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Effects of Combined Resveratrol Plus Metformin Therapy in *db/db* Diabetic Mice

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Abstract

Background: The worldwide prevalence of Type 2 diabetes mellitus is associated with other conditions that trigger metabolic syndrome. Although several studies on the benefits of resveratrol have been carried out, few have assessed this drug in combination with metformin.

Objectives: This study looks at the effects that combined metformin/resveratrol therapy has on body weight gain and liver and renal damage of *db/db* diabetic mice. It also addresses biochemical findings.

Method: Diabetic mice were treated with resveratrol (20 mg/kg/day), metformin (150 mg/kg/day) and combined metformin/resveratrol therapy for 5 weeks. Histopathological tissue analyses and biochemical parameters (glucose, insulin, triglycerides and cholesterol), functional liver enzymes (AP, AST and GGT) and renal parameters (urea and uric acid) were examined.

Results: Our data clearly showed that combined metformin/resveratrol treatment reduced obesity, glucose and triglyceride levels, as well as improving renal function and partially improving liver function in diabetic mice.

Conclusion: The combined therapy may enhance remedial effects in diabetic patients as well as in other metabolic disorders, such as metabolic syndrome.

Keywords: Type 2 diabetes mellitus; Metabolic syndrome; Metformin; Resveratrol; Glucose; Triglycerides; Steatosis

Introduction

Type 2 Diabetes mellitus (T2DM) is a globally prevalent chronic illness associated with high morbidity, disability, and mortality rates, especially in developing countries [1]. T2DM is a multifactorial disease that combines hereditary and environmental factors. Diabetes mellitus is characterized by two major metabolic dysfunctions: decreased insulin secretion by pancreatic beta cells, and peripheral resistance to insulin action [2]. T2DM in combination with other metabolic alterations is known as metabolic syndrome. This is a common disorder characterized by increases in waist circumference, blood pressure and triglyceride levels, as well as a reduction in high-density lipoprotein cholesterol (HDL-C) levels and evidence of glucose intolerance [3].

Metformin is unanimously considered a first-line glucose-lowering agent in the pharmacological management of type 2 diabetes, used either alone or in combination with other antihyperglycaemics [4]. There are no clinically relevant pharmacological interactions with metformin because this compound is not metabolized and does not inhibit the metabolism of other drugs [5]. The antihyperglycaemic properties of metformin are mainly attributed to its suppression of hepatic glucose production, especially hepatic gluconeogenesis, and slightly increased peripheral tissue insulin sensitivity. This drug has hypoglycemic effects by reducing basal and postprandial blood glucose [4].

Resveratrol is an antioxidant found in several plants, especially in the skin of red grapes, and has numerous health-promoting effects in both animals and humans. It has been recently found to have beneficial effects in animals with experimental insulin-deficient diabetes, including antihyperglycemic action and protection of pancreatic β-cells, and could potentially support the conventional treatment of type 1 diabetes [6-8]. Resveratrol regulates numerous intracellular signaling pathways in both humans and other animals, resulting in disparate cellular functional alterations with distinct clinical implications. Although resveratrol is still not an approved drug for use in humans, it has yielded beneficial effects in patients suffering from a wide variety of diseases, including diabetes, arthritis, cancer, epilepsy, proliferative retinopathy and renal failure [9-13].

Metformin appears to be more effective at treating insulin resistance than resveratrol. However, the potential beneficial effects of combined metformin/resveratrol therapy on glucose homeostasis has been previously reported given it increases insulin signaling in adipose tissues and muscle [7]. Our aim was to evaluate the pharmacological effects of resveratrol and metformin, alone and in combination, in obese *db/db* diabetic mice.

Material and Methods

Animal maintenance

Six-week-old male *db/db* diabetic mice (BKS.Cg-+ Lepr^{db}/+Lepr^{db}/OlaHsd) were obtained from Envigo Laboratories Inc. Mexico (Cat. No. T.2018S.15). The animals were housed in a temperature and humidity controlled environment and allowed food (Standard Purina Chow Diet, Mexico) and water *ad libitum*. The experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals [14].

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Pharmacological treatments and sample collection

After acclimatization, the animals were divided into the following groups, each one consisting of 5 animals. Non-diabetic lean mice comprised the control group. The diabetic mice were randomly assigned to four groups: group 1 received 500 µL of water orally; group 2 received metformin at a daily dose of 150 mg/kg body weight p.o. in 500 µL of water; group 3 received resveratrol in doses of 20 mg/kg body weight/day p.o. in 500 µL of water, and group 4 received a combined metformin/resveratrol treatment (150/20 mg/kg). During treatment, all groups were under a controlled diet and received water *ad libitum*. All treatments were administered for 5 weeks. Metformin hydrochloride was purchased from Wanbury Limited (Maharashtra, India), and resveratrol (resVida®) was supplied by DSM Nutritional Products (Mexico, Mexico). Both were dissolved in sterile deionized water prior to experimental use. Mice body weight was monitored throughout the study. After treatment, animals were starved overnight and sacrificed under light chloroform anesthesia. Blood and tissue samples (pancreas, kidney, liver and fat) were collected from each animal and kept at -4°C until further study.

Biochemical analysis

Serum from blood samples was collected by centrifugation, and triglycerides, cholesterol, glucose and insulin levels were quantified using colorimetric methods (Triglycerides SL, Cholesterol PAP SL, Glucose PAP SL, ELITech, Mexico and rat/mouse insulin ELISA kit from Millipore, USA). Liver enzymes, aspartate amino transferase (AST), gamma glutamyl transpeptidase (GGT) and alkaline phosphatase (AP) were also quantified using a colorimetric method (AST, GGT and AP SL, ELITech, Mexico). Renal function was analyzed by measuring urea and uric acid levels in blood using commercial kits (Urea SL and Uric Acid Mono SL, ELITech, Mexico) and in accordance with manufacturer protocol.

Histopathological analysis

Tissue fragments of pancreas, kidney, liver and fat were fixed in 10% formaldehyde solution, dissolved in phosphate-saline buffer (pH 7.4), dehydrated in alcohol and embedded in paraffin. Four-micrometer paraffin sections were stained with hematoxilin and eosin (H&E) and subjected to histopathological examination.

Data Analysis

Weight control values from mice in each treatment group were analyzed and compared; we employed descriptive statistical parameters as means ± standard error of the mean (SEM). The biochemical data (hepatic enzymes, renal parameters, triglycerides, cholesterol, glucose and insulin) were analyzed with SPSS 10.0 software (SPSS Inc., Chicago, Ill, USA) using ANOVA, as well as Dennett's test for multiple comparatives. Differences were considered significant if the p value was less than 0.05.

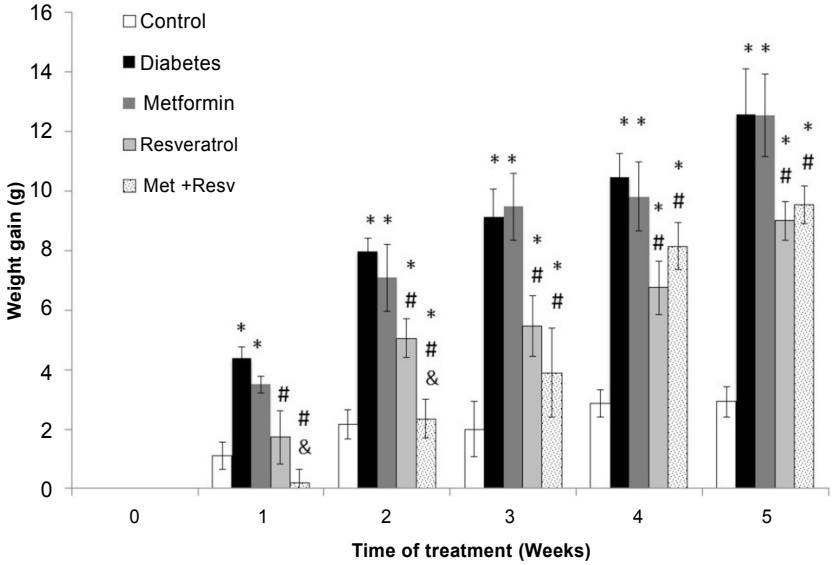
Results

Body weight gain of *db/db* diabetic mice

Figure 1 shows body weight gain for all groups. This was significant in diabetic mice when compared to the control group ($p<0.05$). Diabetic mice treated with metformin showed a weight gain similar to that of untreated diabetic mice. Animals treated with resveratrol alone showed a significant body weight reduction when compared with diabetic mice, as well as those mice treated with metformin alone ($p<0.05$). However, animals treated with metformin and resveratrol showed a significant reduction in weight gain, mainly during the first three weeks ($p<0.05$). During the last two weeks of treatment, their weight gain was similar to that of animals treated only with resveratrol. All animals had a gradual increase in body weight, but weight-gain reduction was better among those groups treated with resveratrol, either alone or combined.

Biochemical findings in *db/db* diabetic mice

Table 1 shows the biochemical parameter analysis of all groups at the end of treatment. All diabetic animals had high levels of glucose as compared with non-diabetic lean mice ($p<0.05$). However, a significant decrease in glucose levels was observed in groups treated with metformin, resveratrol, and both drugs combined when compared with untreated diabetic mice ($p<0.05$). The lowest observed value (308 ± 37 mg/dL) belonged to those animals treated with the metformin/resveratrol combination ($p<0.05$).



Note: Data are means ± S.D. n=5, *p<0.05 compared with control group, #p<0.05 compared with diabetic group and p<0.05 compared with single treatment group.

Figure 1: Body weight gain of *db/db* diabetic mice.

Group	Glucose mg/dL	Triglycerides mg/dL	Cholesterol mg/dL	Insulin ng/mL
Control	189.0 ± 4.3	148.0 ± 13	101.0 ± 5.5	1.8 ± 0.6
Diabetes (D)	486.0 ± 32*	505.0 ± 89*	167.0 ± 20*	10.0 ± 2.1*
D + Metformin	362.0 ± 31*#	325.0 ± 63*#	142.0 ± 21*	7.1 ± 3.2
D + Resveratrol	387.0 ± 20 *#	312.0 ± 95*#	90.0 ± 25*#	8.1 ± 1.6*
D + Metformin + Resveratrol	308.0 ± 37 *#	288.0 ± 86*#	136.0 ± 8.34*	10.6 ± 3.5*
Data are means ± S.D. n=5, *p<0.05 compared with control group, #p<0.05 compared with diabetic group.				

Table 1: Biochemical parameters analyzed in diabetic mice.

All diabetic animals had high triglyceride levels when compared with non-diabetic lean mice ($p<0.05$). However, all treatments significantly decreased serum triglyceride levels ($p<0.05$). The combined metformin/resveratrol treatment reduced 57% of these values when compared with diabetic mice ($p<0.05$). The resveratrol treatment lowered cholesterol the most effectively ($p<0.05$). Although animals treated with metformin and combined therapy also showed a reduction in cholesterol levels, there were no significant differences with diabetic mice (Table 1).

On the other hand, diabetic mice showed increased insulin levels when compared with the control group ($p<0.05$). However, no significant changes were observed in the insulin levels of animals treated with metformin, resveratrol and combined therapy as compared with those of diabetic mice.

Hepatocellular damage and cholestasis analysis in *db/db* diabetic mice

Table 2 shows the analysis of liver enzymes in animals treated with metformin, resveratrol and combined metformin and resveratrol. As we can see, the *db/db* diabetic mice showed a significant increase in AP (1.95-fold), GGT (2.12-fold) and AST (1.65-fold) levels when compared with the control group ($p<0.05$). Diabetic mice treated with resveratrol, metformin and combined therapy showed a significant reduction in AP levels ($p<0.05$) when compared with diabetic mice. Only the resveratrol treatment reduced AST levels in diabetic mice ($p<0.05$).

Renal function analysis in *db/db* diabetic mice

Renal function analysis of *db/db* diabetic mice showed a significant increase in urea and uric acid levels ($p<0.05$) (Table 2). Diabetic animals treated with metformin, resveratrol or combined therapy showed a significant reduction in urea and uric acid when compared with untreated diabetic mice ($p<0.05$), though this decrease did not compare to that of the control group.

Histopathological findings

Figure 2 shows the representative histological changes observed in the livers of the different treatment groups. By week 5, diabetic mice showed hepatocellular injuries characterized by centrilobular, micro and macrovesicular fatty infiltration, necrosis, ballooning degeneration and pleomorphic nuclei; the same liver architecture was observed in those mice treated with metformin. Resveratrol treatment showed a significant improvement in liver architecture, non-fatty infiltration and hepatocellular degeneration. Although the combined metformin/resveratrol therapy likewise showed an improvement in liver architecture, we also observed areas with fatty infiltration (Figure 2). These results correlated with improvements in hepatocellular damage and cholestasis (Table 2). Figure 3 shows a representative microphotography of the pancreas. As we can see, the size of the islets of Langerhans in the pancreatic tissues of diabetic mice was large and more abundant in the lobule when compared to normal mice tissues; the acini (exocrine pancreas) did not show significant changes. Diabetic mice treated with metformin showed reduced islets of Langerhans and more abundant acini when compared with diabetic mice, the size of their islets and acini were quite similar to those found in control mice. Animals treated with resveratrol showed large and small islets and a reduction of the exocrine pancreas; pancreas characteristics were similar to those of diabetic mice.

Combined therapy yielded similar results. However, the size of the islets was smaller than in diabetic mice and those animals treated with resveratrol alone. The exocrine pancreas did not show significant changes. All treatments showed an absence of necrosis and fibrotic changes. Figure 4 shows the morphological aspect of the kidneys of diabetic mice. There was an absence of Bowman's capsule, glomerular atrophy, while tubular necrosis and inflammatory infiltration were present. Animals treated with either metformin or resveratrol showed normal kidney tissue architecture when compared with the control group (normal renal tubules and glomerulus). The combined therapy yielded similar results.

Finally, Figure 5 shows the difference in adipocyte size under each of the treatments. Diabetic mice showed a significant increase in the size of all fat cells. Metformin, resveratrol and combined therapy greatly decreased the size of adipocytes when compared with untreated diabetic mice.

Discussion

This study examined the benefits of metformin, resveratrol and metformin/resveratrol combined treatment in *db/db* diabetic mice. These treatments were administered for 5 weeks, at the end of which we evaluated different parameters such as glucose, triglycerides, cholesterol, insulin, liver and kidney function, as well as ultrastructural changes in tissues (pancreas, kidney, liver and fatty tissue) to determine the benefits of resveratrol alone and in combination with a reference drug (metformin).

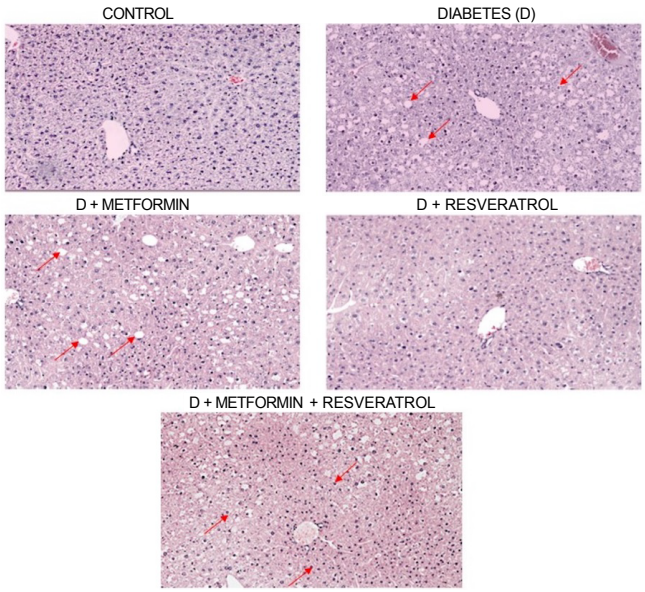
The diabetic mice model was employed given its usefulness in the study of diabetes and other co-morbidities. *db/db* diabetic mice have an autosomal recessive mutation in the Leprdb gene in chromosome 4 plus a Leptin receptor deficiency. They are obese and exhibit several metabolic characteristics such as hyperinsulinemia, depletion of insulin-producing islets, hyperglycemia, hyperlipidemia, hypertriglyceridemia, insulin resistance, and hyperglucagonaemia. They also show non-alcoholic steatohepatitis, nephropathy and pancreatitis [15,16].

The effects of resveratrol on metabolic health have been well-studied during the past decade. Preclinical studies have established this compound has beneficial effects in animals with experimental insulin-deficient diabetes, including antihyperglycemic action and protection of pancreatic β-cells. It has the potential to support the conventional treatment of type 1 diabetes, though its beneficial effects are not exclusive to glucose metabolism. Previous research demonstrates that resveratrol can reduce glucose, triglyceride and cholesterol blood levels, as well as ameliorate renal and hepatic function in obese Zucker

Group	Alkaline phosphatase (U/L)	Glutamyl transpeptidase (U/L)	Aspartate amino transpherase (U/L)	Urea (mg/dL)	Uric acid (mg/dL)
Control	122.0 ± 16.0	6.2 ± 0.09	230.0 ± 15.0	78.0 ± 4.0	4.2 ± 0.75
Diabetes (D)	239.0 ±13.1*	13.2 ± 2.19*	380.0 ± 110.0*	105.0 ± 6.0*	18.0 ± 0.5*
D + Metformin	171.2 ± 14.3*#	12.0 ± 3.84*	260.0 ± 10.0*	66.0 ± 2.0 #	8.7 ± 0.6*#
D + Resveratrol	152.5 ± 4.7*#	10.8 ± 1.09*	252.0 ± 30.0*#	51.6 ± 0.5 #	7.5 ± 1.2 *#
D +Metformin + Resveratrol	178.0 ± 16.0*#	17.0 ± 0.08*	340.0 ± 98.0*	54.0 ± 6.0 #	10.6 ± 0.4 *#

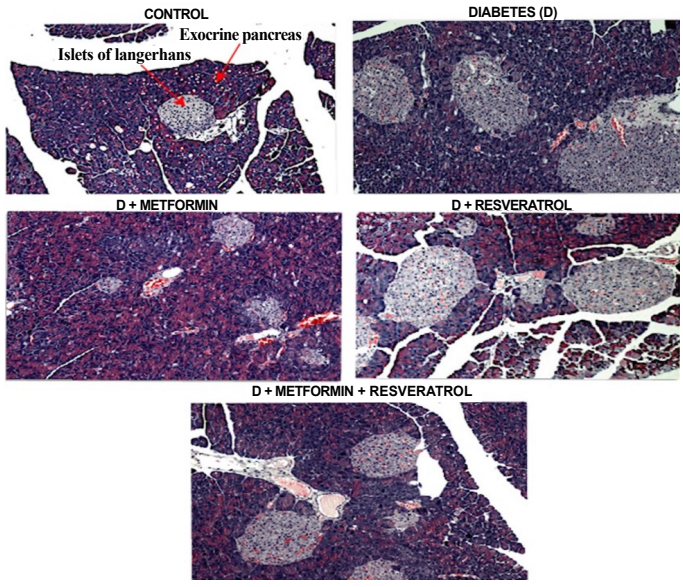
Data are means ± S.D. n=5, *p<0.05 compared with control group, #p<0.05 compared with diabetic group.

Table 2: Renal and hepatical parameters analyzed in diabetic mice.



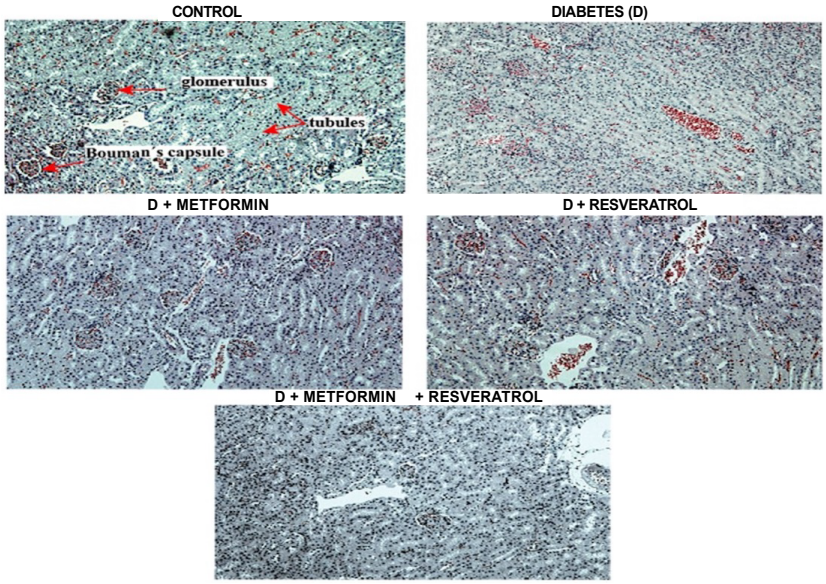
Note: Our analysis does not exhibit signs of toxicity. The arrows point to fat accumulation. The integrity of tissues was analyzed after staining with hematoxylin and eosin (magnification 20X).

Figure 2: Representative light microscopy of liver tissues from male *db/db* mice groups for control, diabetic, metformin, resveratrol and metformin+resveratrol treatments.



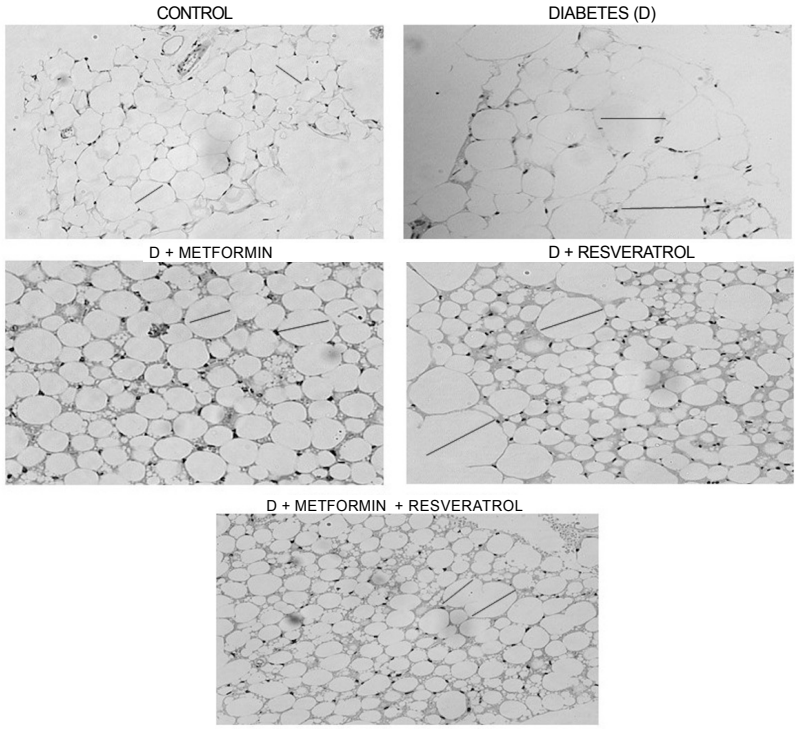
Note: The integrity of tissues was analyzed after staining with hematoxylin and eosin (Magnification 20X).

Figure 3: Representative light microscopy of pancreas tissues from male *db/db* mice groups for treatments control, diabetic, metformin, resveratrol and metformin+resveratrol treatments.



Note: The integrity of tissues was analyzed after staining with hematoxylin and eosin (magnification 20X).

Figure 4: Representative light microscopy of kidney tissues from male *db/db* mice groups for control, diabetic, metformin, resveratrol and metformin+resveratrol trreatments.



Note: The integrity of tissues was analyzed after staining with hematoxylin and eosin (magnification 20X).

Figure 5: Representative light microscopy of fat tissues from male *db/db* mice groups for control, diabetic, metformin, resveratrol and metformin+resveratrol treatments.

rats when administered 5 mg/kg/day for 30 days [17]. Thus, we decided to evaluate the combination of this compound with a reference drug, metformin, in diabetic mice with additional metabolic disorders.

Animal models of obesity have demonstrated resveratrol can have beneficial effects on glucose homeostasis, diabetes and metabolic dysfunction [18]. Different researchers have shown that resveratrol decreases blood glucose and increases insulin secretion [8,19]. Rivera et al. found that 10 mg/kg/day of resveratrol doses over 30 days improved insulin sensitivity and decreased hyperglycemia in Zucker fat rats [20]. Administration of resveratrol to streptozotocin/nicotinamide-induced

diabetic rats significantly decreased insulin resistance [21]. Our results agree with previous reports and demonstrate a significant decrease in glucose levels in *db/db* diabetic mice treated with metformin, resveratrol and combined therapy. However, although all three therapies reduced glucose levels, the pharmacological treatment produced different effects on the pancreas. The histological findings showed reduced islets of Langerhans in animals treated with metformin, while resveratrol and metformin/resveratrol treatments yielded large and numerous islets and a reduction of exocrine acini. In type 2 diabetic human disease, insulin resistance leads to an increased demand for insulin, β-cells secrete more hormone and blood insulin levels are initially elevated. Although the histological findings showed large islets of Langerhans in diabetic mice, as well as in those animals treated with resveratrol alone and combination therapy, these results were not functional because the insulin serum levels did not show a significant reduction. Animals treated with metformin showed a significant reduction in the size and number of islets, but these findings did not correlate with insulin levels.

Excessive body weight and obesity can exert negative metabolic health effects, partly via accumulation of fat in the liver [22]. Such accumulation, when unrelated to alcohol intake, is a strong independent marker of dyslipidaemia and insulin resistance [22]. Pre-clinical studies have revealed promising results regarding the beneficial effects of resveratrol in preventing and reversing obesity-induced metabolic disturbances [23]. Specifically, rodents supplemented with resveratrol have shown improved mitochondrial function, insulin sensitivity and liver fat accumulation [18]. Here we used obese diabetic mice with liver steatosis and demonstrated that resveratrol administration (20 mg/kg/day) and combined treatment (resveratrol/metformin) produced a significant reduction in weight-gain, mainly during the first three weeks, which metformin did not do. The weight-gain reduction induced by resveratrol alone and metformin/resveratrol was associated with a significant reduction in the size of adipocytes at the end of treatment; we believe that the decrease in weight-gain was directly related to the reduction in the size of fat cells.

Hepatic fat accumulation is a well-known complication of type 2 diabetes. Fat is stored in the form of triglycerides and may be a manifestation of increased fat transport to the liver, enhanced hepatic fat synthesis, and decreased oxidation or removal of fat from the liver. Obesity entails excessive fat accumulation accompanied by lobular inflammation and steatonecrosis. Our study showed diabetic mice with high levels of triglycerides and cholesterol, fatty infiltration and necrosis in the liver, and alterations in hepatic enzymes. Previous studies report that metformin and resveratrol diminished the activity of pathophysiological enzymes such as aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (AP) [24,25]. Our results are consistent with previous reports, although our study only showed a reduction in AP levels under all treatments ($p<0.05$). Only resveratrol treatment reduced AST levels in diabetic mice, and those animals showed amelioration of hepatic steatosis. Animals treated with combined therapy showed a reduction in liver hepatic steatosis. There were no observable changes in liver transaminase levels.

It has been reported that resveratrol ameliorates hyperglycemia-mediated renal dysfunction or diabetic nephropathy by reducing urinary levels of urea, creatinine, albumin and albumin to creatinine ratio, as well as by increasing levels of proinflammatory proteins [26]. Previous studies show diabetic rats treated with resveratrol (5 mg/kg) for 30 days had a significant decrease in urea, uric acid and creatinine levels [17]. Our results showed that all treatments decreased urea and uric acid levels, and those findings correlated with the histological changes in kidney tissue.

This study demonstrated that metformin/resveratrol combination therapy reduces obesity, glucose and triglyceride levels, as well as improving renal function and partial liver damage in diabetic mice. These data suggest said combined therapy may enhance remedial effects in diabetic patients as well as those suffering other metabolic disorders, such as metabolic syndrome. Because of we used a mouse model the sample amount obtained was small and was not enough for performing others experimental analyzes contemplated by the authors.

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