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Research Article
Effect of Tributyrin Supplementation on Glucose Levels, Liver and Kidney Integrity in an Experimental Model of Diabetes Mellitus

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Abstract

Background and Objective: Studies report the beneficial effects of short-chain fatty acids on intestinal health, hyperglycemia and kidney disease. However, it is unknown whether tributyrin, a prodrug of butyric acid, produces the same effects. Due to the limitations of current treatments for patients with chronic diabetic disease, other alternative therapies could provide a better quality of life for the patient. Therefore, the objective was to evaluate the effect of tributyrin in an animal model with this pathology. **Materials and Methods:** Twenty-seven male Wistar rats weighing 200-250 g were assigned randomly into three groups. The animals received food and water *ad libitum*. The G_1 and G_2 animals were chemically induced into a model of chronic diabetes, with liver and kidney damage, using streptozotocin. At G_1 and G_3 (healthy animals): Tributyrin (Tb: 450 mg kg^{-1} PO SID) was administered for 4 weeks. To G_2 (control group): Only physiological saline solution 4.5 mL kg^{-1} PO SID was given. Hemogram, blood biochemistry and urinalysis were performed at the beginning and every week. In addition, a glucose tolerance test was performed before and at the end of treatment. At the end of the study, histopathology examinations were performed. **Results:** Supplemented tributyrin increased insulin sensitivity, decreased blood glucose and improved liver and kidney tissue. **Conclusion:** Oral tributyrin supplementation in a rat model of chronic diabetes mellitus improved blood glucose levels, produced a hepatoprotective effect and delayed kidney disease. Therefore, tributyrin is considered a complementary therapy to the usual treatment of type 1 diabetes mellitus.

Key words: Short-chain fatty acids, hepatoprotective, diabetic nephropathy, food supplement, chronic diabetes mellitus, butyric acid prodrug, hyperglycemia

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Type 1 diabetes mellitus (DM) is a chronic metabolic disease. Some of the main complications are kidney damage or diabetic nephropathy, liver damage and alterations to the heart and blood vessels. These arise as a consequence of oxidative stress caused by chronic hyperglycemia that leads to the activation of different mechanisms of cell damage at the kidney level, such as the activation of signalling for the synthesis of Nuclear Factor kappa B NF-kB[1,2], the synthesis of transforming growth factor-beta TGF-β[3], tumour necrosis factor α[4], angiotensin II activation, polyol pathway activation, which decreases the synthesis of antioxidants at the cellular level, deposition of collagen fibres at the mesangial level, hypertrophy in the mesangial cells, with increased gene transcription and the secretion of extracellular matrix proteins, such as collagen, laminin and fibronectin, among others[5].

Recent studies have shown that high-fibre diets promote an increase in Short-Chain Fatty Acids (SCFA), which are directly involved in the intestine-kidney axis, decreasing intestinal permeability and thus meta-inflammation in diabetic patients[6-8]. Studies in which SCFA have been administered intravenously and intraperitoneally in individuals with DM1 show positive effects since they block cytokine synthesis mechanisms, thanks to their effect as histone deacetylase inhibitors, in addition to having effects on insulin production and on type 4 glucose receptors (GLUT4), which improves glycemia, liver metabolism and consequently delays renal cell degeneration and end-stage renal disease[9,10].

The use of SCFA has been limited since they present pharmaceutical forms of rapid absorption and metabolism that complicate their arrival in target organs. They have a short half-life (<90 min)[11] and routes of administration are not very viable for regular use via the intraperitoneal or intravenous route. Among the SCFA, butyric acid (BA) affects the control of hyperglycemia in DM. Tributyrin (Tb), the prodrug of butyric acid, has a state-of-the-art pharmaceutical form composed of three butyric acid molecules bound to glycerol, which reduces its bioavailability in the first segments of the digestive tract. As a result, butyric acid molecules (the active form of tributyrin) are released from the small intestine by pancreatic lipase and other cellular lipases, allowing a higher concentration of BA in the posterior gastrointestinal tract and increasing the area under the drug concentration curve and lifetime[12-14].

In addition, Tb functions as a primary nutrient that provides energy to colonocytes. Tributyrin acts as a cellular mediator that regulates multiple functions, such as gene expression, cell differentiation, tissue development, immune modulation, stress reduction, oxidative stress, the control of enteric pathogens, the removal of inflammation, the improvement of growth and the control of satiety, among others[14,15].

To our knowledge, no studies are evaluating the effect of oral Tb on haematological and biochemical parameters, effects on glycemia and histologically on liver and kidney protection in animals with DM1. Given the potential of this prodrug to improve health in individuals with chronic DM1, it was considered extremely important to evaluate its hypoglycemic capacity and its hepato and nephroprotective effects. Therefore, this study aimed to assess the impact of orally administered Tb in rats with chemically induced chronic DM1.

MATERIALS AND METHODS

The experimental study of the animals, and the clinical pathology analyses, were carried out in the Animal Experiment Unit of the Faculty of Chemistry at the National Autonomous University of Mexico. Mexico, from November, 2019 to May, 2020. The statistical analysis and interpretation of the results were carried out in the Department of Veterinary Physiology of the Faculty of Veterinary Medicine and Zootechnics.

Materials: The tributyrin used in this research was donated from the Bioquifa S. de R.L. of C.V. Mexico, streptozotocin (STZ, Sigma Aldrich, Mexico) and isoflurane (Sofloran® VET, 250 mL, Pisa Agropecuaria, Mexico) were purchased from the distribution companies.

Animals: Twenty-seven (8 weeks old) male Wistar rats weighing 200-250 g from the Animal Facility of the Institute of Cellular Physiology, UNAM, were used. The Institutional Subcommittee approved all experimental procedures for the Care and Use of Experimental Animals (SICUAE-FMVZ-UNAM) with the number MC-2018/2-1, by Mexican legislation NOM-062-ZOO-1999. Animals were kept in a temperature-controlled room at 20°C and received water and Formulab Diet 5001 food (LabDiet, St. Louis, MO) *ad libitum*.

Study design: A completely randomized experiment with repeated measures over time, with three groups, was performed. Type 1 diabetes mellitus was chemically induced by a dose of 45 mg kg^{-1} of streptozotocin in groups G_1 and G_2.

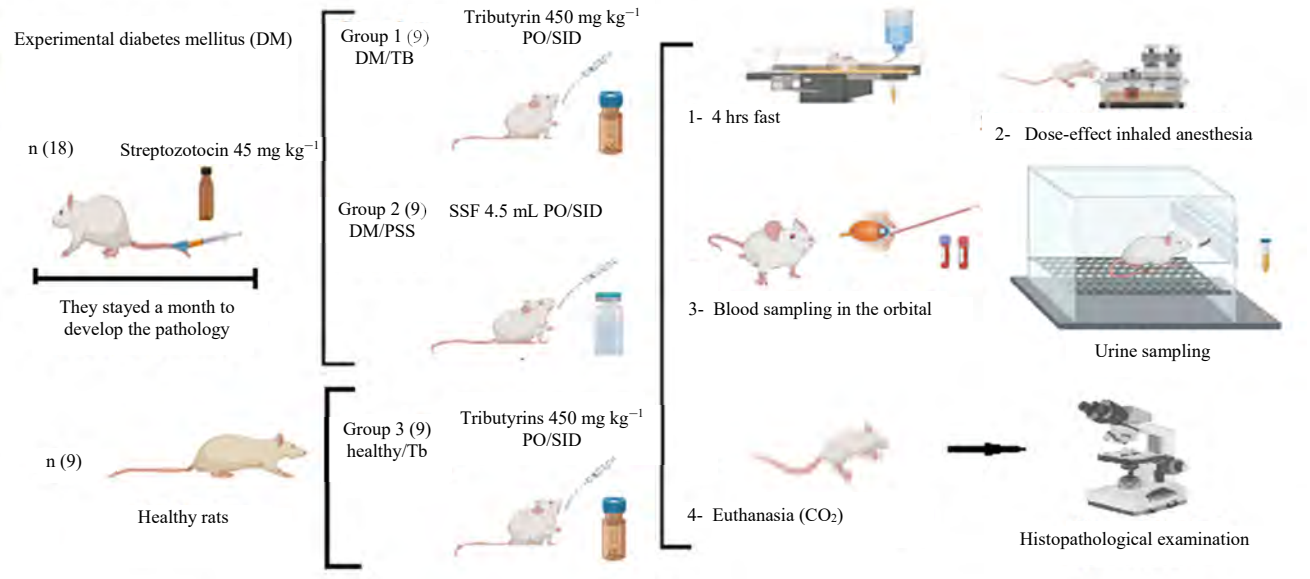

Fig. 1: Study design for *in vivo* testing of the effect of tributyrin in an animal model with type 1 diabetes

The induction of diabetes mellitus1 (MD1) was performed with 45 mg kg^{-1} Streptozotocin (STZ) intravenous injection into the tail vein (IV). After 24-48 hrs later, animals with glucose values greater than 250 mg dL^{-1} or 13.88 mmol L^{-1} were considered diabetic and remained for 1 month until liver and kidney damage developed. The animals were randomly assigned into three groups, two of them received Tb supplemented orally and one group received a physiological saline solution, a glucose curve was obtained before and at the end of treatment. HG, blood biochemistry and urinalysis were performed every week throughout the treatment (every week for 28 days). The animals were used as their controls, basal glucose concentrations were measured

Source: Picture created with biorender.com

They were maintained in this state for 1 month until they established chronic course damage to the kidney and liver tissues. Groups G_1 and G_3 were administered 450 mg kg^{-1} PO SID of Tb. The G_2 animals were treated only with physiological saline solution 4.5 mL kg^{-1} PO SID (Fig. 1). At the end of the study, after euthanasia of the animals, using CO_2 intoxication, a histopathological examination evaluated the liver, pancreas, kidneys, duodenum and colon integrity (Fig. 1).

Experimental diabetes mellitus: The animals were divided into three randomly assigned groups of nine animals each. We induced type 1 diabetes in G_1 and G_2 by IV administration of a 45 mg kg^{-1} dose of streptozotocin (STZ) applied in the tail vein. After 24-48 hrs of administration, glucose values were evaluated in animals fasting for 4 hrs, by puncturing the orbital venous sinus of the animals, previously anaesthetized with Isoflurane (Sofloran® VET, 250 mL,) a. effect dose and with the help of a glucometer (One touch-ultra mini, Johnson & Johnson) and test strips (One touch-Ultra). Diabetes was confirmed in animals that presented glucose values above 250 mg dL^{-1} and were maintained for 4 weeks to establish diabetic disease with kidney and liver damage[16]. In the third group, healthy rats were kept (without diabetes).

Evaluation of clinical parameters: A weekly blood sample (1100 µL) from all animals was taken throughout the Tb supplementation study to perform a complete blood count, blood chemistry focusing on kidney and liver profiles, glucose levels and urinalysis. Blood sampling was performed after the rats were anaesthetized (Isoflurane-Soflorant 250 mL at the effect dose) with a capillary tube without anticoagulant that was introduced into the medial canthus of the rats' eye to access the orbital sinus. Subsequently, moisturizing ointment based on sodium hyaluronate (Humectant®) is applied to the eye to reduce complications of eyeball irritation. We stored the samples in 1 mL microtainer tubes (BD Microtainer) with EDTA for blood biometry analysis and without EDTA for blood biochemistry tests.

Glucose tolerance tests: Glucose tolerance tests were performed twice, one before supplementation and the second at the end of the study, each trial consisted of 50% dextrose solution at a dose of 2 g of glucose per kg via oral, after fasting for 4 hrs. In addition, we performed blood sampling at 0, 30, 60, 90 and 120 min[17]. Samples were obtained from coccygeal vein puncture using a glucometer (One touch-ultra mini, Johnson & Johnson) and test strips (One touch-Ultra) in the three experimental groups.

Histopathological analysis: At the end of the study, after the euthanasia of the animals, using CO_2 intoxication, carried out the necropsy of 27 rats according to the standard operating procedure for carrying out these procedures in the Animal Experiment Unit (UNEXA). After registering the animals in the admission log, performed organ inspection to identify macroscopic lesions and tissue samples of the liver, pancreas, kidneys, duodenum and colon were collected. Once fixed in 10% buffered formalin, they were included in cassettes and recorded. Next, the samples were dehydrated in the histokinette and later had paraffin, from which 3-micron thick sections with the microtome. Then, they were placed in the flotation bath and later placed in the incubator. Then, conventionally stained the sections using the hematoxylin and eosin technique, according to the standard operating procedure for the histopathology technique. Finally, we observed the lamellae under the microscope.

Statistic analysis: Data were analyzed by ANOVA using SAS PROC GLM (version 9.3, SAS Ins. Inc., Cary, NC) and a Tukey-Kramer post hoc test. The model included treatment, sample, animal, sample/animal and interactions. The results express the mean of least squares and the standard error with a significance of $p<0.05$.

RESULTS

Hemogram: In all groups of rats, the values of Hematocrit (Ht), Platelets (Pl) and Lymphocytes (Ly) have within the normal parameters for the species (Table 1).

The values of leukocytes, neutrophils and eosinophils exceeded the typical reference values, but moderately, in the three groups of rats, this may be due to the dehydration of the animals during handling. In diabetic animals (G_1 and G_2), the number of leukocytes increased due to pancreatitis and necrosis of pancreatic beta cells after using streptozotocin.

The value of Hg and MCHC (mean corpuscular haemoglobin concentration) was moderately increased in the three groups of rats. In G_2 and G_3, this may be due to DM being a systemic disease impacting the hematopoietic system. Other causes of the increase may be dehydration of the animals or hemolysis during sampling due to the use of the capillary since the three groups are slightly increased.

The value of MCV (mean corpuscular volume) was moderately increased, by 12-15%, in groups G_2 and G_3, which indicates that there may be a deficiency type of vitamin B due to liver disease.

Blood biochemistry:

Liver function indicators

Cholesterol: Groups G_1 and G_2 presented slightly higher values than average for this species, there was no significant statistical difference between groups (Table 2).

Triglycerides: Groups G_1 and G_3 presented values higher than typical values for this analyte. G_2 showed statistically higher values than the groups.

Hyperlipidemia is common in untreated or poorly regulated diabetic animals. This elevation occurs by an alteration in the metabolism of fats due to the lack of insulin action. It should be noted that the experimental animals in this study did not receive insulin.

Table 1: Complete blood count (CBC) in the three experimental groups in the three experimental groups

Groups	Ht (%)	Hg (g L^{-1})	Er×10 12/L	MCV (fL)	MCHC (g L^{-1})	Pl×109/L	Lk×109/L	Ne (%)	Ly (%)	Eo (%)
RV	0.39-0.53	137-176	7.27- 9.65	48.9-57.9	329-375	638- 1177	1.96-8.25	6.2- 26.7	66.6-90.3	0.2-3.5
G_1	0.51±0.003	195 .4±1.2^b	8.86±0.8	55.8±5.9	388.4±6.6	828.4±26.5^a	14.58±2.1	27.6±1.1	67.8±1.2	4.5±0.4
G_2	0.50±0.003	199.2±1.1^b	10.1±0.7	66.6±5.5	396.6±6.2	920.1±24.8^a	16.84±1.9	31.0±0.9	64.8±1.1	4.8±0.4
G_3	0.48±0.003	188.0±1.3^a	8.55±0.8	65.0±6.6	407.6±7.5	978.9±29.8^b	14.63±2.4	27.4±1.2	67.1±1.4	5.0±0.4

Literals a, b and c show a significant statistical difference between groups, RV: Reference values, Ht: Hematocrit, Hg: Hemoglobin, Er: Erythrocytes, MCV: Mean corpuscular volume, MCHC: Mean corpuscular hemoglobin concentration, Pl: Platelets, Lk: Leukocytes, Ne: Neutrophils: Ly: Lymphocytes, Eo: Eosinophils, G_1: Diabetized rats administered tributyrin (Tb: 450 mg kg^{-1} PO SID) for 4 weeks, G_2: Group of experimentally diabetic rats, received PSS 4.5 mL kg^{-1} PO SID. G_3: Group of healthy rats administered tributyrin (Tb: 450 mg kg^{-1} PO SID) for 4 weeks

Table 2: Mean values of liver function tests reported in the three experimental groups are shown

Groups	CO (mmol L^{-1})	TG (mmol L^{-1})	ALT (U L^{-1})	AST (U L^{-1})	ALP (U L^{-1})	TB (Umol L^{-1})
RV	1.20-2.38	0.23-1.3	17-50	39-93	62-255	0.85-2.56
G_1	2.5±0.1	2.05±0.3^a	202.1±23.9^b	206.9±23.8^b	746.5±57.6^b	3.4±0.3^b
G_2	2.8±0.1	4.1±0.3^b	269.0±24.3^b	299.9±24.1^b	1005.2±58.5^c	3.8±0.3^b
G_3	2.3±0.1	1.24±0.3^a	83.8±24.5^a	116.9±24.4^a	248.9±59.0^a	2.8±0.3^a

Literals a, b and c show a significant statistical difference between groups, RV: Reference values, CO: Cholesterol, TG: Triglycerides, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, ALP: Alkaline phosphatase, TB: Total bilirubin, G_1: Diabetized rats administered tributyrin (Tb: 450 mg kg^{-1} PO SID) for 4 weeks, G_2: Group of experimentally diabetic rats, received PSS 4.5 mL kg^{-1} PO SID, G_3: Group of healthy rats administered tributyrin (Tb: 450 mg kg^{-1} PO SID) for 4 weeks

Table 3: Mean values of the renal function analysis, reported in the three experimental groups are shown

Groups	BUN (mmol L^{-1})	Ct (µmol L^{-1})	Urea (mmol L^{-1})	TP (g L^{-1})	Alb (g L^{-1})	Glo (g dL^{-1})	A:G
RV	11.42-19.3	37-53	14-22	63-86	33-49	24-39	1.58-2.67
G_1	7.9±0.3[a]	45.9±0.8	16.8±0.7[a]	67.3±0.5[b]	29.5±0.3[b]	40.1±0.5[a]	1.7±0.01[b]
G_2	9.3±0.3[b]	47.6±0.8	20.1±0.7[b]	71.1±0.5[a]	31.0±0.3[a]	41.6±0.5[a]	1.8±0.01[a]
G_3	7.04±0.3[a]	46.6±0.8	15.1±0.8[a]	75.6±0.5[a]	33.9±0.3[a]	37.8±0.5[b]	1.8±0.01[a]

Literals a, b and c show a significant statistical difference between groups, RV: Reference values, BUN: Blood urea nitrogen, Ct: Creatinine, TP: Total protein: Alb: Albumin, Glo: Globulin, A:G: Albumin: Globulin ratio, G_1: Diabetized rats administered tributyrin (Tb: 450 mg kg^{-1} PO SID) for 4 weeks, G_2: Group of experimentally diabetic rats, received PSS 4.5 mL kg^{-1} PO SID, G_3: Group of healthy rats administered tributyrin (Tb: 450 mg kg^{-1} PO SID) for 4 weeks

Table 4: Mean urinalysis values reported in the three experimental groups are shown

Groups	pH	TP (g L^{-1})	Glu (mmol L^{-1})	Ket (mmol L^{-1})	Bil (µmol L^{-1})	Blood (Hg)	Dt (erit µL^{-1})	Ct (mg dL^{-1})
RV	7.3-8.5	0.019-0.028	0.11-0.28	0.0-0.0	0.0	0.0	1.040-1.070	33.55-39.75
G_1	7.0±0.1[b]	0.15±0.1[a]	31.9±1.8[c]	0.5±0.4	0.0	23.5±6.9	1.04±0.002	25.2±2.5[b]
G_2	6.9±0.1[b]	0.16±0.03[b]	37.8±1.8[b]	1.6±0.4	0.0	31.4±7.0	1.04±0.002	40.7±2.5[c]
G_3	7.9±0.1[a]	0.11±0.1[a]	0.01±1.8[a]	0.5±0.4	0.0	13.6±7.1	1.04±0.002	16.0±2.4[a]

Literals a, b and c show a significant statistical difference between groups, RV: Reference values, pH: Hydrogen potential, TP Total protein, Glu: Glucose, Ket: Ketones, Bil: Bilirubins, Dt: Density, Ct: Creatinine, G_1: Diabetized rats administered tributyrin (Tb: 450 mg kg^{-1} PO SID) for 4 weeks, G_2: Group of experimentally diabetic rats, received PSS 4.5 mL kg^{-1} PO SID, G_3: Group of healthy rats administered tributyrin (Tb: 450 mg kg^{-1} PO SID) for 4 weeks

Alanine aminotransferase (ALT):

Aspartate Aminotransferase (AST), Alkaline Phosphatase (ALP) and Total Bilirubin (TB): The values found in these enzymes were higher than usual in all groups. The values corresponding to group G_2 presented higher statistical significance ($p<0.05$) for groups G_1 and G_3. In the case of ALP, higher values were presented numerically concerning the other groups in the rest of the enzymes.

It is reported that increased ALT values can occur in cases of pancreatitis or liver disease, when ALT, AST, TB and ALP are elevated, the patient likely has a liver disorder.

Renal function indicators: The values obtained for creatinine, urea, total proteins and the protein and albumin ratio were within the ordinary matter in all groups (Table 3).

BUN: The three experimental groups presented a value lower than usual for the species. Urea is a waste product your body makes when breaking down protein. Liver disease or damage can lower your BUN level.

Albumin: The G_1 and G_2 presented lower values than usual for the species. Albumin is a low molecular weight serum protein and filtered by the kidney in a lower percentage (1%) than its plasma level value, it is reabsorbed and metabolized in the proximal tubule, for this reason, it is a good marker of tubular dysfunction. A lower albumin level can occur in liver disease or inflammation.

Globulin: Groups G_1 and G_2 presented slightly higher values for the species, unlike group G_3, which showed typical values. (Table 3). Globulins are a group of blood proteins produced in the liver. Globulins play an essential role in liver function, blood coagulation and the immune system. Although slightly increased in groups G_1 and G_2, the globulin value did not affect the albumin:globulin ratio.

Urinalysis

pH: Group G_3 presented values within normal ranges and groups G_2 and G_3 showed more acidic values. The pH values are statistically different in the three groups, with G_2 the most acidic value. Distal tubular acidosis is associated with metabolic acidosis and pH <7 because of the impaired renal acidification mechanism (Table 4).

Bilirubin and urinary density: The three groups presented average values according to the species' ranges (Table 4).

Glucosuria: Group G_2 (DM/PSS) presented the highest values of glucose in the urine, followed by group G_1 (DM/Tb), Its appearance may be due to two factors: (1) Decreased tubular reabsorption (proximal tubulopathy) and (2) Blood levels that exceed the renal threshold, such as diabetes mellitus or other hyperglycemic states. In group G_3 (Healthy/Tb), no one had glycosuria (Table 4).

Creatinine: Groups G_1 and G_3 have a lower value than usual in eliminating creatinine through the urine, by that recommended for this species. The G_2 slightly increased. Low levels of creatinine in the urine can indicate kidney disease, as a complication of diabetes. Increased creatinine levels may be due to a temporary condition such as dehydration or developing kidney failure.

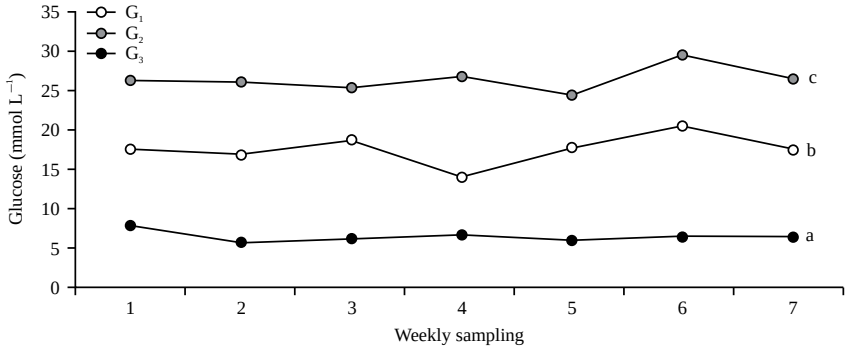

Fig. 2: Blood glucose levels in diabetic rats treated with tributyrin or PSS

Glucose reference value: 5.7-8.4 mmol L^{-1}. Each week the glycemic profile was performed after fasting for 4 hrs. A value of ≥13.88 mmol L^{-1} is considered diabetes, in the case of the group treated with Tb, the glucose value remains 1.8-2.6 higher than the reference values and in the case of diabetic rats treated with the negative control (use of placebo: PSS), its value rises from 3.1-3.8 times the maximum reference value. Therefore, insulin is recommended in conjunction with Tributyrin to control glucose levels

Total proteins

Ketones and hemoglobin in urine: Of these parameters, the three groups presented increased values in urine. These values may suggest a problem in the urinary tract of the animals, which may be due to the effect of the inhaled anaesthesia they received with isoflurane. In the case of animals with DM and that did not receive Tb, the Creatinine value is increased and in the case of Ketones, they are increased to a greater degree (three times more) than the animals that did receive Tb (G_1 and G_3). In the case of the increased Glucose value, which occurs in G_1 and G_2, formed by diabetic animals, unlike G_1 that received Tb, the deal was reduced by 15% compared to animals that did not receive Tb.

Proteinuria can be the expression of renal disease, as it occurs in nephrotic and nephritic syndromes, reflux nephropathy, or renal failure.

Blood glucose: Groups G_1 and G_2, made up of experimentally diabetic animals, exceeded the standard blood glucose reference value and showed a significant difference for group G_3 (healthy animals treated with Tb). In addition, G_1 presented statistical significance concerning G_2, which gave the highest importance. (Fig. 2). Persistent hyperglycemia was experimentally caused in rats as a result of receiving streptozotocin, which induces apoptosis of pancreatic beta cells.

Glucose tolerance curves: The glucose curve or (Oral Glucose Load Test). It is a test that measures the body's ability to metabolize glucose. This test can be used as a screening test for diabetes. The test was performed on the animals on two occasions, the initial curve before the start of tributyrin supplementation (Fig. 3a). The second curve was performed at the end of 4 weeks of supplementation (Fig. 3b). Figure 3 represented the blood glucose values by treatment by study. In this, the G_3 group presented glycemic values within ranges, followed by the G_1 group. Finally, the G_3 group showed the highest values of glucose.

Tributyrin supplementation slightly improved glycemic control, which can be seen in glucose values that remain lower in the first 30 min, in rats that already presented complications of diabetic nephropathy or liver damage, which is demonstrated in the values obtained in the analysis by treatment by the group in comparison with the values obtained before supplementation (Table 2, 3).

Histopathological analysis: At the end of the experiment, the study rats were euthanized using a CO_2 chamber, subsequently, the necropsy was carried out without finding relevant macroscopic lesions. Histopathology of the following tissues was performed: Liver, pancreas, kidneys, duodenum and colon. Hematoxylin and eosin H&E staining was performed on the tissues.

The macroscopic description of the tissues was altered in two individuals from the G_1 group (Fig. 4a), where a slightly pale liver surface was observed, in liver cells, there was moderate vacuolar degeneration compared to the G_2 and G_3 groups. Regarding the microscopic description, the group of G_1 rats presented mild multifocal vacuolar degeneration in addition to mild diffuse lymphoplasmacytic enteritis, the presence of mild inflammation of the small intestine was also reported in the group of rats (Fig. 4b), clarifying that this injury does not compromise the state of animal health. With this staining, no further histological changes were observed in the large intestine (Fig. 4c), pancreas (Fig. 4d), kidney (Fig. 4e) or spleen (4f).

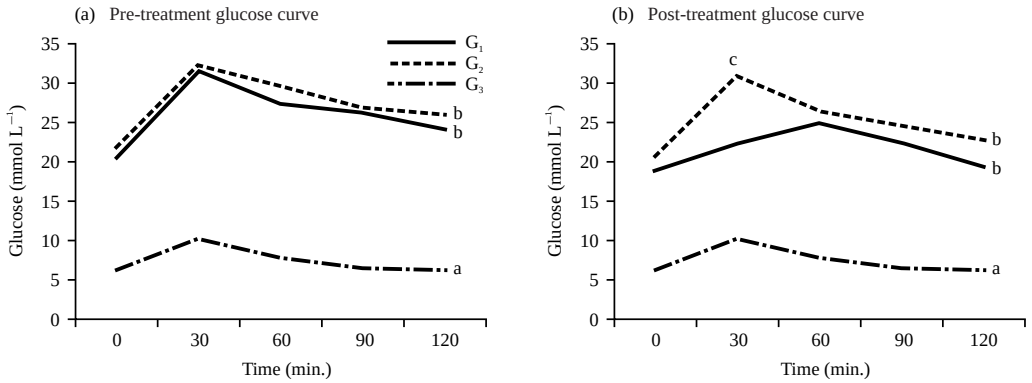

Fig. 3(a-b): Average blood glucose concentrations (mg dL^{-1}) are shown after the glucose tolerance test in the experimental rats, (a) Before initiation of tributyrin or placebo (PSS) treatment, the glucose tolerance curve was measured in rats after a 4 hrs fast and (b) was compared with the glucose tolerance curve at the end of the 4 weeks treatment

Literals a, b, c show a significant statistical difference between groups. Glucose reference value: 5.7-8.4 mmol L^{-1} samples were obtained from coccygeal vein puncture using a glucometer (One touch-ultra mini, Johnson & Johnson) and test strips in the three experimental groups, G_1: Diabetized rats administered tributyrin (Tb: 450 mg kg^{-1} PO SID) for 4 weeks, G_2: Group of experimentally diabetic rats, received PSS 4.5 mL kg^{-1} PO SID, G_3: Group of healthy rats administered tributyrin (Tb: 450 mg kg^{-1} PO SID) for 4 weeks

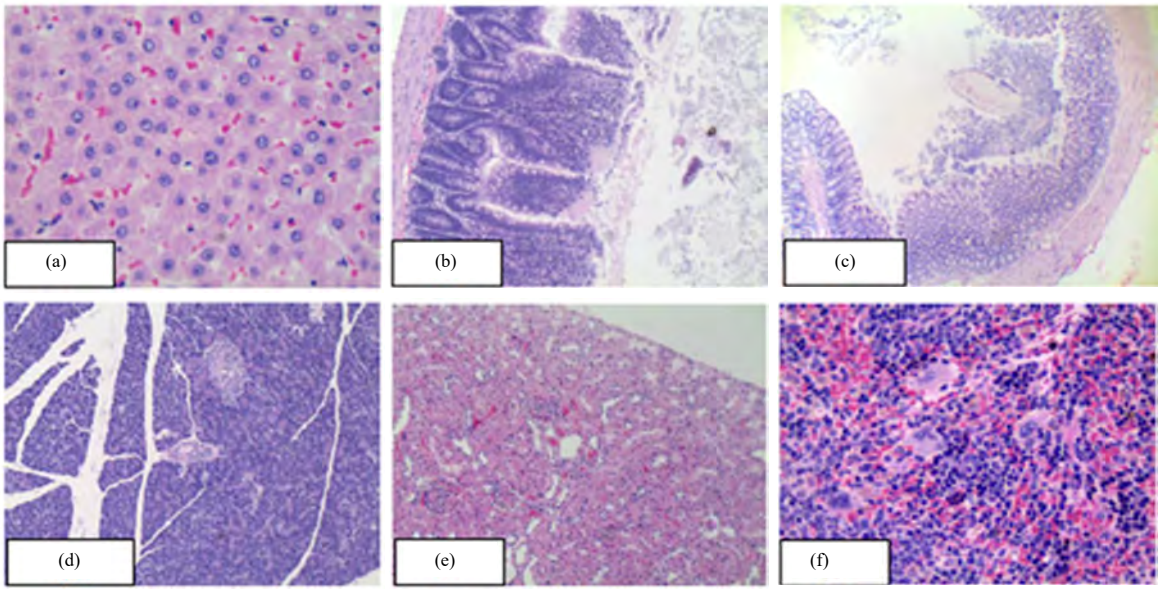

Fig. 4(a-f): Selection of photomicrographs of group G_1, (a) Healthy liver, without lesion H&E 40X, (b) Small intestine with mild inflammation H&E 4X, (c) Healthy large intestine, without lesion H&E 4X, (d) Healthy pancreas, without injury H&E 10X, (e) Healthy kidney, without damage H&E 10X and (f) Healthy spleen, without lesion H&E 40X

In Fig. 5a, it is shown in the liver that seven of the animals in this group presented moderate vacuolation in the cytoplasm of hepatocytes. In Fig. 5b. Small intestine: In four animals, low numbers of lymphocytes and plasma cells were observed expanding the lamina propria. Figure 5c-e shows the healthy large intestine, pancreas and kidney without injury. In Fig. 5f, numerous megakaryocytes are seen in the spleen.

In group G_3, liver: In three of the animals, very slightly vacuolized hepatocytes were observed, one of them also had a mild focal lymphocytic infiltrate (Fig. 6a). In Fig. 6b, Small intestine: In two of the animals, slight lymphoplasmacytic inflammation was observed in the submucosa. The rest of the organs were found without apparent histological changes (Fig. 6c-e). Spleen: Numerous megakaryocytes are observed (Fig. 6f).

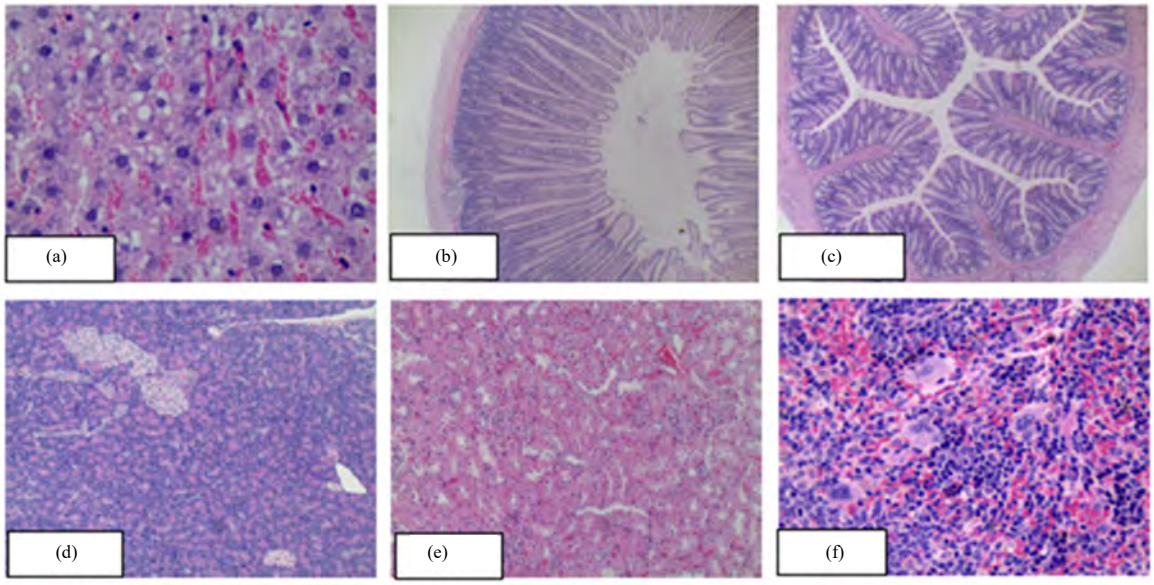

Fig. 5(a-f): Selection of photomicrographs of group G_2, (a) Liver with vacuolar degeneration H&E 40X, (b) Small intestine with mild inflammation H&E 4X, (c) Healthy large intestine, without lesion H&E 4X, (d) Healthy pancreas, without injury H&E 10X,(e) Healthy kidney, without damage H&E 10X and (f) Healthy spleen, without lesion H&E 40X

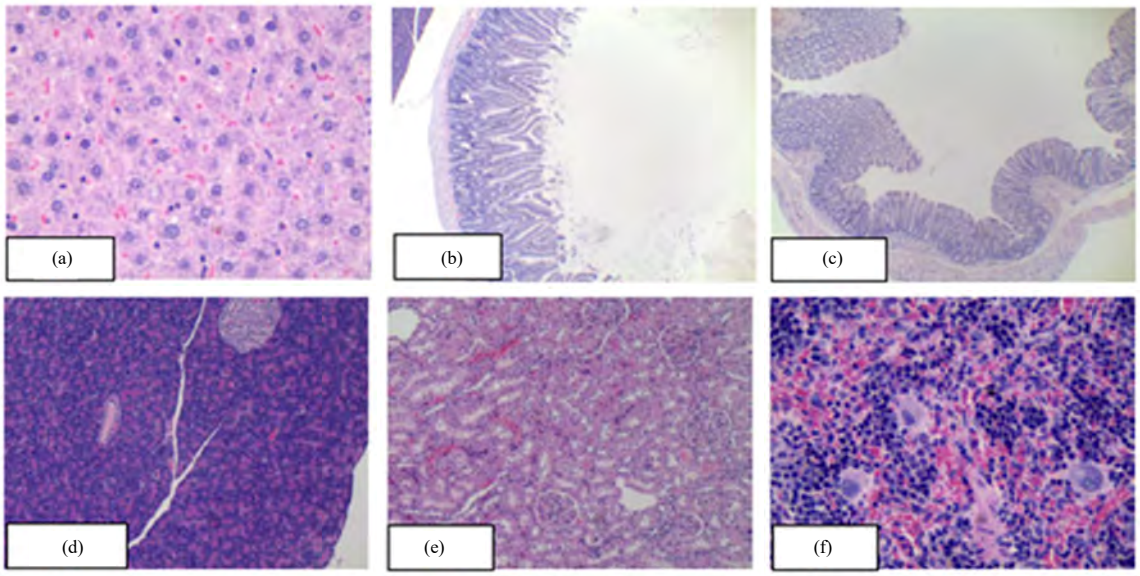

Fig. 6(a-f): Selection of photomicrographs of group G_3, (a) Liver with vacuoles in the cytoplasm, healthy tissue H&E 40X, (b) Small intestine with few lymphocytes and plasma cells, healthy tissue H&E 4X, (c) Healthy large intestine, without lesion H&E 4X,(d) Healthy pancreas, without injury H&E 10X, (e) Healthy kidney, without damage H&E 10X and (f) Healthy spleen, without lesion H&E 40X

DISCUSSION

Recent findings suggested that the population census of DM has increased significantly during the last decade and can be considered a growing epidemic at a global level, currently, 8.8% of the adult population is diagnosed with this pathology. If these trends are not reversed, it is predicted that by 2040 around 693 million people between the ages of 18 and 99, representing 9.9% of the world population, will have DM[18]. In addition, increasing dietary SCFA such as Sodium butyrate (Sb)

has been shown to have beneficial effects on the ratio of energy to colonocytes and satiety control[14,15], which is a compound with potential properties to improve the metabolism of sugars in people with DM. This study in diabetic male Wistar rats weighing 200-250 g, Tb, a prodrug of butyric acid, increased insulin sensitivity, lowered blood glucose and creatinine, protected liver structure and maintained liver density. And urinary pH, the above data suggest that tb can be a complementary therapy and accompany the usual treatment of DM to improve patients' quality of life. Tb can release a higher concentration of butyric acid (its active form) and achieve higher bioavailability in the intestines. It has also been published that the effect of butyric acid, in addition to reducing inflammation, regulates the synthesis of cytokines and decreases the proliferation of inflammatory cells[11].

In the three groups of rats, the values of Ht, Pl and Ly, were observed within the normal parameters for the species. Moderate leukocytosis was present in the three groups of animals, it may be due to dehydration, or maybe secondary to inflammation or states of metabolic stress-acidosis, in the case of diabetic animals, maybe due to the inflammatory response due to pancreatitis and necrosis of the beta cells of the pancreas due to the action of streptozotocin. The slightly higher Hg and MCHC values in all groups may be due to hemolysis during sampling due to the use of the capillary or secondary to the hemolytic crisis of any aetiology. According to what was published by Khan and Jena[3], who administered Sb in juvenile diabetic rats, found no significant difference in plasma and total albumin values and protein values compared to the respective controls[3], these results are similar to those obtained in the present investigation when administering Tributyrin, a prodrug of butyric acid. Other studies indicate that butyrate can affect the inflammatory response of the host, which, together with oxidative stress, is the main pathological factor in response to chronic hyperglycemia[19,20].

This study allowed us to observe that oral Tb supplementation for four weeks in rats with experimental diabetes mellitus (DM) helped improve glycemic control, which is consistent with what has been reported in the use of short-chain volatile fatty acids, such as butyrate of sodium[3,19,20] or by the use of propionate[21], this can be confirmed both in the treatment results by group (Fig. 2), in which it was possible to reduce the glucose value obtained by 61-76% in G_1, from those obtained by G_2, formed by diabetic animals that were not supplemented with BT, but without reaching the average value. The glucose curves (Fig. 3) expressed better glycemic control during the first 30 min, these results are similar to those reported using sodium butyrate. However, to achieve the restoration of normal glucose levels, it is recommended to take insulin treatment simultaneously, which reaffirms that Tb is a supplement that can accompany the standard treatment of DM (insulin). Unfortunately, this circumstance was not evaluated in this study.

The liver is the main organ responsible for primary metabolism, related to carbohydrates, proteins, lipids, porphyrins and bile acids[22]. With the hepatic (enzymatic) profile, it is possible to detect if there is inflammation or cell necrosis and to assess its extension. The enzymes analyzed in this study are Aspartate aminotransferase (AST formerly TGO) is located in the mitochondria and cytoplasm of cells, while alanine aminotransferase (ALT formerly TGP). is only in the cytoplasm, hence, in moderate hepatocellular damage, ALT reaches levels higher than AST, while the latter predominates in cases in which there is some accentuated hepatocellular damage and necrosis. (Table 2) In addition, ALT has a longer half-life than AST in serum, so elevated values often persist for an extended time to detect hepatocellular damage[23].

These enzymes act on phosphoric acid's aliphatic, aromatic and heterocyclic esters. In patients with DM, depending on the severity of the disease, an increase in the levels of ALT, AST, TB and ALP may occur due to the possibility that the patient has a liver disorder, also due to pancreatitis as a result of the highly selective cytotoxicity for pancreatic islet β cells of streptozotozin[24,25], in experimental DM groups (G_1 y G_2). According to the results of blood biochemistry, experimentally diabetic animals, G_1 and G_2, presented liver damage. The ALT and AST values did not show significant differences between groups G_1 and G_2, however, Tb supplementation helped reduce the values of these enzymes in G_1 concerning G_2: ALT reduced it by 33%, AST by 44% and ALP by 34%.

Dyslipidemia is a concomitant condition of diabetes and there is an increase in fats in the blood (cholesterol and triglycerides). It can cause other diseases, especially in the heart and blood vessels[26]. In the present study, in the case of cholesterol levels, after Tb treatment, cholesterol values improved slightly, by 12%, concerning what was found in G_2, in the case of triglycerides, it reduced values by 50%. In total bilirubin 11%, these values confirm that Tb did produce a beneficial effect on liver function.

As corroborated, the results suggest that Tb has a partial positive effect on liver function. Also, Miyoshi *et al.*[27] reported that Tb, administered orally, inhibits the secretion of Tumour Necrosis Factor (TNF)-α by attenuating the activation and expression of nuclear factor-κB (NF-κB), protecting against liver damage in rats. Furthermore, tributyrin, the prodrug of butyrate, is reported to increase the level of plasma butyric acid in the hepatoportal system and attenuate liver injury and

subsequent inflammatory responses. On the other hand, Miyoshi *et al.*[28] mention that Tb administered orally reduced the increase in plasma triglycerides and the total cholesterol and cholesterol levels bound to Low-Density Lipoproteins (LDL-C). In addition, it prevents the elevation of these through greater oxidation of fatty acids in endotoxemic rats, the authors mention that tributyrin increased the expression of PPAR and histone H3 in the liver at basal levels. The G_3 group (healthy animals) that received Tb presented an elevation of the enzymes: ALT, AST and a slightly higher value of Total Bilirubin. It may be due to the isoflurane anaesthesia to which the animals were subjected on five occasions or to hemolysis during sampling due to the use of the capillary[23].

The measurement of urea and creatinine is essential for the monitoring and prognosis of established kidney disease[29,30]. In the present study, the values of creatinine, urea, total proteins and the albumin: Globulin ratio in all groups were typical (Table 3). In the case of creatinine, the values rise even when there is greater than 50% damage to glomerular filtration. In addition, slightly reduced Blood Urea Nitrogen (BUN) levels were in all groups, suggesting incipient liver damage. In the case of albumin, in groups G_1 and G_2, they presented lower values than usual in the species; this affectation can occur in malnutrition, liver disease, or inflammation. In the case of globulin G_1 and G_2, they presented slightly higher values, suggesting damage to liver function, blood coagulation or the immune system. The G_1 showed better deals in the BUN value by 17 and 5% in Albumin and 3% in the globulin value, for G_2. That suggests that the Tb dose or the treatment time was insufficient for better kidney protection regarding these parameters.

It has been published that butyrate of sodium supplementation in DB rats helps to decrease blood creatinine values, as reported by Machado *et al.*[1] and Lau *et al.*[7]. According to Gonzalez *et al.*[19], the Sb reduced elevated serum urea and proteinuria in nephrectomized rats and mild to moderate impact on renal failure. However, it did not have a significant impact effect on serum creatinine[19]. The author mentioned that these positive effects could be secondary to improving metabolic parameters (glucose tolerance, insulin tolerance and gluconeogenesis). On the other hand, the results obtained in the present work were similar to those published by Khan and Jena[3], who showed that Sb treatment improved kidney function and damage in juvenile rats[3].

The urine test gives us information about all the organs and systems of the body[31]. Urinary sediment examination is essential for the early recognition of infectious, inflammatory and neoplastic conditions that affect the body and the urinary system[32].

In the urine pH value, which in the rat is 7.3-8.5, groups G_2 and G_3 presented more acidic values (7 and 6.9, respectively), which suggests that the animals gave distal tubular acidosis or metabolic acidosis, with time and the supplemented dose of Tb, it was not enough to alkalinize the urine. The three experimental groups presented increased total protein values; however, the G_1 group supplemented with Tb managed to reduce the absolute protein values in the urine by 6% compared to G_2. Glucose values increased in G_1 and G_2, but in G_1, reduced this value by 18%, thanks to the Tb supplement. The three groups presented increased ketones, creatinine and haemoglobin values in the urine, however, G_1 managed to reduce the concentration of haemoglobin by 33% compared to G_2 (Table 4).

No positive effects on blood protein values were observed in the present study. Other authors report that butyric acid and its prodrugs help control protein levels in urine, however, it was not evident in our experiment, perhaps due to the time or the applied dose of Tb[33,34,19].

In ketones, when blood glucose levels rise due to insulin deficiency, preventing cells from taking advantage of it, the body begins to use the fats consumed to produce energy, this process has waste that is harmful to the called ketones[26] and in this aspect, G_1 managed to reduce three times the value found for this analyte compared to G_2, which was not supplemented with Tb.

Supplementation with tributyrin helped metabolic control in rats with diabetic nephropathy. Evidenced by better management of urinary pH, maintaining creatinine in urine at an average value and decreased glycosuria, however, it shows partial positive effects on matters of ketone bodies and haemoglobin in urine.

Tb protects the liver structure, which is confirmed by histological studies. On histopathological examination of animals with experimental DB, it was accompanied by multifocal mild vacuolar hepatic degeneration and diffuse mild lymphoplasmacytic enteritis. The most significant lesion was observed in the liver of the animals of group 2, where the hepatocytes present vacuolar degeneration, compared to the animals of groups G_1 and G_3. The lesion was much milder and presented in a significant number smaller proportion (Fig. 4-6).

On the other hand, most of the animals in group 2 presented mild inflammation of the small intestine, however, this is a nonspecific lesion and does not compromise the health status of the animals. Furthermore, the megakaryocytes observed in the spleen of all animals are average when it comes to young animals-no histological changes were observed in the pancreas, kidney or large intestine[35,36].

In the experiment, isoflurane was used as an anaesthetic to facilitate the intravenous administration of streptozotocin and to obtain samples for blood chemistry and CBC analysis from retroorbital sinuses of rats. Variable results have been published regarding the toxicity of inhalational anaesthetics, which usually appear in the liver and kidney, where they are metabolized. However, the metabolism of isoflurane occurs in a small percentage, so the amounts of fluoride ions and trifluoroacetic acid generated in this degradation (in the urine) are insufficient to cause damage to renal cells[37-40]. As for acute exposure, having a low lipid solubility, ISO has minimum retention of the drug in the adipose deposits of the organism, little hepatic metabolism and minimum renal excretion of metabolites, according to Kharasch *et al*.[41] and Andrew *et al*.[42].

The ISO has also been reported to have harmful effects on insulin secretion and glucose homeostasis. Therefore, they are not recommended for measuring metabolic parameters in animal models with abnormal glucose metabolism. Also, the anaesthesia time could be related to changes in blood biochemical parameters. The present study used to anaesthetize the animals for a few minutes every week throughout the experiment, so the animals were subjected to anaesthesia five times. Aronson[38], evaluated the effects of anaesthesia in people for more than 10 hrs with isoflurane and reported that the concentrations of glucose increased during and after surgery, but insulin increased only after surgery, glucagon fell in the experimental groups. However, the results of Daş, *et al*.[43], who anaesthetized adult pigs with ISO did not find any changes in glucose, lactate, cholesterol, urea, glucagon, insulin and cortisol. But did report that triglycerides increased within the first 45 min, which may explain the results in the experimental animals of G_3.

For their part, Sano *et al*.[44] measured the effect of ISO anaesthesia in Sprague-Dawley rats when the fasting serum glucose level increased and the plasma insulin concentration decreased in the groups anaesthetized with isoflurane at 45 min of induction of anaesthesia.

To predict insulin sensitivity and pancreatic β-cell function, found that serum concentrations of total cholesterol, LDL cholesterol, HDL cholesterol and triglycerides did not differ in the groups of rats, which were anaesthetized with ISO were not affected, however, the glucose metabolism if affected by ISO, since it induces hyperglycemia, by activating KATP channels and thus inhibiting insulin release from pancreatic β cells.

Published research shows the beneficial effect on DM of sodium butyrate obtained as a diet rich in fibre, probiotics or the exogenous administration of butyrate as a drug. In the present study, the administration of tributyrin, as a butyric acid prodrug, is another option to achieve glycemic control and other effects such as histone deacetylase inhibition. This enzyme plays a critical role in modulating fat beta cells, modifying the pathogenesis of diabetes mellitus and its complications. The results obtained in this research are encouraging. However, it is necessary to continue the investigation, investigate the long-term effects and approach glycosylated albumin measurement. The long-term study would allow histological observations on the impact of tributyrin on renal, hepatic and pancreatic damage due to fibrosis.

CONCLUSION

In conclusion, the potential benefits of tributyrin and its therapeutic implications for clinical application were presented. Results suggested that tributyrin can be considered a complementary supplement to the standard treatment of diabetes mellitus, which will help improve the patient's health, it can investigate further by varying the dose administered and the time of treatment and using different experimental and clinical settings for the treatment of DM1 and DM2.

SIGNIFICANCE STATEMENT

The present study found that oral tributyrin supplementation in rats with diabetes mellitus with a chronic course, improved glycemia allowing a hepatoprotective effect and delayed kidney disease. Tributyrin has excellent potential to be a complementary therapy to the usual treatment of diabetes mellitus.

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