

## **Empagliflozin alone and in combination with metformin ameliorate diabetes type 2-oxidative stress**

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### **Abstract**

Currently, one of the major public health concerns is diabetes mellitus, which is known as a silent killer due to the failure to diagnose it at an early stage. A growing body of research indicates that metformin and empagliflozin have antioxidant properties in addition to their hypoglycemic properties. Our research looked into the potential antioxidant effects of metformin and empagliflozin on diabetic rats by decreasing oxidative stress. For eight weeks, 42 adult male Sprague Dawley rats were divided into six groups: normal control, diabetic control group, diabetic group received dimethyl sulfoxide as a vehicle(solvent) of the empagliflozin, 250 mg/kg metformin only, 10 mg/kg empagliflozin only and a combination of both. A single injection of 40 mg/kg streptozocin administered intraperitoneally was after administering 10% fructose in drinking water for two weeks to cause type 2 diabetes in rats. The evaluation of oxidative stress involved the quantification of serum malondialdehyde (MDA) and superoxide dismutase (SOD) levels upon completion of the experimental period. Additionally, blood glucose levels were determined using blood samples from the tail, and body weight was monitored on a weekly basis. Our findings showed that compared to the diabetic group, the therapy groups had considerably lower blood glucose, and serum MDA levels, while higher levels of SOD, and less decrease in body weight. Furthermore, even better results were obtained when empagliflozin and metformin were administered together. As a result of our findings, diabetic rats treated with empagliflozin and metformin had lower levels of oxidative stress.

**Keywords: Diabetes, empagliflozin, metformin, oxidative stress, serum malondialdehyde, superoxide dismutase.**

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### **INTRODUCTION**

Diabetes mellitus (DM), regarded as a silent killer, is one of the current main worldwide health challenges [1]. Based on data provided by the International Diabetes Federation (IDF), the global population of individuals aged 20 to 79 diagnosed with diabetes is estimated to be 537 million as of 2021. Projections indicate that this figure is

anticipated to increase to 643 million by 2030 and further escalate to 783 million by 2045. About 90% of diabetic people are now diagnosed with type 2 diabetes (T2DM), also known as non-insulin-dependent diabetes, distinguished by insulin resistance and relative insulin insufficiency due to secretory defects [2]. Globally, scientists found they are more obese than non-obese people and people with multiple genetic factors (polygenic). An

unhealthy diet and a sedentary lifestyle are also risk factors [3]. T2DM management by First, non-pharmacological treatments such as lifestyle modification such as diet besides regular exercise and physical activity. Secondly, pharmacological treatments that increase insulin levels and insulin sensitivity by the cell, such as insulin and hypoglycemic drugs [4]. Empagliflozin (EMPA) is a hypoglycemic medication that belongs to the sodium-glucose co-transporter-2 (SGLT2) inhibitor. EMPA has an insulin-independent mechanism of action by highly selective SGLT2 inhibition by suppressing glucose reabsorption in the kidney and enhancing glucose excretion in urine, increasing natriuresis, glycosuria, and a decrease in blood glucose levels [5]. EMPA has remarkable antioxidant effects by ameliorating oxidative stress via decreased renal malondialdehyde (MDA) and increased renal activities of superoxide dismutase (SOD) [6]. Metformin (MET) is a pharmacological therapy belonging to Biguanide. The action of MET is not related to insulin secretion but increases insulin sensitivity in muscle, liver, and GIT [7]. The recent guidelines outlined in the 2022 edition of the American Diabetes Association (ADA) have demonstrated the efficacy of this medication in the prevention of diabetes mellitus (DM) among adults diagnosed with pre-diabetes, despite its primary application in the treatment of individuals already diagnosed with diabetes [8]. Metformin is a pharmaceutical agent that exhibits antiaging properties by promoting an extended health span and lifespan. This is achieved through its ability to modulate cellular metabolism via anti-hyperglycemic mechanisms, enhance insulin sensitivity, reduce oxidative stress, and protect endothelial and vascular functions. Consequently, metformin indirectly mitigates mortality rates associated with a range of diseases, including diabetes [9].

Type 2 diabetes can develop a wide range of diabetic complications; the risk varies depending on glycemic control and the severity of the disease [10]. Cute consequences like hyperglycemia or chronic conditions that are either macrovascular

diseases like atherosclerosis, hyperosmolar nonketotic coma, diabetic ketoacidosis, or microvascular diseases like neuropathy, nephropathy, and retinopathy [11]. Nowadays, studies aiming to minimize diabetic complications can be done by glycemic control and taking a hypoglycemic drug that has an antioxidant effect, such as EMPA and Met [12,13].

## MATERIALS AND METHODS

### Ethical approval:

The current study received ethical approval from the University of Kufa's Faculty of Pharmacy in Iraq, specifically from the ethics committee (EC:6018) on February 27, 2022.

### Study design:

This study was carried out in the animal house of the Kufa Faculty of Science. The experiment study persisted for 12 weeks. The time frame spanned in this work is from December/2022 through March/ 2023. A 42 male type of Sprague Dawley rats (*rat Rattus*) were used with 180-200g in weight and divided into six groups (n=7): Normal control (NC), diabetic control (DM), diabetic received DMSO as a vehicle (DM+ Vehicle), diabetic treated with empagliflozin (DM+ EMPA), diabetic treated with metformin (DM+ Met), diabetic treated with empagliflozin and metformin [(DM+ EMPA+Met)]. These animals were housed at temperature controlled at  $24 \pm 2^{\circ}\text{C}$  with the 12hr light: 12hr dark cycle. Rats got a standard pellet feed with tap water. The experiments were started following two weeks of acclimatization in the quarantine room. Animals in the control group were not exposed to any application. Experimental DM was induced by giving 10% fructose in the drinking bottle as drinking water for 14 days, then Streptozocin dissolved in 0.1mM sodium citrate buffer with (pH 4.5) and injected intraperitoneally into animals immediately. The blood glucose level of animals was measured after 72 hr. using a glucometer, and animals  $\geq 250$  mg/dl blood glucose level was considered experimental diabetic animals. Then treatment administration in a dose (10mg/kg/day) orally for eight weeks for DM+

empagliflozin (250mg/kg/day) orally for eight weeks [14], for DM+ metformin [15], and (10mg/kg/day) and (250mg/kg/day) via intragastric route for eight weeks for DM+ empagliflozin + metformin after 12 weeks the animals of the study have been euthanized and sacrificed using intraperitoneal injections of ketamine/ xylazine (75 /5 mg/kg) [16].

#### **Material:**

Apure fructose powder 99% (cat#8100), Streptozocin (STZ; Cat. No. S8050), empagliflozin (Cat. No. E2280), and metformin (Cat. No. D9351) were collected from Solarbio, China. The glucometer (SN: GB27598969) and Test Strip were ACCU-CHEK Active (Roche), Germany. The Malondialdehyde (MDA) Assay Kit (Cat No: BC0020) and Superoxide Dismutase (SOD) Activity Assay Kit (Cat No: BC0170) were purchased from Solarbio, China. Other reagents and chemicals used in this study were of analytical grade.

#### **Methods:**

##### **Body weight measurement:**

Every week for 12 weeks of the experiment, body weight was measured and recorded using an animal balance.

##### **Serum assay:**

After 12 weeks, the study animals were euthanized and sacrificed using intraperitoneal injections of ketamine/ xylazine (75 /5 mg/kg) [16]. Blood samples were obtained via direct collection from the heart puncture using a 5cc syringe. Then, the blood was put in a gel tube at room temperature without anticoagulant. Finally, the serum was collected by centrifuging the blood for 15 minutes at 3000 rpm [17].

##### **Measurement of biochemical parameter**

A glucometer (Roche, Germany) measured blood glucose weekly via blood

from the tail. Serum SOD and MDA levels were assessed by a spectrophotometric kit (Solarbio, China).

#### **Statics analysis:**

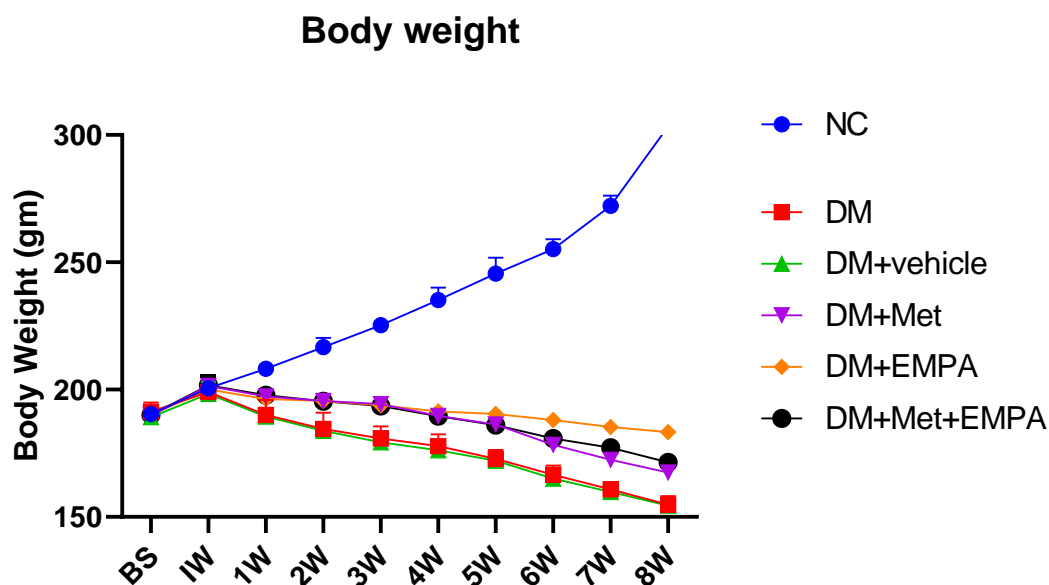
GraphPad Prism version 8 is the statistical analysis program to determine the significance values. The data was reported in terms of the mean and standard error of the mean (SEM). The One and Two-way Analysis of Variance (ANOVA) method was employed to conduct a comparative analysis of the various groups. Tukey's multiple comparisons approach was subsequently employed to assess the statistical significance among the groups. The threshold for statistical significance was established at a p-value below 0.05.

## **RESULTS**

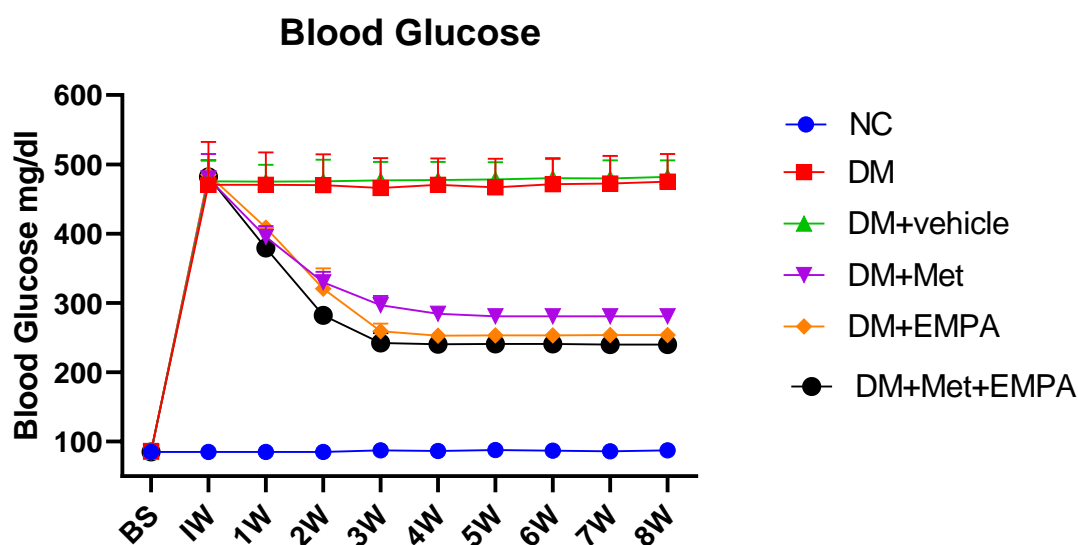
### **Empagliflozin and Metformin ameliorate hyperglycemia but not body weight**

The body weight significantly increases in normal control compared to diabetic and diabetic + vehicle. However, EMPA and Met treatment significantly increase body weight compared to diabetic control and vehicle. Conversely, the body weight values significantly decreased compared with the normal control group, there was a non-significant difference between diabetic control and vehicle as shown in Figure 1.

Serum blood glucose is significantly increased in diabetic control and vehicle compared to the normal control group. However, EMPA and Met biochemical results show a significant decrease in blood glucose levels compared to diabetic control and vehicle. The most reduction is when giving this medication together. There was a non-significant difference between diabetic control and vehicle. as shown in Figure 2.



**Figure 1.** Mean body weight (mg) across groups (n=7 rats per group). Data are presented as mean± standard error of means (SEM), ANOVA two ways Tukey's multiple comparisons test  $p \leq 0.05$ . Baseline BS, Week W, Normal control (NC), Diabetic control (DM), Diabetic treated with the vehicle (DM + Vehicle), Diabetic treated with Metformin (DM + Met), Diabetic treated with empagliflozin (DM + EMPA), Diabetic treated with empagliflozin and metformin (DM+ EMPA+ Met).



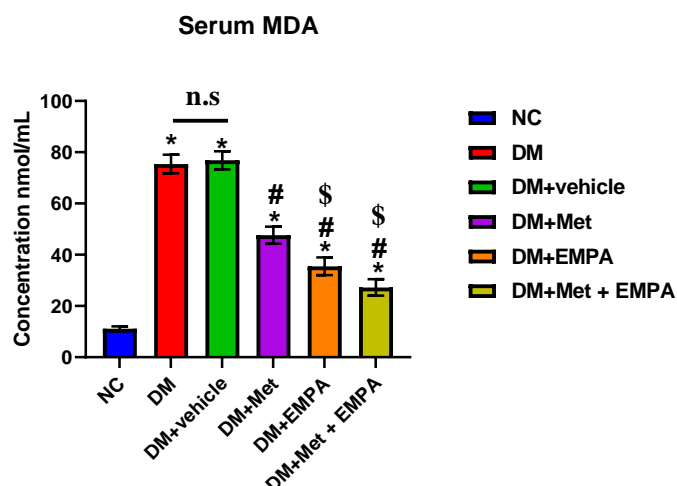
**Figure 2.** Mean blood glucose (mg/dL) across groups (n=7 rats per group). Data are presented as mean± standard error of means (SEM), ANOVA two ways Tukey's multiple comparisons test  $p \leq 0.05$ . Baseline BS, Week W, Normal control (NC), Diabetic control (DM), Diabetic treated with the vehicle (DM + Vehicle), Diabetic treated with Metformin (DM + Met), Diabetic treated with empagliflozin (DM + EMPA), Diabetic treated with empagliflozin and metformin (DM+ EMPA+ Met).

### Biochemical findings

#### Empagliflozin and Metformin ameliorate oxidative stress:

Serum levels of MDA were significantly increased in diabetic control and vehicle groups compared to normal control. The

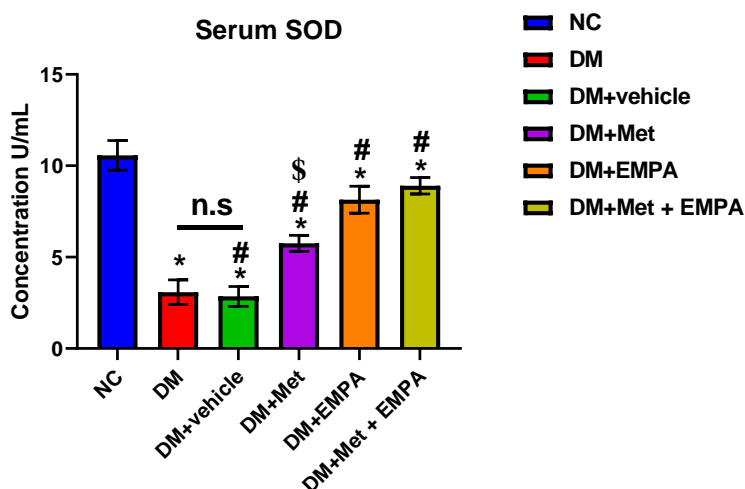
treatment with EMPA and Met significantly reduced MDA compared to diabetic control and vehicle groups. The most significant result is when giving this medication together, there was a non-significant difference between diabetic control and vehicle group. as shown in Figure 3.



**Figure 3.** Serum MDA (nmol/mL) level. STZ-induced diabetic animals were treated with empagliflozin (10mg/kg/day) and/or metformin (250mg/kg/day) for eight weeks. The results are presented as the mean  $\pm$  SEM (n= 7 rats/group). \* $p \leq 0.05$  versus normal control. # $p \leq 0.05$  versus diabetic control. \$ $p \leq 0.05$  versus combined treatment.

Serum levels of SOD were significantly decreased in diabetic control and vehicle groups compared to normal control. The treatment with EMPA and Met significantly increased SOD compared to diabetic control

and vehicle groups. The most significant result is when giving this medication together, there was a non-significant difference between diabetic control and vehicle. as shown in Figure 4.



**Figure 4.** Serum SOD (U/mL). STZ-induced diabetic animals were treated with empagliflozin (10mg/kg/day) and/or metformin (250mg/kg/day) for eight weeks. The results are presented as the mean (n= 7 rats/group). \* $p \leq 0.05$  versus normal control. # $p \leq 0.05$  versus diabetic control. \$ $p \leq 0.05$  versus combined treatment.

## DISCUSSION

Diabetes is among the most prevalent metabolic diseases worldwide [1]. One of the main consequences of diabetes, diabetic nephropathy (DN), can cause end-stage renal failure and even mortality [18]. Streptozocin (STZ) is used clinically as a chemotherapeutic agent in treating cancer. However, due to the drug's serious side effects, including its ability to cause diabetes, it is now primarily used in induced diabetic animal models [19]. A methyl nitrosourea analog with a diabetogenic mechanism, streptozocin selectively enters  $\beta$ -cells via the GLUT2 glucose transporter and then splits into two molecules, glucose, and methyl nitrosourea moiety, which is a toxic molecule leading to DNA damage, alkylation, and pancreatic cell death [20]. Our study showed that in diabetic animals, there is an increase in water intake, a decrease in body weight, and reduced insulin sensation due to the important physiological roles of Insulin in the liver, skeletal muscles, and adipose tissue. Hence, insulin action inhibition leads to decreased glucose entering the cells of these organs and more glucose remaining in the bloodstream as a result of increasing blood glucose levels, increasing lipolysis, glycogenolysis, and proteolysis to obtain enough energy, decreasing body weight [21,22]. The treated animals appeared more active and decreased blood glucose levels compared to an animal model, indicating that they responded to the treatment; the treated animals also lost less body weight than diabetic animals [23,24].

The global concept of "Oxidative Stress" (OXS) results from a temporary or permanent imbalance between antioxidants and reactive oxygen species, so the damaged molecules build up in the cell [25]. Usually, there is an equilibrium between oxygen species and antioxidant generation in cells or tissues to prevent damage. Reactive oxygen species are by-products resulting from oxygen metabolism, environmental factors, and xenobiotics; There exist free radicals that are readily accessible, including Superoxide radicals ( $O_2^{\bullet-}$ ), hydrogen peroxide ( $H_2O_2$ ), hydroxyl radicals ( $\bullet OH$ ), and singlet oxygen ( $^1O_2$ ). Many

cellular structures, like cell membranes, lipids, proteins, and nucleic acids, are harmfully affected by free radicals [26].

It has been reported that in many diseases like diabetes, free radicals' build-up leads to DNA damage, protein modification, and lipid peroxidation; this situation is called oxidative stress. Free radicals participate in diabetic complications, and many organs in the body, like the kidney, are subjected to the OXS and damage [27].

In diabetes, the insulin function loss leads to metabolic disturbance, causes inflammation, and liberations of ROS which leads to lipid peroxidation, an increase in MDA level, and a decrease SOD level which is considered as a marker of oxidative stress [28,19,12]. Empagliflozin decreases lipid peroxidation by increasing the activity of the antioxidant enzyme SOD in the animals treated with EMPA [29].

Metformin alleviates oxidative stress through the modulation of the AMPK/SIRT1-FoxO1 pathway, which is involved in the regulation of oxidative stress in diabetic kidney disease (DKD). This intervention reduces oxidative stress, enhances autophagy, and decreases abnormal cell proliferation in renal mesangial cells cultivated under high glucose conditions [30].

## CONCLUSION

The results of this study demonstrate that treating diabetes conditions with empagliflozin plus metformin efficiently lowers oxidative stress in diabetic rats via antioxidant pathways.

## CONFLICT OF INTEREST

The authors assert that there are no conflicts of interest.

## ACKNOWLEDGMENT

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