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Glucose markers in healthy and diabetic bitches in different stages of the oestral cycle

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Glukosmarkörer hos friska och diabetessjuka tikar i olika löpstadier

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ABSTRACT

The female dog has a unique reproductive endocrinology, with high levels of progesterone throughout the 70-day long luteal phase. In this project, markers of glucose metabolism were studied and compared from the aspects of breed group and time in oestral cycle. The parameters studied were: glucose, insulin, progesterone, fructosamine and the newly introduced analysis of HbA1c. HbA1c was also further evaluated and discussed as a diagnostic tool in canine medicine.

No differences in terms of glucose markers were found between a group of pure-breed dogs of various breeds compared to a group of dogs with high risk of dioestrus diabetes, namely the Norwegian and Swedish elkhounds.

No differences were found between the two sample occasions, anoestrus and dioestrus, concluding that there is no need for relating test results to the time of the oestrous cycle in middle-aged intact female dogs.

There were statistically significant higher values of all glucose markers in diabetic dogs than in non-diabetic dogs. When it came to HbA1c, however, there was a larger overlap between the two groups, compared to fructosamine. The HbA1c analysis was easy to perform, but was found to have a low precision. It was not possible to compare the results of HbA1c with other studies, since there is no agreement of a standardized method. The author therefore concludes that if HbA1c is to be used in veterinary medicine, reference values for the specific laboratory and analytic method needs to be set up.

SAMMANFATTNING

Tikens reproduktionsendokrinologi är unik i sitt slag i och med de höga progesteronvärden som kvarstår under hela lutealfasen. I denna studie jämfördes glukosmarkörer mellan två olika rasgrupper samt utifrån olika stadier av östralcykeln. De markörer som inkluderades i studien var glukos, insulin, progesteron, fruktosamin samt HbA1c. HbA1c utvärderades också vidare som ett diagnostiskt hjälpmedel inom veterinärmedicinen.

När en grupp hundar med förhöjd risk att utveckla diabetes (grå- och jämthundar) jämfördes med en grupp av hundar av blandade raser hittades inga skillnader i nivån på någon av glukosmarkörerna.

Inga skillnader hittades heller då prover från anöstrus jämfördes med prover från diöstrus, vilket leder till slutsatsen att fasen i östralcykeln ej behöver beaktas vid tolkning av provresultat.

Signifikanta skillnader återfanns hos samtliga glukosmarkörer då diabetessjuka hundar jämfördes med friska hundar. Vad gäller HbA1c var det dock många diabetessjuka som befann sig inom samma intervall som de friska hundarna. Dessutom gick det inte att jämföra dessa resultat med de från tidigare studier, då HbA1c-diagnostiken ej ännu är standardiserad inom veterinärmedicinen. Författaren anser därför att om HbA1c ska användas som ett diagnostiskt hjälpmedel för diabetessjuka hundar, måste metoden standardiseras för att meningsfulla resultat ska kunna erhållas.

INTRODUCTION

The syndrome diabetes mellitus, hereafter referred to as diabetes, has for a long time been known as a frequently occurring disease in humans as well as dogs and other companion animals. In Sweden, 1,2 % of all dogs are estimated to develop diabetes mellitus before the age of twelve years. The Swedish and Norwegian elkhounds are breeds with elevated risk of developing diabetes, and in which almost only females are affected by the disease (Fall, 2009).

Concerning the techniques for diagnosing diabetes and monitoring the treatment, there has been a huge development since the time when diabetes was diagnosed through a sweet taste of the urine. One very important instrument for diagnosing and monitoring treatment of diabetes in humans has for a long time been the measurement of glycosylated hemoglobin molecules, HbA1c. These are formed by an irreversible non-enzymatic binding of glucose to haemoglobin, and the percentage of glycosylated molecules increases if the patient has hyperglycemia. HbA1c reflects the glycemia, during the life of hemoglobin, which is approximately 120 days in humans and dogs.

For mainly economical and practical reasons, HbA1c has not been used in dogs until recently. However, this has now become possible through the development of less expensive instruments. Earlier tests has shown that these analytical instruments may be possible to use in veterinary diagnostics, but that still more needs to learn about normal values of HbA1c in dogs.

One of the aspects of canine diabetes not extensively tested is whether the reproductive hormonal cycle of bitches affects HbA1c and other glucose markers, something that this study aims to investigate. We hypothesize that dioestral bitches have higher values of glucose, fructosamine and HbA1c than anoestral bitches, due to the antagonistic insulin effect of progesterone and possibly of mammary-derived growth hormone. The purpose of this project is to:

- a) Test the hypothesis that glucose markers are elevated in dioestrus compared to other phases of the oestral cycle
- b) Investigate if there are differences to be found in glucose markers levels between a high-risk diabetes breed for dioestrus diabetes (elkhounds) compared to a mixed dog population
- c) Further evaluate the use of HbA1c in dogs

BACKGROUND INFORMATION

Blood glucose

Glucose is a monosaccharide, which is used by the body as an energy source and as a precursor in the production of lipids and proteins. Glucose is used as the prime energy source in most cells (Johnson, 2008). Glucose is supplied to the body by food intake, and is also synthesized in organs such as the liver.

Normal blood glucose value is essential to the well-being of an animal. There are mainly two hormones, insulin and glucagon, produced by alpha and beta cells in the endocrine pancreas, which acts together to maintain normal values of blood glucose. The release of these hormones is controlled by the glucose sensing system in the alpha and beta cells.

Hormones increasing blood glucose

Glucagon is a catabolic hormone released from alpha cells when blood glucose is low. The effect of glucagon is to increase blood glucose by glycogenolysis and gluconeogenesis. Other hormones also act to increase blood sugar, namely cortisol, growth hormone, epinephrine and norepinephrine (Johnson, 2008). These hormones both act to increase the secretion of glucose from the liver, but also diminish the effect of insulin in target cells.

Insulin

Insulin is secreted by beta cells in response to increasing blood glucose levels. This hormone has the opposite effect in the body compared to glucagon, as it enables the intake of glucose into cells and stimulates the storage of glucose in forms of glycogen and fat (Johnson, 2008).

The oestral cycle and progesterone

The oestral cycle of a bitch consists of 4 different phases: prooestrus, oestrus, dioestrus and anoestrus. Progesterone is a hormone which is important to maintain a pregnancy. During the oestral cycle, progesterone values change dramatically. The concentration of this hormone is rising during prooestrus and oestrus to reach its maximal level approximately three weeks after the onset of dioestrus. During anoestrus the bitch is hormonally inactive considering reproductive hormones and the values of progesterone are low (Concannon et al, 1975).

The unique thing in bitches compared to females of other species is the maintenance of high progesterone values during dioestrus, independent of whether the bitch is pregnant or not (Eigenmann et al, 1983). Concannon et al (1973) found no differences in maximal progesterone values in dioestrus between pregnant and non-pregnant bitches. In most other species, the progesterone values decrease as soon as the body has recognized that there is no pregnancy to maintain.

Diabetes Mellitus in Dogs

Pathophysiology

Canine diabetes mellitus is an endocrine disorder, in which the dog has defects in insulin secretion, insulin action, or both. A normal insulin production and response enables glucose molecules in the blood to enter into the body tissue cells. A lack of insulin or insulin response results in high concentrations of blood glucose, but low levels in intracellular glucose. As a result of the hyperglycemia, glucose is excreted in the urine causing polydipsia and polyuria. The lack of glucose in tissues causes increased gluconeogenesis and glycogenolysis, which gives further rise to the blood glucose, as well as causing weight loss and

polyphagia. When the insulin/glucagon ratio is low, the liver will start to form ketones as an alternative source of energy, eventually leading to ketoacidosis.

Classification of canine diabetes mellitus

Canine diabetes can result from a wide range of causes resulting in either insulin deficiency or insulin resistance. There are several different ways used to classify diabetes into different groups, for example based on whether they require insulin treatment or not, or whether the disease results from insulin deficiency or insulin resistance (Catchpole et al, 2005). However, according to the latest research, these classification methods are not concerned to be satisfactory for dogs as both will leave a large proportion of individuals unclassified due to the fact that the different groups overlap. Almost all dogs with diabetes eventually will need insulin treatment, making it hard to distinguish a non-insulin dependent form from an insulin dependent one and insulin resistance diabetes often will turn into insulin deficiency diabetes, resulting in the same problem. In her doctoral thesis, Tove Fall therefore proposed a new classification scheme for diabetes based upon the cause of disease (Fall, 2009). The main groups of classification in this new scheme can be reviewed in table 1.

| Table 1. Classification of Diabetes Mellitus (Fall, 2009) |
|--|
| Juvenile diabetes mellitus |
| Progesterone-related |
| Secondary to pancreatitis |
| Endocrine tumours |
| Iatrogenic |
| Immune-mediated diabetes mellitus |
| Idiopathic diabetes mellitus |

Progesterone-related diabetes

The main concern of this project is progesterone-related diabetes which may be divided into gestational and dioestrus diabetes. This type of diabetes arises when a patient has enough insulin-secreting beta cells to cover their day to day life, but who has no residual capacity and hence could not handle if the tissue gets a bit more resistant to insulin, e.g. by raised levels of progesterone due to the iatrogenic supply or during the dioestrus phase in bitches.

In intact bitches, diabetes often is diagnosed during dioestrus (Catchpole et al, 2005). Progesterone can give rise to an insulin resistance, through a post binding defect (Ryan, 1988). The insulin resistance leads to chronic hyperglycemia which damages the insulin-producing cells, resulting in insulin deficiency (Fall, 2009). According to Rijnberk, high endogenous progesterone levels may also result in high levels of mammary gland-derived growth hormone which may enhance the insulin resistance (2003).

Eigenmann et al showed that ovariohysterectomy improved glucose tolerance by lowering levels of GH (1983). In a study from 2005, it was stated that the response to insulin decreased with approximately 1/3 (35%) by iatrogenic supply of progesterone (Batista, 1978). It would therefore be expected that dogs in the

end of dioestrus have higher values of fructosamine and HbA1c levels than anoestrous dogs.

In Sweden, dioestrus diabetes accounts for the majority (60%) of all diabetes cases diagnosed in female dogs.

Age and breed predisposition

Diabetes may occur in any dog, the disease is, however, shown in several studies to be more frequently occurring in females than in males, in older dogs than in younger, and in some breeds more than others. According to a Swedish study of 180,000 dogs, the Australian terrier has an incidence rate of 183 cases per 10,000 dog years at risk, while the incidence for Golden Retrievers turned out to be 0 cases per 10 000 years at risk (Fall, 2008).

In the study by Fall in 2008, Swedish elkhounds were among the four breeds that were most commonly affected by diabetes and Norwegian elkhounds were in the middle risk range. What was most notable with these two breeds, however, was that almost only intact female elkhounds were diagnosed with diabetes. The proportion of intact females of the total amount of diabetes cases in these breeds were 96% in Swedish and 100% in Norwegian elkhounds. Looking at the proportion of females in diabetes cases of all breeds, this was calculated to 72%. In 2009, Wallberg found that 96% of all diabetic elkhounds debuted during dioestrus. However, this was not related to a higher progesterone level in elkhounds than in normal dogs.

The mean age of onset of diabetes in the total dog population is 8.6 years, and somewhat lower in Swedish elkhounds; 7.8 years, (Fall, 2007).

Diagnostic methods

The suspicion of diabetes in a dog should arise if all or some of the classic signs (polyuria, polydipsia, polyphagia, weight loss) are shown. Different diagnostic tests are discussed below.

Blood glucose

A high blood glucose level could indicate diabetes mellitus, but the level is constantly altering and a high level may indicate either short-term physical stress, recent intake of food or exaltation as well as disease. Davison et al (2003) has concluded that to diagnose diabetes by glucose measurements, there should be a minimum of 16 tests taken to establish the true mean blood glucose value. A device that is inserted subcutaneously which measures blood glucose continuously has been evaluated and found useful in a hospital setting. Continuous blood glucose measuring is, however, laborious and expensive. This is why the need for an instrument that will measure glucose levels over longer periods arises. For this purpose, fructosamine is almost exclusively used in dogs in Sweden today, while HbA1c is extensively used in human diabetic patients.

A glucose tolerance test is done to evaluate a patient that is only mildly hyperglycemic with varying state of glucosuria. The test is done by infusing the dog with glucose intravenously and then measuring the levels of glucose

continuously during the following hour. In a non-diabetic patient, the glucose values returns to normal within this hour, but in a diabetic patient the high values persists after this first hour.

Fructosamine

Fructosamine is formed when serum proteins are glycated, which they do continuously even in healthy dogs, but in a larger proportion when values of glucose and/or proteins in the blood is elevated. The reaction is irreversible and high values indicate high levels of blood glucose during the latest 1-3 weeks before sampling, based on the lifespan of albumin (Jensen, 1992).

Values of fructosamine are not fluctuating like blood glucose in response to stress or intake of food, and therefore serve as a good compliment to measuring of single blood glucose values. However, high values of fructosamine can arise during other circumstances than diabetes, e.g. if not blood glucose but blood proteins are above a normal level, for example in a dehydrated animal (Reusch et al, 2001). Fructosamines can also be falsely high in dogs with a slow metabolism of proteins, e.g. hypothyroid patients, which was proved in several studies, among others one by Reusch et al in 2002. Reusch explained the elevation in fructosamine with the longer time that proteins are in the blood and may react with amino acids. Fructosamine value can also be falsely low due to hypoproteinemia, hyperlipidemia and azotemia (Reusch, 2001).

Insulin/C-peptide

According to Nelson et al (2004), measurement of insulin is only recommended in a dog with suspicion of secondary diabetes, to detect high insulin levels in combination with hyperglycemia. The disadvantage of measuring insulin as a diabetes marker is that it can vary due to for example amount of time from latest intake of food. It can also be affected by certain types of drugs. This problem can be bypassed by measuring insulin in fasted animals or after a controlled stimulation with glucagon or arginine (Fall, 2008). Higher insulin values than normal indicates secondary diabetes mellitus while lower values indicate a primary insulin deficiency or secondary diabetes turned into an insulin deficiency. Low insulin values can occur due to glucotoxicity in a patient with secondary diabetes.

If glucagon is injected intravenously into a normal dog, this will give a short rise in insulin blood concentrations. However, if injected into a diabetic dog, there will be no rise in insulin concentration, since there is a deficiency in the production of insulin. (Montgomery et al, 1996). The test is often used in humans. According to several authors, instead of measuring insulin after glucagon injection, one of its precursor units called C-peptide is preferred (Montgomery et al 1996, Fall 2000), since insulin treatment will not affect the amount of this peptide. The test is carried out by injecting 1 mg glucagon intravenously and then measure either insulin or c-peptide before and 10 minutes after glucagon administration. Since basic levels of insulin and c-peptide is low in patients that lack insulin production and high in patients who have sufficient insulin production, but a deficiency in insulin response, this test can help to separate different kinds of diabetes from one another (Fall, 2007).

Progesterone

If an intact bitch is diagnosed with diabetes mellitus, progesterone can be measured to see if the dog is in luteal phase or not.

HbA1c

Formation of HbA1c

Hemoglobin is an important part of the blood, as it serves as the oxygen carrier in the red blood cells. A proportion of the total amount of hemoglobin molecules in the blood is always bound to glucose, as a result of the chemical properties of these two blood constituents. Glucose can bind to hemoglobin in the red blood cell in any time of its lifespan by a non-enzymatic reaction (Bunn et al, 1978), and once formed the only way for the HbA1c to dissolve is when the red blood cell is taken out of circulation (Peacock, 1984). The binding is irreversible and the proportion of hemoglobin molecules bound to glucose will rise in relation to increasing amounts of blood glucose. There are many different types of glycosylated hemoglobin molecules, based on where in the hemoglobin molecule the glucose is situated. HbA1c is a measurable fraction of glycosylated hemoglobin, in which the N-terminal of the B-Chain of the hemoglobin molecule is glycosylated. Such a glycosylation alters the properties of the hemoglobin molecule more than glycosylation at other sites, why these molecules are the best for analyzing. (Peacock, 1984). The lifespan of a red blood cell is up to 120 days, why HbA1c levels reflects the medium blood glucose values for up to 3 months back in time. Marca et al studied the effect of acute hyperglycemia on HbA1c and found that such a thing will not affect the levels of neither HbA1c nor fructosamine values (2000c).

HbA1c in human medicine

HbA1c has been used in human diagnostics since 1977 (John et al, 2007). In a review article from 1984, a human medicine expert writes that glycosylated hemoglobin “offer the best available means of assessing diabetic control” (Peacock, 1984). When first introduced, the analysis was based on differences in charge between glycated and non-glycated hemoglobin molecules, but since 1981 there also has been test based on structural differences between the two (John et al, 2007). When first analyzing HbA1c in humans, the results differed and were not comparable between different laboratories, why standardization methods have been taken into operation. The normal value of HbA1c with these standardized methods in humans is 4-6%. In an article in Clinical Endocrinology and metabolism, Saudek et al found that HbA1c was preferred among doctors as a diabetic instrument for several reasons; the results are now standardized and reliable and it does not alter after intake of food, it reflects glucose values over a long time and it is uncommon that factors other than glucose will interfere with the HbA1c measurements (2008).

Factor interfering with HbA1c results in humans

In humans, it is found that HbA1c values could differ between groups of people based on origination. In one study, it was found that Americans from Africa had higher HbA1c values than rest of the American population. This is an interesting

finding, as this study is going to explore if there is differences between different groups of dog breeds.

HbA1c can be falsely low in patient with erythrocytes of a relatively shorter lifespan; as the younger the blood cells, the less they are exposed to a persistent hyperglycemia (Peacock, 1984). Examples of such conditions are hemolytic anemia or bleeding. Saudek et al (2008) therefore suggests that HbA1c should not be used in patients with anemia. Oppositely, if erythrocyte lifespan is prolonged, HbA1c will become falsely elevated due to a longer exposure of red blood cells to hyperglycemia. An example of this condition is in patients which have undergone splenectomy.

HbA1c analysis may also be disturbed by certain vitamins, iron deficiency and hemoglobinopathies (Saudek et al, 2008). It also depends on methods of determination as well as calibration of the assay (Hetil, 2009). High levels of HbA1c leading to a diabetes mellitus diagnosis, should therefore be confirmed with other types of diagnostic testing (Saudek et al, 2008).

Previous HbA1c measurements in dogs

The use of HbA1c in canine samples has not been investigated thoroughly, but there are a number of studies reported. A study of HbA1c was carried out by Catchpole et al in 2008 in which the results were that there was a good correlation between high fructosamine values and high HbA1c values, that diabetic dogs had statistically significant higher values of HbA1c than non-diabetics, but that there were no difference in HbA1c values according to if the dog had a untreated diabetes or was under treatment with insulin. The normal value of HbA1c was established at 3,7 to 5,6 per cent. Diabetic dogs had HbA1c values between 4,9 and 13 per cent. However, in an earlier study by Davison, Catchpole et al, it was found that the correlation between HbA1c and fructosamine was not so good (2002). In this study the normal values somewhat different from the 2008 study, with a median normal value of 2,1-3,7 %, while diabetics had values ranging from 2,5-7 %. The same method (boronate affinity chromatography) for measuring HbA1c as in this study was used in the study from 2002, while another method was used by Catchpole in 2008.

In 2000, a test measuring HbA1c in 86 dogs was carried out in and there was no difference in HbA1c level found between different age groups of dogs or between males and females. In the study differences were found between the different phases in the oestrous cycle, but the values were still in the reference interval given in the study why authors of the article concluded that there would be no need for taking the test at a certain time of the oestral cycle. (Marca & Loste, 2000)

MATERIALS AND METHODS

Dogs

Three different groups of dogs were included in the study. One group of clinically healthy elkhounds, one group of clinically healthy pure-bred dogs of various

breeds and one group of dioestrous diabetes pure-breed dogs of various breeds. The dogs in group 1 and 2 were sampled by the same veterinarian and frozen in -20°C for 0-6 months, before sent on ice to SLU where it was stored in at -70°C. The inclusion criteria for the different groups were as follows:

Group 1

Included in this group are clinically healthy female intact dog, minimum 4 years old, from any part of Sweden. Breeds included were Swedish or Norwegian elkhounds. One of the dogs in this group turned diabetic during the study and was consequently excluded from further analysis.

Group 2

Included in this group are clinically healthy female intact dogs, minimum 4 years old, from any part of Sweden. The dog could belong to any pure-bred dog except elkhounds (Swedish or Norwegian), Beagles or Border Collies.

Blood samples from Group 1 and 2 were taken at two sample occasions, once during anoestrus and once during dioestrus. The dioestrus test was taken 6-8 weeks after the end of oestrus, and the anoestral test was taken at least 3 months after heat. All groups in group 1 and 2 were fasting at the times of sampling.

Group 3

In this group there are dogs of any breed diagnosed with diabetes in the progesterone phase (1-3 months after end of heat). Diagnosis was based on clinical signs, glucosuria and high blood glucose and fructosamine levels. No treatment with insulin was started at the date of sampling. Blood samples from this group of dogs were taken at the day of diagnosis, or in some cases a couple of days later, but still before onset of treatment. 12 dogs were found to match the criteria. The samples were taken between the years of 2005 and 2008 and stored at -70°C. Fasting status was unknown.

The breeds and ages of the dogs are shown in Table 2.

The samples

From all dogs blood samples in both EDTA and serum test tubes were taken. Serum was separated from the blood clot within one hour. Sera and whole blood was stored in freezer until analysis.

| | N | breeds | age (median, range) | Weeks after estrus for sample 1 (dioestrus) |
|----------------|----|--|---------------------|---|
| Group 1 | 24 | 8 Norwegian elkhounds 16 Swedish elkhounds | 6.0, 4.1-10.1 | 4.4, 2.1-7.6 |
| Group 2 | 18 | 2 Rough-haired collie 1 Dalmatian 1 Finnish lapphund 1 Finnish Spitz 1 Finnish hound 2 German Shepherd 5 Golden Retriever 1 Hamilton hound 1 Hovawart 1 Labrador retriever 1 Papillon 1 Tervueren | 6.1, 3.8-12.2 | 4.0, 2.0-5.4 |
| Group 3 | 12 | 1 Alaskan husky 2 Australian terrier 1 Bichon frisée 2 Mixed breed 3 Border collie 1 Borzoi 1 Hälleforslund 1 Leonberger | 10.0, 7.0-11.9 | 4.7, 1.3-10.0 |

Table 2. Groups of dogs included in the study

The measurements

Glucose, fructosamine, insulin and progesterone were analysed at the University Animal Hospital Laboratory at the Swedish University of Agricultural Sciences (SLU). The methods used and the reference values can be seen in table 3. HbA1c was measured on by the author by the method described beneath.

| Value | Method | Name of assay | Reference value |
|---------------------|---------------------------|-----------------------------|---|
| Fructosamine | Colometric assay | ABX Pentra Fructosamine | 200-400 µmol/l |
| Insulin | Sandwich ELISA | Mercodia Insulin ELISA | 5-25 mU/L |
| Glucose | glucose hexokinase method | Thermo Clinical Labsystems | 3,5-6,0 mmol/l |
| Progesterone | enzyme immunoassay | Immunlite 2000 Progesterone | Anoestrus: <1 nmol/L Dioestrus: High |

Table 3: Reference values of glucose markers

Analysing the results

All results were collected into a Microsoft Excel Spreadsheet and analyzed in JMP and Graphpad Prism. Wilcoxon rank sum test was used to assess differences among groups. Box plots were drawn to illustrate the results.

Measuring HbA1c with Cholestech GDX Analysis

For the measurement of HbA1c in this study, a device called Cholestech GDX Analyze was used. The Cholestech GDX analyzer uses EDTA blood and analyzes it through a method called boronate affinity chromatography. The measurement is carried out in three steps, in which the first step lyses the blood which makes the glycosylated hemoglobin can attach so called boronate affinity resin. After 40 seconds the sample is poured into the cartridge test tunnel where the unglycosylated hemoglobin molecules will be trapped into a special chamber, while the glycosylated hemoglobin molecules will sink to the bottom of the tunnel as they are bound to the boronate affinity resin. In step 2, a washing solution is poured onto this bottom fragment and in the third step a solution that finishes the binding between the resin and the glycosylated molecules is added, so that the glycosylated hemoglobin molecules can be measured. The machine will then divide the result of the glycosylated molecules with the total (glycosylated and nonglycosylated) hemoglobin amount. The result is then mathematically corrected and finally the machine shows the percentage of hemoglobin molecules that are glycosylated. Before and after the measurements were done, the machine was evaluated using human blood with a pre-known glycosylated hemoglobin fraction. In this study, the machine showed good correspondence.

All blood samples were measured twice with the same procedure, and a mean value was calculated from the two received values. In cases where the value from measurement number one and two differed more than 0.3%, HbA1c was calculated a third time. The mean value was then calculated from the two values closest to one another, out of these three times. If all three measured values differed more than 0.3 % from one another, the tested sample was excluded from the calculations.

RESULTS

The results are shown in Figure 1.

Glucose markers by breed

There were no statistically significant differences in levels of glucose, insulin or fructosamine, HbA1c or progesterone between healthy elkhounds (group 2) and dogs of other breeds (group 1). The lack of differences was consistent during both oestrus and dioestrus.

Comparison by phase in oestrous cycle

Progesterone was statistically higher in dioestrus than in anoestrus. This was an expected but still an important finding since it gives evidence of that the dogs really were in dioestrus at the time of one sample and in anoestrus at the time of

the other sample. There were no statistically significant differences to be found concerning insulin, glucose, fructosamine and HbA1c in any of the breed groups, when comparing anoestrus with dioestrus.

Comparison healthy dogs to diabetic dogs

There was a statistically significant difference in HbA1c ($p < 0.001$), glucose ($p < 0.001$) and fructosamine ($p < 0.001$) values in dioestrous diabetics compared to clinically healthy dogs in dioestrus. There was no difference in insulin or progesterone levels between the two groups.

DISCUSSION

Are glucose markers values higher for dogs in dioestrous?

The hypothesis of this work was that the insulin resistance caused by progesterone and possibly growth hormone would yield higher values of HbA1c and fructosamine during dioestrus than during anoestrus. There were no such differences to support our hypothesis seen in this study. The result may have been different if all dogs were sampled later in dioestrus, since HbA1c and fructosamine are formed continuously during high levels of blood glucose. The lack of difference, however, makes diagnosing diabetes by HbA1c and fructosamine measurement less complicated, since it doesn't form a need for different reference intervals for dioestrus dogs.

Is HbA1c reliable to use for canine medicine?

Comparison with earlier results

In this comparison, elkhounds and dogs of other breeds, as well as anoestrus and dioestrus HbA1c values are calculated together, since no differences were seen between the groups. The mean value of all HbA1c measurements for healthy dogs in this study was 4,8 (range from 3,6-9,4), which corresponds well to the result of Catchpole (2008) (median value of reference in his study interval 4,7). However 8 out of 80 HbA1c measurements of healthy dogs would be higher than the set reference interval of Catchpole without showing any signs of diabetes and 1 would be lower than the reference interval. Compared to Davison's study in 2002, the values measured in this study was much higher and only 1 out of 80 measurements on healthy dogs was inside the Davison's interval, pointing out the need for different reference interval with different devices.

When it comes to the diabetic patients, the correspondence to Catchpoles studies is not as good, as in his study diabetic patients had Hba1c values with a median value of 9,3 and in this study no diabetic patient had such a high value. HbA1c values in diabetics in this study ranging from 4,8 % to 6,7 %, which more resembles the results of Davison, where diabetic patients ranged from 2,5-7 %.

One reason for the lack of comparable result may be that most studies are done in hospitalized animals, while this study was done in outward animals. Catchpole et al concluded that diseased animals have a higher glucocorticoid level, higher blood glucose and supposedly higher HbA1c values than other dogs, but that there are also other studies with opposite results (2008).

The dogs in this study were all newly diagnosed, which may have caused lower HbA1c than in dogs that had had the disease for longer time.

Standardization

It seems to be inappropriate to compare HbA1c measurements between different laboratories and measurement methods, since large variations can be seen. This fact was supported by Marca & Loste, when they pointed out the lack of extensive use of HbA1c measurements in dogs as a reason for this (2000). When HbA1c first started to be used in human medicine, there were similar problems with lack of meaningful implementation of HbA1c measurements (Little et al, 2001). Conclusions were drawn that the materials used in the assays were prepared in different ways, i.e. for calibration and hence gave different results (Little et al, 2001), and the American Diabetes Society was negative to using HbA1c for diagnostics (Saudek et al, 2003). In America, a standardization program was begun, which would make results comparable between different laboratories. Referring to the background information of this assay, HbA1c measurements are now preferred among many medical doctors. It is in the authors' strong belief that if HbA1c measurements are to be used in veterinary medicine, the assay would benefit from a standardization program for canine samples.

Between run variation

In this study, the HbA1c value was measured twice, and if the difference between these two times were more than 0,3%, the same test was run a third time. This happened 30 out of 92 times (33%), according to the authors' belief showing a low precision for the use of the machine for canine blood samples.

It can be declared that the poor between run precision is a disadvantage of the method, findings supported by Marca et Loste in 2000, although they used another method. Marca et Loste proposed better calibration of the assay as a solution to the problem.

Overlap

HbA1c values of diabetics had a median value of 5.9 while non-diabetics in mixed breeds had a median value of 4.7, leading to a significant difference between the two groups. However, 5 out of the 12 dogs (42%) diagnosed with diabetes had values of HbA1c that would also fit into the interval of the non-diabetic dogs.

Many authors who investigated HbA1c in dogs found that HbA1c is significantly elevated in diabetics compared to non-diabetics (Marca et al, 2000; Catchpole et al, 2008; Davison et al, 2002)

However, several authors have also found a degree of overlap between glycosylated hemoglobin values of healthy and diabetic dogs (Davison et al 2002; Delack & Stogdale, 1983). This was also found in this study. In the study of Delack & Stogdale, all diabetic animals were in the reference range. That study was measuring total HbA1 and not only HbA1c. However, HbA1c constitutes approximately 80 % of the total HbA1, why these results are considered interesting.

Human methods for dog use

According to the manufacturer of the Cholestech GDX analyzer, values of HbA1c would be reliable between 4 and 15 %. 8 out of 92 (9%) of values are below this range, none above.

As far as the author knows, there is no HbA1c measuring method especially made for dogs. The similarities between human HbA1c and canine HbA1c has been clarified by several authors. However, Davison et al suggest that there might be differences in the physiochemical properties between these two molecules (2002). The author believes that this may interfere with the accuracy of all methods used for HbA1c measurements in dogs. Davison is also pointing out the need for an algorithm in HbA1c measurements evaluated especially for dogs (2002). Marca et Loste found that when using canine blood in HbA1c measurements, the assay had to be calibrated far more often than human blood using the same method (2000), which supports the idea of important differences between human and canine HbA1c.

The effect of storage on HbA1c

One theory that the author has for explaining the somewhat different HbA1c values achieved in this study is that the storage of the blood resulted in changes that resulted in faulty measurements. The blood in the study was first stored in -20 degrees for a couple of months, followed by storage in -70 degrees, It has been shown that levels of fructosamine can change statistically during storage of canine blood and that these changes may be of clinical importance (Thoresen et al, 1995). There is one notice of that HbA1c is stable in -20 degrees for 77 days (Marca et al, 2000) and another study where no changes were seen after storage in 4 degrees for 7 days (Easley, 1986). However, no further information about this has been found, for which reason the storage factor cannot be completely disregarded.

Lack of hematocrite values

According to the Cholestech GDX manufacturer, the hematocrite concentrations that would be appropriate for the analysis should range from 30 to 60 % in human blood. There was no measurements of hematocrite done in this study, which could be a source of errors, since it has been declared that dogs with anemia should be excluded from measurements of HbA1c (Haberer 1998). However, in 2000, Marca et al found that the differences in HbA1c between anemic and non-anemic dogs were not significant. (Marca et al, 2000, CJVR). Furthermore, the dogs in group 1 and 2 of this study were all healthy dogs without clinical symptoms, and the influence of hemtocrine could probably therefore be minimized.

Fructosamine or HbA1c?

Whether to diagnose and monitor diabetes best with fructosamine or HbA1c remains to be further elevated. Loste & Marca concluded that the main difference is the time aspect, since HbA1c reflects glucose levels during a longer time than does fructosamine (2000). In this and other studies there is an extensive degree of overlap in HbA1c values of healthy and dioestrous dogs, but there has also been reports on the same problem with fructosamine. Reusch et al (2001) found that some diabetic cats and dogs have fructosamine values in the reference interval,

mainly because of lack of elevated blood glucose during a longer time, or due to the earlier explained changes associated with other concurrent diseases. Patients with such hypoproteinemia, hyperproteinemia and azotemia should not be sample for fructosamine (Reusch, 2001). In the same way, it can be argued that patients with for example anemia or hemoglobinopathy should not be sampled for HbA1c.

CONCLUSIONS

Concerning the main question in study, whether dioestral bitches have higher markers of glucose than anoestral bitches, no significant difference between the two groups were found.

This is an important finding, since if HbA1c is to be used in diabetic dogs, it states that there will be no need for a different reference interval for cycling bitches than for other dogs. The result is consistent with the one for fructosamine, glucose and insulin, which also lacks significant differences between the two groups.

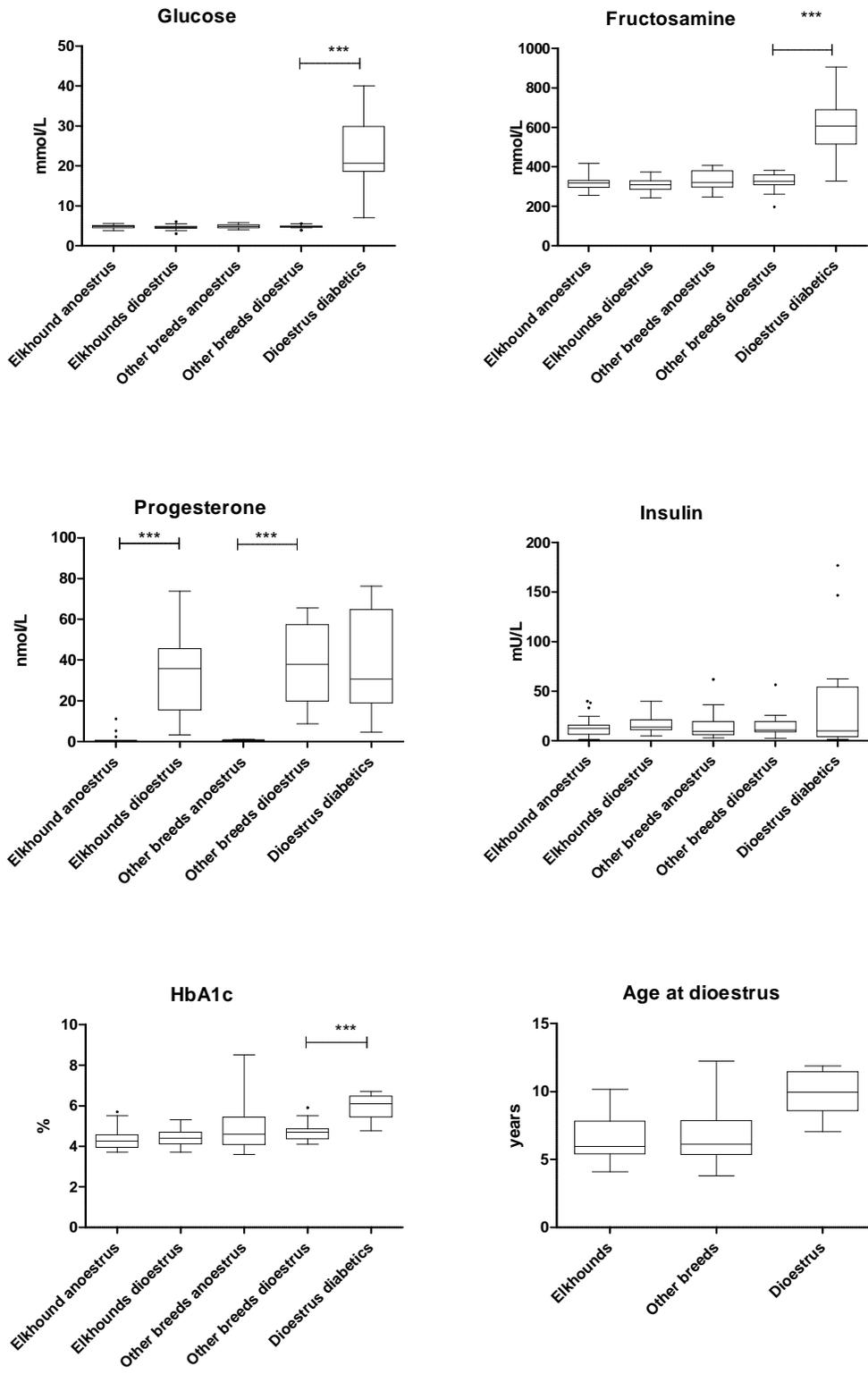
Throughout the study, no difference in either glucose, HbA1c, fructosamin, insulin or progesterone was found between elkhounds and other dogs, leading to no further answer why these dogs are more commonly affected by diabetes.

The evaluation of HbA1c measurements for canine samples in this study came to the conclusion that if to be used further in veterinary medicine, HbA1c measurement must be improved and standardized, since today, there are too many possible sources of errors which will prevent the method from yielding meaningful results. The problems found in this study is the lack of agreement between different studies, the high degree of overlap between healthy and diabetic dogs, the possibility that devices developed for human use may not be direct applicable to other species and the poor within-run precision. Although fructosamine measurements also suffer from errors, these are according to the authors' conclusions fewer and the degree of overlap between healthy and diabetic animals is less extensive, why fructosamine are preferred until solutions to these problems are attained. When so, fructosamine and HbA1c may both be used, and choice between them could be done according to the existence concurrent diseases and the duration of glucose metabolism most wanted to be evaluated.

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FIGURE 1. STUDY RESULTS



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