

The PhD. program

Name: Animal breeding

Part of science: Agricultural science

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## **1. PRECEDING SCIENTIFIC ACHIEVEMENTS**

### **1.1. Importance of the examination of glycated proteins**

Monitoring of the glycated proteins [glycated haemoglobin (GHb) and serum fructosamine (SeFa)] is already used routinely for approximately 15 years in the clinical laboratory control of human diabetes, but at the same time in cases of domestic/farm animals the measurements of these parameters are not so common.

In companion animals the tests of the glycated blood proteins are mainly used for controlling the diabetes therapy.

The blood glucose is bound permanently to plasma proteins (SeFa), and the protein part of the haemoglobin (GHb), in proportion to its concentration in the blood (SUHONEN et al., 1989, GEBHART et al., 1995).

This linkage is maintained until the protein (3-20 days) or the red blood cell (60-145 days, depending on the species) disintegrates. It means, that we can achieve a permanent and reliable picture of the blood glucose level of the previous weeks before taking the blood sample, while the actual blood glucose level of the animal might be elevated over the real value by the sympathoadrenal activation. This is why the blood glucose in itself is not a reliable parameter to reflect the actual glucose status of the animal. (BISSE, 2003, GOPALKRISHNAPILLAI et al., 2003).

The glycated blood parameters (GHb, SeFa) are perfectly suitable in order to evaluate the carbohydrate metabolism of the examined animals. This way it is possible to correct certain feeding errors, which might cause health problems later.

Since the metabolic diseases might cause not only health, but also economic problems, it would be very important to complete the carbohydrate and fat metabolism parameters measured currently during the laboratory analyses with the measurement of glycated proteins (JAKAB, 1983, JERMENDY, 1988, LAKNER et al., 1997).

#### **1.1.1. Serum fructosamine (SeFa)**

The formation of the serum fructosamine is a post-translational, non-enzymatic glycosylation; during this process glucose binds to the plasma proteins in two steps. By the uptake of one water molecule, the two components first form aldimine during a reversible process, and subsequently, following the so-called Amadori rearrangement, the stable ketoamine, fructosamine is formed (BAYNES et al., 1984).

In case of diabetes, the concentrations of SeFa elevate significantly, and in healthy people a slight increase can be demonstrated in parallel with ageing, too (TAS and ZEIN el DIN, 1990). Since tests for measuring SeFa are less common in veterinary laboratories, mainly the human standard parameters (up to 2.8 mmol/l in healthy individuals) are considered relevant. (JENSEN, 1992, BERGAMINI, 1993, THORESEN and BREDAL, 1999).

#### **1.1.2. Glycated haemoglobin (GHb)**

In humans the post-translational, non-enzymatic glycosylation of the haemoglobin occurs by the binding of glucose to the N-terminal valin of the haemoglobin  $\beta$ -chains, similarly to the formation of SeFa (MIEDEMA and CASPARIE, 1984).

The importance of this process is that the membrane of the red blood cells is selectively permeable for certain ions; it lets the glucose through proportionately to its presence. It means, that in humans its percentage in the blood depends on the concentration of the plasma glucose (or in different animal species proportional to it) (BELL, 1971).

RAHBAR (1968) found its connection with the diabetes. In the course of cation-exchanger column chromatography of HbA, there is a smaller (6-8 %) "rapid" HbA<sub>1</sub> subfraction before the "slow" or main fraction, called HbA<sub>0</sub> (ZIMMERMANN, 1989).

The sections of the HbA<sub>1</sub> in order of disjunction are HbA<sub>1a</sub> and HbA<sub>1b</sub>, which originate from binding with different sugars and other molecules, e.g. carbamide. The third subfraction is the HbA<sub>1c</sub>, a GHb originating from the non-enzymatic binding of glucose to haemoglobin (BÁRDOS and OPPEL, 1988, PETERSON et al., 1998, BÁRDOS et al., 1990, OPPEL et al., 2000 a).

The first HbA<sub>1c</sub> measurements on animals (healthy and diabetic mice) were performed by KOENIG and CERAMI (1975) as a model of determination of human GHb.

The normal range of GHb in humans is 4.5-6.5%, in animals the standard is 1.9-6.5%, depending on the species (HASEGAWA, 1991, HARTL and FERRAND, 1993).

### **1.1.3. Glycated haemoglobin levels in rabbits**

The determination of the glycated parameters of the different species is important, because the lifespan of the red blood cells, and their permeability against glucose also differs from one another (BELL, 1971).

As an example, in our study we examined rabbits, in case of this species the membrane permeability of the red blood cells is lower than in humans (HIGGINS et al., 1982).

In our department we determined the following standard values for the rabbit species: GHb(%)=3.42±0.69, SeFa(mmol/l)=3.69±0.15, glucose(mmol/l)=5.84±0.15 (OPPEL, 1993).

## **1.2. Glucose homeostasis in healthy individuals**

In healthy individuals the blood glucose level moves within relatively narrow bounds, as a result of different regulating mechanisms. The standard/normal value of the blood glucose level is variable according to the species, but in case of mammals it can be found generally between 2.6 and 8.1 mmol/l (5.3 mmol/l in rabbits) (BONATH et al., 1982).

The pancreas has a determinant role in the formation and maintainance of this normal value through the insulin/glucagon effect. As a result of some diseases, like the diabetes mellitus, the mechanism of the carbohydrate regulation system of the body is disturbed. In order to avoid the hypoglycaemic coma in humans, the blood glucose level must be maintained over 2.6 mmol/l (AMERICAN DIABETES ASSOCIATION, 1998 a). The permanently high blood glucose level is also damaging to the health, since hyperglycaemia causes the non-enzymatic glycosylation of certain proteins, which is an irreversible process, and causes altered functioning of the proteins.

## **1.3. Diabetes mellitus**

Diabetes might develop as a result of missing insulin production (absolute insulin deficiency), or the inhibited functioning of the insulin-dependent tissues (relative insulin deficiency). Because of the disturbance of the carbohydrate uptake, the cells are unable to take up glucose, so the blood glucose level increases, which has several harmful effects to the health.

There are more types of the diabetes mellitus. We can distinguish two types of the primer diabetes, type I. is insulin-dependent (IDDM), and type II., which is not insulin-dependent (NIDDM) (FEKETE et al., 1992, AMERICAN DIABETES ASSOCIATION, 1998 a).

In case of one type of the secunder diabetes the insulin secretion is inhibited due to the necrosis or surgical excision of the pancreas. The other form of it is not associated with the pancreas, but the predomination of the insulin antagonist hormones (STH-hypertrophy, Cushing disease) is the cause of the disease.

### **1.3.1. The tardive complications**

The permanently high blood glucose level is the reason of the subsequent complications, damaging tissues and organs. The high blood glucose level causes intense glycosylation of the proteins, and as a result of this, it changes the physicochemical and morphological characteristics of the proteins. The severity of the complications depends on the degree of the high blood glucose level, the duration of this condition and the ability of the organism to tolerate this. There are microangiopathic and complex subsequent complications.

### **1.3.2. The antidiabetic effect of barley**

In order to complement the insulin therapy, a dietetic menu is recommended. It is a well-known fact, that the basis of the diabetes therapy is a high-fibre, low-energy diet. This diet helps to decrease the fluctuation of the blood glucose level. The fibre content of the barley is very high (30-80 g/kg dm.), but it has a relative low energy content compared to other crops (gross E=15.10 MJ/kg dm.) (ANDERSSON, 1998). The barley has a high  $\beta$ -glucane content (26-59 g/kg dm.), which is also favourable in the dietetic therapy of the diabetes (TOR-AGBIDYE, 1992, WURSH and PI-SUNYER, 1997).

## **2. AIMS OF THE STUDY/OBJECTIVES**

- Clarification of the conservability and storage conditions of blood samples during the glyated protein tests in domestic rabbit
- Drawing up a simplified, cost-saving method for the measurement of serum fructosamine from the glyated blood parameters
- Separating the adult and fetal haemoglobin, determination of their ratio in domestic rabbit
- Surveying the physiological data of the glyated parameters of the rabbit (age, sex), and establishing the correlations between the blood glucose and the glyated proteins (serum fructosamine, glyated haemoglobin)
- Examination of the effect of glycosylation in the domestic rabbit, and the effects of the energy content of the feed on the blood glucose and protein content of the plasma, including the glyated parameters, too

- Examination of the effect of artificially induced (with alloxan) diabetes on the glycated and other parameters of the plasma, in connection with the histopathological deformations
- Studying the antidiabetic effect of the barley

### 3. MATERIALS AND METHODS

#### 3.1. Preliminary examinations

##### 3.1.1. Examination of the conservability of blood samples. The effects of the storage methods and time span on the glycated parameters

The blood samples of the 15 does from our first examination were repeatedly tested following the laboratory analyses of that day (SeFa, GHb tests).

After establishing the results of the last tested blood sample, we compared the glycated protein values of the different storage methods and periods (+4°C - 1 day, -20°C - 1, 3, 7 days) with the results of the analyses of the fresh blood samples, examined on the day of blood taking.

##### 3.1.2. A new micro method for the determination of serum fructosamine

The aim of this study was to develop a precise, money-saving automated method for the measurement of SeFa.

We used the classical method of JOHNSON et al (1982), the nitroblue tetrazolium method was modified. ELISA microplates with 96 U-formed wells were used. 20 µL plasma and standard samples were pipetted into the wells separately in three parallels, using Finnpiettes. The plates were incubated in a shaking machine at 38 °C for 5 minutes, then read at 550 nm, to determine the initial absorbance (A1). The plates were again incubated for 5 minutes, and after determination in the same way, A2 values were measured. The concentrations of the samples were determined from the differences of the two values of absorbance, with the help of the known standard value. The macro version of the new method (200 µL plasma) could also be used well.

##### 3.2.1. Examination of the sympathoadrenal stress of the domestic rabbit

**Objective:** examination of the effects of the stress caused by the repeated blood taking procedures on blood glucose level, and glycated proteins

**Experimental design:** 5 adult (4-5 kg) female New Zealand White rabbits were examined at the KÁTKI (Research Institute for Small Animal Breeding, Gödöllő) rabbit farm

**Blood taking schedule:** 5 repeated blood takings (0., 30., 60., 120. and 240. minutes)

**Determined blood parameters:** blood glucose (G), serum fructosamine (SeFa), glycated haemoglobin (GHb), haematocrit (Ht), haemoglobin (Hb), albumin (Alb), total protein (TP)

##### 3.2.2. Age-dependent examination of rabbits fed diets of different nutrient content

**Objective:**

- Studying the effects of diets of different nutrient content on the glycated and other blood parameters in adult and newborn animals

- Examination of the plasma serum fructosamine and blood plasma glucose levels and their correlation in the kindling/postnatal period

### **Experimental design:**

Our study was carried out in the rabbit farm of KÁTKI. All of the rabbits were pregnant. After the deliveries the suckling bunnies were studied automatically in the groups of the does. There were three experimental groups formed (n=5 individuals) according to their ad libitum diets.

*Group I: 16% crude protein, 9.5 MJ/kg DE, 14.00% crude fibre (commercial fattening mash)*

*Group II: 18% crude protein, 10.5 MJ/kg DE, 13.50% crude fibre (diet for does)*

*Group III: 18% crude protein, 12 MJ/kg DE, 12.10% crude fibre (high energy diet)*

**Blood taking methods and frequency:** The experiment started 4 weeks before delivery, there were blood takings weekly up to the 7th week, so also at delivery, and lasted to the 7th week after delivery. From the 7th week of the experiment we carried out the blood takings once every two weeks. We took blood samples every time from all rabbits (n=15), and later from 3 suckling animals per groups. The blood takings were carried out every time between 8 and 10 am. On every occasion we measured the feed consumption and body weight too.

**Determined blood parameters:** blood glucose (G), serum fructosamine (SeFa), glycated haemoglobin (GHb), haematocrit (Ht), haemoglobin (Hb), albumin (Alb), total protein (TP)

### **3.2.3. Ratio of the fetal and adult haemoglobin, and determination of the glycated parameters in newborn and young rabbits**

**Objective:** Determination of the quantity and ratio of fetal/adult haemoglobin in rabbits, and establishment of the age (days) when fetal haemoglobin is changing to adult haemoglobin.

**Experimental design:** This experiment was also carried out in the KÁTKI rabbit farm, under normal keeping and feeding regime, using New Zealand White rabbits (n=33). There were blood takings carried out from 3 individuals of each age-groups (11 categories altogether). The animals drawn into the experiment were 0, 1, 2, 7, 14, 28, 31, 34, 46, 56 and 70 days old.

**Determined blood parameters:** blood glucose (G), serum fructosamine (SeFa), glycated haemoglobin (GHb)

**Other laboratory work:** Stained smears from the blood samples were prepared and evaluated (NIERHAUS, 1967, NIERHAUS and BETKE, 1968). After the microscopic cell counting we determined the ratio of the fetal/adult haemoglobin in the individual age-groups.

### **3.2.4. Correlations between the glycated haemoglobin and blood glucose in rabbits**

**Objective:**

- Determination of the correlation ratio of these two parameters, and the time intervals needed for its establishment.
- Examination of the changes of 50 ml blood taken at the antibody production on the metabolism of the above parameters.

**Experimental design:** In the course of the experiment there were 7 adult New Zealand White does examined in our animal house.

**Implementation:** Initial blood samples were taken from each rabbit in heparinized tubes (5 ml/animals from the marginal ear vein) (value 0.). After this, blood taking were continued, so from each animal 50 ml blood were taken for one occasion. In the following period 5 ml blood samples were taken from each rabbit on days 1, 7, 14, 21 and 35. The blood samples were analysed in the laboratory on the day of the blood taking.

**Determined blood parameters:** haematocrit (Ht), haemoglobin (Hb), blood glucose (G), glycated haemoglobin (GHb)

### **3.2.5. Examination of the effects of the diabetes mellitus in rabbits as model animals, in a barley feeding trial**

**Objectives:****Experimental design:****Feeding:****Implementation:****Determined blood parameters:**

### **3.3. Laboratory examinations**

**3.3.1. Haematocrit determinations:** micro-haematocrit capillary tube

**3.3.2. Haemoglobin determinations:** Drabkin method (SÓS, 1974)

**3.3.3. Glycated haemoglobin determinations:** with REANAL kit, by BÁRDOS et al. (1990), modified animal (FLÜCKIGER and WINTERHALTER, 1976).

**3.3.4. Serum fructosamine determinations:** with our new macro method. (OPPEL et al., 2000 b, c).

**3.3.5. Blood plasma glucose determinations:** with GOD/POD method (REANAL)

**3.3.6. Plasma albumin determinations:** with BCG method (SÓS, 1974)

**3.3.7. Total protein determinations:** with Biuret method (SÓŠ, 1974)

**3.3.8. Cytological determinations:** the human method of NIERHAUS (1967) and NIERHAUS and BETKE (1968) were applied for rabbit.

#### **4. NEW SCIENTIFIC RESULTS**

#### **5. CONCLUSIONS AND PROPOSALS ARISING FROM THE EXPERIMENTAL RESULTS**

##### **Examination of the conservability of the blood, mainly concerning the effects of the storage methods and periods on the glycated parameters**

In case of SeFa, there was no significant difference between the measurements of fresh blood and blood samples stored on  $-20^{\circ}\text{C}$  for one week. This means, that standard measurements can be carried out from fresh and stored blood, too.

In case of glycated haemoglobin, the results of the first day after blood taking ( $+4^{\circ}\text{C}$ ) were significantly higher ( $p<0.001$ ) than the results of the fresh blood. The results of the examinations on  $-20^{\circ}\text{C}$  indicated even more significant differences ( $p<0.05$ ) compared to the test results of fresh blood.

Consequently in case of GHb the fresh blood analysis is advisable.

##### **Setting in a new measurement methodology of serum fructosamine**

SeFa concentrations were measured in human plasma samples by the standard LaRoche kit method and by our new microplate ELISA method in two parallels. The SeFa concentrations were in close, significant correlation ( $r=0.942$ ) ( $p<0.001$ ), and were fitting well to the regression line. In case of three parallels the correlation was even closer,  $r=0.972$ . The possibility of the implementation of the method was also tested on mammals. The close correlation of the first tested human samples was well in accord with the results of the two similar animal (cattle, dog, rabbit and horse) test methods ( $r=0.93-0.97$ ). On the basis of these data, the measurement of SeFa can be carried out with our simplified macro method at low cost in smaller, less equipped laboratories, too.

##### **Examination of the sympathoadrenal stress of rabbits**

It is very important to determine the glycated parameters because the blood glucose values can not be considered as standard values testing carbohydrate metabolism, since some changes, e.g. the increase of the activation of the sympathoadrenal system can alter its actual value. In the meantime the values of the glycated parameters are stable, reliable, without alterations. The examination of the effects of the repeated blood takings on the carbohydrate metabolism in

rabbits by determining the glycated protein and blood glucose levels also called the attention to this.

### **Age-dependent examination of the rabbit fed different nutrient-content diets, simultaneously determination of the correlation between serum fructosamine and blood glucose levels**

The most important tendencies of the three groups of rabbits examined in the kindling period, fed slightly different diets are as follows. The higher total protein values were measured mainly in group II. of the groups II and III fed higher protein-content diets. The measured total protein level is not due to the higher protein-content diet, since the albumin levels are almost identical in the three experimental groups. Thus the difference is owing to the increase of the globulin fraction, with a probable health problem in the background.

The decrease found in the parameters of the carbohydrate metabolism (blood glucose, SeFa) might be surprising in case of does fed higher energy-content diet, it indicates that not only the quantity of the carbohydrate component is reflected in the feed conversion efficiency, but also its ratio to the protein component. The control group (I.) was fed a commercial fattening diet, while the does of group III. were given not only higher energy-content feed, but more protein supply in comparison with the diet of group I. This means their ratio was different only in group II. in favour of the protein.

According to our results, the studied production factors (survival rate of the suckling animals, average kindle size) were the most favourable in does fed the higher carbohydrate- and protein-content diet. The daily weight gain of the does was the most balanced also in this group, so it can be ascertained that the experimental feed is slightly more expensive due to the higher nutrient-content of it, but the positive breeding parameters make this extra cost yet profitable.

It can be ascertained that the glycated proteins (mainly GHb) were the steadiest indicators of the protein- and carbohydrate contents of the diet fed. The measurement of the GHb is also important, because the traditionally determined total protein value is based not only on the nutrient content of the feed, but other factors (e.g.: inflammation, immunoglobulins) may alter its value. In addition, the actual changes of the sympathoadrenal activity alter the blood glucose levels.

In the course of evaluation of the effects of feeding on laboratory parameters, it was necessary to determine certain physiological values measurable in different periods (which can not be found in the literature), in order to compare them with the alterations.

The parameters of suckling rabbits of all the three groups clearly reflected the maternal values in the first period of life. Later, the studied age-dependent physiological parameters were taking shape continuously, as their own blood-forming functions were developing.

According to our study it can be proved that it is worth measuring the parameters of the carbohydrate metabolism (G, SeFa, GHb) not only by themselves, but collectively too. We found that in rabbits the glucose is in close correlation ( $r=0.91$ ) with the serum fructosamine of one week later.

### **Ratio of the fetal/adult haemoglobin and determination of the glycated parameters in newborn and young rabbits**

At birth the red blood cells contain exclusively HbF (100 %), at about the 2nd-7th days of life the 50% of HbF changes to HbA. The HbA level increases steadily in the red blood cells of the young rabbit, so at the 28th day of life its ratio is 95.60%, at the 31st day 98.04% and finally at the 70th day only HbA can be found in the red blood cells. After this period the HbF totally disappears from the blood circulatory system of the young rabbits.

The increase of the glycated haemoglobin levels from birth to the age of 10 weeks ( $p < 0.001$ ) in rabbits is caused by the different structure of the HgF and HbA. The fetal haemoglobin consists of two  $\gamma$  protein chains besides the two  $\alpha$  chains, which causes greater affinity for oxygen to the Hb molecule (ANDERSEN, 1977), but it shows no glycosylation ability (ABRAHAM et al., 1983).

### **Determination of the correlation between the glycated haemoglobin and blood glucose in rabbit**

In our study we established that the blood glucose is in close correlation ( $r=0.85$ ) with the glycated haemoglobin of two weeks later in rabbits. The two-week correlation calculated in rabbits can be well explained knowing the average lifetime of the red blood cells.

### **Examination of the effects of the diabetes mellitus in rabbits as model animals**

The experimental results of the effects of diabetes mellitus induced artificially by alloxan in rabbits can be used as additional information to the therapy of the naturally arising diabetes (e.g.: pets). In this experiment we could verify the antidiabetic effect of the barley, which question was already raised by other investigators before (MAHDI et al., 1994, WURSCH and PI-SUNYER, 1997). This conclusion is based on the fact that all parameters of the partly barley-fed experimental group were the most favourable, and in this group the symptoms of the diabetes developed more slowly. This means that the barley with its relatively low energy-content and high fibre-content is suitable for the dietary treatment of the diabetes.

In our experiment we managed to control the barley feeding precisely (by accurate measurement of the remaining feed at animals in kept in individual cages), but it would be worth granulating the experimental feed for the future studies. This would make the technical implementation easier.

It would be reasonable also to use higher number of individuals, since in spite of the careful insulin treatment; such grave changes cause inevitably the death of some experimental animals. According to the histopathological results we can say that the studied nine-weeks period is not enough for the development of the tardive complications of the diabetes, so in order to examine these changes, the period of the experiment should be increased.

The proof of the significance of the examination of glycated proteins also means the possibility to obtain information of the carbohydrate status of three different periods at the same time, from only one blood taking. It allows us to acquire more exacting results and this way the principles of animal protection of can be better asserted.