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Postgraduate School of Veterinary Science**

**Evaluation of oral hypoglycaemic drugs
and examination of certain factors
in the development of cardiovascular consequences
in alloxan-induced diabetes mellitus in dogs**

Theses of Ph.D. Dissertation

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INTRODUCTION

Diabetes mellitus is one of the most common hormonal diseases in dogs. It is also frequent in humans and the number of patients increases progressively. Type-2 diabetes is considerably more frequent in humans.

Diabetic complications are strongly involved in human mortality reasons. Cardiovascular alterations are of great importance among these complications.

The diabetes of dogs is equally important both in veterinary and in experimental human medicine. The management of diabetic dogs is an important issue for the veterinarian. The therapy is based almost exclusively on insulin.

The present study compiles three experiments. In the first experiment we examined the effectiveness of oral antidiabetic drugs in alloxan diabetic dogs.

There are three widely used drug families in human type-2 diabetes: alpha-glucosidase inhibitors, biguanids and sulfonylureas. Each of them is represented in our study.

The alpha-glucosidase inhibitor acarbose, the biguanid-type metformin and the sulfonylurea gliclazide were investigated. According to our results, metformin was the only drug which exerted significant blood glucose lowering effect in postprandial blood glucose level comparing to that of before feeding.

The second and the third experiment of the study are of human and of veterinary interest at the same time.

Endothelin-1 is an endogenous peptide with different effects. Besides being a potent vasoconstrictor, it exerts positive inotropic and arrhythmogenic effect on the heart and it can contribute to the development of certain pathological states.

The aim of second experiment was to determine the changes in endothelin-1, in N-terminal fragment of atrial natriuretic peptide (NT-ANP) and in atrial natriuretic peptide (ANP) levels in the peripheral blood, in the coronary arteries and in the pericardial fluid in healthy dogs and in dogs with experimentally induced diabetes in response to acute hemodynamic burden that was produced with arteriovenous shunts. According to our results the endothelin system responds differentially to acute hemodynamic load in diabetic and in healthy dogs which can be regarded as a potentially adaptive mechanism. Pericardial space represents a functionally different compartment than the coronary sinus and the peripheral plasma.

As endothelin-1 is presumably implicated in natriuretic peptide secretion, we controlled the changes in NT-ANP and in ANP levels parallelly with that of ET-1. The alterations in NT-ANP levels followed the hemodynamic state sensitively, while ANP levels did not show consequent changes.

In our third experiment the arrhythmogenic effect of endothelin-1 was examined in diabetic and in metabolically healthy dogs.

Our results showed that a diabetic heart is more prone to the arrhythmogenic effect of endothelin-1.

AIMS OF STUDIES

- Examination of the effect of the alpha-glycosidase inhibitor acarbose, the insulin sensitiser metformin and the insulin secretiser gliclazide on blood glucose level in alloxan-diabetic dogs.
- Evaluation of the changes in the levels of endothelin-1, atrial natriuretic peptide and N-terminal fragment of atrial natriuretic peptide in response to acute hemodynamic load in experimental diabetes in dogs and the determination of the possible relations between them.
- Examination of the arrhythmogenic effect of low-dose endothelin-1 on diabetic and on healthy heart in dogs. Comparison of the reactivity of healthy and diabetic heart.

EXPERIMENTS

Experiment 1: Evaluation of oral hypoglycaemic drugs in alloxan-induced diabetes mellitus in dogs

Study conditions

Seventeen clinically healthy, mixed-breed, medium-sized dogs of both sexes were used in this study.

The animals were housed in individual cages at a mean environmental temperature of 20 °C for 12 hours in artificial light and 12 hours in darkness. The dogs received complete dog food twice a day at 7 a.m. and 3 p.m. and water *ad libitum*.

Diabetes was induced by a single intravenous infusion of 560 µmol/kg bw alloxan. Blood glucose level of dogs selected for the study were moderately elevated (6.5–8.3 mmol/l) when measured on the 7th day.

On days 8-15, the dogs did not get any drug. Their blood glucose level was measured twice a day. The first sampling was done just before feeding (FBG), and the second 60 minutes later for the determination of postprandial blood glucose (PPBG) level. Daily fasting and postprandial blood glucose levels, measured in that period, served as control data.

On day 16 the dogs were randomly divided into three groups. The first group of six dogs was administered 200 mg acarbose (9.1±0.8 mg/kg bw) daily. The second group of five dogs was treated with metformin 1700 mg per dog per day (60.9±6.4 mg/kg bw). The third

group of six dogs were given 160 mg gliclazide daily (6.1 ± 0.7 mg/kg bw). The daily dose of drugs were divided into two equal parts and administered at the time of feeding. FBG and PPBG data were determined every day in the following five weeks.

Haemogram, serum alanine transaminase (ALT), alkaline phosphatase (ALP), amylase, lipase, triglyceride (TG) and total cholesterol (TC) were tested before the induction of diabetes and subsequently at weekly intervals during the experiment.

The insulin level was measured weekly with the exception of the insulin secretiser gliclazide group, where samples were taken every third day.

The differences between postprandial and fasting blood glucose levels were used for the statistical evaluation. The difference between blood glucose levels after and before feeding (PPBG-FBG) resulted in blood glucose difference (BGD) data. Daily BGDs were averaged for the control and the treated period in each dog. Their difference resulted in treatment difference (TD) for each dog. The average of TDs gave the mean decrease (MD) for each drug. The significance of MD was statistically analysed by the two-sampled paired *t*-test. The data were checked using the Shapiro-Wilk normality test.

Results and discussion

During the treatment period, FBG and PPBG levels varied in the normal or slightly elevated range (4.0–8.5 mmol/l) in the metformin and

acarbose groups, while in the gliclazide group severe hyperglycaemia (up to 22.4 mmol/l) was detected several times in three dogs.

The MD was 0.49 ± 0.58 mmol/l ($P=0.09$) for acarbose treatment, 1.28 ± 0.69 mmol/l ($P=0.01$) for metformin and 0.08 ± 1.33 mmol/l ($P=0.88$) for gliclazide.

According to the statistical evaluation, out of the investigated agents metformin was the only drug that caused a significant decrease in blood glucose level.

The haemogram of the dogs remained in the normal range during the experimental period. In two cases transient eosinophilia occurred in the metformin group, which was not proved to be associated with the treatment.

The higher initial amylase, ALT and ALP activities in some dogs can be attributed to the alloxan treatment, which overloaded the hepatic capacity.

The plasma concentrations of lipase, TG and TC were normal in all dogs throughout the experiment.

The insulin data most of the time remained in the reference range in the metformin group, and were below that several times in the acarbose and in the gliclazide group.

Acarbose, metformin and gliclazide are oral antidiabetic drugs extensively used in the human medicine either alone or in combination with one another or with insulin. In our experiment we examined their possible role in veterinary medicine.

Our experimental animals were treated with alloxan, an agent that selectively destroys beta cells. The damage of these cells is dose dependent, so low dosages of alloxan can produce non-insulin dependent diabetes mellitus (Cooperstein and Watkins 1981), that we used in our study. Our experimental animals had moderately elevated FBG concentrations, which were also indicative of NIDDM.

Our first examined drug, the acarbose did not induce a statistically significant decrease in postprandial blood glucose compared to control. Acarbose acts at the “beginning” of glucose metabolism; namely, it can decrease the absorption of glucose from the gastrointestinal tract, but it does not directly influence either the insulin secretion or the glucose uptake of the cells. Even then it can reduce daily insulin requirements (Balfour and McTavish 1993; Rios 1994). As acarbose slows digestive and absorptive processes, a smaller amount of glucose enters the systemic circulation, reaching the pancreatic beta cells in lower concentrations (Coniff et al. 1995), which means that acarbose reduces PPBG (Gerard et al. 1981; Dimitriadis et al. 1985; Rios 1994). Acarbose is not absorbed systematically. The effect of acarbose can be influenced by the diet; namely, at a higher carbohydrate content the drug presumably can exert greater effect. Acarbose might exert its effect slower as there may be a threshold proportion of brush border enzymes that must be inhibited before the effects of acarbose can be identified (Robertson et al. 1999). According to the literature, insulin secretion was decreased in acarbose-treated healthy dogs, which could be attributed to

the drug-induced delay in the absorption of carbohydrates (Robertson et al. 1999).

As the target of this drug is different, it would be worth studying in combination with insulin or metformin mainly in obese dogs.

The insulin sensitiser metformin, our second drug examined, significantly decreased the PPBG as compared to FBG.

In our study FBG data remained on the same level (4.0–8.5 mmol/l) during the 4–5 weeks of the experiment.

The actual mode of action of metformin is not known, although it has several effects on glucose metabolism at different sites of action. Metformin presumably acts in different ways in dogs: it increases glucose uptake and oxidation in peripheral tissues, increases insulin receptor activity, delays glucose absorption from the bowel, and some of these effects result in a decrease in PPBG. Metformin improves insulin sensitivity in the peripheral tissues, but it is not effective in the absence of insulin (Stumvoll et al. 1995), although treatment with this drug has been reported to be associated with reduced plasma insulin, indicating that biguanides attenuate insulin resistance (Lenhard et al. 1997).

In our experimental model the sensitivity of insulin receptors was presumably not altered as in the case of a real type 2 diabetic patient. However, even in our experimental model metformin could significantly reduce the PPBG level. It did not decrease insulin secretion, which in our case was not originally high as it would have been in the case of insulin resistance.

In view of the above facts, careful patient selection is important. Metformin can be appropriate for controlling pre-diabetic state in dogs with secondary diabetes mellitus, when insulin production still exists, e.g. in oestrous diabetes or in obese dogs with decreased glucose tolerance, until neutering or until dietary modifications restore the physiological balance in an optimal case.

Gliclazide, the third drug of this study elicited no significant decrease in PPBG compared to FBG.

Gliclazide induces insulin secretion in the pancreatic beta cells and inhibits glucogenolysis and gluconeogenesis in the liver. Through improving insulin binding to surface receptors also enhances the insulin sensitivity of target cells and even increases the number of insulin receptors in tissues of action.

Conclusions

- The insulin sensitiser metformin decreases postprandial blood glucose level in dogs.
- It would be worth studying metformin further in dogs.
- It would be important to characterise the type of diabetes in which this drug can be used and to determine the optimal dosage and probably a combination therapy.

Experiment 2: Endothelin and natriuretic peptide levels in cardiac hypertrophy in experimental diabetes in dogs

Study conditions

21 healthy and 20 diabetic dogs were involved in the study. Diabetes was induced by a single intravenous infusion of 560 μ mol/kg bw alloxan and dogs were investigated 8 weeks after the induction of diabetes. Blood glucose level of the diabetic dogs was over 15 mmol/l.

Cardiac hypertrophy was induced with shunted circulation. Arterio-venous side to side shunts (3-4 cm) were placed between the arteria and vena femoralis on both sides in both distal femoral regions of the dogs. The control animals were sham operated.

Dogs were divided into six groups: healthy control (sham operated) (n=10), healthy dogs with 1 day shunted circulation (n=6), healthy dogs with 3 days shunted circulation (n=5), diabetic control (sham operated) (n=10), diabetic dogs with 1 day shunted circulation (n=5) and diabetic dogs with 3 days shunted circulation (n=5).

Blood pressure, mean arterial blood pressure and heart rate were recorded. Dogs were sacrificed 1 or 3 days after the shunt operations. The hearts were excised and weight.

The levels of endothelin-1 (ET-1), the N-terminal atrial natriuretic peptide and the natriuretic peptide were measured in the pericardial fluid, in the coronary sinus blood and in the peripheral blood with radioimmunassay. The differences of data were analyzed by analysis of variance followed by the Mann-Whitney test.

Results and discussion

Diabetic dogs had lower blood pressure as compared with healthy controls, which can be explained by the profound fluid and electrolyte depletion in untreated diabetes. Shunted circulation decreased the blood pressure further after 1 day in diabetic dogs, while it had no effect on the blood pressure in the healthy ones.

Both right and left ventricular weights increased upon shunting. Heart weight/body weight ratios and similarly left ventricular weight/body weight ratios increased upon 1 day of shunted circulation in the diabetic group, while similar changes were detected only after 3 days shunted circulation in the healthy group. Beside the increase in the heart-weight, the quantity of NT-ANP also elevated upon shunting in diabetic and in healthy dogs indicating that our model initiated cardiac hypertrophy.

The cardiac hypertrophic process is elicited by an increase in cardiac load, which causes the elevation of contractile strength. The process is followed by the elevated secretion of different autocrine and paracrine factors for example ET-1 and angiotensin II (Sadoshima et al. 1993, Yamazaki et al. 1995). These factors considered to have primary role in hypertrophic process (Kim and Iwao 2000).

In a study a 9% rise was found in the left ventricular weight/body weight ratios in rats after 12 hours in angiotensin II induced pressure overload model, which was further increased up to 16% after 72 hours (Lakó-Futó et al. 2003).

In our experiment the ET-1 level significantly decreased upon shunting in the coronary sinus and in the peripheral plasma in diabetic dogs, while there was no change in the healthy groups. The lowest ET-1 level was measured in the pericardial fluid out of the three compartments in the diabetic group, and it was not altered upon shunting. In healthy dogs the ET-1 level was higher in the pericardial fluid and in the coronary sinus than in peripheral plasma, while it was significantly higher in the peripheral plasma in diabetic dogs than in healthy control.

In healthy dogs the higher pericardial ET-1 concentrations are in accordance with literature data about different species (dogs, rats and humans) (Horkay et al. 1998). The six-fold higher elimination rate of ET-1 from the peripheral plasma than from the pericardial fluid can contribute to the phenomenon (Shiba et al. 1989, Szokodi et al 1998). The analogy in the changes in the ET-1 content of the coronary sinus and the peripheral plasma, whereas there was no change in the pericardial fluid suggests that the pericardial space is a functionally different compartment. ET-1 levels in the coronary sinus were slightly higher than in the systemic plasma, suggesting that the heart actively contributes to the maintenance of plasma levels of ET-1.

ET-1 is considered to be involved in the secretion of the natriuretic peptide (Ruskoaho et al 1997). In our study we measured the concentrations of endothelin, ANP and NT-ANP in all three compartments.

Levels of NT-ANP showed similar patterns in all three compartments: it was low in the control groups and raised in shunted animals.

The ANP level was significantly increased in the peripheral plasma after 3 days of shunted circulation in the healthy and in the diabetic groups and in the pericardial fluid in healthy dogs after 1 day of shunted circulation.

The endothelin and the natriuretic peptide are essential in regulating blood pressure. Their effects are controversial: the endothelin is a potent vasoconstrictor, while the natriuretic peptide causes vasodilatation.

ANP and NT-ANP are widely used for the objective signalment of cardiac function, diagnosing heart failure, examining its progression and controlling the effectiveness of therapy. Their elevation is a sensitive signal of deterioration of heart function (Moe 2006) in humans and also in dogs (Boswood et al. 2008, Haddad et al. 2008, Schellenberg et al. 2008). The NT-ANP is produced in equimolar quantity like the active derivate ANP, but has a longer half life and thus more suitable for examination, which was also supported by our results.

Endothelin has a series of cardiovascular effects. The potent vasoconstrictor and the direct arrhythmogenic effect can be harmful both in the short and in the long term, and the induction of tissue proliferation is harmful in the long term. On the other hand ET-1 is a positive inotropic agent and influences the inotropic responses by modulating the

Frank-Starling mechanism as well, which can be beneficial in acute situations.

Therefore we assume that the acutely observed changes in plasma ET-1 levels did not have any short term consequences in diabetic dogs.

Conclusions

- The endothelin system responds differentially to an acute hemodynamic load in alloxan diabetic and in healthy dogs.
- Reduction of plasma ET-1 levels in response to an acute hemodynamic load in diabetic dogs can be regarded as a potentially adaptive mechanism, since it reduces the chances of vasospasm and cardiac arrhythmias.
- The pericardial space represents a functionally different compartment in the endothelin system.

Experiment 3: Effect of experimental diabetes on endothelin induced ventricular arrhythmias in dogs

Study conditions

Experiments were carried out on 19 healthy and 4 diabetic dogs. Diabetes was induced with a single intravenous injection of 560 $\mu\text{mol/kg}$ bw alloxan and dogs were investigated 8 weeks after the induction of diabetes. The blood glucose level of the diabetic dogs was over 15 mmol/l.

Group 1 (n=4), group 2 (n=11), and group 3 (n=4) consisted of metabolically healthy dogs, while group 4 (n=4) contained diabetic dogs.

In group 1 physiological saline was administered. In group 2 33 pmol/min ET-1 was infused. In group 3 the administration of 33 pmol/min ET-1 was accompanied by simultaneous intracoronary glucose infusion (25 mmol/l) for characterization of possible effects of local hyperglycemia. In group 4 33 pmol/min ET-1 was administered to the diabetic dogs.

Infusions (physiological saline, ET-1 and glucose) were administered from 0 to 40th minutes of the experiment. Experiments were terminated in groups 2-4 when ventricular fibrillation evolved and in the control group after 40 minutes.

Mean arterial pressure, heart rate and coronary blood flow was continuously registered and standard electrocardiogram was made.

Student t-test was used for statistical evaluation.

Results and discussion

The hemodynamic parameters, the mean arterial pressure, the heart rate and the coronary blood flow were practically unaltered in the control group during the experiment.

In group 4 (ET-1-treated diabetic dogs) the mean arterial pressure was significantly lower than in group 2 (ET-1-treated healthy dogs) in the 40th minute, which can be attributed to the ventricular fibrillation which emerged sooner in group 4 than in group 2.

In group 3 (ET-1 and local hyperglycaemia) the heart rate elevated significantly comparing to that of in group 2 at the 40th minute, which can be explained by the time of the appearance of tachycardia: $26,3 \pm 6,7$ minutes in group 3, and $30,0 \pm 12,4$ minutes in group 2.

There was no arrhythmia in the saline infused control (group 1). In healthy dogs (group 2 and 3) a substantial number of ventricular extrasystoles, occasionally couplets and triplets appeared after about 24 minutes of ET-1 infusion. These arrhythmias emerged significantly earlier (at 18,7 minutes) in the diabetic (group 4) than in the healthy (group 2 and 3) dogs.

At around 30 minutes, ET-1 administration provoked recurring non sustained tachycardias. At about 35 minutes following the start of infusion, sustained tachycardias developed leading finally to ventricular fibrillation and the termination of the experiment.

Local hyperglycaemia, which was produced by simultaneous administration of 25 mmol/l glucose with ET-1 (group 3) did not evoke the earlier appearance of extrasystoles. There were no significant

alterations between the groups considering the number of ventricular fibrillations or the total elapsed time until the termination of the experiments.

ET-1 is an endogenous peptide with widespread types of action. It exerts its influence also on the heart and is also known as a potent arrhythmogenic agent. This phenomenon can be considered as a result of the developing myocardial ischemia, however its direct arrhythmogenic effect was also described. Diabetes mellitus is accompanied by a series of metabolic disfunctions. The endothelin system is also altered: higher ET-1 levels were observed in the blood of diabetic patients as it was seen in our former experiment.

According to experimental data high dose (60 pmol/min) intracoronary ET-1 infusion resulted in myocardial ischemia. As myocardial ischemia is an arrhythmogenic factor itself, the direct and indirect arrhythmogenic effect of ET-1 also affected and shortened further the time till ventricular tachycardia evolved (Yorikane et al. 1991).

Low dose (30 pmol/min) intracoronary ET-1 infusion resulted in ventricular tachycardia without the signs of myocardial ischemia, which can be attributed to the direct arrhythmogenic effect of ET-1 (Tóth et al. 1995).

In our experiment the coronary blood flow did not change near the ET-1 dose applied, demonstrating that we did not provoke myocardial ischemia and the arrhythmogenic property of ET-1 observed was a direct effect.

According to literature data the hyperglycaemia observed in diabetes results in the elevation of ET-1 concentration and in the same time the activity of the endothelin converting enzyme, which contributes to the production of ET-1, also rises (Cardillo et al. 2002). The hyperglycaemia-induced alterations in the endothelin system can sensitize the diabetic heart and may induce malignant arrhythmias.

According to our results the local hyperglycaemia did not influence the time of appearance of the extrasystoles. That is possibly why high glucose-induced alterations cannot be observed as acute effects, the development of diabetic complications takes a longer time.

In our experiment, ventricular arrhythmias emerged earlier in diabetic dogs, which proves that the diabetic heart is more prone to arrhythmogenic effects. However there was no difference in the number of ventricular fibrillations and the termination time of the experiments.

Conclusions

- The diabetic heart is more sensitive to the arrhythmogenic effect of low-dose intracoronary ET-1 infusion.
- Local hyperglycaemia is not an arrhythmogenic factor itself, although the pathological derangements upon its prolonged existence sensitise the heart to arrhythmogenic effects.

NEW SCIENTIFIC RESULTS

- The insulin sensitiser metformin, applied in humans, decreases postprandial blood glucose level in dogs.
- The endothelin system responds differentially to an acute hemodynamic load in alloxan diabetic and in healthy dogs.
- The reduction of plasma ET-1 levels in response to an acute hemodynamic load in diabetic dogs can be regarded as a potentially adaptive mechanism, since it reduces the chances of vasospasm and cardiac arrhythmias.
- A diabetic heart is more sensitive to the arrhythmogenic effect of low-dose intracoronary ET-1 infusion.
- Local hyperglycaemia is not an arrhythmogenic factor itself, although the pathological derangements upon its prolonged existence sensitise the heart to arrhythmogenic effects.

PUBLICATIONS RELATED TO THE DISSERTATION

PÓSA I., HORKAY F., SERES L., SKOUMAL R., KOVÁTS T., **BALOGH É.**, DECHATEL R., TÓTH M., KOCSIS E.: Effects of experimental diabetes on endothelin-induced ventricular arrhythmias in dogs. *J. Cardiovasc. Pharmacol.* 2004. 44. 380–382.

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