The identification of some relevant markers in the diagnosis of diabetes mellitus by spectrofluorimetry

(SUMMARY OF THE PhD THESIS)

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CURRENTLY, DIABETES MELLITUS IS A FREQUENT DISEASE IN BOTH ANIMALS AND HUMANS, WHICH SHOULD BE EFFECTIVELY MONITORED AT AN EARLY STAGE TO PREVENT ADJACENT COMPLICATIONS. THIS IS FEASIBLE BY EARLY AND ACCURATE DIAGNOSTIC AND MONITORING METHODS. AN EFFECTIVE MONITORING OF DIABETES FOR THE REDUCTION OF ITS COMPLICATIONS CAN ONLY BE ACHIEVED BY STRICT CONTROL OF GLYCEMIA. HIGH GLUCOSE LEVELS IN THE BODY, AS WELL AS PROTEIN GLYOSYLAION IN BLOOD VESSELS MOST FREQUENTLY INDUCE THE FORMATION OF ADVANCED GLYCATION END PRODUCTS, WHICH LEAD TO BLINDNESS AND CARDIAC DISORDERS (LAKOWICZ, 2006). ADVANCED GLYCATION END PRODUCTS (AGEs), ALONG WITH OTHER ELEMENTS SUCH AS KYNURENE (KYN), RETINOL-BINDING PROTEIN (RBP), NICOTINAMIDE ADENINE DINUCLEOTIDE (NADH), FLAVIN ADENINE DINUCLEOTIDE (FAD), REPRESENT A CLASS OF COMPOUNDS WITH FLUORESCENT PROPERTIES, SUBJECTED TO SPECTROFLUORIMETRIC ANALYSIS IN VARIOUS SYSTEMIC DISEASES. UNLIKE THE STUDY OF DIABETES MELLITUS IN HUMANS, THESE FLUOROPHORE COMPOUNDS HAVE NOT YET BEEN INVESTIGATED IN DIABETIC ANIMALS.


KEYWORDS: AGEs, KYN, NADH, FAD, SPECTROFLUORIMETRY, DIABETES MELLITUS
STRUCTURE OF THE THESIS

The paper entitled „The identification of some relevant markers in the diagnosis of diabetes mellitus by spectrofluorimetry”, contains 149 pages, is written in compliance with the norms and is structured in two parts.

The first part, the literature review was divided into 2 chapters and extends over 34 pages. In this part were summarised information about the advantages of using the spectrofluorimetry, about the main fluorophores (AGEs, KYN, NADH, FAD, RBP) which were investigated by this technique in the different systemic diseases, and also in human and animals with diabetes mellitus, and finally were presented information about the study of these compounds in the context of the apparition of diabetes mellitus complications in humans and animals.

The second part of the thesis extends over 88 pages and was divided into 3 chapters. In this part of personal contributions were presented the objectives and the aim of this research, the materials and methods, the results and the discussions, and respectively, the comparison of these results with those from the literature review and ultimately were presented the partial conclusions. The results were illustrated on several 19 figures and 10 tables.

RESULTS OF THE RESEARCH

In this study, we investigated by spectrofluorimetry two species of animals: non-diabetic and diabetic rats and dogs, and non-diabetic and type 2 diabetic humans. With respect to animals, all procedures used complied with the Romanian legislation regarding correct manipulation of laboratory animals, the entire experimental procedure being approved by the Ethics Committee of the University of Agricultural Sciences and Veterinary Medicine in Cluj-Napoca, Romania. In the case of humans, the protocol of this study was approved by the Ethics Committee of the “Iuliu Hațieganu” University of Medicine and Pharmacy in Cluj-Napoca, Romania, and was in accordance with the Declaration of Helsinki and institutional guidelines. All participants were informed about the investigational nature of this study and gave their informed consent prior to the application of any experimental procedure.

In Chapter 3 of this thesis, entitled “Spectrofluorimetric investigation of advanced glycation end products, kynurenine, pentosidine and retinol-binding protein in diabetic rats compared to healthy rats”, we investigated by spectrofluorimetry the serum, plasma and urine of non-diabetic rats and rats with induced diabetes. The analyzed fluorophore compounds were: AGEs, the metabolite of tryptophan – KYN, pentosidine, and retinol-binding protein (RBP). Our aim was to determine relevant
biochemical markers in diabetes induced in rats, as well as the attempt to turn this spectrofluorimetric technique into a routinely used method in specialized laboratories.

The results obtained in the case of serum and plasma samples indicate the presence of a statistically significant difference between normoglycemic rats and rats with streptozotocin-induced type 1 diabetes, regarding AGE levels. Thus, the height of the emission bands obtained at approximately 432 nm, attributed to these fluorophore compounds, significantly increased in diabetic rats compared to non-diabetic rats. The observations of our study support the important role of high glucose concentrations in the formation of these AGEs; thus, high blood glucose concentrations in rats with induced diabetes led to a significant increase in the height of emission bands attributed to AGEs. Another compound subjected to spectrofluorimetric investigation in the blood serum of rats was KYN. Our results evidence an increase of kynurenine emission in the serum of diabetic rats compared to healthy rats. Thus, rats with induced diabetes showed an increased tryptophan metabolism, an increased activity of the enzyme directly responsible for the conversion of tryptophan to kynurenine, which was demonstrated by this significant increase in serum kynurenine levels compared to non-diabetic rats.

The analysis of the results obtained following spectrofluorimetric investigation of urine samples showed, for the first time in the literature, the presence of lower AGE contributions and higher KYN contributions to total urine fluorescence, with a subunitary AGEs to KYN ratio in the case of healthy rats. In contrast, in rats with streptozotocin-induced diabetes, higher AGE contributions compared to KYN contributions were evidenced, with a supraunitary ratio of the two compounds. Also, the ratio of the two contributions was significantly higher in diabetic rats compared to healthy rats. In the case of diabetic rats, we evidenced the fact that the AGEs to KYN ratio was strongly influenced by insulin administration. Thus, the studied ratio decreased significantly in diabetic rats in the first three days after administration of a single insulin dose, but remained significantly higher in these rats compared to healthy rats. At 4 to 6 days from insulin administration, the studied ratio increased, reaching values almost similar to those of diabetic rats before insulin administration. Previous studies showed that both insulin and glycemic control can influence this contribution of AGEs to total urine fluorescence. Also, a strong correlation between KYN contribution to total urine fluorescence and blood hyperglycemia values was observed.

Subsequently, we investigated by spectrofluorimetry the fluorophores pentosidine and retinol-binding protein. We wanted to investigate their status in blood serum obtained from healthy rats, as well as the changes caused by the induction of type 1 diabetes. Thus, spectrofluorimetric analysis of serum samples, for the investigation of these fluorophores, revealed significantly higher levels of these fluorophore compounds in diabetic rats compared to healthy rats. The contribution of
these two products to total serum fluorescence was also significantly higher in the case of diabetic rats compared to normoglycemic rats. This aspect was evidenced by the calculation of the area under the curve of the peaks attributed to these compounds.

Based on these results, it can be said that the use of this method for the study of these fluorophore compounds in diabetes mellitus rats represents a practical and accessible modality that can differentiate between non-diabetic and diabetic animals.

**Chapter 4** was divided into **two subchapters**; the first subchapter was entitled "Evaluation of the advanced glycation end products to kynurenine ratio in non-diabetic compared to diabetic rats and humans", and the second subchapter was entitled "The fluorophores advanced glycation end products (AGEs) to NADH ratio is predictive of chronic diabetic kidney disease and cardiovascular disease".

**In the first subchapter**, we calculated the AGEs to KYN ratio in non-glycemic humans and rats and respectively, in diabetic humans and rats. We chosen to calculate this ratio because as far as we know there is no existing dependence function between the integral of the band areas derived from the Gaussian spectra deconvolution and the total number of serum AGEs and KYN' molecules and also because we wanted to have a better quantification of AGEs and KYN' contributions to serum fluorescence. Moreover, we obtained new correlations and associations between the contributions of AGEs and KYN to the total fluorescence of the serum and the level of hyperglycemia in order to improve the understanding of metabolic processes and the pathogenesis of diabetes mellitus. 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 two investigated fluorophores (AGEs/KYN) has a mean predictive value for one of the complications of diabetes, i.e. diabetic polyneuropathy, while the contributions of the two 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. Also, we evidenced an association between the AGEs to KYN ratio and the diabetic polyneuropathy.

Another interesting observation of this study is the fact that in the case of human subjects, the ratio of contributions of the two 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 two 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 two compounds led in the case of each to a significant correlation with serum glucose values. 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 two 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.

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 (Pawlak și colab, 2009).

The obtained results suggest a high potential for the investigated ratio to be considered as a new biomarker for the presence of diabetic polyneuropathy.

In the second subchapter of this chapter, we identified new serum fluorophore biomarkers in the case of patients with type 2 diabetes mellitus (T2DM), in order to facilitate a certainty diagnosis, as well as to evidence new correlations with certain complications of this disease. According to the literature, the metabolism of AGEs (advanced glycation end products) and NADH (nicotinamide adenine dinucleotide) is altered in two complications of diabetes mellitus (DM), i.e. chronic kidney disease (CKD) and cardiovascular disease (CVD) (Cooper, 2004; Hanssen et al., 2015; Kihovd et al., 2007). So far, there are no available data regarding the concomitant evaluation of AGEs and NADH in patients with T2DM. All these aspects led us to assess these fluorophores (AGEs and NADH) in the serum, as well as their ratio (AGEs/NADH) in patients with T2DM compared to non-diabetic patients. We also aimed to evaluate the relationship of these compounds with two diabetes complications, CKD and CVD.

The most important conclusion of this study was the fact that the fluorophore ratio had a moderate predictive value for the presence of both CKD and CVD, while AGEs or NADH taken separately had no predictive value. Patients with diabetic CKD had higher values of the AGEs to NADH ratio compared to patients with normal renal function. We also evidenced the fact that the ratio of these fluorophores was positively associated with triglyceride levels and negatively associated with HDL-cholesterol levels. AGEs taken separately were not associated with lipid levels, while NADH was negatively associated with triglyceride levels.
A positive association between the AGEs to NADH ratio and blood glucose levels was observed, although no significant association was found when investigating diabetes control by using HbA1c, diabetes duration, age, sex or smoking/non-smoking status.

Our results indicated significantly higher NADH levels in patients of the control group compared to patients diagnosed with T2DM. Similar results were reported in an interventional study, when significantly higher NADH levels were detected in the muscle tissues of healthy participants compared to subjects with T2DM. The authors (Ritov et al., 2010) explained this by the presence of a sustained exercise program and caloric restriction, which further improved insulin resistance.

The results of this study suggest the fact that the AGEs to NADH ratio can be considered as a new biomarker for the presence of diabetic CKD and CVD. This last subchapter is a reprint of the article CIOBANU D.M., OLAR L.E., STEFAN R., VERESIU I.A., BALA C., MIRCEA P.A., ROMAN G., 2015, Fluorophores advanced glycation end products (AGEs)-to-NADH ratio is predictor for diabetic chronic kidney and cardiovascular disease, Journal of Diabetes and its Complications, 29:893-897; with the permission of Elsevier.

Chapter 5 of this thesis, entitled “Spectrofluorimetric determination of advanced glycation end products (AGEs), kynurenine (KYN), nicotinamide adenine dinucleotide (NADH), flavine adenine dinucleotide (FAD) in the serum of non-diabetic dogs and dogs suspected of diabetes mellitus”, comprises data on the spectrofluorimetric investigation of the contributions of AGEs, KYN, NADH and FAD to total fluorescence of serum obtained from dogs suspected of diabetes, which presented glucose, triglyceride and cholesterol values above the normal limits for this species, as well as from dogs with values of these parameters within normal limits.

Over a 10-month period, 48 serum samples were collected from 11 non-diabetic dogs and 37 dogs (13 females and 24 males) suspected of diabetes. All dogs included in this study presented to the veterinary clinic for routine vaccination and examination.

The results obtained evidenced significantly higher contributions of all investigated fluorophores to total serum fluorescence in the case of dogs suspected of diabetes mellitus compared to the control group. These results emphasize and reinforce the idea according to which this metabolic and nutritional disease may cause significant changes in the concentrations of all analyzed serum biomarkers, and consecutive alterations of their fluorescence.

The spectrofluorimetric investigation of the cellular coenzymes NADH and FAD showed an increased activity of these coenzymes in the serum of dogs with high glucose, triglyceride and cholesterol levels. Unlike the other fluorophores investigated in this study, the contributions of NADH to total serum fluorescence were correlated with blood glucose levels and the age of the dogs included in the study. The explanation of the correlation between NADH contributions to total serum fluorescence...
fluorescence and the age of the dogs is provided by the high glucose concentrations present in the case of the investigated dogs. In the literature, age is reported to be a factor triggering insulin resistance in the case of healthy elderly patients, the direct relationship between high glucose levels and the patients’ age being emphasized (Broughton and Taylor 1991; Ko et al., 2006). The major influence of the blood glucose concentration and age of dogs on NADH contributions to total serum fluorescence was also evidenced. We mention the fact that this type of correlations was established only for the NADH enzyme cofactor, and not for the other investigated biochemical compounds.

**GENERAL CONCLUSIONS**

1. The statistically significant results obtained following spectrofluorimetric analysis of biological fluids collected from diabetic compared to non-diabetic animals and humans highlight the major importance of this technique in evaluating the levels of fluorophore compounds in the two categories of investigated subjects.

2. The AGEs to KYN ratio can be considered as a new biomarker for diabetic polyneuropathy in the case of human subjects; the AGEs to NADH ratio can be considered as a new biomarker for the presence of diabetic CKD and CVD.

3. In the case of the studied dogs, all investigated fluorophores (AGEs, KYN, NADH, FAD) had higher contributions to total serum fluorescence in animals suspected of diabetes compared to healthy animals, NADH being correlated with age and blood glucose concentration.

4. Our results are augmented by the enhanced sensibility and specificity of this spectrofluorimetric method in what concerns the evaluation of AGEs, KYN, FAD, NADH contributions in dogs, and also the contribution of AGEs and KYN in the humans of this research.

5. The correlation between glucose and the contribution of AGEs to the total fluorescence of serum in rats highlights the importance of this carbohydrate in the AGEs formation.
RECOMMANDATIONS

We recommend the use of this spectrofluorimetric method in the diabetes mellitus investigation in humans and animals.

We recommend the use of rats as an experimental model of diabetes mellitus induction with streptozotocin, as well as the use of this model in getting some valuable information about the pathogenesis of diabetes mellitus by spectrofluorimetry.

We recommend the use of the deconvolution operation in the analyse of fluorescence spectra in order to calculate the fluorophore contributions to the total fluorescence of the biological fluids, but also for differentiating the two species analysed by this technique (non-diabetics and diabetics).

BIBLIOGRAPHY

