Metformin reduces insulin resistance and the tendency toward hyperglycaemia and dyslipidaemia in dogs with hyperadrenocorticism

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Abstract

Hypercortisolism induces a state of insulin resistance that can occur concurrently with fasting hyperglycaemia, dyslipidaemia and diabetes mellitus. Metformin reduces hepatic glucose production and insulin resistance of the skeletal muscle and adipose tissue. The aim of this study was to evaluate the effects of metformin on the control of metabolic disorders of dogs with hyperadrenocorticism (HAC). Twenty-three dogs with HAC were randomly divided into two groups, consisting of a control group and a metformin group (10 mg metformin/kg/12 h). Both groups received the same treatment for HAC. At baseline and 3 months, blood glucose, total cholesterol, triglycerides and insulin concentrations, in addition to urinary cortisol:creatinine ratio, Homeostatic Model Assessment (HOMA) for insulin sensitivity and β-cell function were measured. Dogs treated with metformin showed significantly reduced glycaemia, cholesterolaemia and triglyceridaemia. They also presented reduced hyperinsulinism and insulin resistance, as well as improved pancreatic β-cell function. The implementation of metformin as an adjuvant therapy is effective for the normalisation of metabolic disorders of dogs with HAC.

Keywords: Dyslipidaemia, Hyperadrenocorticism, Insulin resistance, Metformin.

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Introduction

Cortisol antagonises various functions of insulin, and high levels may predispose an individual to insulin resistance, a condition in which the peripheral tissues have a reduced response to insulin (Andrews and Walker, 1999; Miceli *et al*., 2014; Mazziotti *et al*., 2017). Indeed, the antagonism of insulin by cortisol is so potent that the glucocorticoid effects are referred to as "diabetogenic" (Van Raalte *et al*., 2009; Van Raalte and Diamant, 2014). Chronic hypercortisolism stimulates hepatic gluconeogenesis, interfering with the insulin signalling cascade and glucose uptake in the skeletal muscle and adipose tissue, in addition to affecting the insulin synthesis and secretion of pancreatic β-cells, as well as inducing their apoptosis (Schäcke *et al*., 2002; Vegiopoulos and Herzig, 2007). These metabolic disorders are clinically characterised by fasting hyperglycaemia, hypercholesterolaemia, hypertriglyceridaemia, abdominal obesity, hepatic steatosis and diabetes mellitus (Peterson *et al*., 1984; Pivonello *et al*., 2010; Di Dalmazi *et al*., 2012). Between 5% and 22% of dogs with HAC develop diabetes mellitus (Peterson *et al*., 1981; Nichols, 1994; Ishino *et al*., 2010; Gomes Pöppl *et al*., 2016; Miceli *et al*., 2017).

Homeostatic Model Assessment indices (HOMA) are used to evaluate both peripheral insulin sensitivity (HOMA_{insulin sensitivity}) and the function of pancreatic β-cells to produce insulin (HOMA_{β-cell function}) (Matthews *et al*., 1985; Verkest *et al*., 2010; Miceli *et al*., 2014).

Metformin is a biguanide that is commonly used as an oral antidiabetic medication for the treatment of type 2 diabetes mellitus in humans (Herman *et al*., 2005; Aroda *et al*., 2015; American Diabetes Association, 2016). Metformin reduces hepatic glucose production and insulin resistance in peripheral tissues (especially the skeletal muscle and adipose tissue), therefore, metformin is considered an "insulin-sensitising" drug (Natali and Ferrannini, 2006).

The effects of metformin are not immediate as it acts by modifying the gene expression of key enzymes involved in metabolic regulation (Foretz *et al*., 2014). In the liver, metformin inhibits gluconeogenesis and glycogenolysis, and stimulates glycogenesis (Zhou *et al*., 2001). In the adipose tissue and skeletal muscle, metformin increases the number and affinity of insulin receptors, the tyrosine kinase activity of the insulin receptor, and the translocation and intrinsic activity of GLUT-4 (glucose transporter type 4) (Natali and Ferrannini, 2006; Geerling *et al*., 2014).

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In veterinary medicine, metformin can be used to treat cats with diabetes mellitus, but only in cases where the patient has a functional reserve of pancreatic β-cells (Nelson *et al*., 2004). In dogs, experimental studies have been performed to evaluate the hepatic effects of metformin (Chu *et al*., 2000), but it is not routinely used as a treatment. In dogs with HAC, the effects of metformin treatment remain unknown. The purpose of this study was to evaluate the therapeutic actions of metformin for controlling the tendency toward hyperglycaemia, hypercholesterolaemia, hypertriglyceridaemia, hyperinsulinism and insulin resistance in dogs with HAC.

Materials and Methods

Study population

Twenty-three dogs (n = 23) with pituitary-dependant hyperadrenocorticism (PDH) were studied. The diagnosis of PDH was based on history (polydypsia, polyuria and polyphagia, panting, persistent anestrous and any other typical sign of the disease), physical examination (alopecia, prominent abdomen, thin inelastic skin, muscular atrophy), routine laboratory findings (stress leukogram, mild hyperglycaemia, dyslipidaemia, increased alkaline phosphatase or alanine transferase, low urine specific gravity) and an increased urine cortisol:creatinine ratio (UCCR) in two consecutive morning urine samples collected by the owner at home. After collection of the second urine sample, three doses of dexamethasone (0.1 mg/kg) were administered PO at 8-hour intervals, and a third urine sample was collected the following morning, according to Kooistra and Galac (2012). The diagnosis was further supported by visualisation of the adrenal glands by ultrasonography and the pituitary gland by nuclear magnetic resonance imaging. Abdominal ultrasonography revealed bilateral adrenal hyperplasia and no evidence of an adrenocortical tumor in any of these 23 dogs. Nuclear magnetic resonance imaging revealed a pituitary mass in all dogs. Dogs with an infectious disease (systemic or local), pancreatitis, diabetes mellitus, hypothyroidism, tumours (except pituitary adenoma) or any other systemic disorder capable of inducing insulin resistance were excluded (Hess, 2010). Also, dog with abnormalities on Complete Blood Count (CBC), urinalysis or biochemical profile other than those expected with PDH (stress leukogram, low urine specific gravity, mild hyperglycaemia, dyslipidaemia, increased alkaline phosphatase or alanine transferase), were excluded. Likewise, no dog with increased Blood Urea Nitrogen (BUN) was included. In all cases, the common therapy used in Argentina was administered, consisting of combined treatment with cabergoline (0.07 mg/kg/week, divided into doses administered every 48 hours) and ketoconazole (20 mg/kg administered every 24 hours) (Castillo *et al*., 2008; Lien and Huang, 2008).

The 23 dogs with HAC were randomly divided into two groups: (1) *control group* (n = 10; six females and four males; all were non-castrated; median age of 8.5 years, range 6 to 14 years); or (2) *metformin group* (n = 13; eight females and five males; two castrated females; median age of 9 years, range 7 to 13 years), for which metformin was administered at a dose of 10 mg/kg every 12 hours with food. In cases of intolerance, metformin was administered gradually (first week: 2.5 mg/kg/12 h, second week: 5 mg/kg/12 h, third week: 7.5 mg/kg/12 h, and finally the total dose of 10 mg/kg/12 h). All the non-castrated females had low progesterone levels (< 2 nmol/L), both at the beginning and at the end of the treatment (data not shown), so that the progesterone insulin antagonism was ruled out. None of the dogs showed any clinical signs or biochemical abnormalities (both serum and urine) characteristic of liver or kidney disease that might have exacerbated the adverse effects of metformin.

Hormone and biochemical measurements

Glucose, triglycerides, total cholesterol, insulin and UCCR were measured. Blood samples were collected at the same time for all dogs after a 12-hour fast from solid food, both at the time of diagnosis (basal time point) and 3 months after starting treatment. Blood glucose was assessed using an automated laboratory method (Metrolab Autoanalyzer; Merck, Germany) according to the manufacturer's instructions. Blood samples for glucose analysis were collected in glass tubes containing sodium fluoride and EDTA as the anticoagulant (Anticoagulant G; Wiener Laboratory, Argentina). Blood samples for triglycerides and total cholesterol analysis were collected in another tube and measured by an automated laboratory method (Metrolab Autoanalyzer; Merck), according to the manufacturer's instructions.

Insulin concentrations were measured by a specific Enzyme-Linked ImmunoSorbent Assay (ELISA) for dogs and pigs (Porcine/Canine Insulin ELISA; Alpco, USA), with intra- and interassay variation coefficients (canine performance) of 4.2% and 4.3%, respectively, and sensitivity of 0.007 ng/mL.

Urine cortisol was measured by radioimmunoassay (RIA) using a commercial kit (DPC Corporation, San Diego, CA, USA). The inter- and intra-assay coefficients of variation for cortisol were 8% and 5%, respectively. Creatinine was measured by an automated method (Metrolab Autoanalyzer; Merck) according to the manufacturer's instructions.

HOMA for insulin sensitivity and β-cell function

Both fasting insulin sensitivity and the role of pancreatic β-cells were assessed by HOMA. The HOMA$_{insulin\ sensitivity}$ and HOMA$_{β-cell\ function}$ were calculated with nonlinear formulas (Levy *et al*., 1998) using HOMA Calculator version 2.2.2 (Diabetes Trial Unit, University of Oxford, UK;

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http://www.dtu.ox.ac.uk/index.php?maindoc_/homa/) according to the methods previously described (Verkest *et al.*, 2010; Miceli *et al.*, 2014). In those cases in which insulin concentrations exceeded the maximum value allowed by the HOMA calculator, the mathematical formula (Matthews *et al.*, 1985) was used to calculate the aforementioned indices:

HOMA$_{insulin\ sensitivity}$: ([Glucose] x [Insulin]) / 22.5
HOMA$_{\beta\ -cell\ function}$: (20 x [Insulin] / [Glucose] − 3.5)

Statistical analysis
The intra- and intergroup comparisons were performed using nonparametric tests, the Wilcoxon rank test and Mann-Whitney U test, respectively. Data are expressed as the median and interquartile range. The level of significance was set at $P < 0.05$. The statistical analysis were performed using GraphPad Prism 6.0 software (GraphPad software Inc.).

Ethical approval
The Ethics Committee of the Faculty of Veterinary Science (CICUAL) and the Office of Science at the University of Buenos Aires approved the present study, according to the laws on experimentation in animals in Argentina, as well as the recommendations of the World Health Organization. Signed consent was obtained from the owners of all dogs prior to their participation in this project.

Results

Clinical appearance
In 5/13 dogs treated with metformin (38.46%), we observed some degree of drug intolerance. The most common adverse effects were gastrointestinal symptoms, including diarrhoea, inappetence and vomiting. Diarrhoea was the most frequently observed adverse effect. In these five cases, metformin was administered at gradually increasing doses, progressing from 2.5 mg/kg/12 h during the first week, then 5 mg/kg/12 h, then 7.5 mg/kg/12 h, and finally the total dose of 10 mg/kg/12 h. No dogs were excluded from the study, and none showed signs of lactic acidosis. No dogs developed diabetes mellitus, at least during the first 3 months of treatment.

Blood glucose, triglycerides and total cholesterol concentrations
When comparing the basal time blood glucose [control group: median 5.93 mmol/L (interquartile range 4.1–6.2), metformin group: 6.38 mmol/L (5.3–6.9)], triglycerides [control group: 1.6 mmol/L (1.3–2.6), metformin group: 2.3 mmol/L (1.9–3.7)] and total cholesterol concentrations [control group: 7.8 mmol/L (6.1–11.5), meformin group: 9.9 mmol/L (7.3–12.3)] between the groups, no significant differences were observed (Fig. 1 and 2). In the metformin group, blood glucose ($P < 0.01$), triglycerides ($P < 0.05$) and total cholesterol ($P < 0.01$) concentrations were significantly reduced after 3 months of treatment (Table 1).

Table 1. Blood glucose, triglycerides, total cholesterol and insulin concentrations and urine cortisol:creatinine ratio (UCCR) in dogs with hyperadrenocorticism (with or without metformin) at basal time and after 3 months of treatment.

Parameter	Control group		Metformin group	
	Basal	3 months	Basal	3 months
Glucose (mmol/L)	5.93 (4.1-6.2)	5.71 (5.2-6.8)	6.38 (5.3-6.9)	4.76**,a (4.1-5.5)
Triglycerides (mmol/L)	1.6 (1.3-2.6)	2.0 (1.6-2.6)	2.3 (1.9-3.7)	1.1*,b (0.7-1.8)
Total cholesterol (mmol/L)	7.8 (6.1-11.5)	7 (5.6-7.8)	9.9 (7.3-12.3)	7.2** (6.2-10.7)
Insulin (pmol/L)	185 (97-406)	165* (80-340)	180 (90-430)	110**,c (75-299)
UCCR (10-6)	74 (22.4-282)	15*** (8.5-30)	55 (25-675)	13*** (7-280)

Values are expressed as median and interquartile range. Reference values: (Glucose: 3.36–6.72 mmol/L); (Triglycerides: 0.5–1.5 mmol/L); (Total cholesterol: 2.6–6.5 mmol/L); (Insulin: 35.9–179.5 pmol/L); (UCCR: <10 × 10^{-6}). Significant intragroup difference between basal and 3 months indicated by: (***: $P < 0.001$); (**: $P < 0.01$) and (*: $P < 0.05$). Significant intergroup difference at 3 months indicated by: (a: $P < 0.01$); (b: $P < 0.05$) and (c: $P < 0.05$).
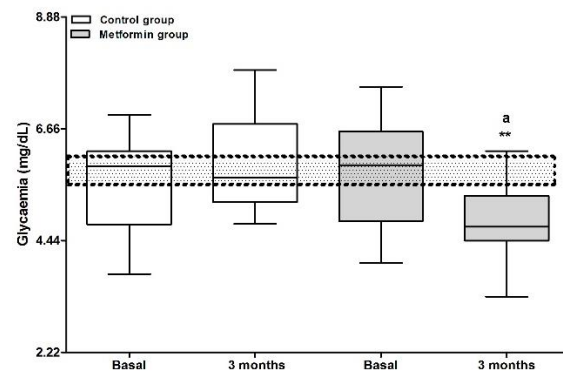

Fig. 1. Comparison of the blood glucose concentration of dogs with hyperadrenocorticism, with or without metformin treatment, at basal time and after 3 months of treatment. ** $P < 0.01$ vs. basal time; [a] $P < 0.01$ vs. 3 months of control group. The dotted line indicates the glucose upper limit of the reference range (6.72 mmol/L) and into shadow area the values considering as fasting glucose intolerance.

In the control group, no significant differences were observed between the basal time measurements and those taken at 3 months for the three variables analysed. Comparing the concentrations between each group at 3 months, only blood glucose [control group: 5.71 mmol/L (5.2–6.8), metformin group: 4.76 mmol/L (4.1–5.5)] and triglycerides concentrations [control group: 2.0 mmol/L (1.6–2.6), metformin group: 1.1 mmol/L (0.7–1.8)] differed significantly ($P < 0.01$ and $P < 0.05$, respectively), with lower concentrations than the control group.
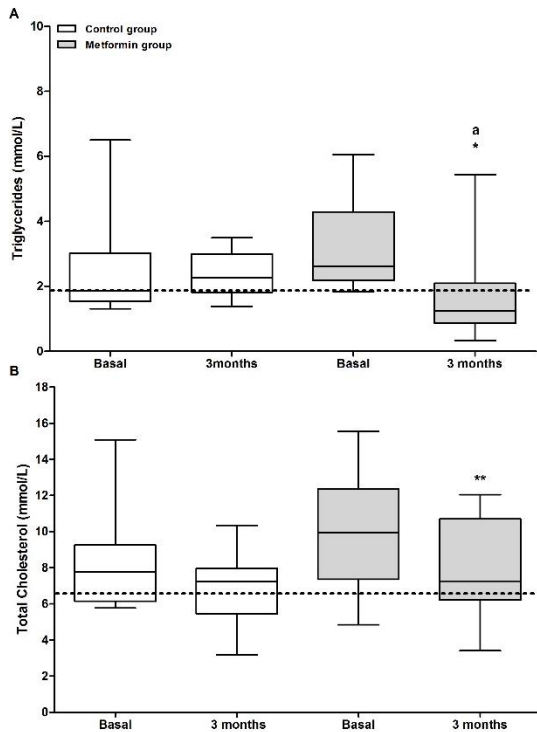
Fig. 2. Comparison of the blood triglycerides (A) and Total cholesterol (B) concentrations of dogs with hyperadrenocorticism, with or without metformin treatment, at basal time and after 3 months of tratment. A) * P < 0.05; [a] P < 0.05 vs. 3 months of control group; B) ** P < 0.01. The dotted line indicates the upper limit of the reference range (A: 1.5 mmol/L; B: 6.5 mmol/L).

Analysing the individual variation, the blood glucose concentration decreased in 13/13 dogs in the metformin group, and the triglycerides and total cholesterol concentrations decreased in 11/13 dogs (84.61%), with the concentrations increasing in the remaining two dogs. In the control group, blood glucose levels were decreased in 4/10 dogs (40%), increased in 4/10 dogs (40%) and remained constant in 2/10 dogs (20%). Triglycerides concentrations decreased in 3/10 dogs (30%), increased in 2/10 dogs (20%) and remained constant in 5/10 dogs (50%). Total cholesterol concentrations decreased in 5/10 dogs (50%) and remained constant in 5/10 dogs (50%) of the control group.

Insulin concentration and urine cortisol:creatinine ratio

When the basal time insulin concentrations (Fig.3) were compared amongst the groups [control group: 185 pmol/L (97–406), metformin group: 180 pmol/L (90–430)], no significant differences were observed. However, at 3 months, the insulin levels were significantly lower in the metformin group [110 pmol/L (75–299)] than in the control group [165 pmol/L (80–340)] (P < 0.05).
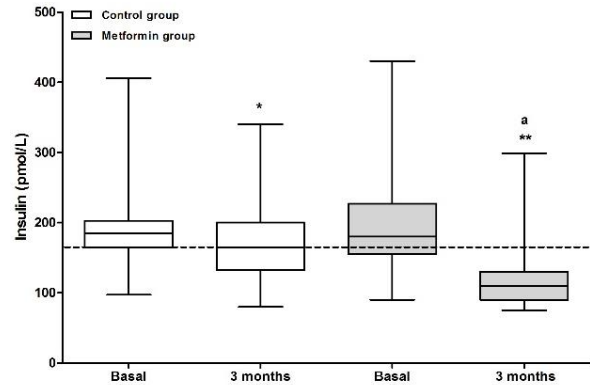

Fig. 3. Comparison of the blood insulin concentration of dogs with hyperadrenocorticism, with or without metformin treatment, at basal time and after 3 months of treatment. * P < 0.05; *** P < 0.001; [c] P < 0.05 vs. 3 months of control group. The dotted line indicates the upper limit of the reference range (173 pmol/L).

For the intragroup variation, insulin levels were significantly decreased after 3 months of treatment both in the control group (P < 0.05) and in the metformin group (P < 0.01), with the most pronounced decrease observed in the metformin group.

When comparing the basal time UCCR between the groups, no significant differences were observed. The UCCR decreased significantly after 3 months of treatment in both groups (P < 0.001) (Table 1).

Homeostatic model assessment (HOMA) for insulin sensitivity* and *β-cell function

At basal time, the HOMA$_{insulin\ sensitivity}$ (Fig. 4) did not show any significant differences between the groups. At 3 months, the HOMA$_{insulin\ sensitivity}$ of the metformin group was higher than the control group (P < 0.05). For the intragroup variation, the HOMA$_{insulin\ sensitivity}$ was significantly increased at 3 months in both the control group (P < 0.01) and the metformin group (P < 0.001), with the most pronounced increase observed in the metformin group.

At basal time, the HOMA$_{β-cell\ function}$ (Fig. 4) showed no significant differences between the groups. At 3 months, the HOMA$_{β-cell\ function}$ of the metformin group was lower than the control group (P < 0.05). In regard to intragroup variation, the HOMA$_{β-cell\ function}$ decreased significantly at 3 months in both the control group (P < 0.01) and the metformin group (P < 0.001), with the most pronounced decrease observed in the metformin group.

Discussion

In both dogs and humans with HAC, the comorbidities associated with hypercortisolism (hypertension, nephropathy, pulmonary thromboembolism, pancreatitis and diabetes mellitus) are the cause of patient death (Nichols, 1997; Ferraú and Korbonits, 2015; Miceli *et al*., 2017).
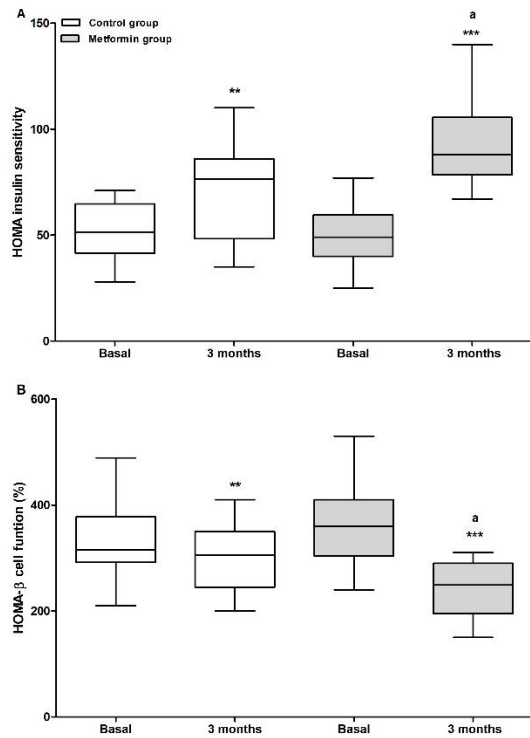
Fig. 4. Homeostatic model assessment (HOMA) for insulin sensitivity **(A)** and β-cell function **(B)** in dogs with hyperadrenocorticism, with or without metformin treatment, at basal time and after 3 months of treatment. **(A):** (**: P < 0.05) and (***: P < 0.001); 3 months vs basal intragroup; (a: P<0.05) vs 3 months of control group. **(B):** (** P < 0.01) and (*** P < 0.001); 3 months vs basal intragroup; (a: P<0.05) vs 3 months of control group.

Therefore, therapies should not only be aimed at reducing hypercortisolism, but also at the prevention and/or control of comorbidities that may occur in the short- or long-term if the metabolic disorders associated with HAC are not normalised. Paradoxically, even after reducing hypercortisolism, the adverse effects of comorbidities associated with excess glucocorticoids can persist for several months (Barahona *et al*., 2009, 2010). A previous study by our group reported that the use of insulin detemir at low doses helped control the metabolic disorders associated with HAC, preventing the development of diabetes mellitus (Miceli *et al*., 2012). Similarly, in the present study, we observed that metformin was associated with decreased blood glucose, triglycerides and total cholesterol concentrations in dogs with HAC. In diabetic humans, it is known that metformin treatment decreases fasting hyperglycaemia, and to a lesser extent, postprandial hyperglycaemia, without increasing the risk of hypoglycaemia (Natali and Ferrannini, 2006; Aroda *et al*., 2015). In dogs, Chu *et al*. (2000) demonstrated that metformin is effective for decreasing hepatic glucose

production, not so much by inhibiting gluconeogenesis, but rather by reducing glycogenolysis. The mechanism of action of metformin remains controversial, and it is not currently known exactly which receptor it binds. However, several studies have shown that metformin acts on AMPK (cAMP-activated protein kinase), an enzyme complex that is activated by increasing the AMP:ATP ratio (Rena *et al*., 2013; Foretz *et al*., 2014; Hongying and Ling, 2016). In contrast, some dogs with HAC that were not treated with metformin showed increased levels of glucose and triglycerides, despite having started the treatment for HAC. These increases may be due to individual differences of each dog to either the severity of the disease, resistance to therapy with ketoconazole or cabergoline, or persistence of the adverse effects of hypercortisolism (Vegiopoulos and Herzig, 2007; Castillo *et al*., 2008; Barahona *et al*., 2010; Miceli *et al*., 2012, 2017). To date, no studies have described the effects of metformin in dogs with HAC, but there have been several reports in diabetic cats. Nelson *et al*. (2004) evaluated the use of metformin to treat cats with diabetes mellitus, obtaining satisfactory results only in animals with a good insulin reserve. This is also the case for dogs with HAC that also present insulin resistance and hyperinsulinism (Montgomery *et al*., 1996; Miceli *et al*., 2014), which explains why the insulin-sensitising effect of metformin would be useful. Treatment of HAC with ketoconazole and cabergoline was found to be effective for reducing UCCR, and thus, controlling the clinical signs of the disease. Once hypercortisolism is controlled, the degree of hyperinsulinism, insulin resistance (indicated by increased $HOMA_{insulin\ sensitivity}$) and pancreatic β-cell functionality (indicated by decreased $HOMA_{\beta-cell\ function}$) is simultaneously decreased. In this study, we used ketoconazole rather than retinoic acid (isotretinoin 9-cis) (Castillo *et al*., 2006) because patients receiving retinoids may present dyslipidaemia as an adverse effect (Lilley *et al*., 2013). The most interesting observation of the current study was that the administration of metformin, combined with ketoconazole treatment for HAC, caused a further reduction in the hyperinsulinism and insulin resistance of patients, as well as improved pancreatic β-cell functionality. By reducing hepatic glucose production (Chu *et al*., 2000; Hongying and Ling, 2016), metformin indirectly decreases the insulin requirement to maintain glycaemic homeostasis. Furthermore, due to its insulin-sensitising effects on adipose tissue and skeletal muscle (Natali and Ferrannini, 2006), metformin also decreases the peripheral insulin resistance in dogs with HAC. The use of metformin in dogs with HAC was associated with the same adverse effects observed in humans, including diarrhoea, inappetence and vomiting (Hostalek *et al*., 2015). No lactic acidosis or haematological disorders were

observed, at least 3 months after therapy. Adverse gastrointestinal effects were controlled by gradually increasing the metformin dose. When the gradual dosage of metformin and the contraindications in humans, such as liver and kidney disease (Foretz *et al*., 2014; Hostalek *et al*., 2015), are considered, metformin represents a drug that can not only effectively improve insulin sensitivity and reduce the glycaemia of humans, cats and dogs, but is also safe to use. For future research, the long-term effects of metformin and its beneficial effects on other systems or diseases, such as cardiovascular disease, obesity, inflammation or cancer, will be assessed (Mahmood *et al*., 2013). The implementation of metformin as an adjuvant therapy is effective for normalising and improving glycaemia, triglyceridaemia, cholesterolaemia and insulinaemia in dogs with HAC, at least within the first 3 months of treatment. In addition, metformin reduced the insulin resistance and improved the pancreatic β-cell function in dogs with HAC. Dogs represent an excellent model for HAC study, and the results obtained have an interesting potential for translation to human medicine.

Acknowledgments

Conflict of interest

The Authors declare that there is no conflict of interest.

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