The Fluorescence Identification of Advanced Glycation End Products in Streptozotocin-Induced Diabetic Rats' Plasma Samples

Loredana Elena OLAR^{1*}, Razvan ȘTEFAN¹, Cristian BERCE², Dana CIOBANU², Ionel PAPUC¹

¹ University of Agricultural Sciences and Veterinary Medicine, Faculty of Veterinary Medicine, 3-5 Mănăștur street, 400372, Cluj-Napoca, Romania

² „Iuliu Hatieganu” University of Medicine and Pharmacy, 8 Babeș street, 400012, Cluj-Napoca, Romania

Corresponding author: olarloredanaelena@yahoo.com

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Abstract

The plasma samples obtained from non-diabetic and diabetic rats were examined by fluorescence spectroscopy in order to find specific spectroscopic biomarkers for the monitoring and the development of diabetic complications. We studied 10 non-diabetic rats (8 middle-aged rats and 2 younger). A quantity of 0.2 ml blood plasma from healthy rats was analysed spectrofluorimetrically and served as controls for the advanced glycation end products (AGEs) analysis (370 nm/433 nm). Diabetes mellitus was induced by the intravenous injection of streptozotocin. One week later, the advanced glycation end products (AGEs) content of plasma were determined again by using the same method. The emission intensities were relatively the same in healthy rats' plasma samples. The results showed that emission bands appearing at 404 nm and 433 nm were significantly increased in intensity in all tested diabetic rats compared with all examined samples obtained from healthy rats. As statistically demonstrated, upon diabetes induction, the emissions intensities of diabetic rats in what concerns the plasma content of AGEs increased (1777 ± 527 vs. 1072 ± 262 ; $p=0.001$). The fluorescence emission spectra of studied plasma samples are able to discriminate the diabetic rats from the healthy controls.

Keywords: AGEs, diabetes, fluorescence, plasma

INTRODUCTION

Diabetes mellitus (DM) is a common metabolic disorder in animals that can lead to a large number of complications, though they are not as frequent as in humans, it can affect a large proportion of the animal population.

In veterinary medicine the control of diabetic animals through haematological and biochemical tests is difficult. Measurements of glycated haemoglobin for the determination of plasma glucose concentration are not routinely made in animals' DM (Comazzi *et al.*, 2008). In recent years, increasing attention has been focused on the fluorescence determination of AGEs in diabetic subjects.

Fluorescence spectroscopy is one of the most accurate techniques to assess the existence of AGEs in various biological liquid environments, i.e. blood, urine or various tissues (Genevieve *et al.*, 2013; Maza *et al.*, 2007; Maza *et al.*, 2012; Piwowar *et al.*, 2008). Regarding their fluorescence properties, AGEs are supposed to give an emission at around 440 nm when the excitation is performed at 370 nm (Schmitt *et al.*, 2005; Piwowar *et al.*, 2008; Henle *et al.*, 1999).

Advanced glycation endproducts (AGEs) are produced during the course of diabetes and are associated with normal ageing (Munch *et al.*, 1997; Maza *et al.*, 2007). They are generated by a non-enzymatic glycation, in which reducing sugars

(glucose) are covalently attached to free amino groups of macromolecules (Bierhaus *et al.*, 1998).

In addition to circulation in the blood, AGEs accumulate in tissues (Stitt, 2001), blood and urine of diabetic patients (Comazzi *et al.*, 2008). Moreover, the circulating AGEs increase in the blood of diabetic patients in association with renal complications (Yanagisawa *et al.*, 1998), also they are thought to play an important role in the development of cardiovascular disease in type 1 diabetes mellitus (Van Eupen *et al.*, 2013). As the main complication of canine DM- the diabetic cataract leads to glycosylation of structural proteins, in the presence of a normal renal function, this AGE products are rapidly cleared but they accumulate in individuals with renal insufficiency (Stitt, 2001).

Advanced glycation endproducts have in common a yellow brown pigmentation, a characteristic fluorescence spectrum and the ability to form protein-protein cross-links (Makita *et al.*, 1992). The isolation and identification procedures have led to the recognition of several AGEs such as: FFI (4 furanyl-2-furoyl-14-imidazole), AFGP (1-alkyl-2-formyl-3, 4-diglycosylpirole), pyrraline, carboxymethyllysine, imidazolone, pentosidine (Makita *et al.*, 1992), some of this structures are non-fluorescent like N- (carboxymethyl)lysine, pyrraline or imidazolone, or fluorescent like pentosidine or the crosslinks (Munch *et al.*, 1997).

The aim of this study was to measure blood plasma fluorescence intensities of AGEs in healthy and streptozotocin induced diabetic rats.

MATERIALS AND METHODS

Plasma from healthy rats (8 middle-aged rats and 2 younger) was analysed spectrofluorimetrically and served as controls for the AGE analyses. After that, diabetes was induced by the intramuscular injection of streptozotocin (5,5 mg/100g body weight) dissolved in citrate buffer (pH 4.5) (Gopal and Indira, 2010). AGEs were determined again at 1 week interval from the diabetic rats having blood glucose levels between 200 and 550 mg/dl, by using the same method. The blood samples (0,2 ml) were collected from orbital veins of healthy/diabetic rats in EDTA coated vacutainers (Henle *et al.*, 1999). Then the blood samples were centrifuged (1000g for 10 min) to obtain pure plasma and were diluted 1:500 (Munch *et al.*, 1997) in physiological serum 0,9%

in order to obtain the fluorescence signal. For a correct interpretation of the graphs, physiological serum signal was subtracted from the total spectra of the plasma.

Fluorescent emission spectra of plasma samples were measured with FP-8200 spectrofluorometer (Jasco, Japan) at room temperature in 1 cm quartz cell and recorded in a region from 400 to 600 nm (excitation at 370 nm) (Piwowar *et al.*, 2008; Schmitt *et al.*, 2005) using a high sensitivity. The wavelength accuracy of the spectrofluorimeter was ± 2 nm.

Data analyses were performed using Microsoft Office Excel 2010 for Windows. Continuous data were expressed as means $\pm$ standard deviation. Group comparisons of all variables were performed using t- test for paired samples. A *p* values less than 0.05 was considered statistically significant.

The protocol was approved by the local Ethical Committee of University of Agricultural Sciences and Veterinary Medicine in Cluj-Napoca, being in accordance with Romanian laws.

RESULTS AND DISCUSSIONS

The results showed an increased level of AGE in plasma diabetic rats' samples when compared with the plasma healthy rats samples. A freshly prepared rats plasma, diluted 1:500 with physiological serum, exhibits a good fluorescence in the visible range 400-450 nm, when excitation is performed at 370 nm.

The fluorescence spectra of healthy (Fig. 1) and diabetic (Fig. 2) plasma rats samples showed two emission bands appearing at around 404 nm and 433 nm.

The emission intensities (Fig. 1) were relatively the same in healthy rats' plasma samples, the high of the emission maximum at around 404 nm was located between 727- 1653 a.u while the maximum of the peak around 433 nm was between 748 and 1475 a.u.

Upon diabetes induction, the height of the emission bands (Fig. 2) increased as it follows: the emission maxima of the peak around 404 nm was between 1195-5081 a.u and the height of the peak around 433 nm was between 1043 a.u and 2635 a.u.

We observed statistically significant higher emissions of blood plasma AGEs (370 nm/433 nm) in diabetic rats compared with healthy rats

(1777 ± 527 vs. 1072 ± 262 ; $p=0.001$). Also, the peak at around 404 nm (2852 ± 1414 vs. 1227 ± 313 ; $p<0.001$) had statistically significant higher emissions in diabetic rats when compared with healthy rats.

We found that the emission intensities of the diabetic rats' plasma samples are more elevated than the emission intensities of healthy rats' plasma samples. These results are in agreement

Fig.1. Fluorescence emission spectra of AGEs (emission at 433 nm) in healthy rats' blood plasma samples after excitation at 370 nm. The emission spectra were recorded in a region from 400 nm to 600 nm.

Fig. 2. Fluorescence emission spectra of AGEs (emission at 433 nm) in diabetic rats' blood plasma samples after excitation at 370 nm. The fluorescence emission spectra of AGE-peptide in plasma of diabetic rats were recorded in a region from 400 nm to 600 nm.

with previous studies obtained not only by measuring plasma fluorescence of total AGEs in patients with diabetic complications (Henle *et al.*, 1999), but also serum AGEs in type 2 diabetic patients (Munch *et al.*, 1997; Dolhofer-Bliesener *et al.* 1995).

Kalousova *et al.* (2002) previously reported that the elevation of serum AGEs was found only in patients with diabetes mellitus type 2 but not in diabetes mellitus type 1 when compared with healthy subjects. We were able, however, to show that the elevation of plasma AGEs occurs in rats with diabetes mellitus type 1.

Although, most studies suggest that using an excitation of 370 nm, the fluorescence of the plasma is determined mainly by emission of AGE (Piwowar *et al.*, 2008; Henle *et al.*, 1999) our study shows that besides AGEs which have an emission at around 433 nm, also one other fluorescent compound appears at around 404 nm. According to (Zallesskaya and Maslova, 2010) in the visible region of the plasma spectrum may appear the fluorescence of tryptophan and tyrosine– the albumin and globulin constituents. Along with this, may appear other non-protein components of the plasma such as: bilirubin, coenzymes nicotinamide adenine dinucleotide (NAD) and its derivatives (NADP), indican, riboflavin (Zallesskaya and Maslova, 2010). The visibility of these components in the plasma spectrum depends on their concentration, the absorption coefficients at the excitation wavelength λ_{ex} , the fluorescence quantum yield and the physicochemical properties of the environment.

CONCLUSION

We found that the emission intensities of AGEs in diabetic rats' plasma samples are significantly higher than the emission intensities of AGEs in healthy rats' plasma samples. Also, we observed higher emission intensities of the peak at around 404 nm in diabetic rats' plasma samples compared with healthy rats' plasma samples.

PERSPECTIVES

This variation in AGEs concentration between the healthy and diabetic rats sustains the fact that this studied products should be considered as possible therapeutically targets in the prevention of chronic diabetes complications.

Future studies should explore the fluorescence of the plasma when excitation is performed at 370 nm in order to find proper assignment for the emission band at around 404 nm.

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