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Modulatory role of resveratrol on some biomarkers of oxidative stress and inflammation in diabetic nephropathy in Wistar rats

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ABSTRACT

Diabetic nephropathy (DN) is a chronic debilitating microvascular complication of diabetes, and often leads to end-stage renal disease, a common cause of morbidity and mortality with high burden worldwide. Both oxidative stress and chronic inflammation had been implicated in the pathogenesis of DN. Resveratrol is a natural polyphenolic antioxidant with several beneficial effects in medicine due to its antioxidant, antidiabetic, anti-inflammatory, anticancer, neuroprotective among other properties. This study aimed to investigate the modulatory role of resveratrol in experimentally-induced diabetic nephropathy. Ethical approval was obtained from the institution's Ethics Committee for Research Animal Care and Use. Twenty male Wistar rats were randomly assigned into five groups (n=4): normal control; normoglycemic + 1ml/kg carboxymethylcellulose (CMC); diabetic control; diabetic + 100mg/kg resveratrol; and diabetic + 1mg/kg lisinorpril. Twelve weeks after the induction and treatment, the animals were humanely sacrificed. Serum was collected for biochemical analysis. Values at $P<0.05$ were considered statistically significant. There was a marked decrease in both serum creatinine and tumour necrosis factor-alpha levels in the resveratrol group compared to the diabetic control. Although not significant, there was slight decrease in malondialdehyde concentrations and increase in superoxide dismutase and catalase activities in the resveratrol group when compared to the diabetic group. Resveratrol ameliorated renal dysfunction in DN by reducing oxidative stress and inflammation in male Wistar rats,

Keywords: anti-inflammatory; anti-oxidant; diabetic nephropathy; oxidative stress; resveratrol.

INTRODUCTION

Diabetes mellitus (DM) is a multi-factorial, chronic and progressive heterogeneous metabolic disorder characterized by chronic hyperglycemia due to defects in the metabolism of carbohydrate, fat and protein[1,2]. It is due to either insulin deficiency or the impaired effectiveness of insulin's action, or both [2,3]. The global prevalence of DM is 9.3% (463 million) as at 2019, with a projection to about 10.9% (700 million) by 2045 [4] and it is still rising [5]. As at 2011, an estimated 366 million people had DM worldwide, with type 2 constituting about 90% of the cases [6]. In Africa, Nigeria is the fourth country with the highest burden of diabetes with an estimated figure of over 1.7 million people living with the disease [7].

DM is the second leading cause of blindness and renal disease worldwide [8]. Chronic hyperglycemia produces systemic effects, which include glucose-induced vascular inflammation, and impaired cellular immunity by stimulating inflammatory cytokines and inhibiting leucocytes formation [9]. Persistent hyperglycemia also causes excessive levels of superoxide in endothelial cells, which can activate the various pathways of microvascular damage [10]. The structural and functional disruption in organ system vasculature leads to macro- and micro-vascular complications, which ultimately results in organ failure. These complications tend to affect organs such as eyes, kidneys, heart and brain [1, 11]. They affect majority of patients with diabetes in both developed and developing countries. These include diabetic nephropathy, retinopathy, and neuropathy, which are responsible for morbidity in most patients [12].

Diabetic nephropathy (DN) is a chronic progressive disease of the kidney, a major complication of diabetes and also called "microalbuminuria", as a result of chronic hyperglycemia [13]. It is characterized by persistent albuminuria (usually greater than 300 mg/24hr, or 300 mg/g creatinine), progressive decline in glomerular filtration rate (GFR), arterial hypertension and an increased cardiovascular morbidity and mortality [14]. It is reported to affect approximately one-third of diabetic individuals [15] and to be the most common cause of end-stage renal disease (ESRD) worldwide [16, 17]. DN develops 10-30 years after the onset of diabetes. It is more common among African-Americans, Asians, and Native Americans (13). The prevalence of albuminuria is around 30–35% in both types of diabetes (type 1 and 2). The DN development rarely occurs before 10 years of duration of type 1 diabetes, while approximately 3% of diagnosed type 2 diabetic patients are already affected by overt nephropathy [18].

Many chemokines are involved in the inflammatory response in DN. Elevated serum levels of TNF receptors (TNF-R) 1 or 2 are strongly associated with a risk of renal functional decline or ESRD [19]. TNF-α is an inflammatory cytokine with many determinant actions in inflammatory response by several tissues and pleiotropic effects. TNF-α with the help of TNFR-1 plays a valuable role to activate and recruit the immune cells to propagate the inflammation. TNF-α activates transcriptional pathways that induce oxidative stress, and then this stress and inflammation interact with each other to promote the degeneration of cells [20].

Oxidative stress occurs in living organisms when the antioxidant defense system is insufficient against free radicals [21]. Reactive oxygen species (ROS) specifically attack and oxidize cell components such as polyunsaturated lipids, proteins and nucleic acids. ROS at a low amount is normal but in excess is very hazardous. A balance between antioxidants and free radicals generation must be achieved in order to lead a healthy life, but if there is any imbalance between these two oxidative stress results [22].

Oxidative stress plays an important role in the development of vascular complications in diabetes particularly type 2 diabetes mellitus [23].

Antioxidants can be exogenous or endogenous. Exogenous antioxidants can be found from natural sources (vitamins, flavonoids, anthocyanins, some mineral compounds), but can also be synthetic compounds, like butylhydroxyanisole, butylhydroxytoluene, gallates, etc. [24]. The most common exogenous antioxidants include vitamins A, E and C [25]. Endogenous antioxidants are products of the body's metabolism that can either be enzymatic or non-enzymatic, protein (glutathione, alpha lipoic acid, coenzyme Q, ferrin, uric acid, and bilirubin) or non-protein (superoxide dismutase [SOD], catalase [CAT], and glutathione peroxidase [GPx]) [26]. The endogenous antioxidants especially the protein types are considered as the first line of defence against oxidative stress on the body. They help in sequestration of transition metal ions, scavenging and quenching of ROS and reactive nitrogen species (RNS), ending of chain reactions by free radicals, and molecular repairing of radical's damages [27].

Resveratrol (3,4',5-trihydroxystilbene) is a natural polyphenolic non-flavonoid phytoalexin that belongs to the stilbenoids group of secondary metabolites [28]. Stilbenes are known for their ability to protect plants from UV light, the effect of chemical fertilizers, and biotic stresses such as bacterial, fungal, or nematode infections [29]. Resveratrol is synthesized by more than 70 species of plants in response to infection, stress, injury and UV-irradiation [30-32]**.** It was also reported to be found in important dietary sources such as grapes, red wine, peanuts, berries, jackfruit and soy [30,33]. Resveratrol via its radical scavenging and metal ion chelation abilities [31], act as a direct antioxidant and an indirect cellular antioxidant system inducer through modulation of several cellular antioxidant pathways, thereby balancing cellular redox status. It is antidiabetic (34-36), cardioprotective [37-39]; neuroprotective [40, 41], anti-inflammatory [42, 43], and anti-cancer [44, 45].

In some of type 2 diabetic patients, complications such as DN might have developed at the time of diagnosis, and strict glycemic control may not be attainable. Therefore, development of treatment modalities aimed at preventing occurrence or progression of DN becomes necessary [46]. Furthermore, there are only few studies that demonstrated the role of resveratrol on inflammation and oxidative stress especially in the setting of diabetic nephropathy. Therefore, the aim of this study is to investigate the modulatory role of resveratrol on inflammation and oxidative stress in hyperglycemia-induced nephropathy in Wistar rats.

MATERIALS AND METHODS

Materials: Digital glucometer (Accu-Chek Active, Roche, Germany), Simas margarine, syringes and needles, weighing balance, cages, drinking bottles, measuring cylinder and conical flask, dissecting kit and tray.

Drugs and reagents: Distilled water, 20% fructose solution, streptozotocin, 0.1M citrate-buffered saline (pH 4.5), carboxymethylcellulose, resveratrol (99% pure trans-resveratrol, from Sigma, USA), lisinorpril tablets, ketamine hydrochloride, diazepam, TNF-α enzyme-linked immune-sorbent assay (ELISA) biochemical assay kit.

Experimental animals: Twenty (20) healthy adult male Wistar rats, weighing 120-180g were obtained from the Animal House of the Department of Human Physiology, Faculty of Basic Medical Sciences, College of Basic Medical Sciences, Ahmadu Bello University, Zaria, Nigeria. They were housed in proper portable cages and were maintained and allowed free access to food and water. The animals were left to acclimatize for a week before commencement of the experiment and were fed on standard commercial vital feeds with water.

Ethical approval: Ethical approval on guidelines for care and use of laboratory animals in scientific research was obtained from Ahmadu Bello University Committee on Animal Use and Care (ABUCAUC/2021/33).

Induction of type 2 diabetes mellitus: Type 2 diabetes was induced in three of the groups by feeding the rats high-fat diet (HFD) (Normal feed + Simas margarine in the ratio 10:1) and 20% fructose solution as drinking water for 6 weeks. Rats where fasted overnight after which they were given a single intraperitoneal injection of streptozotocin (STZ) at a low dose of 35 mg/kg diluted in 0.1 M citrate-buffered saline (pH 4.5). Fasting blood glucose levels of the rats were checked Accu-Chek glucometer 3 days post STZ injection and confirmed 7 days after to validate diabetes. The HFD and fructose solution administrations were continued for the remaining 6 weeks after STZ injection. Rats with fasting blood glucose levels ≥ 200 mg/dL were considered diabetic [47].

Animal grouping: The animals were randomly selected and divided into five groups of four (n=4) rats per group.

Group I: They are the normal control. They received 1ml/kg distilled water orally in addition to standard rat chow throughout the experiment.

Group II: They are normoglycaemic and received 1ml/kg carboxymethylcellulose (CMC) orally in the second 6-week duration.

Group III: They are the diabetic control that received 1ml/kg CMC orally in the second 6-week duration.

Group IV: They are the diabetic rats that received 100mg/kg resveratrol (48) in the second 6-week duration.

Group V: They are the diabetic rats that received 1mg/kg lisinorpril (49) in the second 6-week duration.

Blood sample collection: After the six weeks intervention period, the rats were fasted overnight and then anaesthetized with 50 mg/kg ketamine hydrochloride and 25 mg/kg diazepam. Blood was collected via cardiac puncture in plain bottles for the biochemical assay.

Determination of superoxide dismutase (SOD) activity: Superoxide dismutase (SOD) is widely distributed in plants, animals and microorganisms and cultured cells. Catalytic superoxide anion causes deuteration to form H_2O_2 and O_2. SOD aside being a superoxide anion scavenging enzyme, it is also a major producing enzyme of H_2O_2 which plays an important role in the biological antioxidant system.

Superoxide anion (O_2) is produced by xanthine and xanthine oxidase reaction system. The xanthine and xanthine oxidase reaction system is used to generate O_2 and nitroblue tetrazolium (NBT) reduction is used as an indicator of O_2 production by forming blue formazan which absorbs at 560nm. SOD will compete with NBT for O_2. SOD can remove O_2 thereby, inhibiting the formation of formazan, the deeper the blue of the reaction liquid, the lower the SOD activity and vice versa. In other words, the percentage inhibition of NBT reduction

is a measure of the amount of SOD present (50).

Determination of catalase (CAT) activity: Catalase (CAT) assay is widely found in animals, plants, microorganisms and cultured cells. It is the most important H_2O_2 scavenging enzyme and plays an important role in the active oxygen scavenging system.

H_2O_2 has a characteristic absorption peak at 240nm, and CAT can decompose H_2O_2, so the absorption at 240nm of the reaction solution is decreased with the reaction time. The CAT activity can be calculated according to the rate of change of absorbance (51).

Determination of malondialdehyde (MDA) concentrations: Oxygen free radicals acts on lipid unsaturated fatty acid to form lipid peroxides; the lipid peroxides decompose into a series of complex compounds, including MDA. The level of lipid oxidation can be detected by detecting the level of MDA.

MDA is condensed with thiobarbituric acid (TBA) to form a red product with a maximum absorption peak at 532nm. After colorimetry, the content of lipid peroxide in the sample can be estimated and the time absorbance at 600nm is measured. The amount of MDA was calculated using the difference in absorbance at 532nm and 600nm (52).

Determination of serum TNF-α levels: Biochemical analysis was carried out based on the ELISA kit manufacturer's instructions. The kit was used to test the level of rat tumor necrosis factor alpha (TNF-α), based on the principle of double anti-body sandwich technology ELISA.

The standard and sample were added to the wells that were pre-coated with objective antibodies. Then HRP-conjugate reagent was added to form an immune complex, followed by incubation and washing, removal of unbounded enzyme, and addition of substrates A and B. The colour of the solution was turned into blue and finally changes into yellow at the effect of acid. The color depth or light was positively correlated with the concentration of TNF-α.

Biochemical analysis of serum creatinine levels: Creatinine reacts with picric acid to produce a colored compound, creatinine alkaline picrate. The change in absorbance is proportional to the creatinine concentration [53].

Statistical Analysis: Data collected were expressed as mean ± standard error of mean (SEM). All data were also analyzed using one-way analysis of variance (ANOVA) followed by Tukey's *post-hoc* test to compare the level of significance between the groups. In all cases, values of $p \leq 0.05$ were considered significant.

RESULT

There was a significantly elevated level of serum creatinine observed in the diabetic control group (2.30 ± 0.12) compared to all the other groups (table 1). The lowest level was seen in the normoglycemic (0.65 ± 0.06 mEq/L) and in the resveratrol (0.65 ± 0.06 mEq/L) groups.

The result in figure 1 showed a slight depletion of the SOD activity in the diabetic group (17.95 ± 0.95 IU/mL), although not significant compared to the normal control (21.63 ± 1.62 IU/mL). This was mildly ameliorated with the administration of resveratrol (18.78 ± 1.23 IU/mL).

There was no statistically significant change in CAT activity in all the groups (figure 2). However, treatment with resveratrol at a dose of 100mg/kg (21.08 ± 1.85 IU/mL) shows an increase even more than that with lisinorpril at a dose of 1mg/kg (20.55 ± 0.61 IU/mL) when compared to the diabetic control group (18.85 ± 0.61 IU/mL).

There was a marked decrease ($p < 0.05$) in MDA concentrations in groups treated with lisinorpril at a dose of 1mg/kg (792.23 ± 49.58 nmol/ml) when compared to the diabetic control group (1054.60 ± 16.74 nmol/ml). There is a decrease but not statistically significant in the group treated with resveratrol at 100mg/kg (996.38 ± 1.40 nmol/ml) when compared to the diabetic control group (figure 3).

A remarkable increase ($p<0.05$) in serum TNF-α concentrations was noted in the diabetic control group (49.94±5.71 ng/mL) when compared with the normal control group (34.84±1.65 ng/mL). However, this was greatly reduced by resveratrol (38.38 ± 1.82 ng/mL) and especially lisinorpril (29.82 ± 3.94 ng/mL) administrations (table 2).

Table 1: Effect of resveratrol on serum creatinine levels in high-fat, high-fructose and streptozotocin-induced diabetic Wistar rats (n = 4).

Group	Creatinine (mEq/L)
Normal Control (1 mL/kg DW)	0.80 ± 0.04
Normoglycaemic (1 mL/kg CMC)	0.65 ± 0.03[a]
Diabetic Control (1 mL/kg CMC)	2.30 ± 0.12[b]
Diabetic (100 mg/kg resveratrol)	0.65 ± 0.06[a]
Diabetic (1 mg/kg lisinorpril)	1.13 ± 0.17[a]

Values with different superscripts are significantly different at $p<0.05$. DW = distilled water; CMC = carboxymethylcellulose.

Table 2: Effect of resveratrol on serum TNF-α in high-fat, high-fructose and streptozotocin-induced diabetic Wistar rats (n = 4).

Group	TNF-α (ng/mL)
Normal Control (1 mL/kg DW)	34.84 ± 1.65
Normoglycemic + 1 mL/kg CMC	35.43 ± 1.66[a]
Diabetic + 1 mL/kg CMC	49.94 ± 5.71[b]
Diabetic + 100 mg/kg resveratrol	38.38 ± 1.82[a]
Diabetic + 1 mg/kg lisinorpril	29.82 ± 3.94[a]

Values with different superscripts are significantly different at $P<0.05$, a = compared to diabetic control; b = compared to normal control. ANOVA followed by *post hoc* test done.

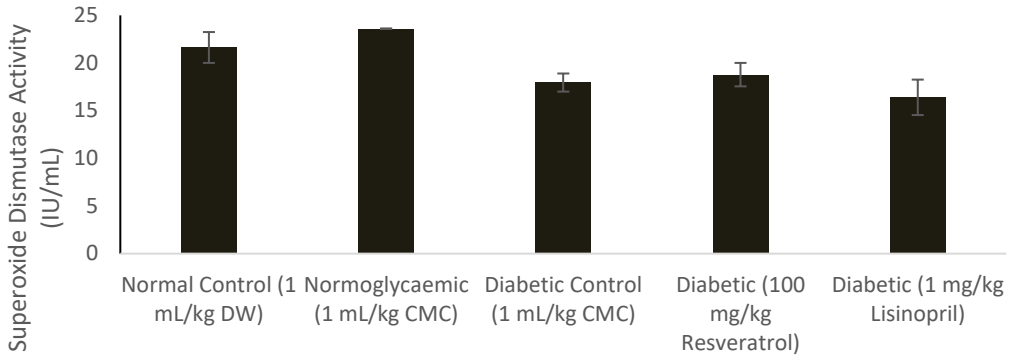

Figure 1: Effect of resveratrol on superoxide dismutase activity in high-fat, high-fructose and streptozotocin-induced diabetic Wistar rats (n = 4). DW = distilled water; CMC = carboxymethylcellulose.

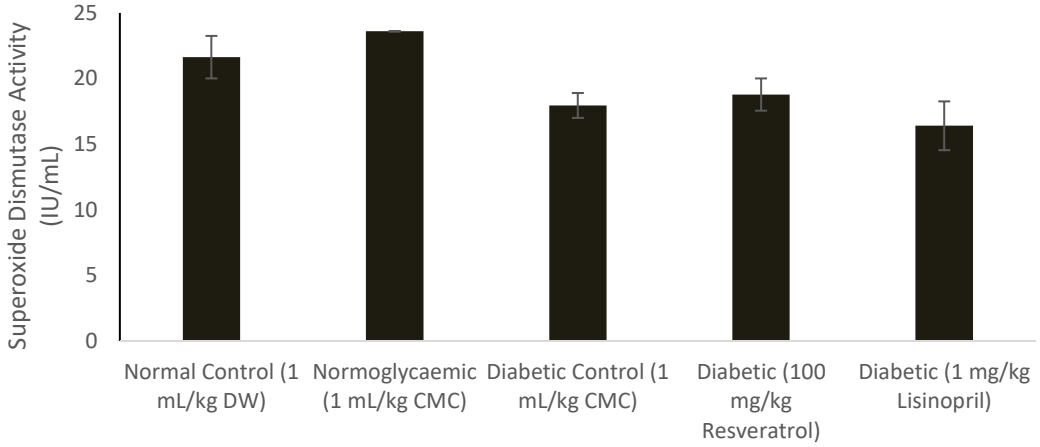

Figure 2: Effect of resveratrol on catalase activity in high-fat, high-fructose and streptozotocin-induced diabetic Wistar rats (n = 4). DW = distilled water; CMC = carboxymethylcellulose

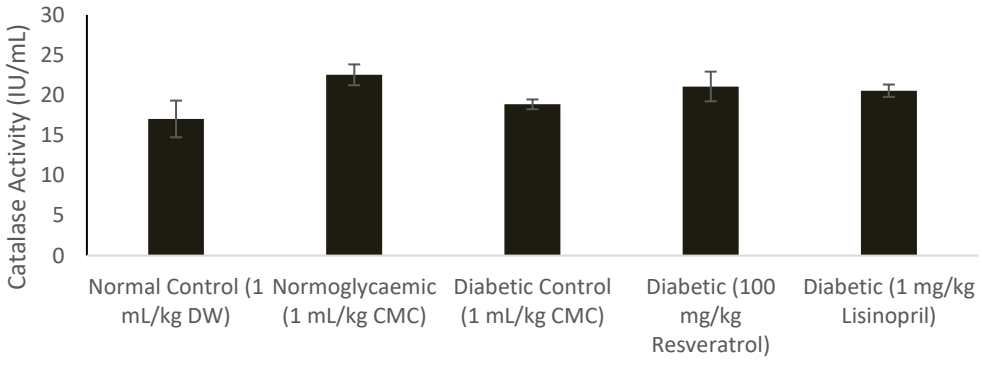

Figure 3: Effect of resveratrol on malondialdehyde concentrations in high-fat, high-fructose and streptozotocin-induced diabetic Wistar rats (n = 4). DW = distilled water; CMC = carboxymethylcellulose.

DISCUSSION

Experimental induction of type 2 diabetes mellitus resulted in diabetic nephropathy, which was assessed by measuring the serum creatinine levels on the 12th week of the experiment. This is in agreement with Attallah et al. [46]. Measurement of serum urea and creatinine are easily available tests which can aid in detection and prevention of diabetic kidney disease at an early stage and can limit the progression to end stage renal disease (ESRD) [54]. Serum creatinine and blood urea nitrogen concentrations are the best guidelines for estimating progression, prognosis, instituting dietary restrictions in the renal disease [55]. Impairment of urea and creatinine level due to increased blood glucose level indicates reduction in kidney function in diabetic patients [56].

In a randomized clinical trial, resveratrol was shown to ameliorate DN by decreasing urinary albumin excretion probably through anti-oxidant mechanism[57]. Resveratrol have been shown to have reno-protective effect in some animal studies by decreasing albuminuria [58], an easy marker for progressive diabetic kidney disease [57].

The administration of resveratrol from our result normalized the serum creatinine levels that was elevated in the hyperglycaemia-induced nephropathy in the rats. In other words, diabetic nephropathy was significantly improved by resveratrol treatment in the diabetic rats. Administration of resveratrol (20 mg/kg/day) for 8 weeks to diabetic Wistar rats in a study [59], resulted in significantly reduced plasma glucose, creatinine, and urinary protein excretion. Resveratrol treatment also attenuated the diabetic-induced mesangial cell hyperplasia and mesangial matrix expansion [59].

Treatment of animals suffering from diabetic nephropathy with resveratrol attenuates hyperglycemia, hyperlipidemia and improves kidney structural integrity and function in another study [60]. Resveratrol administration decreased urinary albumin and serum creatinine levels, indicating improved kidney function. In addition, renal oxidative stress, inflammatory cell infiltration, cytokine production, and MDA content were reduced with resveratrol administration, while antioxidant enzyme activity and SIRT1 expression were increased. These showed that resveratrol treatment has protective effects against diabetic nephropathy [60].

Lisinorpril belongs to the class of angiotensin converting enzyme (ACE) inhibitors. ACE inhibitors have demonstrated several reno-protective and other beneficial effects in patients with diabetes mellitus [61]. But from our results, resveratrol was more beneficial in terms of reno-protection compared to lisinorpril.

Resveratrol from the present study was found to be an effective protective mechanism against the oxidative stress as depicted in the assays of levels of some of the biomarkers of oxidative stress such as catalase (CAT) and superoxide dismutase (SOD) and a biomarker for lipid peroxidation, malondialdehyde (MDA). The variation in the levels of these enzymes makes the tissues susceptible to oxidative stress leading to the development of diabetic complications [22]. The modulation of SOD activity and MDA levels by resveratrol indicates its antioxidant effects and has proven to be beneficial in reducing oxidative stress in rats from the findings of Elbe *et al.,* [62].

From the results obtained in the present study, no significant increase in SOD and CAT activities was observed in the group treated with resveratrol as compared to the diabetic control group. The MDA level was also not significantly decreased in the group treated with resveratrol as compared to the diabetic control group but this is in line with the work of Arman *et al.,* [22]. This probably suggests that reactive oxygen species (ROS)

level elevation in diabetic nephropathy may be due to decrease in destruction of ROS and in the production of oxidative stress biomarkers (CAT, SOD).

The result of this study shows a statistically significant increase in the TNF-α level in the diabetic control group when compared with the normal control group. There was also a marked increase when comparing the diabetic group treated with resveratrol group. This is in line with the finding by Palsamy and Subramanian [63], which reported significant decreases in the level of TNF-α and other pro-inflammatory cytokines in the diabetic renal tissues of rats treated with resveratrol compared with the diabetic control group. According to Xiao *et al.,* [64] the increased levels of IL-1B, IL-6 and TNF-α in kidney tissue and serum under hyperuricemic conditions, indicates a regulation of the inflammatory response plays a crucial part in resveratrol protecting against hyperglycemia-induced nephropathy.

From the result of this study, there was a remarkable increase in TNF-α level in the group treated with lisinorpril when compared to the diabetic control group. Finding by Stenvinkel *et al* [61], demonstrated that patients receiving ACE inhibitors treatment had lower plasma TNF-α levels when compared with patients not on ACE-inhibitor treatments. However, Schinlder *et al.,* [65] reported a differential effect of different ACE inhibitors on the TNF-α level. Captopril dose-dependently depressed IL-1B-induced synthesis of TNF-α as does enalapril and cilazapril while ramipril, lisinopril and spirapril had no significant effect on TNF-α synthesis.

Apart from the antioxidant and anti-inflammatory pathways, resveratrol antidiabetic effect can also be linked to its ability to increase insulin sensitivity. Administration of resveratrol at a dose of 2.5mg/kg-400mg/kg for 6 months significantly improves insulin sensitivity and/or reduce insulin concentration in type 2 diabetic individuals or animal models [66]. In human clinical trials, the single-dose oral administration of resveratrol for 12 months tends to bring about a decrease in blood glucose level and an enhance insulin sensitivity in diabetic patient [67].

Resveratrol has been reported to have a protective effect in patients with chronic kidney disease and diabetes [60]. Its bioavailability is usually enhanced by combination with other bioactive components which produce synergic therapeutic effects and expand the metabolic effects of the combined agents [68].

CONCLUSION

Rats with diabetic nephropathy treated with resveratrol shows promise in reducing the oxidative stress biomarkers (SOD, MDA and CAT) but it is not statistically significant. From the research work carried out, the administration of the resveratrol significantly decreases the TNF-α levels in plasma when compared with the diabetic control group. The administration of resveratrol and pioglitazone ameliorated diabetic nephropathy in hyperglycaemia-induced nephropathy in Wistar rats by reducing the serum creatinine levels.

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