Advanced glycation end products (AGE) in the pathogenesis of ischaemic cardiomyopathy in diabetic patients – preliminary report

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Abstract

Background: Glycaemic disorders occurring in diabetes type 2 lead to non-enzymatic coupling of glucose to proteins, nucleic acids and lipids, thereby resulting in advanced glycation end products (AGE) which disturb myocardial mechanics.
Aim: The aim of the work was morphological evaluation of AGE in the left ventricle of the heart in patients with diabetes type 2, and establishing whether, and in what way, AGE play a role in ischaemic cardiomyopathy in diabetic patients.
Material and methods: The diabetes group encompassed 9 hearts explanted in patients with diabetes type 2 undergoing orthotopic heart transplant. The control group comprised 9 fragments of the left ventricular muscle with no clinical features of diabetes; the specimens originated from hearts designated for homogenic valve harvesting. The localization of AGE was detected immunohistochemically, and intensity was marked with a semi-quantitative scale.
Results: The positive reaction in cardiomyocytes was of a cytoplasmatic character, predominantly granular, whilst in the diabetic group it was mixed, diffuse-granular. Tiny blood vessels and stromal fibroblasts displayed AGE expression. The diabetic group was characterized by a significantly stronger reaction in cardiomyocytes, stromal fibroblasts and in the walls of arterioles, as compared with the control group. The intensity of reaction did not correlate with patient age in either group; however, a significant correlation was found between reaction intensity and the duration of diabetes.
Conclusions: Intensified AGE presence in left ventricular structures in diabetic patients indicates a likelihood of AGE in the pathogenesis of cardiac failure.
Key words: advanced glycation end products, heart failure, ischaemic cardiomyopathy, immunohistochemistry.

Streszczenie

Wstęp: Zaburzenia glikemii zachodzące w cukrzycy typu 2 prowadzą do nieenzymatycznego sprzęgania glukozy z białkami, kwasami nukleinowymi i lipidami, wytwarzając końcowe produkty zaawansowanej glikacji (ang. *advanced glycation endproducts*, AGE), zaburzające mechanikę mięśnia sercowego.
Cel pracy: Celem pracy była ocena morfologiczna AGE w lewej komorze serca u chorych z cukrzycą typu 2, a poprzez to odpowiedź na pytanie, czy i jak końcowe produkty zaawansowanej glikacji mogą u chorych z cukrzycą odgrywać rolę w postępie kardiomiopatii niedokrwiennej.
Materiał i metody: Grupa cukrzycy obejmowała 9 serc eksplantowanych u chorych z cukrzycą typu 2 podczas zabiegu ortotopowego przeszczepienia serca. Grupę kontrolną stanowiło 9 wycinków mięśnia lewej komory, pochodzących z serc przeznaczonych do preparatyki zastawek homogennych, bez klinicznych cech cukrzycy. Lokalizację AGE wykrywano immunohistochemicznie, nasilenie oznaczano w skali półilościowej.
Wyniki: Dodatni odczyn w kardiomiocytach miał charakter cytoplazmatyczny, przy czym w grupie kontrolnej dominował charakter ziarnisty, a w grupie cukrzycy dyfuzyjno-ziarnisty. Drobne naczynia i fibroblasty zrębu ujawniały ekspresję AGE. Grupa cukrzycy charakteryzowała się znamiennie silniejszym odczynem w kardiomiocytach, fibroblastach zrębu mięśnia sercowego oraz w ścianach drobnych tętniczek w porównaniu z grupą kontrolną. Nasilenie reakcji w obu grupach nie korelowało z wiekiem chorych. Stwierdzono istotną korelację nasilenia reakcji z czasem trwania cukrzycy.
Wnioski: Nasilona obecność AGE w strukturach komory lewej u chorych z cukrzycą wskazuje na prawdopodobieństwo ich AGE w patogenezie niewydolności serca.
Słowa kluczowe: zaawansowane końcowe produkty glikacji, niewydolność serca, kardiomiopatia niedokrwienna, immunohistochemia.

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Introduction

Chronic heart failure is one of the most frequent causes of cardiological death, particularly in the elderly population. Ischaemic aetiology of heart failure is predominant in this group of patients. The so-called ischaemic cardiomyopathy occurs as a result of inadequate myocardial perfusion originating from the presence of pathological changes which compromise blood flow in coronary arteries. Despite progress in the treatment of ischaemic heart disease, according to the ISHLT register ischaemic cardiomyopathy still constitutes an indication for transplant in approximately 40% of patients undergoing this procedure [1]. Diabetes is particularly conducive to the occurrence of intramuscular ischaemic changes, both at the level of the large epicardial arteries and the distal coronary arterioles. One of the metabolic diseases described most in depth, diabetes is characterised by scarce specific histological substrate. Regardless of the aetiology, the most prominent is hyperglycaemia, which leads to non-enzymatic coupling of glucose as a reducing sugar with proteins, nucleic acids and lipids, thereby producing the so-called advanced glycation end products (AGE) [2, 3]. These compounds occur in an insoluble form, creating multicellular deposits responsible for impaired endothelial function and nephropathy – a form of diabetic microangiopathy [4]; the soluble pool is responsible for the inflammatory reaction and creates a number of receptor protein complexes (RAGE) [5]. As a long-term phenomenon, glycation has a particularly damaging effect on long-living proteins, especially collagen IV, basal membranes of capillary vessels, myelin, tubulin and extracellular matrix proteins – collagen type I, III, IV, VI, laminin, elastin. The best known glycation end products are pentosidine and N-(carboxymethyl)-lysine (CML) [6-8]. Glycation of cellular growth factors such as TGFβ and CTGF results in intensified production, which leads to organ fibrosis [9-12]. As the structural proteins bind with AGE, protein cross-linking takes place, leading to changes in biochemical and mechanical properties which result in stiffening and elasticity loss [13-16], whilst in the myocardium diabetic cardiomyopathy occurs [17].

The clinical evaluation of AGE was based on biochemical examination of their presence in the serum [13, 18]. Histopathological evaluation concerned mainly cases of diabetic macro- and microangiopathy [19, 20]. The aim of our work was morphological evaluation and identification of the location of glycation products in the left ventricular structures in patients with diabetes type 2, with the objective to establish whether, and in what way, AGE may play a role in ischaemic cardiomyopathy in diabetic patients.

Material and methods

The study group consisted of 9 hearts explanted in patients with diabetes type 2 during orthotopic heart transplant due to ischaemic heart disease. All these subjects were male, aged 49-63 years, mean 57±5.4 years. Diabetes duration was 1-21 years, mean 7.6±6.6 years. The patients had been treated with oral hypoglycaemic agents or insulin. The control group was composed of multi-organ donors – 9 men aged 18-27 years, mean 22.2±2.9 years, not suffering from diabetes – and the tissue samples of the left ventricle were collected during homogenic valve preparation. The decision against using a donor heart for transplant was not related to the condition of the harvested organ in any of the cases.

All tissues were fixed in 10% neutralised formalin. Transmural fragments of left ventricular muscle layer from an unchanged site were taken for histopathological examination and subjected to routine paraffin procedure. After dewaxing, the 8 µm thick paraffin fragments were treated with Target Retrieval Solution pH 9.0TE at a temperature of 90-95°C for 40 minutes. The slices were subsequently incubated in a humid chamber with anti-AGE peroxidase labelled antibody (RDI-ADVGLY-HRP, clone 6D12, RDI Division of Fitzgerald Industries Intl, 34 Junction Square Drive, Concord MA 01742-3049 USA) at a concentration of 2 µg/ml for 12 hours at 4°C, eventually developing the location in reaction with Envision+/HRP and DAB+ (DAKO), twice for 5 minutes at room temperature. The cell nuclei were counterstained with Mayer's haematoxylin. The slides were dehydrated and placed in water-free medium.

Microscope evaluation encompassed the location and intensity of reaction in muscle fibres, with regard to the inner layer (endocardium and subendocardium), middle layer and outer (subepicardium). Then the remaining tissues were evaluated: the connective tissue – its cells and fibres, blood vessels and their wall elements. The reaction intensity (range $_{Intens}$) was evaluated on a semi-quantitative scale from 0 to 3 (0 – no reaction, 1 – weak reaction, 2 – moderate reaction, 3 – very strong reaction), the level of reaction in cardiomyocytes being the number of cells with a positive reaction (0 – no cells; 1 – non-numerous or single cells, approx. 10%; 2 – the majority of cells, >10-70%; 3 – almost all cells, approx. 80-100%).

This classification of intensity ranges facilitated the calculation of the total range value for cardiomyocytes (AGE $_{Cardiocyte}$) and the left ventricular muscle (AGE $_{Total}$) by multiplying the range of reaction intensity in cardiocytes by the range denoting the value of reaction in a given layer and finally by summing them up according to the formulae:

$$AGE_{Cardiocyt} = range_{Cardiocyt} \times (range_{Endoc} + range_{Middle} \times range_{Epicard})$$

$$AGE_{Total} = AGE_{Cardiocyte} + AGE_{Fibrobl} + AGE_{Capillaries} + AGE_{Arterioles} + + AGE_{Arteries} + ...$$

Moreover, the character of the positive reaction was evaluated and classified into diffuse, granular and mixed.

The results were processed statistically using nonparametric methods. The assumed level of statistical significance was $p \leq 0.05$.

Results

The positive immunohistochemical reaction with the anti-AGE antibody in cardiomyocytes was of a cytoplasmic character, both in the study group and in controls. In the

Tab. I. Character of immunohistochemical anti-AGE staining in analyzed groups

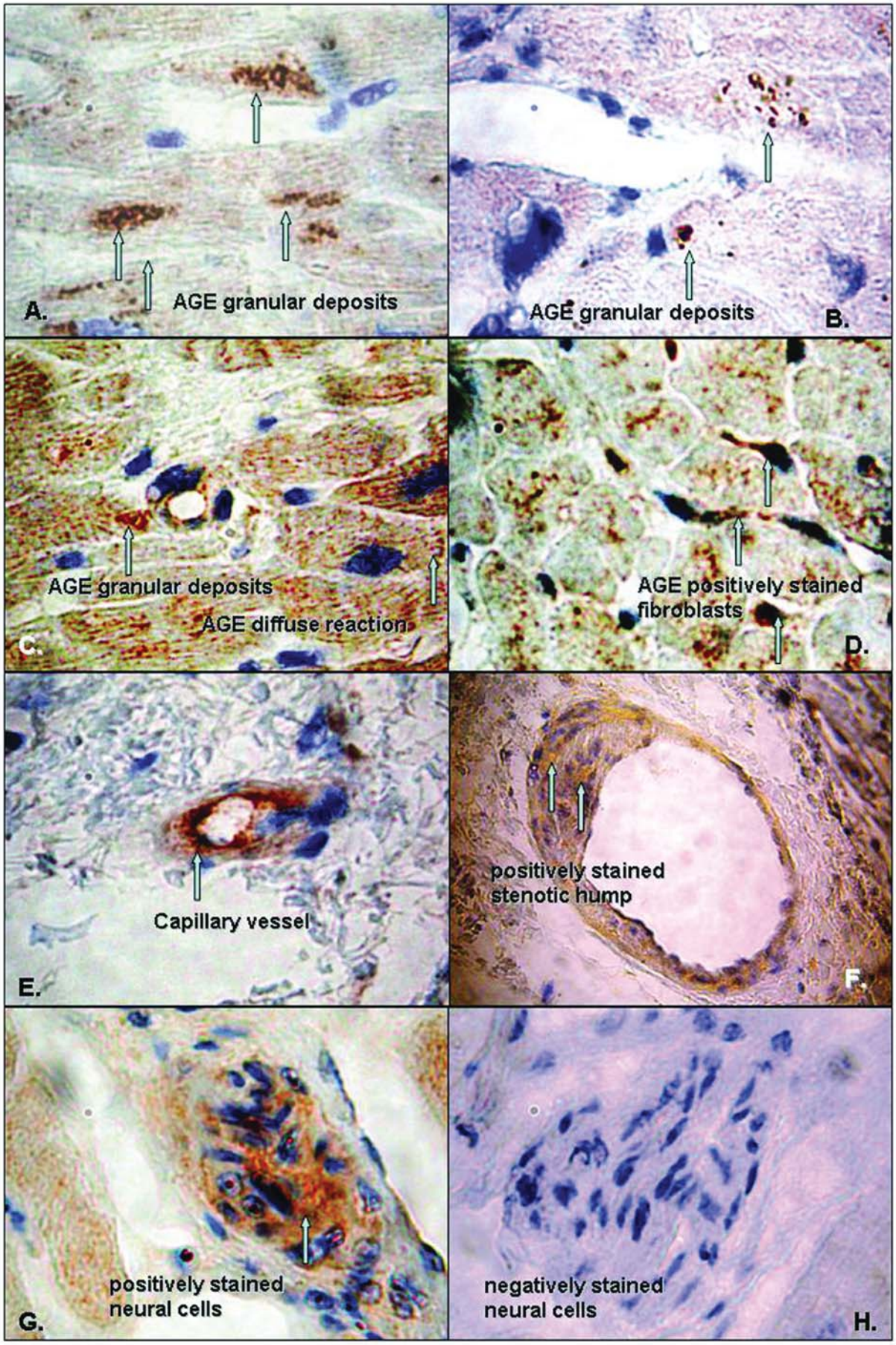
Group	Staining pattern	
	granular	diffuse and granular (mixed)
Control group	9 (100%)	0
Diabetes type 2 group	4 (44%)	5 (56%)
Statistical significance Fisher exact test	p=0.0294 S	

Tab. II. Semi-quantitative characteristics of myocardial constituents in immunohistochemical staining with anti-AGE

Myocardial constituents	Group	No. of cases	Mean range	Standard deviation	Median	Minimum	Maximum	Comparison of groups p Mann-Whitney test
Cardiomyocytes	control	9	1	0	1	1	1	0.0039 **S**
	diabetes	9	1.8	0.4	2	1	2	
Fibroblasts	control	9	0.3	0.5	0	0	1	0.0314 **S**
	diabetes	9	1.2	0.8	1	0	2	
Capillaries	control	9	0.1	0.3	0	0	1	0.2224 NS
	diabetes	9	0.6	0.7	0	0	2	
Arterioles	control	9	0.3	0.5	0	0	1	0.0315 **S**
	diabetes	9	1	0.5	1	0	2	

control group most frequently it had the form of single or numerous clusters of AGE granules located centrally in the cardiomyocyte (Fig. 1A, B). In the diabetes group, however, the reaction was predominantly of a mixed character with diffuse cytoplasmic staining and numerous single granules as well as numerous granules forming conglomerates (Fig. 1C). The diffuse reaction had subtle granules located densely in a linear manner forming axes parallel to the cardiomyocyte long axis, which may coincide with myofibrils. Left ventricle stromal fibroblasts displayed a sporadically dense positive cytoplasmatic reaction only in the diabetes group (Fig. 1D). There was no positive reaction

Fig. 1A-H. A. Control group. Longitudinal cardiomyocytic cross-section with positively stained granular AGE deposits in central part of sarcoplasm (arrows). Cell nuclei stained blue with haematoxylin, negatively for AGE. Immunohistochemical staining anti-AGE-HRP/DAB+ Mayer haematoxylin. Magn. 600 ×. **B.** Control group. Transverse cardiomyocytic cross-section with few positively stained granular AGE deposits in sarcoplasm (arrows). Capillary vessel endothelial cells (left-central part of figure) with no positive reaction product. Cell nuclei stained blue with haematoxylin, negatively for AGE. Immunohistochemical staining anti-AGE-HRP/DAB+ Mayer haematoxylin. Magn. 1000 ×. **C.** Diabetes type 2 group. Oblique cardiomyocytic cross-section, dominating diffuse sarcoplasmic positive AGE staining and few granular conglomerates (arrows). Centrally visible small arteriole with fine granular endothelial localization of positive reaction product. Cell nuclei stained blue with haematoxylin, negatively for AGE. Immunohistochemical staining anti-AGE-HRP/DAB+ Mayer haematoxylin. Magn. 600 ×. **D.** Diabetes type 2 group. Transverse cardiomyocytic cross-section with positively stained fine granular and diffuse AGE reaction in cardiomyocytes. Interstitial fibroblast cytoplasm filed with dense AGE deposits (arrows). Immunohistochemical staining anti-AGE-HRP/DAB+ Mayer haematoxylin. Magn. 600 ×. **E.** Diabetes type 2 group. Oblique cross-section. Centrally visible capillary vessel with intense diffuse reaction in endothelial cells (arrow). Few perivascular cells (fibroblasts) negatively stained for AGE, with blue-stained nuclei. Connective tissue fibres negatively stained for AGE deposits. Immunohistochemical staining anti-AGE-HRP/DAB+ Mayer haematoxylin. Magn. 600 ×. **F.** Diabetes type 2 group. Intramyocardial arteriole with upper left intimal stenotic "hump" (recent plaque). Almost all vascular wall cells present diffuse slight and moderate positive staining, increased in stenotic hump cells (arrows). Immunohistochemical staining anti-AGE-HRP/DAB+ Mayer haematoxylin. Magn. 80 ×. **G.** Diabetes type 2 group. Small subepicardial autonomic ganglion. All neural cell cytoplasm filled with diffuse AGE-positive reaction product (arrow). Immunohistochemical staining anti-AGE-HRP/DAB+ Mayer haematoxylin. Magn. 400 ×. **H.** Control group. Small subepicardial autonomic ganglion. Neural cells negatively stained for AGE. Immunohistochemical staining anti-AGE-HRP/DAB+ Mayer haematoxylin. Magn. 400 ×


A.
AGE granular deposits
AGE granular deposits
B.
AGE granular deposits
AGE diffuse reaction
C.
AGE positively stained
fibroblasts
D.
Capillary vessel
E.
positively stained
stenotic hump
F.
positively stained
neural cells
G.
negatively stained
neural cells
H.

Tab. III. Semi-quantitative characteristics of myocardial layers and scoring in immunohistochemical staining with anti-AGE

Myocardial constituents	Group	No. of cases	Mean range	Standard deviation	Median	Minimum	Maximum	Comparison of groups p Mann-Whitney test
Internal layer	control	9	1	0	1	1	1	0.1135 NS
	diabetes	9	1.7	0.9	1	1	3	
Medial layer	control	9	1	0	1	1	1	0.0039 **S**
	diabetes	9	1.9	0.6	2	1	3	
External layer	control	9	0.8	0.4	1	0	1	0.0018 **S**
	diabetes	9	1.8	0.4	2	1.0	2	
$AGE_{Cardiocyt}$	control	9	2.8	0.4	3	2	3	0.2224 NS
	diabetes	9	10.0	4.4	10.0	3.0	16.0	
AGE_{TOTAL}	control	9	3.3	0.5	3	3	4	0.0315 **S**
	diabetes	9	12.7	5.1	13.0	5	20	

located in the extracellular matrix (Fig. 1E). Capillary vessels and arterioles showed a cytoplasmatic reaction in endothelial cells in the diabetes group (Fig. 1C, E), whilst intramuscular arterioles with stenotic atheromatous humps in the intima displayed a positive diffuse reaction in both groups (Fig. 1F). A rare element of the picture was autonomic nerve plexi noticeable in single slides of each of the groups; accordingly, they were not taken into account in the table summary. Furthermore, a strong positive reaction was observed in the cytoplasm of nerve cells in the diabetic group, and its lack in the control group (Fig. 1G, H).

The character of the immunohistochemical reaction in groups is summarized in Table I. Granular reaction was predominant in the control group, whilst the diabetes group was characterised by a mixed, diffusive-granular form. This relationship was statistically significant. Further microscopic and statistical analyses encompassed the intensity of reaction and its location in particular parts of the myocardium. The diabetic group was characterized by a significantly stronger reaction in cardiomyocytes, stromal fibroblasts and in the arteriolar walls as compared with the control group (Table II). Analysis of myocardial layers showed statistical significance between the groups in the middle and outer layers (subepicardium). The inner subendocardial layer displayed higher but not significant reaction intensity (Table III). Despite the high value, the total cardiomyocyte range value ($AGE_{Cardiocyte}$) did not display statistically significant differences either; however, the total range of the left ventricle (AGE_{Total}) showed statistically significant differences (Table III). The intensity of the anti-AGE reaction was not age-dependent (Table IV), but there was a very strong and statistically significant correlation between reaction intensity and diabetes duration in the case of the inner subendocardial layer of the left ventricle muscle and both summary indices, $AGE_{Cardiocyte}$ and AGE_{Total} (Table V). The above-listed differences in correlations regarding the various muscle layers of the left ventricle suggest the need to examine dependencies within groups; however, no statistically significant differences were observed (Table VI).

Discussion

The data obtained indicate the existence of a significant relationship between the presence of AGE in the left ventricular muscle and diabetes type 2. The mixed cytoplasmic diffuse-granular reaction was significantly more frequently observed in cardiomyocytes in the diabetes type 2 group. A similar phenomenon had previously been noticed in proximal tubules in patients with diabetic nephropathy [21], where the diffuse reaction was prevalent, and the mesangial cells displayed both types of reaction [22, 23]. AGE deposition is not exclusive to the working myocardial cells. Also, supportive elements such as fibroblasts and components of arteriolar walls showed significant intensification of changes (Table II), thereby suggesting the widespread nature of the glycation process. The comparison of total cardiomyocyte scores with scores encompassing the remaining tissue elements of the left ventricle (Table III) indicates the prevalent significance of myocardial elements such as arterioles, capillaries and fibroblasts. The phenomena of AGE deposition in the arteriolar walls and atherosclerotic coronary changes correspond to observations made by Sakata et al. [24], although these concerned advanced atherosclerotic plaque; AGE reaction products, primarily carboxymethyl-lysine-(CML), were deposited on the edges of cell-free foci, thereby suggesting the effect of translocation of cellular reaction products from dead cells. A similar increase in AGE epitope concentration was described earlier not only in diabetes but

Tab. IV. Correlation of patient age with immunohistochemical staining intensity in analyzed groups

Myocardial constituents	Control group		Diabetes type 2 group	
	Spearman R	probability/significance	Spearman R	probability/significance
Cardiomyocytes	0	1 NS	0.2609	0.4976 NS
Fibroblasts	0	1 NS	0.5076	0.1630 NS
Capillaries	–0.259	0.4991 NS	0.1315	0.7359 NS
Arterioles	–0.275	0.4739 NS	0.1381	0.7231 NS
Internal myocardial layer	0	1 NS	0.0976	0.8026 NS
Medial myocardial layer	0	1 NS	–0.0452	0.9081 NS
External myocardial layer	–0.259	0.4995 NS	0.2609	0.4976 NS
$AGE_{Cardiocyt}$	–0.259	0.4991 NS	0.1121	0.7740 NS
AGE_{TOTAL}	–0.045	0.9068 NS	0.2605	0.4984 NS

Tab. V. Correlation of diabetes type 2 duration with immunohistochemical staining intensity

Myocardial constituents	Diabetes type 2 group	
	Spearman R	probability/significance
Cardiomyocytes	0.5040	0.2031 NS
Fibroblasts	0.5477	0.1599 NS
Capillaries	0.4564	0.2556 NS
Arterioles	0.5774	0.1339 NS
Internal myocardial layer	0.8229	0.0121 **S**
Medial myocardial layer	0.4261	0.2924 NS
External myocardial layer	0.5039	0.2029 NS
$AGE_{Cardiocyt}$	0.7395	0.0360 **S**
AGE_{TOTAL}	0.8571	0.0065 **S**

Tab. VI. Comparison of myocardial layers according to immunohistochemical anti-AGE staining intensity

Comparisons	Probability/significance Wilcoxon test	
	control group	diabetes type 2 group
Internal myocardial layer vs. medial layer	1 NS	0.3613 NS
Internal myocardial layer vs. external layer	1 NS	0.6858 NS
Medial myocardial layer vs. external layer	1 NS	1 NS

also in renal insufficiency [25], atherosclerotic complications and in some dementia disorders. Examination of coronary artery atherosclerotic restenosis following intravascular stent implantation revealed accelerated and intensified AGE accumulation in 6-month follow-up in an experimental animal model [26]. In this regard, it seems that a significant metabolic disorder leading to disturbed glycaemia levels – local in the case of stents and generalised in diabetes – leads to permanent intracellular disorders. As a result, an insoluble netting AGE form is created which hardens the intracellular structures, including contractile fibrils. The experimental study of Petrova et al. [27] confirmed the increasing stiffness of the heart muscle with increasing concentrations of AGE and its cellular receptor RAGE, though this phenomenon was associated with disturbed Ca++ ion transport and phosphorylation of intracellular signal-regulating kinases [28]. Our earlier observations may also indicate modification of connective tissue, intensifying the above-mentioned phenomenon [29]. The diffuse and microgranular reaction observed in our study, located along myofibrils, as well as the already discussed tendency of AGE to bind with long-living proteins [1, 2, 5-7], seems to indicate clearly the cross-linking of the cardiomyocyte contraction apparatus and therefore impairment of its function through insoluble AGE deposits.

Another issue is the relation between time and intensification of glycation product deposition. Our study encompassed two quite narrow time-frames, between 18 and 27 years in the control group and between 49 and 63 years in the study group, encumbered with diabetes, atherosclerosis and circulatory failure. No relationship was observed in either the study group or the control group between age and the AGE deposits in heart evaluated semi-quantitatively. The study of Sato et al. [30] into AGE accumulation in the hippocampus showed a linear relationship between age and AGE deposition in that area. However, it cannot be excluded that this phenomenon may be affected by the possibility of AGE to induce angiogenesis in the heart [31, 32], as well as the influence of diabetes, which were not analysed by the quoted researchers. In this context, the highly significant relationship between AGE scores, the intensity of their deposition in the inner, less vascularised, subendocardial layer, and diabetes duration (Table V) seems

to be significant for the understanding of the discussed processes. However, functional differentiation of the myocardium into layers, in compliance with the premises of Torrent-Guasp [33], did not show statistically significant differences (Table VI), although the most intensive AGE deposition was observed in the middle layer, which is best vascularized (Table III).

Furthermore, it seems essential to emphasise the role of the autonomic nervous system, as shown in Fig. 1G and 1H. The deposition of AGE complexes with constitutional proteins of nerve cells constitutes the pathological background for diagnosing this disorder [34], though a single observation of the opposite patient group requires extension of the study.

This is the first publication describing the histological location of AGE in the heart, based on clinical material from patients undergoing heart transplantation. Earlier studies of this type concerned only serum-soluble forms of AGE [35].

The limitations of our study were: the low number of study groups and a considerable patient age difference in the study and control groups. Particularly the young age of patients in the control group limited the potential effect of this parameter on AGE deposition in non-diabetic patients. However, despite the low numbers, it was shown unambiguously in the study group that AGE intensification is connected with diabetes duration, and not patient age.

Conclusion

The obtained results indicate unambiguously the existence of a relationship between the duration of diabetes and the presence of advanced glycation end products (AGE) in the hearts of patients who due to ischaemic cardiomyopathy required heart transplantation. The presence of intensified AGE was observed in all heart cells and tissues which play an essential role in the development of heart failure – stenosis of epicardial arteries, tiny intramuscular arterioles, cardiomyocytes, fibroblasts and autonomic nerve plexi. Due to the considerable likelihood of AGE participation in the pathogenesis of heart failure in diabetic patients, further research in this field seems warranted.

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