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THE ASSESSMENT OF FLUOROPHORES ADVANCED GLYCATION END PRODUCTS-TO-KYNURENINE RATIO IN HEALTHY AND DIABETIC RATS AND HUMANS

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ABSTRACT. In this study, we calculated the ratio of the serum contributions of two highly studied fluorophore products (advanced glycation end products - AGEs and kynurenine - KYN) in the area of nutrition and metabolic diseases including diabetes mellitus (DM), by using a non-invasive, economical and easy-to-perform method. Blood serum spectrofluorimetric analysis was performed both in the case of normoglycemic (n=10) and diabetic rats (n=10), and in the case of non-diabetic (n=14) and type 2 diabetes mellitus (T2DM) patients (n=52). Our results showed a significant increase in the contributions of the two products in diabetic patients and rats compared to the control group. The ratio of the two compounds was positively correlated with serum glucose levels in the case of rats, and with serum triglyceride values in the case of humans. Also, the presence of DM complications in human subjects and the subsequent calculation of ROC curves led to a predictive value of the investigated ratio (AGEs/KYN) for the presence of peripheral diabetic polyneuropathy (PNP). The obtained results suggest a high potential for the investigated ratio to be considered as a new biomarker for the presence of PNP.

Keywords: *Advanced glycation end products, Kynurenine, Blood serum, Diabetes Mellitus, Fluorescence Spectroscopy*.

INTRODUCTION

According to IDF statistics, the prevalence of diabetes mellitus (DM) has currently reached global epidemic levels. If in 2007, 39 million persons were diagnosed with diabetes, in 2010 this figure increased to 285 million,

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and by 2040 it is estimated that 642 million persons will be affected by this disease [1, 2]. The situation is equally worrying for animals; thus, a 79.7% increase in the prevalence of canine diabetes cases was evidenced in 2015 compared to 2006 [3]. All these statistical data emphasize the need to introduce new biomarkers, as well as to identify new correlations between the existing biomarkers in order to gain a better understanding of the pathogenesis of diabetes mellitus in both animals and humans.

A useful aid in this respect is provided by the introduction of spectroscopy techniques in general and spectrofluorimetric in particular for the analysis of tissues and biological fluids from patients with a suspicion and diagnosis of diabetes mellitus. The main purpose of these techniques is to provide semi-quantitative information about the biodistribution of fluorophores in tissues and biological fluids, as well as to assess their biochemical composition [4]. Two classes of fluorophores investigated using these techniques and correlated with the study of diabetes and its complications are advanced glycation end products (AGEs) and kynurenine (KYN). Although AGEs and KYN have been studied separately in the context of DM, the relationship established between them, i.e. the ratio of the fluorophores has not been investigated. Furthermore, according to the current literature, there are no comparative data available regarding the simultaneous fluorescence investigation of AGEs and KYN and their ratio in the presence of DM and its complications.

AGEs or Maillard products are heterogeneous molecules produced through the non-enzymatic reaction of proteins with reducing sugars [5, 6, 7] (Figure 1). The multitude of physiological processes occurring in the body determines their continuous formation in cells and tissues. However, the AGE formation rate is increased in DM as a consequence of hyperglycemia [8, 6]. A great number of studies suggest the implication of AGEs in the pathogenesis of diabetes complications such as: retinopathy, neuropathy, chronic renal disease and cardiovascular disease [6, 8, 9]. The other investigated compound, KYN, is a fluorescent metabolite of tryptophan (TRP) and one of the parameters frequently associated with protein oxidative stress and other systemic inflammatory disorders. Thus, in inflammatory diseases such as diabetes, frequently associated with depression, there is an activation of the hepatic enzyme indoleamine 2,3-dioxygenase, responsible for metabolization of TRP to KYN, with the production of high KYN concentrations [10]. Previous studies have demonstrated the presence of a relationship between depression and one of the complications of diabetes – neuropathy, among patients with type 2 diabetes mellitus (T2DM) [11]. One of the objectives of this study was to check for the presence of a disturbance of TRP and implicitly KYN metabolism in the case of patients with T2DM diagnosed with this type of complication. We also aimed to simultaneously investigate AGEs and KYN by fluorescence

spectroscopy in order to assess their serum levels in healthy and diabetic rats, as well as in non-diabetic and T2DM human subjects. In addition, their simultaneous evaluation was performed in the context of DM complications in humans. According to the studied literature, these aspects have not been investigated before. In this context, we calculated the ratio between the contributions of the two classes of fluorophores to total serum fluorescence, with a view to considering this ratio as a new biomarker for the presence of some diabetes complications.

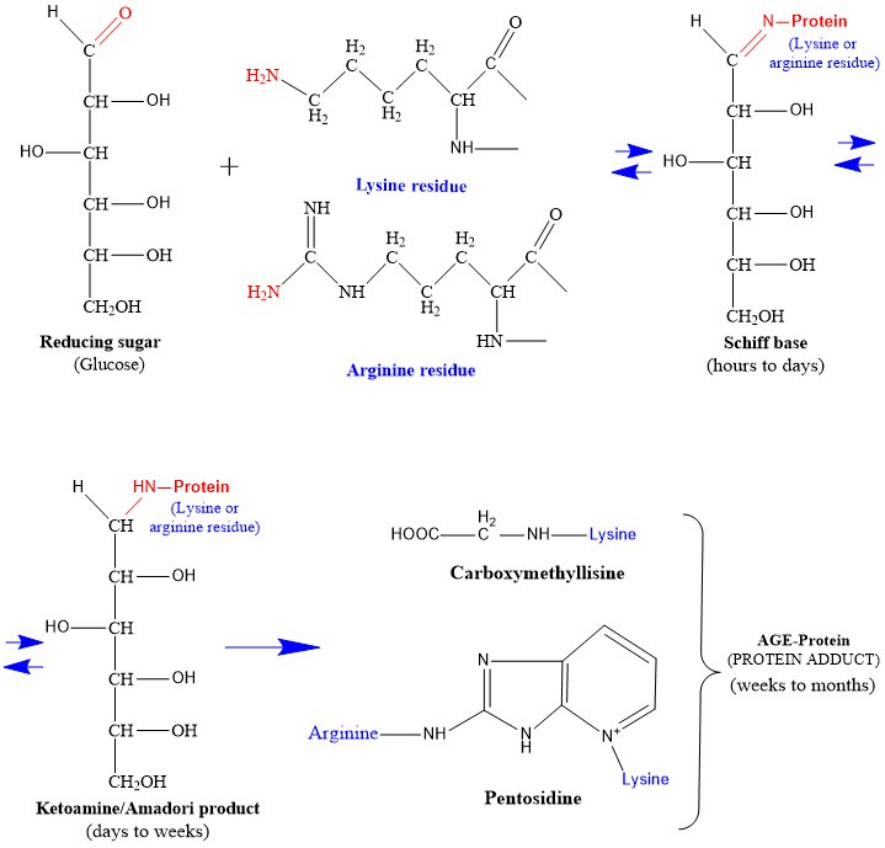

Figure 1. The schematic presentation of advanced glycation end products formation through Maillard reaction. Firstly, a reversible Schiff base is formed within hours through the reaction of reactive carbonyl groups of a reducing sugar with the free amino groups of proteins. Secondly, the Amadori product is formed by multiple chemical rearrangements over a period of days. Finally, through others multiple chemical rearrangements and over a period of weeks, advanced glycation end products such as: carboxymethyllisine and pentosidine are formed.

RESULTS AND DISCUSSION

The characteristics of non-diabetic rats and rats with streptozotocin-induced diabetes were described in the material and method section. Blood glucose levels were significantly higher in rats with streptozotocin-induced diabetes (20.43±6.12 mmol/l) compared to control rats (5.8±0.26 mmol/l). The characteristics of human subjects are shown in Table 1. Significant differences regarding age, duration of diabetes, body mass index, waist circumference, glycated hemoglobin and blood glucose levels were found between healthy and diabetic patients.

Table 1. Characterization of non-diabetic subjects and patients diagnosed with type 2 diabetes mellitus

Variables	Healthy subjects	Patients – type 2 diabetes mellitus
	n=14	n=52
Age (years, IQR)	52.00(40.50-59.00)	59(57-65)
Subjects – men (n, %)	8 (57.14%)	24(46.15%)
Smoking status (n, %)	5 (35.71%)	14 (26.92%)
Duration of diabetes (years)	0	10 (8)
Body mass index (kg/m^2)	25.86±4.54	30.49±4.83*
Waist circumference (cm)	94.21±13.30	108.25±11.20*
HbA1c (%)	<6.5	10.09±2.29*
Blood glucose (mmol/l, IQR)	5.44(4.78-5.78)	9.28(7.89-10.83)
Triglycerides (mmol/l, IQR)	1.12(0.68-1.44)	2.65(1.40-3.64)
Diabetic retinopathy (n, %)	0	20 (38.5%)
Diabetic neuropathy (n, %)	0	27 (51.92%)
Diabetic nephropathy (n, %)	0	20 (38.46%)
Cardiovascular disease (n, %)	0	26 (50 %)
Arterial hypertension (n, %)	0	34 (65.4 %)

Values are expressed as mean ± standard deviation, median and interquartile range or number and percentage, *p <0.05

The contributions of AGEs and KYN to total serum fluorescence were significantly greater in rats with streptozotocin-induced diabetes compared to the control group. Also, the ratio of contributions of the two classes of fluorophores (AGEs/KYN) to total serum fluorescence was significantly higher in rats with streptozotocin-induced diabetes compared to healthy rats (Table 2).

Table 2. Contributions of AGEs (advanced glycation end products), KYN (kynurenine) and the AGEs to KYN ratio in rats and humans

Parameter	Rats			Humans		
	Control	Diabetic	p value	Control	Diabetic	p value
AGEs	15.58±4.07	20.2±2.85	<0.013	16.42±1.27	18.87±3.48	<0.001
KYN	42.16±6.53	10.9±2.18	<0.001	18.41±2.09	21.40±4.42	0.001
AGEs-to-KYN ratio	0.38±0.10	1.89±0.40	<0.001	0.90±0.09	0.93±0.27	0.566

In patients with T2DM, significantly greater contributions of the fluorophores to total serum fluorescence were evidenced compared to the control group. The AGEs to KYN ratio was higher in diabetic patients compared to healthy subjects, but without statistical significance (Table 2).

The positive correlation of the ratio of fluorophores contributions to total serum fluorescence (AGEs/KYN) with serum glucose levels resulted in a Pearson's correlation coefficient value of 0.928 with CI=[0.743, 1.113], for a p value < 0.001. The significant correlation with the serum glucose level was maintained in the case of the two classes of fluorophores investigated separately; thus, for the contribution of AGEs to total serum fluorescence, the Pearson's correlation coefficient value was 0.463 with CI=[0.024, 0.902], with a p value = 0.040, and for the contribution of KYN to total serum fluorescence, the Pearson's correlation coefficient value was -0.867 with CI=[-1.114, -0.621], with a p value < 0.001.

Following evaluation of correlations between AGEs, KYN, their ratio (AGEs/KYN) and biochemical parameters frequently monitored in clinical practice, in the case of human subjects, a significant positive correlation of the ratio of the investigated fluorophores with serum triglyceride levels was established (r=0.43, [95% CI 0.18 to 0.63]; p<0.01). No other correlations were found between the ratio of the studied fluorophores compounds and the other investigated parameters and variables.

No other correlation was established between the contribution of AGEs to total serum fluorescence and the investigated biochemical parameters. Regarding the contribution of KYN to total serum fluorescence, a negative correlation with the triglyceride level was established, with a significantly negative Pearson's correlation coefficient of -0.41 with CI=[-0.63,-0.18] for p=0.01.

To evaluate the presence of possible correlations between the analyzed fluorophores and the development of diabetes complications, we initially considered the mean value of 20.77 as the threshold value for the contribution of KYN to total serum fluorescence; then, its contribution was converted to a dichotomous variable, with divided values above and below the threshold

value. Thus, a significant non-parametric Spearman correlation was obtained between the dichotomous KYN variable and polyneuropathy (PNP), with values of 0.34 at a p level of 0.01.

To investigate the possible correlations between the analyzed ratio (AGEs/KYN) and the presence of diabetes mellitus complications, the ratio (AGEs/KYN) was converted to a dichotomous variable, by using the 0.9233 value as the threshold value. The values obtained in the case of this ratio were above and below this threshold value. Thus, a weak association between the ratio of the fluorophores (AGEs/KYN) and PNP was evidenced, and an asymptomatic Pearson Chi Square test significance of 0.39 with p=0.05 and a contingency coefficient value of 0.246 was obtained (Table 3).

Table 3. Contingency table of the ratio (AGEs/KYN) between the contributions of advanced glycation end products (AGEs) and kynurenine (KYN) to total serum fluorescence and diabetic polyneuropathy (PNP)

			PNP		TOTAL
			ABSENT	PRESENT	
AGEs to KYN ratio	<0.9233	OBSERVED COUNT	19	20	39
		EXPECTED COUNT	23	16	39
	>0.9233	OBSERVED COUNT	20	7	27
		EXPECTED COUNT	16	11	27
TOTAL		TOTAL COUNT	39	27	66
		EXPECTED COUNT	39	27	66

Further, we performed the Mann-Whitney test; a significant difference was obtained between the presence and the absence of PNP regarding the analyzed ratio (AGEs/KYN), the asymptomatic significance value being 0.041.

Following the use of ROC curves, we obtained a mean predictive value for the presence of diabetic polyneuropathy (PNP) based on the assessment of the investigated ratio (AGEs/KYN) (AUC is 0.698±0.072, p=0.007) (Figure 2). Taken separately, the contributions of AGEs and KYN to total serum fluorescence were not predictive for the presence of PNP, which was evidenced by using the ROC curves. Thus, AUC for the evaluation of AGE contribution to total serum fluorescence was 0.519±0.074, with a p value = 0.789, without statistical significance. Also, AUC for the assessment of KYN contribution to total serum fluorescence was 0.255±0.066, with a p value = 0.001. The use of the ROC curve for the analysis of triglyceride levels resulted in an AUC value of 0.533±0.073, p=0.665.

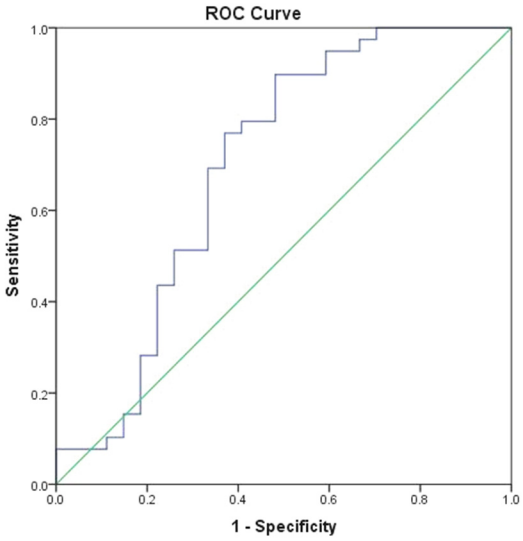

Figure 2. Use of ROC curve to assess the predictability of the AGEs to KYN ratio regarding the development of diabetic polyneuropathy (PNP).

The most important element evidenced by this spectrofluorimetric analysis is the finding that in the case of human subjects (T2DM), the ratio of contributions of the investigated fluorophores (AGEs/KYN) to the total serum fluorescence has a mean predictive value for one of the complications of diabetes, i.e. diabetic polyneuropathy, while the contributions of the products taken separately are not predictors. Thus, lower values of this ratio studied in the case of patients with polyneuropathy can be reported compared to subjects without this type of complication. According to the studied literature, such a predictive value of the ratio of contributions of the fluorophores, AGEs and KYN, for diabetic polyneuropathy, i.e. of the association of these two entities, has not been evidenced so far. This element is useful for investigations aimed at establishing a relationship between the metabolism of the two classes of fluorophores and the presence of diabetes complications, particularly polyneuropathy in diabetic patients. According to the studied literature, inflammatory diseases such as diabetes and implicitly, the development of its complications are determining factors that alter TRP and KYN metabolism and cause depression [12, 13]. These abnormalities in the metabolism of TRP and KYN are sufficiently strong conditions that can lead to an aggravation of diabetes complications and depression symptoms. Thus, according to the study of Bartoli et al. [11], conducted in patients diagnosed with type 2 diabetes mellitus, there is an increased association of neuropathy with the presence

of depression; these authors report that depression in these patients is the consequence of an altered adaptation and functional limitation related to neuropathy. Our study confirms these results, because the presence of neuropathy induces a disturbance of TRP metabolism and implicitly, KYN metabolism in our cases as well. Thus, among patients with T2DM, we evidenced a significant correlation between the contribution of KYN and the presence of polyneuropathy. What makes this study interesting is the fact that the ratio of the investigated fluorophores (AGEs/KYN) has a predictive value for the presence of polyneuropathy in T2DM patients, while in the case of KYN taken separately, such a predictive value for this complication of diabetes could not be established. Like in the case of KYN, recent studies describe the implication of AGEs in the pathogenesis of diabetes complications and implicitly, neuropathy [14, 15]. Peripheral nerves represent one of the favorite sites of AGEs both in the case of human subjects and experimental diabetes in animals [16]. The presence of carboxymethyllisine, one of the products belonging to this large class of AGEs, was described in vascular endothelial cells, pericytes, basement membrane, as well as in axons and Schwann cells belonging to peripheral nerve tissue [17]. Another action mechanism of AGEs which results in the development of neuropathy is through activation of AGE receptors (RAGEs) [18]; the interaction between AGEs and RAGEs leads to endoneural vascular dysfunction and implicitly, to the development of microangiopathies in peripheral nerves [19].

Another interesting observation of this study is the fact that in the case of human subjects, the ratio of contributions of the fluorophore compounds (AGEs/KYN) was significantly correlated with serum triglyceride levels, while no such correlation with the other investigated biochemical parameters could be evidenced. The separate investigation of the fluorophores allowed to obtain an inverse association with serum triglyceride levels only in the case of KYN, while for AGEs, such associations could not be established. In the case of the investigated rats, the studied ratio (AGEs/KYN) was significantly correlated with serum glucose levels, and the separate analysis of the compounds led in the case of each to a significant correlation with serum glucose values. A possible explanation attributed to the correlation between the investigated ratio and serum triglyceride levels in the case of human subjects could be represented by one of the reactions that induce AGE formation; thus, according to the study performed by Bucala et al. [20], the reaction between glucose and amino-containing phospholipids, as well as between glucose and apoproteins initiates AGE formation, finally resulting in the formation of lipid-linked AGEs (AGE-lipid) and apolipoprotein-linked AGEs (AGE-ApoB). Also, the study conducted by Chang et al. (2011) [21] established a positive correlation between AGE level and the patients' lipid profile, as

well as between AGE level and the patients' atherosclerotic characteristics, this type of correlation being much better than those established between the same fluorophore compounds and glucose or HbA1C levels. As regards the effect of KYN on lipid metabolism, this is a currently debated subject [22]. In our study, in the case of patients diagnosed with T2DM, an inverse association of KYN with serum triglyceride levels was obtained; so, it can be concluded that increased contributions of this TRP metabolite induce a decrease of serum triglyceride levels in the case of these patients. Although TRP degradation through the KYN pathway and the effects of the metabolites of this pathway on serum lipid levels were not closely analyzed in this study, the observation of our study is supported by other studies, which report the fact that administration of 3-hydroxyanthranilic acid, a KYN metabolite, causes a decrease in plasma triglycerides in the case of Ldlr (-/-) mice (mice deficient in the LDL receptor) predisposed to the development of atherosclerotic lesions [23]. This reduction in the surface of lipid lesions was explained by a low degree of lipid loading of macrophages, the 3-hydroxyanthranilic acid inhibiting the uptake of oxidized low-density lipoproteins (oxLDL) by macrophages [23].

Another surprise of this study was the correlation established only in the case of rats between both the studied ratio and the contribution of the fluorophores to total serum fluorescence and serum glucose levels. A possible explanation for this correlation only in the case of rats, unlike human subjects, might be the application of multiple treatments over time in the case of humans, with the aim to correct hyperglycemia, the mean duration of diabetes in their case being 10 years. In the case of rats, diabetes was induced and had a short duration, the rats being subjected to spectrofluorimetric analysis immediately after induction of diabetes. The literature provides additional explanations in favor of this aspect. Knowing the difference between early glycation end products and advanced glycation end products could be a useful element. The levels of early glycation end products are deeply influenced by blood glucose concentration. Thus, these levels increase following onset of hyperglycemia, and they normalize when glucose concentration is normalized by treatment [24]. Furthermore, it was observed that with time, in chronic diabetes, these products no longer accumulate in collagen or other stable tissue proteins [25]. In any case, there is the possibility that some of these early glycation end products in collagen or long-life proteins belonging to blood vessels may not dissociate. They are subjected to other chemical reactions resulting in the generation of advanced glycation end products, with the mention that these do not normalize with the correction of hyperglycemia [24].

Our results indicated significantly greater contributions of AGEs to total serum fluorescence both in rats with induced diabetes and patients with T2DM compared to non-diabetic rats and humans. In this case, our results are supported

by other studies which associate the intensification of protein glycation, the increase in AGE formation, respectively, with the development of diabetes mellitus and diabetes complications [9, 8, 6, 44]. Comazzi et al. [8] report an increase of AGEs in the plasma of dogs with well and less well controlled diabetes compared to healthy dogs. An increase of these products was also evidenced in human subjects diagnosed with T2DM, but not T1DM, compared to the control group [26]. However, in both studies mentioned above, AGE concentrations were expressed in AU, which made impossible the comparison or correlation with other species, and the assessment of correlations with diabetes mellitus complications. An element of novelty attributed to our study is the expression of AGE contributions to total serum fluorescence in percentage units, by using the integral of the surface of the band derived from Gaussian spectral deconvolution. This allowed us to compare AGE contributions to total serum fluorescence in the two investigated species, and to assess the degree of correlation with diabetes complications in human subjects.

Regarding the contributions of KYN to total serum fluorescence, the results of our study evidenced significantly greater contributions of this metabolite in patients with T2DM compared to non-diabetic patients, while in the case of rats, significantly greater contributions of this fluorophore to total serum fluorescence were found in the control group compared to the group with streptozotocin-induced diabetes. The results obtained in the case of this TRP metabolite are controversial. Thus, higher contributions of this metabolite in humans are easy to explain; similar results suggest an association of the TRP degradation pathway to KYN and particularly, of KYN with oxidative stress, inflammation and the prevalence of cardiovascular diseases in patients with end-stage renal disease mainly due to DM [27]. These changes, i.e. a significant increase in KYN levels accompanied by a concomitant decrease in TRP levels, were attributed to altered renal function [28], or to a reduced efficiency of dialysis therapy [29]. Also, a significant increase in KYN-modified serum proteins was evidenced in the case of patients diagnosed with T2DM compared to healthy subjects, the presence of cardiovascular disease determining a significant increase in these KYNs [30]. In the case of rats, the decrease of KYN in diabetic animals compared to non-diabetic animals might be explained by the presence of a more intense KYN metabolism in the first category of rats. Thus, in diabetic rats, KYN can be rapidly metabolized through various KYN degradation pathways, with the rapid production of other metabolites. The literature describes significantly increased KYN metabolites such as xanthurenic acid and kynurenic acid in diabetic patients compared to healthy subjects [31, 32, 33]. The mechanisms through which these compounds exert their diabetogenic effect are multiple and varied. One of these mechanisms consists of the fact that these two metabolites of KYN can inhibit proinsulin

synthesis in the pancreatic cells of rats [34]. The absence of much expected significant differences in KYN concentrations between depressive patients and control subjects was also obtained in the study carried out by Myint et al. (2007) [35]. An explanation of extensive TRP degradation in the absence of increased KYN concentrations could be the rapid metabolization of KYN in the case of depressive patients. KYN is further metabolized very rapidly either through the toxic quinolone pathway, generating intermediate products such as 3-hydroxykynurenine, 3-hydroxyanthranilate and finally, quinolonic acid, or through the KYN pathway, with the neuroprotective kynurenic acid as the final metabolite [36, 37].

CONCLUSIONS

In conclusion, it can be said that in human subjects with T2DM as well as in diabetic rats, there is a significant increase in the contributions of AGEs and KYN to total serum fluorescence compared to control groups. The calculation of the ratio of the fluorophore compounds evidenced a significant correlation of this ratio with serum triglyceride levels in humans and with serum glucose levels in rats. By far, the most important result of this study is the identification of this ratio - AGEs/KYN as a predictive factor for the development of PNP in the case of human subjects. Thus, a new potential biomarker for the presence of diabetic PNP is proposed.

EXPERIMENTAL SECTION

1. Biological Material – Rats

We used 10 Wistar rats with a weight between 250 and 300 g. The rats were kept in two stainless steel cages, each cage accommodated 5 rats, and each rat was well individualized. Throughout the study, the rats were maintained under standard environmental conditions: temperature 24±5°C, light/dark cycle (12h/12h), relative humidity 60±4%, free access to food and water during the entire period of investigation. The same rats were used in the first and the second part of the study, after induction of diabetes. All procedures used in this experiment were in accordance with Romanian laws regarding correct manipulation of laboratory animals, and the entire experimental procedure was approved by the Ethics Committee of the University of Agricultural Sciences and Veterinary Medicine in Cluj-Napoca, Romania.

1.1. Blood Collection and Biochemical Determinations

Blood samples (0.2 ml) from the orbital sinus of healthy rats and rats with streptozotocin-induced diabetes were collected in 0.5 ml Eppendorf tubes. Subsequently, the samples were subjected to centrifugation (1000 g for 10 min) in order to obtain blood serum. Glycemia was assessed immediately after sample collection using routine laboratory methods (Hitachi, Roche Diagnostics). Immediately after centrifugation of blood samples, 4 µL blood serum were used for spectrofluorimetric analysis.

1.2. Induction of Diabetes

For induction of diabetes in rats, 60 mg/kg streptozotocin (Sigma, Aldrich) were injected intraperitoneally. Streptozotocin induces diabetes in 1 to 4 days by destruction of insulin-producing pancreatic cells [38]. Hyperglycemia (>300 mg/dl) was detected within 3 days of streptozotocin administration. Rats with glucose values higher than 300 mg/dl were considered diabetic [39].

2. Biological Material – Human Subjects

2.1. Selection of Subjects

This observational study included 66 subjects. Thus, 52 T2DM patients who presented to the Center for Diabetes, Nutrition and Metabolic Diseases in Cluj-Napoca, Romania, from July 2013 to February 2014 were enrolled. The exclusion criteria for the patients included in the study were the following: unstable cardiovascular disease, kidney failure, liver failure, inflammatory diseases, malignant diseases or depressive disorders. The information regarding the patients' personal data was collected using a questionnaire (age, sex, smoking or non-smoking status). T2DM was diagnosed according to the criteria of the American Diabetes Association [40]. The presence of chronic diabetes complications (retinopathy, neuropathy, chronic renal disease, cardiovascular disease) was established by accessing the patients' medical files. The control group, including patients without diabetes or not meeting the above mentioned criteria (n=14), was selected from the Department of Internal Medicine, Medical Clinic I, Clinical Emergency Hospital Cluj-Napoca, Romania.

According to the World Medical Association's Declaration of Helsinki revised in Edinburgh, 2000, and institutional guidelines, the protocol used in this experiment was approved by the Ethics Committee of the "Iuliu Haţieganu" University of Medicine and Pharmacy in Cluj-Napoca, Romania. All participants were aware of the experimental nature of the study and gave their written consent before any study procedure.

2.2. Biochemical Determinations

The blood samples were collected in the morning, before medical examination, after a night of alimentary rest. Glycated hemoglobin, glycemia, cholesterol, triglycerides, HDL cholesterol, creatinine, transaminase were evaluated immediately, using commercially available methods (Hitachi, Roche Diagnostics). A blood sample was taken from each subject and was subsequently subjected to centrifugation, the serum obtained being stored at -80°C until spectrofluorimetric analysis.

3. Spectrofluorimetric Determination of AGEs and KYN

In order to compare and determine the contributions of AGEs and KYN to total serum fluorescence, the blood serum taken from 10 healthy rats and 14 non-diabetic human subjects, as well as from 10 rats with streptozotocin-induced diabetes and 52 patients with T2DM was assessed by spectrofluorimetry. To obtain a good emission spectrum using a minimal blood serum amount, all serum samples were diluted with physiological serum (NaCl 0.9%), in a ratio of 1:500 [41]. Fluorescence intensity was recorded at a maximum emission of ~ 435 nm for AGEs [42, 6] and ~ 485 nm for KYN [43], after an excitation at 370 nm (JASCO FP-8200 spectrofluorimeter). Wavelength accuracy was ±2 nm. The data obtained were processed using the Origin Pro 8.1 software. The relative contribution of AGEs and KYN to total serum fluorescence was established following calculation of the integral of bands derived from Gaussian spectral deconvolution (Figure 3). Thus, using the Gaussian deconvolution

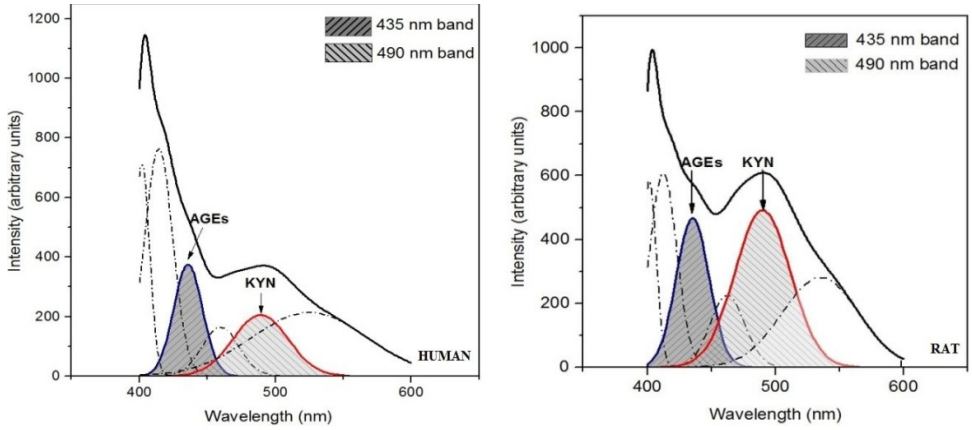

Figure 3. Deconvolution of the serum emission spectrum belonging to a patient with type 2 diabetes mellitus and a rat with streptozotocin-induced diabetes. Representation of Gaussian band components: advanced glycation end products (435 nm) and kynurenine (490 nm), along with the experimental spectrum obtained by spectrofluorimetric analysis.

algorithm as part of the peak analysis option, we carried out deconvolutions of the obtained spectra. Both in humans and rats, we used a combination of six Gaussian bands, whose peak position and width at maximum height were well established, while intensity was allowed to vary so as to match the experimental line shape of the spectra [42]. In the literature, there is currently no dependence function between the integral of the surface of bands derived from Gaussian spectral deconvolution and the total number of AGE and KYN molecules. Thus, for a better quantification of AGE and KYN contributions to total serum fluorescence, we chose to calculate the ratio between the two classes of compounds (AGEs/KYN).

4. Statistical Analysis

Statistical analysis was performed with the SPSS software, the version 22. The Kolmogorov-Smirnov test was used to check for the normality of variable distribution. The mean and standard deviation characterized continuous data with normal distribution. Non-parametric data were characterized by using the median and interquartile range. Dichotomous and categorical data were expressed in absolute and percentage values. The Mann-Whitney test was employed to compare the groups of variables whose data were not normally distributed. Pearson's correlation coefficient was used to assess correlations between parametric variables, and Spearman's correlation coefficient for non-parametric variables. The test used for associations was χ^2; the measure of associations was studied using the contingency coefficient. Also, Fisher's test was employed as an alternative to χ^2. According to the null hypothesis, there was no association between characteristics. The predictive value of the AGEs to KYN ratio regarding the presence of diabetic polyneuropathy (PNP) was studied via ROC (receiver operating characteristic) curves. All results were considered statistically significant at a p value < 0.05.

Limitations

As it can be seen, there are some interesting results in this study, but there are also some limitations. A first limitation would be diet and physical activity, which were not well monitored in this study. This might influence the results obtained; however, the presence of significant differences between diabetic and non-diabetic patients regarding the analyzed compounds excludes the major contribution of these two elements to the results obtained. Another aspect would be the cross-sectional nature of our study. A study designed and carried out over a longer time period could provide more specific causal relationships between AGEs, KYN and the other investigated variables. Finally,

inflammation might play an important role in identifying these relationships between the investigated fluorophores and endothelial dysfunctions present in the case of diabetic patients. We mention the fact that in this study, inflammation markers such as C-reactive protein, interleukin-6 or tumor necrosis factor were not measured. In the future, we propose to conduct further studies in this direction, for the identification of potential mechanisms.

Conflict of Interest: The authors declare that they have no conflict of interest.

Research involving human participants and/or animals

1) *Statement of human rights*

All procedures performed in studies involving human participants were in accordance with the ethical standards of the local Ethics Committee of the "Iuliu Haţieganu" University of Medicine and Pharmacy in Cluj-Napoca, Romania and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

2) *Statement on the welfare of animals*

All procedures were in accordance with Romanian laws and approved by the local Ethical Committee of University of Agricultural Sciences and Veterinary Medicine in Cluj-Napoca, Romania.

Informed consent: Informed consent was obtained from all individual participants included in the study.

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