, TIMP-1, or TIMP-2 activities in both choline-deprived and control rats suggests that the beneficial effects of L-carnitine are not mediated through modulation of tissue expression of MMPs and TIMPs, key enzymes in

the regulation of interstitial tissue composition [85], but probably through alternative mechanisms [86], such as nitric oxide [87], oxidative stress [31], inflammation or PPAR activity [83,88] modulation. It is unlikely that longer exposure to L-carnitine might change the expression of MMPs and TIMPs substantially, since at this time, functional changes are already evident [3].

To our knowledge, this is the first study trying to elucidate the effects of L-carnitine supplementation on heart tissue expression of TIMPs and MMPs in a choline-deficient setting.

In conclusion, we demonstrated that MMPs and TIMPs dysregulation is one of the factors contributing to myocardial dysfunction observed in rats receiving a choline-deficient diet, although it is difficult to clarify if this represents a primary, or a secondary effect. Moreover, the beneficial effects of L-carnitine do not seem to be mediated through MMPs and/or TIMPs regulation. The underlying molecular mechanisms still remain elusive, and further studies will be needed to clarify the precise mechanism of L-carnitine protective effect on the heart in this experimental setting.

Clinical perspectives

Our results highlight the potential significance of relative choline deficiency in heart dysfunction. This might be more relevant for specific populations, such as patients with chronic renal or liver failure in whom choline deficiency is more prevalent [89]. In this setting, baseline cardiac dysfunction due to other factors (such as atherosclerosis or alcohol induced cardiomyopathy) may be exacerbated by concurrent choline deficiency.

Furthermore, recent evidence has shown that choline dietary deprivation could potentially evoke changes in ion gradients during the cardiac cycle [66]. These data, along with the intriguing question raised by the present study regarding the possible silent interference of the nutritional status with the cardiac ECM homeostasis, suggest that the efficacy of commonly prescribed drugs used in cardiac failure management could be affected [17,90]. More importantly, choline deficiency seems to provoke diastolic dysfunction, a heart failure phenotype for which therapeutic options are very limited.

Noteworthy is that the findings of the current immunohistochemical approach revealed that the pattern of MMPs–TIMPs observed in a choline deficiency setting resembles the respective one encountered in known clinical conditions that are strongly associated with diastolic heart dysfunction either as causative or as predisposing factors, such as diabetes [71] and overweigh/obesity [91]. This highlights the need for further investigation on humans to elucidate the role of dietary choline deprivation in the diagnostic and therapeutic implications of this type of heart dysfunction.

Last but not least, there is emerging evidence that nonalcoholic fatty liver disease, which can be induced by a choline-deficient diet, could by itself be associated with subclinical myocardial dysfunction [92]. More research in this field is warranted.

ACKNOWLEDGMENTS

The authors are deeply indebted to all the co-workers for their significant contributions in this field. They would also like to acknowledge their appreciation to Mr Eugenios Danas for his technical assistance. This work was supported by grants from the National and Kapodistrian University of Athens.

CONFLICT OF INTEREST

The authors declare that no conflict of interest exists.

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