

L-arginine and N-acetyl-L-cysteine effects on morphometric parameters in experimental type 1 diabetes-induced myocardial fibrosis

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Aim. To study the morphometric changes of the myocardial connective tissue component under conditions of experimental type 1 diabetes mellitus (T1DM) and to evaluate the effectiveness of correction with L-arginine and N-acetyl-L-cysteine.

Materials and methods. The study was conducted on 47 male Wistar rats (18–20 months old). T1DM was induced by streptozotocin (45 mg/kg). The animals were divided into groups: control, T1DM without correction, T1DM with L-arginine, and T1DM with N-acetyl-L-cysteine (1.5 g/kg/day). The degree of fibrosis was assessed using Masson's trichrome staining and immunofluorescence of type 1 collagen. Microphotographs were analyzed using ImageJ software.

Results. The development of T1DM over 8 weeks led to pronounced myocardial remodeling: interstitial fibrosis increased by 3.8 times, and perivascular fibrosis by 1.9 times compared to the control. Type 1 collagen content increased by 538 %, and its concentration by 332 % ($p < 0.05$). L-arginine administration significantly reduced the progression of both types of fibrosis (interstitial by 0.5 times, perivascular by 0.7 times); however, it was accompanied by a 37 % increase in type 1 collagen concentration compared with the T1DM group. N-acetyl-L-cysteine showed a selective effect, significantly correcting only perivascular fibrosis (0.7-fold reduction) without a significant impact on the interstitial component or type 1 collagen markers.

Conclusions. Experimental type 1 diabetes mellitus leads to maladaptive myocardial remodeling, manifested by fibrosis with a predominance of the perivascular component over the interstitial and critical accumulation of type 1 collagen. Administration of L-arginine reduces the progression of interstitial and perivascular fibrosis, but increases the concentration of type 1 collagen. The use of N-acetyl-L-cysteine effectively corrects perivascular fibrosis, realizing an endothelioprotective effect, but does not affect the severity of interstitial fibrosis, the content and concentration of type 1 collagen.

Keywords:

type 1 diabetes, diabetic cardiomyopathy, myocardium, interstitial fibrosis, perivascular sclerosis, type 1 collagen, L-arginine, N-acetyl-L-cysteine, morphometry.

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Вплив L-аргініну та N-ацетил-L-цистеїну на морфометричні параметри при фіброзі міокарда на фоні експериментального цукрового діабету 1 типу

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Мета роботи – вивчити морфометричні зміни сполучнотканинного компонента міокарда за умов експериментального цукрового діабету 1 типу (ЦД1) і при введенні амінокислот.

Матеріали і методи. Дослідження проведено на 47 щурах-самцях лінії Wistar (18–20 місяців). ЦД1 моделювали введенням стрептозоточину (45 мг/кг). Тварин поділили на чотири групи: контроль, ЦД1 без корекції, ЦД1 з введенням L-аргініну та ЦД1 з введенням N-ацетил-L-цистеїну (1,5 г/кг/добу). Ступінь фіброзу оцінювали за допомогою трихромного забарвлення за Массоном та імунофлуоресцентного визначення вмісту та концентрації колагену 1 типу. Цифровий аналіз мікрофотографій здійснено у програмі ImageJ.

Результати. Розвиток ЦД1 протягом 8 тижнів спричинив виражене ремоделювання міокарда: інтерстиціальний фіброз збільшився у 3,8 раза, а периваскулярний – у 1,9 раза порівняно з контролем. Вміст колагену 1 типу збільшився на 538 %, а його концентрація – на 332 % ($p < 0,05$). Введення L-аргініну достовірно зменшило прогресування обох типів фіброзу (інтерстиціального – в 0,5 раза, периваскулярного – в 0,7 раза), проте супроводжувалося підвищенням концентрації колагену 1 типу на 37 % щодо групи ЦД1. N-ацетил-L-цистеїн виявив вибіркового ефекту, достовірно коригуючи лише периваскулярний фіброз (зменшення в 0,7 раза), без суттєвого впливу на інтерстиціальний компонент та показники колагену 1 типу.

Висновки. Експериментальний цукровий діабет 1 типу призводить до патологічного ремоделювання міокарда, що проявляється фіброзом із переважанням периваскулярного компонента над інтерстиціальним та критичним накопиченням колагену 1 типу. Введення L-аргініну зменшує прогресування інтерстиціального і периваскулярного фіброзу, проте підвищує концентрацію колагену 1 типу. Застосування N-ацетил-L-цистеїну ефективно коригує периваскулярний фіброз, реалізуючи ендотеліопротекторний ефект, але не впливало на вираженість інтерстиціального фіброзу, вміст та концентрацію колагену 1 типу.

Ключові слова:

цукровий діабет 1 типу, діабетична кардіоміопатія, міокард, інтерстиціальний фіброз, периваскулярний склероз, колаген 1 типу, L-аргінін, N-ацетил-L-цистеїн, морфометрія.

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Type 1 diabetes mellitus (T1DM) is an independent factor for the development of cardiomyopathy, characterized by structural and functional remodeling of the myocardium. The key morphological feature of this process is progressive interstitial and perivascular fibrosis, which leads to increased myocardial stiffness, diastolic dysfunction, and reduced coronary reserve [1]. The pathogenesis of cardiac fibrosis in diabetes is associated with chronic hyperglycemia, activation of oxidative stress, impaired bioavailability of nitric oxide (NO), endothelial dysfunction, and fibroblast stimulation resulting in excessive synthesis of collagen, predominantly type 1 [2]. Under hyperglycemic conditions, competition for the substrate (L-arginine) occurs between endothelial NO synthase (eNOS) and arginase. Activation of the arginase pathway can shift L-arginine metabolism from the production of vasoprotective NO to the synthesis of ornithine – a precursor of proline and polyamines that stimulate fibrogenesis [3].

The role of antioxidant therapy in correcting different compartments of fibrosis remains debatable. Donors of local gasotransmitter systems, such as L-arginine and N-acetyl-L-cysteine, demonstrate potential cardioprotective properties, but their impact on interstitial versus perivascular fibrosis may differ significantly [4,5].

Aim

The aim of the study was to study the morphometric changes of the myocardial connective tissue component under conditions of experimental type 1 diabetes mellitus and to evaluate the effectiveness of correction with L-arginine and N-acetyl-L-cysteine

Materials and methods

The study was conducted at the Educational and Scientific Medical and Laboratory Center with Vivarium of Zaporizhzhia State Medical and Pharmaceutical University (Certificate of Technical Competence of the Ministry of Health of Ukraine No 181/23 from 21.12.2023, due to 20.12.2028), following national and European norms for the protection of animals used for scientific purposes, after approval by the local Bioethics Commission at the ZSMPhU (protocole No. 8 from 20 May, 2026) [6,7]. T1DM was modeled in 47 male normoglycemic, normotensive Wistar rats (18–20 months old). The animals were divided into 2 experimental groups: control (n = 7) and DM1 (n = 40) [8]. Induction was performed with streptozotocin (45 mg/kg, Sigma Aldrich) in citrate buffer. Control rats received buffer only. Animals with glycemia >15 mmol/L were included in 2 weeks. From week 6, DM1 rats were divided into: DM1-1 (without the introduction of amino acids), DM1-2 (L-arginine), and DM1-3 (N-acetyl-L-cysteine). Both substances were administered in drinking water (1.5 g/kg/day) for 2 weeks (total duration of simulation – 8 weeks). Euthanasia was performed by decapitation under sodium thiopental (120 mg/kg).

To visualize collagen fibers and assess the degree of myocardial fibrosis, 5 µm thick paraffin sections of the rat heart apex were prepared. The classical Masson's Trichrome staining method was used, as a result of which collagen fibers acquired an intense blue color, muscle tissue – red, and cell nuclei became dark brown.

Quantitative assessment of myocardial interstitial fibrosis was performed with ImageJ software (National Institutes of Health, USA). Photomicrographs were obtained using a light microscope on "PrimoStar" (Carl Zeiss, Germany) with an "AxioCam 105 color" digital camera (Carl Zeiss, Germany) with the same lighting and exposure parameters and saved in TIFF format without compression. After calibration, the Color Deconvolution plugin [9] was used to separate the color components with the "Masson Trichrome vector" selected, and then the collagen channel (aniline blue) was used for analysis. Collagen-positive areas were isolated by "Threshold" segmentation using the standard "Default" method and a light background with visual control to exclude cardiomyocytes and background; the same threshold parameters were applied to all images in the series. After thresholding, a binary mask was created on which the collagen area was determined using the "Analyze Particles" function with a minimum particle size of $\geq 10 \mu\text{m}^2$, a roundness of 0.00–0.70, and exclusion of objects at the edges of the image. The percentage of fibrosis was calculated as the ratio of the area of collagen-positive areas to the total area of tissue in the field of view (58452.6146). For each heart, 50 randomly selected fields of view were analyzed, after which the average value was calculated.

The assessment of the content and concentration of collagen type 1 was carried out using the indirect immunofluorescence method. After standard histochemical processing, tissue antigens were unmasked with 10 mM sodium citrate buffer, pH = 6.0 (Santa Cruz biotechnology, Inc., USA, sc-294091) in a PT-module (Thermo Scientific, USA) for 20 min at +95 °C. After cooling the slides, they were immersed in phosphate-buffered saline (PBS) pH = 7.4 (Santa Cruz biotechnology, Inc., USA, sc-24946) and washed in 3 portions of 5 min. After aspiration of excess moisture in the dark, 300 µl / glass of UltraCruz® blocking reagent (Santa Cruz biotechnology, inc, USA, sc-516214) was applied to the sections, in which the primary and secondary antibodies were subsequently diluted at a concentration of 1:64. After that, the slides were washed again three times in PBS. Then, primary antibodies (goat polyclonal antibodies COL1A1 (C-18): sc-8784, Santa Cruz Biotechnology, Inc., USA) were used, with slide incubation for 1 day at $t = +4 \text{ }^{\circ}\text{C}$. After washing three times in PBS, the slides were incubated for 1 hour in a humidified chamber ($t = +37 \text{ }^{\circ}\text{C}$) with FITC-conjugated secondary antibodies (Santa Cruz Biotechnology, Inc., USA: sc-2356). After washing, the sections were mounted in glycerol for fluorescence microscopy (1040950250, Sigma-Aldrich, Germany).

Immunofluorescence study was performed using an AxioImager-M2 microscope (Carl Zeiss, Germany) equipped with high-emission 38HE filters (green fluorescence – $\lambda_{\text{ex}} = 470/40 \text{ nm}$, $\lambda_{\text{em}} = 525/50$). Analysis of at least 50 image files, obtained using a high-sensitivity AxioCam-ERc 5s video camera (Carl Zeiss, Germany), was carried out in ImageJ. Areas with statistically significant fluorescence were identified in an automatic mode, followed by the calculation of the immunoreactive material content (expressed in Uif), which was defined as corrected total cell fluorescence (CTCF). It was calculated using the formula: $\text{CTCF} = (\text{ID} - (\text{S} \times (\text{M} - \text{SD}))) / 1000$, where

Table 1. Results of the study of the parameters of the connective tissue component of the myocardium of rats of the experimental groups, M ± m

Parameter	Tissue fibrosis in the frame, Masson's trichrome		Collagen type 1, immunofluorescence	
	Perivascular, %	Interstitial, %	Contents, Uif	Concentration, Uif/μm ²
Control, n = 7	10.19 ± 0.48	2.04 ± 0.01	50.13 ± 0.57*	1.01 ± 0.01*
DM1-1, n = 7	19.85 ± 0.93*	7.68 ± 0.11*	319.93 ± 46.08*	4.37 ± 0.36*
DM1-2, n = 7	14.02 ± 0.82**	4.01 ± 0.02**	382.83 ± 25.21*	5.99 ± 0.41**
DM1-3, n = 7	14.38 ± 0.59**	7.95 ± 0.17**	328.52 ± 23.45*	4.66 ± 0.41**

*: statistically significant difference compared to control, $p < 0.05$; #: statistically significant difference between correction groups compared to DM1-1 without amino acids, $p < 0.05$; \$: statistically significant difference between DM1-3 group with N-acetyl-L-cysteine compared to DM1-2 with L-arginine, $p < 0.05$.

ID – the integrated density; S – the area of the region of interest (ROI), μm²; M (Median) – the median pixel value in the ROI, Uif; SD – the standard deviation of grey values calculated in the ROI.

The concentration (C) of the immunoreactive material (IRM) (Uif/μm²) was calculated using the following formula: $C = CTCF / S$, where CTCF (corrected total cell fluorescence) – the immunoreactive material content, Uif; S is the area of the ROI, μm².

Statistical processing was performed using one-way analysis of variance (ANOVA) in the Statistica software (License No. JPZ804I382130ARCN10-J). All continuous variables were tested for normal distribution using the Shapiro-Wilk W-test. Continuous variables are presented as mean ± standard error of the mean (M ± m). A two-tailed p-value <0.05 was considered statistically significant for all tests.

Results

A study of the myocardium of control rats stained with Masson's trichrome revealed the proliferation of connective tissue (blue and purple areas) of the stroma between muscle fiber bundles (red), as well as the appearance of significant layers of fibrous tissue in the walls of blood vessels, which is characteristic of age-related remodeling, accompanied by sclerosis and hyalinosis (Fig. 1A–D, Table 1).

After 8 weeks of T1DM progression, rats in the DM1-1 group formed pronounced interstitial fibrosis and sclerosis of the myocardial stroma, significantly exceeding the corresponding values in the control group (Fig. 1E, Table 1). The severity of these structural changes was accompanied by vascular remodeling with increase in perivascular fibrosis compared to the control (Fig. 1F, Table 1). Additionally, Masson's trichrome staining revealed focal degenerative-destructive and necrotic changes in cardiomyocytes, with their cytoplasm acquiring violet and blue tints. Such areas were most frequently found near vessels with the most pronounced expansion of perivascular spaces due to edematous and pre-sclerotic changes, confirming an ischemic mechanism of damage (Fig. 1E,F, Table 1). Structural changes in the interstitium were realized through an increase in the content and concentration of type 1 collagen (by 538 % and 332 %, respectively, relative to control values, $p < 0.05$) (Fig. 1G,H, Table 1).

Microscopy of myocardial sections from the DM1-2 group rats with L-arginine revealed the preservation of interstitial connective tissue layers between bundles of cardiomyocytes, represented mainly by collagen fibers intensely stained in blue. At the same time, the structure

of muscle fibers was (or became) partially disorganized, with areas of uneven cardiomyocyte staining (Fig. 2A,B, Table 1).

In contrast to the DM1-1 group, the group treated with L-arginine showed a statistically significant moderate reduction in the thickness of perivascular fibrous cuffs, with less pronounced sclerosis and preservation of the vessel lumen. Signs of perivascular space edema were less intense, indicating partial normalization of the microcirculatory bed. The administration of L-arginine to these rats led to a reduction in the progression of interstitial fibrosis, the percentage of which was significantly lower than in the DM1-1 group (Fig. 2A,B, Table 1). While L-arginine had no statistically significant effect on the content of type 1 collagen, its concentration significantly increased by 37 % compared to the DM1-1 subgroup without amino acid administration (Fig. 2C,D, Table 1).

The myocardium of the DM1-3 group rats with N-acetyl-L-cysteine was characterized by moderately pronounced interstitial fibrosis with a diffuse arrangement of collagen fibers between cardiomyocytes; its intensity exceeded control values, though comparison with the DM1-1 group revealed no statistically significant differences (Fig. 2E,F, Table 1). Concurrently, a significant reduction in the thickness of connective tissue layers was noted in the perivascular zones compared to the DM1-1 group, with lower collagen fiber density and the absence of coarse vascular wall sclerosis. Cardiomyocytes mainly retained their transverse striation, although focal dystrophic changes were observed in some areas. Unlike the effects of L-arginine, the content and concentration of Type 1 collagen did not change statistically significantly compared to the subgroup without correction (Fig. 2G,H, Table 1).

Discussion

The obtained results convincingly demonstrate that experimental T1DM is accompanied by pronounced structural remodeling of the myocardium, with progressive interstitial and perivascular fibrosis serving as the key morphological manifestation. The identified increase in the proportion of the connective tissue component and the accumulation of type 1 collagen support the concept of diabetic cardiomyopathy, which possesses a clearly defined fibrotic phenotype [1,2,10]. It is known that excessive deposition of fibrillar collagen in the extracellular matrix leads to increased myocardial stiffness, impaired diastolic filling, and reduced coronary reserve, which determine the functional failure of the heart in diabetes [2,4].

The predominance of perivascular fibrosis in T1DM rats without correction points to primary damage to the microcir-

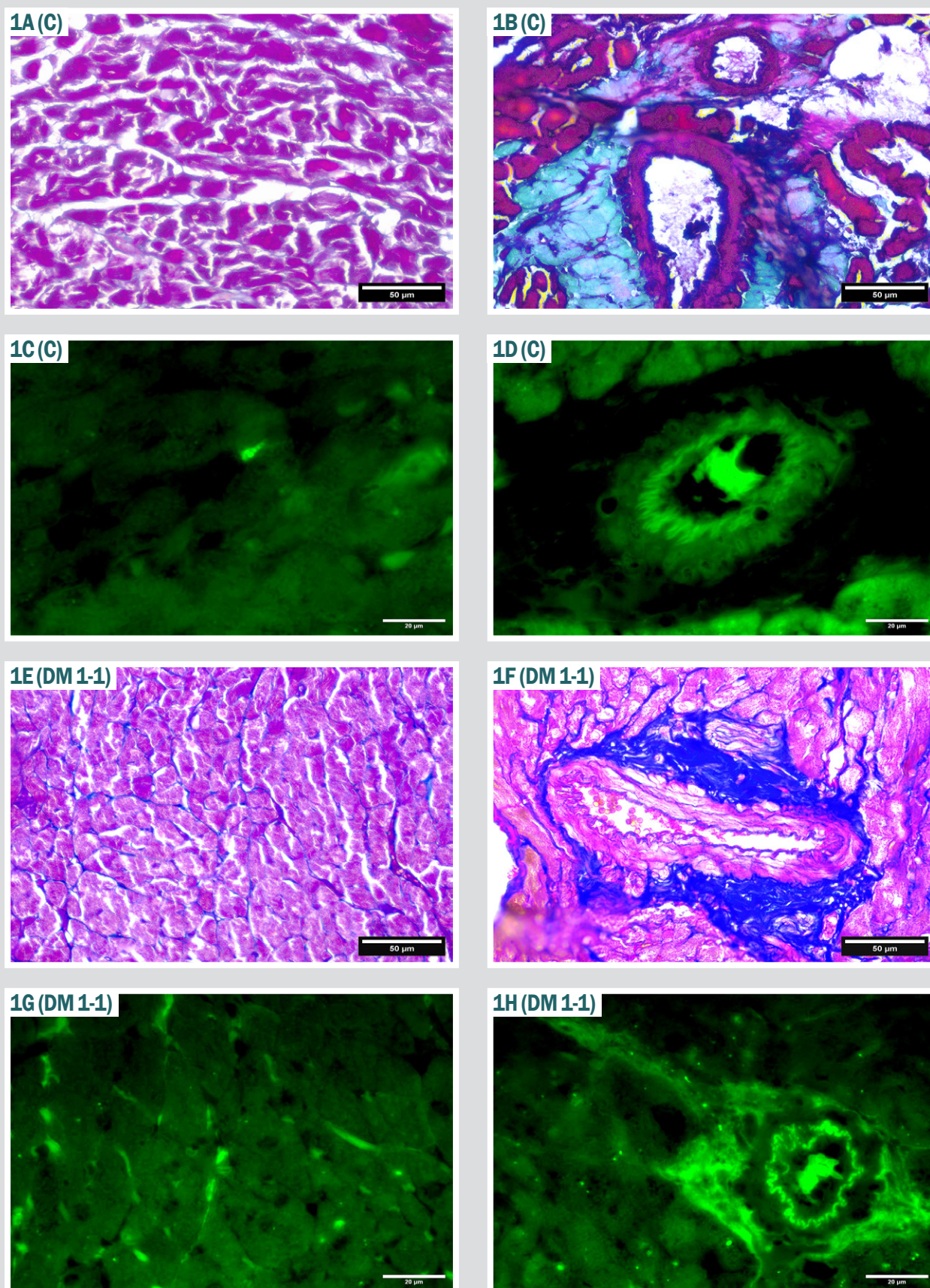


Fig. 1. Microphotographs of the myocardium of rats from experimental groups. Masson's trichrome, $\times 400$: **A, B** – control, **E, F** – DM1 without amino acids; type 1 collagen, $\times 630$: **C, D** – control, **G, H** – DM1 without amino acids.

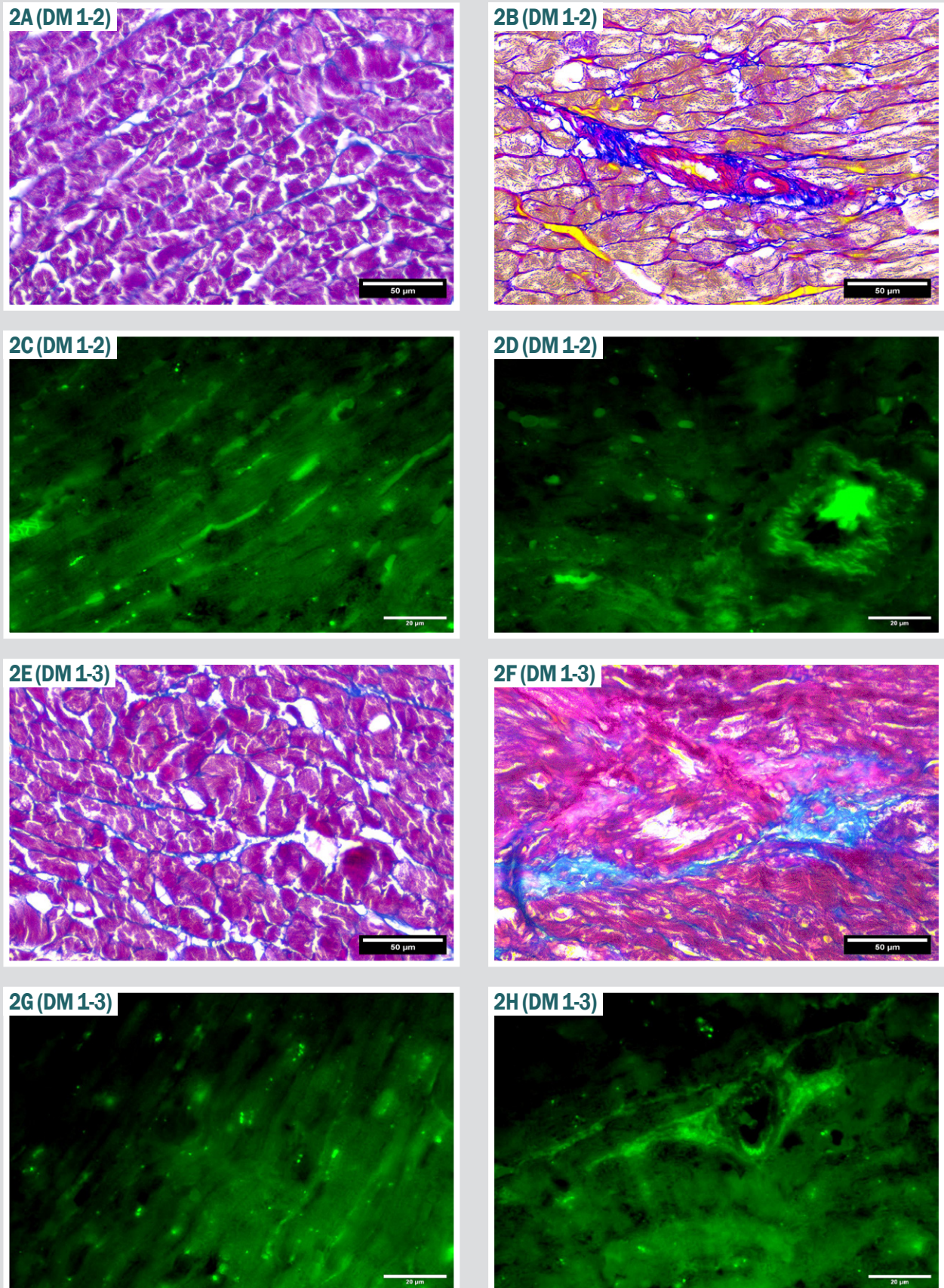


Fig. 2. Microphotographs of the myocardium of rats from experimental groups. Masson's trichrome, $\times 400$: **A, B** – DM1 with L-arginine, **E, F** – DM1 with N-acetyl-L-cysteine; type 1 collagen, $\times 630$: **C, D** – DM1 with L-arginine, **G, H** – DM1 with N-acetyl-L-cysteine.

culatory bed as one of the early pathogenetic mechanisms of diabetic myocardial remodeling. Chronic hyperglycemia promotes the development of endothelial dysfunction, reduces nitric oxide bioavailability, and activates oxidative stress, thereby triggering a cascade of proinflammatory and profibrotic signaling pathways, particularly through the activation of transforming growth factor- β (TGF- β) and cardiac fibroblasts [2,5]. The obtained morphometric data align with literature reports indicating that perivascular fibrosis is a morphological marker of the early stages of diabetic cardiomyopathy and determines the further progression of interstitial sclerosis [10].

L-arginine administration was accompanied by a statistically significant reduction in interstitial and perivascular fibrosis, indicating its positive impact on myocardial remodeling. L-arginine is a substrate for endothelial NO synthase, and increased nitric oxide availability promotes the normalization of endothelium-dependent vasodilation, reduction of leukocyte adhesion, inhibition of smooth muscle cell proliferation, and decrease in vascular sclerosis [3,11]. At the same time, the lack of a statistically significant effect of L-arginine on type 1 collagen content, despite a simultaneous increase in its concentration, may indicate complex processes of extracellular matrix remodeling. It is likely that L-arginine does not so much inhibit collagen synthesis as influence its organization, degradation, or redistribution between the interstitial and perivascular compartments. This may also reflect the stages of fibrosis formation, where a reduction in the total area of fibrosis is accompanied by relative stabilization or even accumulation of more densely organized collagen [11].

In contrast to L-arginine, N-acetyl-L-cysteine administration led to a reduction in perivascular fibrosis without a statistically significant effect on the interstitial component or type 1 collagen parameters. N-acetyl-L-cysteine is a precursor of glutathione and a potent antioxidant that reduces reactive oxygen species levels, stabilizes redox homeostasis, and protects the endothelium from oxidative damage [12]. This may explain the reduction in vascular fibrosis and the improved state of the perivascular space. However, the lack of a substantial impact on interstitial fibrosis suggests that antioxidant therapy has limited efficacy against an already formed fibrous matrix and is insufficient to halt the further progression of this remodeling component [10].

Thus, the results of this study not only confirm the key role of fibrosis in the pathogenesis of diabetic cardiomyopathy but also demonstrate compartment-specific effects of L-arginine and N-acetyl-L-cysteine on myocardial remodeling. This provides a deeper understanding of the mechanisms of action of amino acid donors involved in gasotransmitter signaling and antioxidant defense, highlighting the potential of differentiated or combined therapeutic approaches for attenuating left ventricular fibrotic remodeling in T1DM.

Further research should focus on clarifying the molecular mechanisms behind the L-arginine-induced accumulation of type 1 collagen, particularly regarding the arginase pathway and extracellular matrix remodeling factors. Moreover, evaluating a combined therapy using both L-arginine and N-acetyl-L-cysteine represents a promising direction to achieve comprehensive,

compartment-specific cardioprotection against diabetic fibrosis. It is also essential to correlate these structural alterations with functional cardiac metrics and coronary microcirculation changes in chronological dynamics.

Conclusions

1. Experimental type 1 diabetes mellitus leads to pathological myocardial remodeling, manifested by fibrosis with a predominance of the perivascular component over the interstitial one and critical accumulation of type 1 collagen.

2. L-arginine administration reduces the progression of interstitial and perivascular fibrosis but increases the concentration of type 1 collagen.

3. The use of N-acetyl-L-cysteine effectively corrects perivascular fibrosis but does not affect the severity of interstitial fibrosis or the content and concentration of type 1 collagen.

AI statement

During the preparation of this article, the following artificial intelligence tool was used: Gemini 3.5 Flash. It was used for language editing and translation. All AI-generated or AI-assisted content was carefully reviewed and edited by the authors, who take full responsibility for the content of the article.

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