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SPECTROFLUORIMETRIC INVESTIGATION OF AGEs, KYN, NADH AND FADH IN DOGS SUSPECTED OF HAVING DIABETES COMPARED TO NON-DIABETIC DOGS

INVESTIGAREA SPECTROFLUORIMETRICĂ A AGEs, KYN, NADH ȘI FADH LA CÂINII SUSPECȚI DE DIABET COMPARATIV CU CÂINII NON-DIABETICI

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ABSTRACT | REZUMAT

Diabetes mellitus (DM) is a common disorder in dogs, which is not monitored very effectively or diagnosed on time. Detection of some biochemical abnormalities in the diabetes pathogenesis ever since the beginning of disease suspicion remains an important goal concerning the early therapeutic intervention. This study aimed to use a rapid, economical and non-invasive method for the simultaneous evaluation of some fluorescent compounds whose mechanism of action is closely related to the development of DM and its complications. The spectrofluorimetric analysis was performed in 37 blood serum samples collected from dogs suspected of having diabetes and 11 blood serum samples taken from dogs without suspicion of DM. It was attempted to establish associations and correlations between the following parameters: AGEs (advanced glycation end products), KYN (kynurenine), NADH (nicotinamide adenine dinucleotide), FAD (flavin adenine dinucleotide) and triglycerides, cholesterol, and glucose. In the case of dogs suspected of having diabetes compared to the control group was highlighted a significant increase in the contributions of all analysed fluorophore compounds. Also, a significant correlation was established between the contribution of NADH and the blood glucose level, the age of the investigated dogs, respectively. The results show in dogs, as in humans, the importance of monitoring these serum fluorophore parameters ever since the beginning of diabetes suspicion in order to avoid complications during the progression of diabetes and respectively to find disease-related new pathogenetic mechanisms.

Keywords: canine diabetes, diabetes pathogenesis, fluorophores, spectrofluorimetry

La ora actuală, diabetul zaharat reprezintă o afecțiune destul de comună în rândul câinilor, aceasta fiind de cele mai multe ori nemonitorizată efectiv sau diagnosticată la timp. Detectare unor anormalități biochimice în patogeneza diabetului încă de la începutul suspiciunii de boală rămâne unul din obiectivele importante ale intervenției terapeutice. Scopul acestui studiu a fost acela de a utiliza o metodă rapidă, economică și non-invazivă prin care să se urmărească detecția simultană a unor compuși fluorofori al căror mecanism de acțiune este îndeaproape asociat cu apariția diabetului și a complicațiilor sale. Analiza spectrofluorimetrică a fost efectuată pe un număr de 37 probe de ser sanguin recoltat de la câini suspecți de diabet, respectiv pe 11 probe de ser sanguin recoltat de la câini clinic sănătoși. S-a urmărit evidențierea unor asocieri și corelații între următorii parametrii: AGEs (produșii de glicozilare avansată), KYN (kynurenina), NADH (nicotinadenin dinucleotida), FAD (flavin-adenin dinucleotida) și concentrația de trigliceride, colesterol, glucoză. Astfel, în cazul câinilor suspecți de diabet comparativ cu lotul control a fost evidențiată o creștere semnificativă a contribuțiilor tuturor compușilor fluorofori analizați. De asemenea, o corelație semnificativă a fost stabilită între contribuțiile NADH și nivelul glucozei sanguine, respectiv vârsta câinilor investigați. Rezultatele obținute arată și în cazul câinilor, la fel ca și în cazul oamenilor, importanța monitorizării acestor parametrii fluorofori serici chiar de la începutul suspiciunii de diabet cu scopul de a împiedica apariția complicațiilor odată cu progresia diabetului și respectiv pentru a găsi noi mecanisme patogenetice asociate acestei afecțiuni.

Cuvinte cheie: diabet canin, patogeneza diabetului, fluorofori, spectrofluorimetria

Current guidelines for the diagnosis of diabetes mellitus (DM) in animals include the clinical history, the presence of hyperglycaemia, polyuria, polydipsia, and glycosuria (5, 22, 24). In other words, the efficient communication, the *doctors' and* pet owners' *communicative competence* as a set of personal skills in the communicative process and further the collection of clinical pathology data may point the diagnosis towards certain pathologies (28). Another possible evaluation of diabetic dogs, which can assess the risk of complications associated with this disorder, might be

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conducted by using the spectrofluorimetric techniques (6). Therefore, these techniques allow to evidence and quantify various fluorophore compounds in biological tissues and fluids in a very short time, with a minimum allocated budget (6). Over time, advanced glycation end products (AGEs) in particular have been investigated, and more or less significant differences between their concentrations in different biological fluids and tissues have been evidenced in the case of diabetic compared to non-diabetic dogs (3, 7). The current literature related to the diagnosis of DM in humans describes, in addition to the evaluation of AGEs, the investigation of fluorophores such as the reduced form of nicotinamide adenine dinucleotide (NADH) (6, 10), flavin adenine dinucleotide (FAD) (21), and kynurenine (KYN) (8). The disturbance of AGE metabolism as well as of the tryptophan-KYN metabolic pathway can take place in the following situations: hyperglycemia, oxidative stress and inadequate cell clearance for the body's needs (14, 19). Compared to the measurement of HbA1c or the determination of diabetes duration in human subjects, the assessment of AGEs has a much higher predictive value for diabetes microvascular complications (19). Moreover, some studies suggest the implication of AGEs in the pathogenesis of almost all human diabetic complication highlighting the importance of monitoring these serum fluorophores in humans (6, 18). Thus, the investigation of these two molecules in the case of canine patients suspected of having diabetes might provide the clinician with valuable information, including the inflammation degree evaluation and the detection of some potential complications of this disease (6).

The functional role of intracellular NADH and FAD in cell biology turns these two coenzymes into effective diagnostic instruments, which play the role of natural biomarkers for cellular redox reactions, energy metabolism and mitochondrial abnormalities in various pathophysiological conditions (10). These molecules are naturally fluorescent, consequently, they are considered important in the spectrofluorimetric diagnosis and monitoring of different disorders which have altered cellular processes as the underlying cause (10).

Studies based on the spectrofluorimetric evaluation of the compounds presented above in canine patients suspected of having diabetes are limited. In fact, a search of the literature in this area yielded no articles proposing a simultaneous spectrofluorimetric investigation of these biochemical compounds in the case of dogs suspected of having DM compared to non-diabetic dogs.

In our study, we tested the spectrofluorimetric method's applicability in the monitoring of canine diabetes, ever since the beginning of disease suspicion by performing a simultaneous investigation of these serum fluorophore compounds. More precisely, our goal was to investigate the contributions of AGEs, NADH, FAD and KYN to total serum fluorescence both in dogs with either of the following increased parameters: glucose, triglyceride and cholesterol and with symptoms of diabetes: polydipsia and polyuria, as well as in dogs with serum parameters ranging within physiological limits and without any suspicion of diabetes. Also, we evaluated the associations and correlations between these fluorophores compounds and the biochemical parameters frequently monitored in the case of dogs suspected of having DM: glucose, triglycerides and cholesterol.

MATERIALS AND METHODS

Dogs

The biologic material consisted of 48 blood serum samples collected over 10 months from 11 non-diabetic dogs and 37 dogs (13 females and 24 males) suspected of having DM. All dogs included in this study were brought to the Hospital of the Faculty of Veterinary Medicine Cluj-Napoca for routine vaccination and examination during the period between November 10, 2014 and December 16, 2015. The group of dogs suspected of having diabetes was formed by those animals that showed clinical signs associated with DM such as: polydipsia, polyuria, weight loss and with either of the following increased parameters: glucose, cholesterol and triglycerides. This group of dogs was considered as suspected of having diabetes because the serum biochemistry was monitored only one time at the moment of their arrival in the clinic and we have not obtained any information about the urinalysis. The dogs were not included in the study if they presented any clinical signs associated with a severe infection or disease that could determine a change in dogs' health status. The control group consisted of 11 clinically healthy dogs, in which the parameters presented above ranged within normal limits and the clinical signs associated with the presence of DM were absent. The reference values for healthy control dogs used in this study ranged between 75-120 mg/dl for glycemia, between 102-128 mg/dl for triglycerides, and between 135-278 mg/dl for cholesterol (4, 17). The blood samples from both groups of dogs were collected after a 12-h fast. After blood collection, the biochemical analysis of the serum samples was carried out in order to assess glucose, triglyceride and cholesterol concentrations. These were measured using commercially available methods (Hitachi, Roche Diagnostics). The obtained serum samples were stored at temperatures of -18°C until spectrofluorimetric analysis. The study protocol implemented in this study was approved by the Ethics Committee of the University of Agricultural Sciences and Veterinary Medicine in Cluj-Napoca, Romania. Written informed consent was obtained from the owners at the time their dog was enrolled in this study.

Table 1

The characteristics of dogs suspected of having diabetes mellitus and controls

Variable	Non-diabetic dogs (n=11)	Dogs suspected of having diabetes (n=37)
Age	7.4±3.4	6.9±3.9
Male sex (n, %)	9 (81.8%)	24 (64.9%)
Blood glucose (mmol/l, IQR)	4.98 (3.95-5.53)	8.46 (6.16-10.84)*
Total cholesterol (mmol/l, IQR)	3.33(2.65-5.46)	7.23 (4.86-10.87)*
Triglycerides (mmol/l, IQR)	1.02 (0.78-1.18)	1.17 (0.99-2.13)
AGEs (%)	8.36±1.62	10.7±2.86*
KYN (%)	20.72±2.41	23.1±2.97*
NADH (%)	13.18±2.92	16.05±2.87*
FAD (%)	42.36±4.22	35.54±4.41*

AGEs, advanced glycation end products; KYN, kynurenine; NADH, nicotinamide adenine dinucleotide; FAD, flavin adenine dinucleotide. Values are expressed as means +/- standard deviations or percentages. *p<0.05

Spectrofluorimetric analysis

The spectra obtained following spectrofluorimetric analysis of the serum samples were recorded using a Jasco FP-8200 spectrofluorimeter and a glass cell of 1 cm in diameter. Before spectrofluorimetric analysis, the serum samples (10 µl) were diluted with 5 ml physiological serum, at a dilution of 1:500.

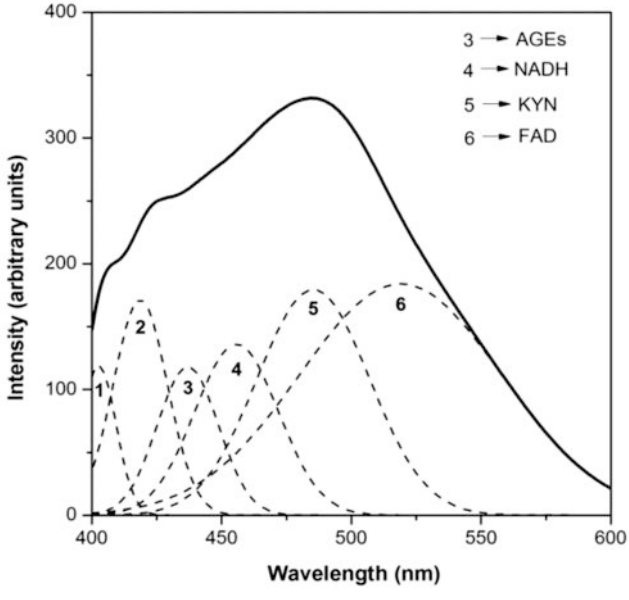

Fig. 1. The fluorescence spectra of a dog serum sample deconvoluted into single Gaussian-shaped bands 1-6

The obtained spectra were recorded in the range from 350 nm to 650 nm, using an excitation of 370 nm and medium sensitivity. The emission of serum fluorophores was obtained at approximately 435 nm for AGEs (12, 23), 460 nm for NADH (6), 480 nm for KYN (20), and 520 nm for FAD (34). In order to quantify the contribution of these fluorophores to total serum fluorescence, the recorded spectra were subjected to curve fitting using the Gaussian deconvolution algorithm of the peak analysis option of the Origin Pro 8.1 software. Thus, the peaks were fitted using a combination of six Gaussian peaks, and the area under the peaks was subsequently calculated (Fig. 1).

Statistical analysis

The obtained data were analysed using the SPSS 20 software. The normal distribution of all variables was evaluated with the Shapiro-Wilk test ($p>0.05$). Continuous *variables* with *normal distribution* were presented as means ± standard deviation. For *non*-normal *variables*, the median and IQR should be reported. The categorical or dichotomous variables were expressed as numbers and percentages. The Mann-Whitney U test was used to compare the two groups of variables: the control group and the group of dogs suspected of having diabetes. The correlation between the investigated fluorophore compounds: AGEs, KYN, NADH, FAD and the biochemical parameters: glucose, triglycerides, cholesterol was assessed by using Spearman's coefficient. The sensitivity and specificity of the measurements performed for the spectrofluorimetric evaluation of AGEs, KYN, NADH and FAD were determined by ROC curve analysis. A *P* value <0.05 was considered statistically significant.

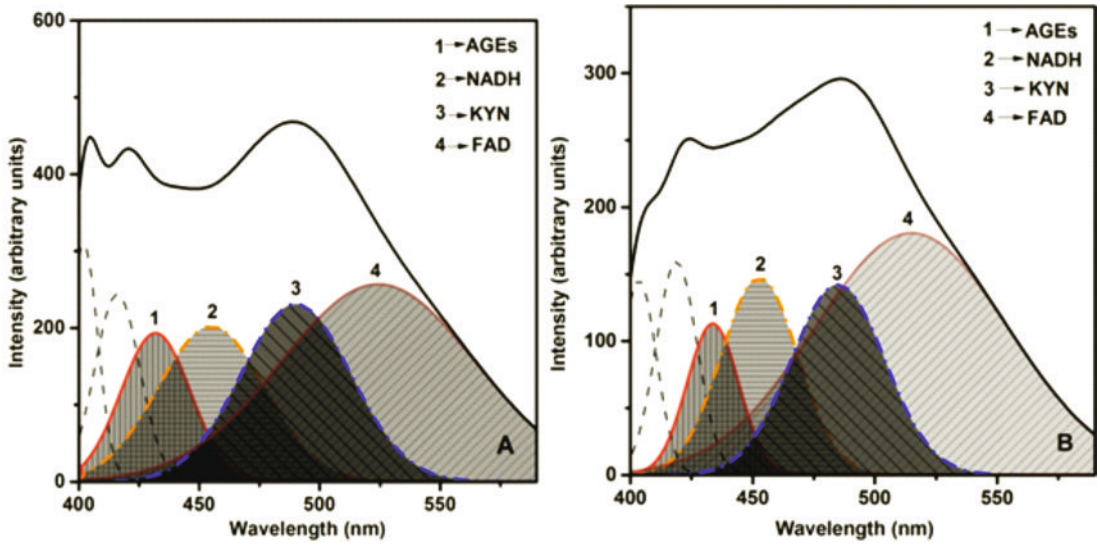

Fig. 2. The curve-fitting deconvolution of the serum fluorescence spectra of a dog suspected of having diabetes mellitus (A) and a non-diabetic dog (B). The spectra were recorded using an excitation of 370 nm and a medium sensitivity, in the range

RESULTS AND DISCUSSIONS

Each dog included in the study underwent a minimum set of tests. The characteristics of the dogs included in the study are shown in Table 1. These tests allowed to evidence higher blood glucose and cholesterol levels in dogs suspected of having diabetes compared to the control group. In contrast, no statistically significant differences were established between the investigated groups regarding age and triglyceride levels. The mean contributions of all fluorophores to total serum fluorescence in the case of both groups of investigated dogs are presented in Table 1.

The spectrofluorimetric analysis of the studied samples together with the band deconvolution method and followed by the statistical analysis allowed to evidence significantly higher contributions of the investigated fluorophores to total serum fluorescence in the case of dogs suspected of having diabetes compared to control dogs (Fig. 2).

A positive correlation was established between the mean contribution of NADH to total serum fluorescence and the blood glucose level ($r = 0.414$ [95% CI - 0.004 to 0.020]; $p<0.05$). Also, a significant positive correlation was evidenced between the mean contribution of NADH to total serum fluorescence and the age of the dogs included in the study ($r = -0.290$ [95% CI - 0.450 to 0.013]; $p<0.05$). No correlation between the mean contributions of AGEs, NADH, FAD and KYN to total serum fluorescence and the levels of triglycerides and cholesterol in the body could be established. Also, there was no correlation between blood glucose levels and the contributions of AGEs, KYN and FAD to total serum fluorescence.

In order to analyse the sensitivity and specificity of the implemented method, ROC curves were calculated. The implementation of this calculation model was aimed at calculating the area under the curve (AUC) of the serum components analysed by spectrofluorimetry. Thus, ROC curve analysis in the case of dogs suspected of having diabetes allowed to evidence the following areas under the curve: 0.742 ($p=0.016$) for AGEs, 0.749 ($p=0.013$) for NADH and 0.713 ($p=0.034$) for KYN. These results indicate the fact that AGEs, NADH and KYN are moderately predictive for the presence of diabetes in dogs.

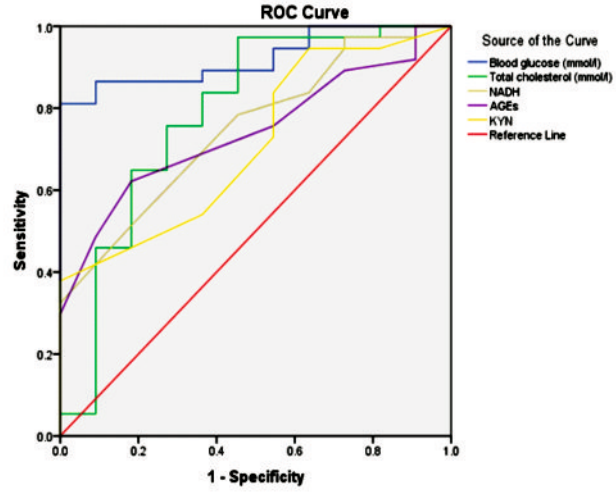

Fig. 3. The ROC curves of investigated fluorophores. ROC curves were used to evaluate the accuracy of the blood glucose, total cholesterol, NADH, AGE, KYN

The aim of this study was to evidence by fluorescence spectroscopy potential changes in the metabolism of serum fluorophores: AGEs, KYN, NADH and FAD in dogs suspected of having diabetes compared to a control group, as well as to correlate these changes with various clinical and biochemical parameters frequently monitored in medical-veterinary clinical practice. The obtained results showed significantly higher contributions of all the investigated fluorophores to total serum fluorescence in the case of dogs suspected of having DM compared to the control group. Therefore, these results demonstrate in dogs, contrary to humans, a significant alteration of the metabolism and composition of the monitored serum compounds once with the suspicion of manifest DM. The high sensitivity and specificity of this spectrofluorimetric method regarding the differentiation between dogs suspected of having DM and controls are also noteworthy. The correlations established between the investigated serum fluorophores and the assessed biochemical parameters led to the following results: a strong correlation between the contribution of NADH to total serum fluorescence and the blood glucose level, and a correlation between the contribution of the same fluorophore and the age of the dogs included in the study. In the case of the other investigated fluorophores, the established correlations were not statistically significant.

The study of the metabolism of tissue and serum fluorophores AGEs, KYN, NADH and FAD in human medicine, unlike veterinary medicine, has been a rather debated subject over time. Furthermore, in human medicine, there is considerable interest in monitoring the serum levels of these fluorophores given the fact that some of them are considered as biomarkers for monitoring the risk of developing diabetes complications (6, 30, 31). A number of factors that influence and induce an alteration in the metabolism of these products have been described. Therefore, in diabetes, the excessive production of glucose can produce several modifications in the body including changes in the metabolism of these fluorophores (31). One of the main differences noted in our study concerns the NADH increase in the case of dogs suspected of having diabetes compared to the controls, being known that glucose metabolism via the glycolytic pathway and the Krebs cycle plays a major role in the storage of electrons in NADH and FADH for the ATP production. Further, the NADH increase can produce the break of the redox balance between NADH and NAD, contributing to the apparition of oxidative stress and a variety of metabolic syndromes, inclusively the cell death (32). Therefore, it was hypothesized that this finding can be used as a protective mechanism against diabetes mellitus as the restoration of NADH/NAD+ redox balance in diabetes is an important goal (31). However, in our study, in addition to the correlation established between NADH and the concentration of glucose, we also observed a correlation between this fluorophore and the age of investigated dogs. In a previous study was found that the levels of NAD^+ (determined as the difference between the total NAD(H) and NADH concentrations) decline with age, highlighting the negative correlation between NAD and the ageing process (13). According to the same study, we can hypothesize that the concentrations of NADH increase in aging, suggesting in humans as in dogs an increase of the fluorophore levels once with age. As NADH, the coenzyme FAD represents an important key metabolic indicator of the cell metabolism which can be associated with the progress of diabetes (1). In our study, the significant differences in the contributions of FADH to total serum fluorescence between control and suspected of having diabetes group of dogs might indicate some changes in the fluorophore composition during the apparition of clinical signs associated with DM. Moreover, the investigation of the optical redox ratio between the emission intensity of FAD and NADH can generate strong information about the changes in their metabolic pathways (1). Therefore, in future studies, we intend to better describe the correlation between these fluorophores and their optical redox ratio, respectively in a more numerous groups of dogs suspected of having diabetes.

Another category of biological molecules with fluorescent properties, which underwent alterations with the development of these metabolic changes in the case of dogs suspected of having diabetes, is represented by the AGEs and KYN. Clinical studies in humans have established the presence of an association of AGEs and KYN with hyperglycemia and increased oxidative stress (2, 9, 16, 26, 29, 33) as well as their association with various metabolic and degenerative disorders: depression, cancer, and diabetes (11, 19, 35). However, most of the clinical studies conducted in diabetic human patients have not yet precisely determined the factors that influence the circulating levels of AGEs, the extent to which the serum concentrations of these fluorophores can reflect and participate in the development of hyperglycaemia, hypertriglyceridemia or hypercholesterolemia, or the extent to which they can contribute to the progression of diabetes (15, 25, 27, 35). According to the literature, AGEs, as HbA1C are glycated proteins used for the evaluation of glucose levels over a period of time (30). However, in humans, future directions recommend AGEs, despite HbA1C, as having a great potential for predicting the risk for diabetes microvascular complications. On this view, more clinical trials with a large number of patients are mandatory in order to implement a method that evaluates the presence of diabetic complications according to AGEs production (30). Regarding the relationship between glucose concentration and

the formation of AGEs, contrary to NADH, we didn't find an association between glucose or any other biochemical parameter and AGEs, suggesting that the levels of NADH are the most suitable when analysing the blood serum of dogs suspected of having diabetes. In this regard, Negre-Salvayre et al. report that increased glucose concentrations have a minimizing effect on AGE formation (15). This limitation is due to the chemical properties of glucose, the main carbohydrate, which is the least recommended for reacting with amines to form AGEs. The literature mentions many other carbohydrates that react much more effectively with amines to form AGEs. Thus, galactose is about 5 times more reactive, fructose 8 times, deoxyglucose 25 times, and ribose 100 times more reactive than glucose (15). A recent study was demonstrated that the AGEs to NADH ratio correlates with fasting blood glucose, triglycerides and HDL-cholesterol levels and is a predictor for the presence of cardiovascular and chronic kidney disease in humans with type two DM (6). Therefore, the therapy aimed to restore the NADH and AGEs profile could prevent the risk of the apparition of these DM complications in humans (6). In dogs, contrary to humans, the incidence of cardiovascular and chronic kidney disease is fairly low (24) . But anyway based on the results of our study it can be declared that the therapy aimed to correct the glucose concentration could re-establish at least the physiologic NADH level and therefore prevent the apparition of some DM complications in dogs.

CONCLUSIONS

Finally, based on the results of the present study, we can confirm that spectrofluorimetry represents a reliable, non-invasive, and cost-effective technique that has a potential for the simultaneous, rapid, and sensitive detection of specific serum metabolic alterations during diabetes development. The present results show that once with the suspicion of manifest DM, the levels of serum fluorophores: AGEs, NADH, KYN, and FAD increase in dogs, revealing the apparition of some metabolic changes in the state of cellular metabolism. Further, in contrast to the other investigated fluorophores, we evidenced a significantly positive correlation between the contribution of NADH to total serum fluorescence and blood glucose levels. Also, the contribution of NADH to total serum fluorescence, unlike the other fluorophores investigated in this study, was correlated with the blood glucose level and the age of the dogs included in the study, highlighting the high accuracy of this fluorophore to differentiate between controls and suspected of having diabetes dogs. Therefore, in further studies, we intend to investigate whether the therapy aimed at correcting glucose concentrations can also improve NADH levels and thus prevent the development of possible secondary complications associated with diabetes in dogs.

Conflict of Interest

The authors declare that they have no conflict of interest.

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.

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