Evaluation of urine samples of diabetic rats treated with metformin and different natural product combinations

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ABSTRACT

Diabetes is highly prevalent worldwide, and urine analyses with dipstick methods are important tools to monitor glucosuria and nephron status in diabetic animals. The aim of this study is to follow glucosuria, ketonuria, and proteinuria changes in hypoglycemic drug use in diabetes and drug and natural product combinations. Male wistar albino rats were used in the study and type 2 diabetes was induced with streptozotocin (65 mg/kg, i.p.) and nicotinamide (110 mg/kg, i.p.). The drug and natural products were administered orally for a period of 84 days (healthy control, diabetic, diabetic+metformin, diabetic+metformin+cherry laurel, diabetic+metformin+rutin and diabetic+metformin+alpha lipoic acid groups), and the urine samples were collected at the end of the experiment. The urinalysis (glucose, ketone, and protein) was done with a dipstick. The results were scored between 0 and 3, and Kruskal-Wallis analysis was applied. There was a significant difference between the untreated diabetic group (DM) and the remaining groups in glucose, but ketone and protein analysis did not show any statistically significant differences. The results showed that drug and drug+natural product combination reduced urinary glucose excretion in diabetes. In conclusion, the use of metformin and/or natural product combinations decreased the glucose output in urine. With an easy and cheap monitoring method such as a dipstick, the metabolic state can be revealed. And the effect of drug and natural product combinations can be monitored.

Introduction

Diabetes mellitus is the most common cause of chronic kidney diseases in the world, and urine analysis is an important laboratory finding in kidney diseases. Urine tests are crucial to get information about renal dysfunction and metabolic disorders. The vital roles of kidneys are filtration and the output of waste products by urine. That's why urine tests can give the metabolic state of the body (25). In normal patients, glucosuria is an undetectable result because kidneys reabsorb all the glucose in a routine metabolic phase (45). If hyperglycemia exceeds the renal threshold, glucosuria can be detected. The most common cause of glucosuria, as it is known, is diabetes. However, in some animals, like cats and birds, severe stress can also be the reason for glucosuria (33). Experimental studies with rats exhibited that severe pain could cause glucosuria in healthy rodents (21). In glucosuria, sometimes the problem could be due to congenital or acquired proximal tubular diseases. In these situations, it is named as normoglycemic glucosuria (41). Congenital diseases could be Fanconi's syndrome, primary renal glucosuria, and congenital renal dysfunction, and acquired diseases could be acute renal failure, toxicosis (heavy metals, nephrotoxic drugs), and chronic renal failure (10). It is stated that enrofloxacin and cephalexin use in dogs could cause false positive glucosuria (35).

Proteinuria has several reasons depending on the type of protein or amino acid. Mostly, a minute amount of protein can be seen in urine, which is named as trace and is a physiological condition. Since test strips often measure albumin, a significant increase in albumin suggests proteinuria when using dipsticks for chemical

analysis. The reasons for proteinuria can be classified as prerenal, glomerular, tubular, hemorrhagic, or inflammatory and protein-losing nephropathy, and renal failure (15). As previously stated, trace protein in urine is physiological and typically corresponds to +1 in dipstick evaluations. However, if the urine is alkaline or a concentrated urine sample, additional methods are required to define proteinuria because the dipstick result may be an artifact; otherwise, it is a pathological finding (37). In a study, nephropathy was induced with doxorubicin in rats, and the urine ketone, glucose, and protein output was analyzed. According to the study, there was no change in ketonuria, but glucosuria and proteinuria gave a statistical difference between the control and nephropathy-induced rats (8). In diabetes, a drop in glycemia gives rise to a decline in HbA1c levels, and every 0.9% decrease in HbA1c lowers the risk of diabetic nephropathy by about 30% (1, 13).

Ketonuria is an important diagnostic parameter for diabetes, but it is not the only reason. Ketone bodies are related to the rise of gluconeogenesis, but if lipid catabolism is increased, ketone bodies will arise in the blood too. Mostly, increased gluconeogenesis is seen in diabetes because there is an impaired ability to use carbohydrates. The other reasons for increased gluconeogenesis could be starvation and fasting (lack of carbohydrates) or loss of carbohydrates (mostly seen in renal or digestive problems). In pregnancy, ketonuria can occur as a physiological condition. The renal threshold to clear the ketone bodies from blood is low, so even a low increase in plasma ketone will result in ketonuria. Since dipsticks often employ the nitroprusside (Rothera) test method, test strips fail to detect ß-hydroxybutyric acid. This situation can sometimes lead to false negative results. For example, extreme dehydration is a reason for hypoxia, and this can increase ß-hydroxybutyric acid levels, which will be undetectable with the dipstick urinalysis method (6, 34, 43, 46).

In physiological conditions, the control mechanism of renal excretion is glomerular filtration, which is a passive transport; tubular resorption, which can be active or passive; and tubular secretion, which is active. In the glomerular filtration process, solutes, depending on their molecular size and electrical charge, can pass the glomerular filtration barrier. When the molecular size is less than 2.5 nm, all solutes can easily pass the barrier, while the size is between 2.5 and 3.4 nm, some of the solutes can pass the barrier, but when the size of the molecules is higher than 3.4 nm, none of the solutes can pass the barrier. It is also hard for the molecules to pass the barrier if they bear a negative charge. Albumin is not anticipated to be present in urine samples, particularly in felines, equines, or bovines, because albumin possesses a negative charge and has a molecular size of 3.5 nm.

Glucose can pass through the filtration barrier, but all of the filtered glucose is reabsorbed in the proximal tubules passively. Smaller proteins and amino acids are filtered too, but these molecules are reabsorbed in the proximal tubules. The transport of glucose is done by carrier proteins. In hyperglycemia, mostly the carrier proteins carry the glucose until there are no more carrier proteins, and at least hyperglycemic glucosuria is formed (12, 15, 40, 41).

With advances in research on the pathophysiology of diabetic kidney diseases, some new treatments targeting kidney inflammation and oxidative stress have gradually entered clinical practice. In fact, some drugs that are useful in mitigating the progression of diabetic kidney diseases have anti-inflammatory properties, such as metformin. Metformin remains the first-line treatment for type 2 diabetes. Natural products with antioxidant effects are effective molecules in preventing oxidative stress and inflammation. The lack of literature on urine analysis findings in diabetic rats gave us the opportunity to evaluate urine results from a different perspective. The goal of this study was to observe how metformin and antioxidant combinations affect urine protein, glucose, and ketone levels in rats with type 2 diabetes, as well as the development of long-term issues like diabetic nephropathy.

Materials and Methods

Chemicals: Streptozotocin (STZ; Sigma cat. no.: S0130-1G), nicotinamide (NA; Sigma cat. no.: 72340-100G), and alpha lipoic acid (ALA; Sigma cat. no.: 62320-25G-F) were supplied by Sigma Aldrich. Rutin flavonoid (R; Alfa Easer cat. no.: A13570.22) was bought from Thermo Fisher Scientific, and metformin (Met; Novartis Glukofen® 1000 mg) was bought from the pharmacy. Dimethylsulfoxide (DMSO) was purchased from Honeywell fluka (cat. no.: 41640-1L), and phosphate buffer solution (PBS) was obtained from Merck (cat. no.: P4417-100TAB). Cherry laurel fruit was obtained from a market in Istanbul. The fruits were grown in the northwest region of the Black Sea, in Zonguldak and Bartin provinces.

Extraction of L. officinalis: The extraction of *L. officinalis* was done according to the method of Agcam and Akyildiz (2). The methanolic extraction solution (methanol: HCl (0.1 N); 85:15, v/v) was prepared with reference to Bronnum Hansen et al. (9). After the cherry laurel was extracted, methanol was evaporated in a vacuum oven and dissolved in DMSO not to exceed 1% of the total solution and diluted in PBS.

Animals and Urine Collection: Eight-week-old 250-350 g weighed male Wistar albino rats were used. It is

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demonstrated that the estrogen hormone in females has a protective effect on beta cell damage (27). This is the rationale behind the choice of male rats for the purpose of this study.

Twelve hours of light and 12 hours of darkness on a regular basis were provided. Water and food were given *ad libitum*. The room temperature was set to 25-27℃. Five rats were in a cage, and each group contained 10 animals. Rats were grouped randomly.

The induction of type 2 diabetes was done with STZ (65 mg/kg) and NAD (110 mg/kg) injection (28). As laboratory animals, 8-week-old Wistar albino male rats were chosen, and the rats were grouped into 6 groups, with 10 rats in each group. Before the induction of diabetes, rats were grouped randomly because their weights were close to each other. In Table 1, the groups and doses of given products can be seen.

To assess diabetes, an OGT test has been performed (24). After diabetes developed, metformin and natural products were given for 84 days (12 weeks). Some of the animals could not complete the study. Urine samples of the rats were collected before the sacrificial process. During the weighing process, the rats handled spontaneously urinated into the weighing cup, and the urine was collected from the weighing cup with the help of a syringe. Samples could not be taken from some rats because their bladders were empty. The urine volume that rats urinate into the weighing cup was not enough to do all the parameters listed on the dipstick. The volume was about 0.2 cc/animal. The weight and glucose measurements after ending the protocol are given in the PhD thesis of Kaya Kartal (24).

Urinalysis: Samples were measured semi-quantitatively with a dipstick (microcult, REF: 116010). Only glucose, protein, and ketone were measured, and the results were numbered from 0 to 3 according to the color on the dipstick. The working principle of glucose measurement in dipstick is based on the enzymatic reaction. First, glucose is oxidized and then forms gluconic acid and H_2O_2 in the presence of glucose oxidase. Ketone body detection in urine samples with the dipstick method uses the nitroprusside test (Rothera test) principle, but in this evaluation, β-hydroxybutyric acid cannot be detected. To measure proteinuria, the reaction is based on the phenomenon known as the protein error of pH indicators, where an indicator that is highly buffered will change color in the presence of proteins (anions) as the indicator releases hydrogen ions to the proteins (17).

Statistical Analysis: The SPSS 21.00 package program was used to determine the statistical differences. For semi-quantitatively measured results, the Kruskal Wallis test was applied. Only glucose gave a statistically significant difference between groups, and $P<0.05$ means statistical significance (13). The results are given as median (Q1-Q3).

Results

There was a statistical significance between DM and all other groups ($P<0.05$). Especially in the control group and the DM+Met+ALA group, no glucose has been found in the urine of a rat. The results are given in Table 2. In ketonuria, no statistical significance was seen ($P>0.05$). The results of urine ketones are 0.0 (0.0-1.0) in control, 1.0 (0.5-1.0) in DM, 2.0 (1.0-2.0) in DM+Met, 1.0 (0.75-1.0) in DM+Met+CL, 1.0 (1.0-2.0) in DM+Met+R, and 1.0 (1.0-2.0) in the DM+Met+ALA group. In urine protein measurement, there was no statistical significance between groups ($P>0.05$). The results of proteinuria were too close in all groups, and proteinuria was observed in all rats.

Table 1. Name of the groups and doses of drugs and natural products and the induction of diabetes.

Groups	n	Flavonoid dose	Metformin dose (30)	STZ+NAD (27)
Control	10	-	-	-
DM	10	-	-	65 mg/kg STZ+110 mg/kg NAD
DM+Met	10	-	500 mg/kg	65 mg/kg STZ+110 mg/kg NAD
DM+Met+CL	10	100 mg/mL	500 mg/kg	65 mg/kg STZ+110 mg/kg NAD
DM+Met+R	10	60 mg/kg (16)	500 mg/kg	65 mg/kg STZ+110 mg/kg NAD
DM+Met+ALA	10	100 mg/kg (21)	500 mg/kg	65 mg/kg STZ+110 mg/kg NAD

DM: Diabetic, DM+Met: Diabetic+Metformin, DM+Met+CL: Diabetic+Metformin+Cherry Laurel, DM+Met+R: Diabetic+Metformin+Rutin, DM+Met+ALA: Diabetic+Metformin+α Lipoic Acid. STZ+NAD: Streptozotocin+Nicotinamide.

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Table 2. Results of urine glucose, ketone, and protein measurement (median (Q1-Q3)).

Parameter	Control	DM	DM+Met	DM+Met+CL	DM+Met+R	DM+Met+ALA	P
Glucose	0.0 (0.0-0.0)[a]	2.0 (2.0-2.0)[b]	0.0 (0.0-0.0)[a]	0.0 (0.0-0.0)[a]	0.0 (0.0-0.0)[a]	0.0 (0.0-0.0)[a]	<0.05
Ketone	0.0 (0.0-1.0)	1.0 (0.5-1.0)	2.0 (1.0-2.0)	1.0 (0.75-1.0)	1.0 (1.0-2.0)	1.0 (1.0-2.0)	>0.05
Protein	2.0 (1.5-2.0)	3.0 (2.0-3.0)	3.0 (1.0-3.0)	3.0 (1.75-3.0)	3.0 (2.25-3.0)	2.0 (2.0-3.0)	>0.05
N	5	5	5	10	8	7	

DM: Diabetic, DM+Met: Diabetic+Metformin, DM+Met+CL: Diabetic+Metformin+ Cherry Laurel, DM+Met+R: Diabetic+Metformin+ Rutin, DM+Met+ALA: Diabetic+Metformin+ α Lipoic Acid· **[a-b]: The difference between the means with different letters in the same row is significant (P<0.05).**

Discussion and Conclusion

Hard (19) researched the urine protein content of humans and rats. According to the review, the protein output of rat urine is much higher than that of human urine. Protein output by urine differs between sexes too because studies showed that male rats' urine protein content is approximately 10-fold higher than female rats' urine protein content. Male rats mostly excrete the urine protein as α2μ-globulin because its secretion is controlled by androgens (44). There is a decrease in urine α2μ-globulin output by age, but the decrease is compensated by an increase in albumin output. So, in young mature rats, the proteinuria is defined as physiological proteinuria, while the latter is defined as pathological albuminuria in later phases (32).

When handled for restraint, most rodents urinate and defecate spontaneously, so collecting urine by placing the rodent in a plastic bag can be a urine collection method. Diabetic rodents urinate 10 times more than normal rodents, which makes it easier to collect the urine samples with the mentioned collection method (30).

In normal conditions, glucose is not detected in urine, but according to studies, stress, pain, and fright can cause glucose output in urine. Glucosuria and ketonuria are mostly present in diabetes, but in some rodents with dental and gastrointestinal (cecum) problems, ketonuria can occur (21). In a study in rats fed with a low-carbohydrate, high-fat diet, conducted by Bielohuby et al. (7), the ketonuria measurements with urine dipsticks (two different companies) and laboratory methods (GC/MS) were compared. According to the study, wet chemistry methods are more reliable than urine dipsticks for ketonuria (7). The output of ketone, even in healthy rats, in the current study could be due to the false positive result of the dipstick. There were no significant differences in ketonuria between the groups, and this reveals that dipstick ketone measurement is not reliable. On the other hand, glucose monitoring via urine dipsticks can be beneficial in diabetes.

Masrika et al. (29) studied male Wistar rats fed with a high-protein, low-carbohydrate, and low-fat diet and evaluated urine samples with a dipstick. The ketone and protein outputs in standard diet-fed rats were significantly higher, even if the rats were healthy. Glucose output was not detected in two of the diets. According to these findings in the current study, ketonuria and proteinuria in healthy rats could be regarded as usual, which could explain why there was no statistically significant difference between the healthy and diabetic groups.

Hoffman et al. (20) compared the urine collection and stress markers in rats housed in normal cages with hydrophobic sand and metabolic cages and did not find any significant difference in urinalysis between the collection methods. Urinalysis was done with a dipstick. According to their results, glucose was not detected in any of the healthy rats, but ketone and protein were detected in small amounts. Another study compared wire-bottom and solid-bottom cages, and according to the urinalysis, which was measured with a dipstick, none of the rats' urine samples contained glucose, but approximately all of the healthy rats had ketonuria, and there was no statistically significant difference between the housing types (39).

According to the studies, it can be said that the collection method and the cages in which the rats are housed did not influence the results of urinalysis. But especially according to the study of Bielohuby et al. (7), ketone measurement can differ when done with a dipstick.

Studies on healthy rats and diet, housing, and urine collection methods and the effects on urinalysis were discussed extensively, but the results of nontreated diabetic rats and those treated with drugs, natural products, and their combinations still needed to be evaluated. The lowest blood sugar levels in treated diabetic rats were found in the DM+Met+ALA and DM+Met+CL groups, which are consistent with urinalysis results (24). So, the analysis of glucosuria and blood glucose is an essential diagnostic parameter in diabetes. Studies showed that metformin in combination therapies

is more useful than using metformin alone in type 2 diabetes (5).

The use of lipoic acid alone is a known antidiabetic agent because of the positive effect on insulin resistance and glucose tolerance. Lipoic acid improves hepatic insulin sensitivity (36), so administration of lipoic acid with metformin shows that its antihyperglycemic effect is improved. Magnesium content of natural products is crucial for regulating glucose impairment because magnesium enhances insulin action and has an insulin-like impact on the metabolism of glucose (38). Cherry laurel has a high magnesium content (11), and the mechanism of action against type 2 diabetes could be due to its mineral content, which still needs further investigation. So, according to the current study, the combination of cherry laurel and metformin has positive effects on blood sugar and glucosuria levels. In a study conducted by Sun et al. (42), the effects of rutin on hyperglycemic rats were studied, and rutin reduced blood sugar and lipids and had positive effects on damaged islet cells and antioxidant activity. Based on the current study, it can be said that rutin has positive effects on decreasing the urine glucose output too if used in combination with metformin.

In addition to the classical complications of proteinuria, such as hypoalbuminemia, edema, and acidosis, there is increasing evidence in laboratory animals and humans that proteinuria can cause glomerular and tubulointerstitial damage and lead to progressive nephron loss (16).

In a study, it was found that the microalbuminuria in diabetic patients shows pseudoesterase activity, while the overt albuminuria group did not show this activity. To measure the activity, urine proteins were first isolated, and pseudoesterase activity was measured with electrophoretic measurements (26). This is a good biomarker for understanding renal failure in diabetic patients, but the dipstick method is an easier, less time-consuming, and low-cost method when electrophoresis is not readily accessible.

In a study, the dipstick method for urinalysis in glucosuria was checked in dogs and cats. The dipstick readings were done with visual observation and with an automated approach, and the results showed that visual observation is more sensitive than the automated approach. Glucosuria measurement with a dipstick can lead to false negatives, so if glycemia occurs but no glucosuria is observed, other approaches for glucosuria should be carried out for verification. Dipstick methods for glucosuria in cats were more useful than in dogs (3). Another study with cats used four different dipstick models for glucosuria and found that not all dipstick models are useful to detect glucosuria, but for cats with diabetes, to control the glucosuria, a dipstick with high analytical sensitivity is still useful (47).

A study with diabetic male mice revealed the importance of circadian rhythm for hyperglycemia. In this study, the blood glucose levels between the diabetic and diabetic+treated groups were close, but in urinary glucose, the glucosuria of diabetic mice was higher than that of the diabetic treated group, but there was no statistical difference, and it was concluded that the change in circadian rhythm can lead to a worse effect on diabetes (4).

In the DM+Met+R group, in one rat, the urine glucose level was scored as +2; this is due to the blood glucose level (583 mg/dL). The other animals in this group did not show a high blood glucose level like this. At the beginning of the experiments, there was no statistical difference between groups according to the weights, but after the experiment, the DM+Met+R group showed a significant difference between the other groups (24). There may have been a decrease due to the debilitating effect of rutin flavonoid. The studies showed that the rutin flavonoid decreases the food intake in rats (22).

In conclusion, metformin and metformin + natural product combinations in glucosuria measurement show statistically significant differences between nontreated DM groups. There was still a glucose output in some animals, but the results were closer to those of the control group. However, using metformin alone and using it with different natural products did not produce any statistically significant differences. Urinalysis with dipsticks is mostly used in various housing, urine sample collection methods, and different diets. Urine collection from rats using the spontaneous collection method and dipstick analysis is easy and cheap. So, it can be used in some metabolic disorders to see the metabolic state of animals. The hypoglycemic effects of metformin and antioxidant administration in type 2 diabetes are critical for adjusting treatment strategies, preserving renal function, and improving prognosis. When we look at the additive effects of natural products on the hypoglycemic action of metformin, it is understood that it has no modifying effect on metformin use. Proteinuria measurement is not preferable for revealing diabetic nephropathy in rats due to the results we found. Diseases are mostly induced in laboratory animals like rats, and the results are interpreted for humans or other animals (dogs, cats). In veterinary medicine, dipsticks for urinalysis are commonly used, and according to this study, glucose monitoring from urine could be a choice for diabetic animals since it causes less stress. But for ketonuria and proteinuria, using the dipstick needs more study in the veterinary field to be sure of following diabetic nephropathy and ketoacidosis.

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Ethical Statement

This study was carried out after the animal experiment was approved by Ankara University Local Ethics Committee of Animal Experiments (Decision number: 2021-5-29).

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

Y.K.K. and T.S. conceived and designed the study, performed the sample collection, conducted the analyses, wrote drafted the manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

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