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Combination of telmisartan with sildenafil ameliorate progression of diabetic nephropathy in streptozotocin-induced diabetic model

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ABSTRACT

Diabetic nephropathy (DN) is a leading cause of end-stage renal disease in the world. Several signaling pathways are involved in the pathogenesis of DN including elevation in level of angiotensin II, formation of advanced glycation end products (AGE), activation of protein kinase c (PKC), and lipid accumulation. These pathways activate one another mutually leading to oxidative stress, increasing expression of transforming growth factor beta-1(TGF-β 1) and release of interleukins and adhesion molecules, so the aim of this study is to interrupt more than pathogenic pathway to ameliorate the progression of DN. In the present study, white male rats (N = 48) were divided into six groups (8 rats each), the first two groups served as normal control and a control vehicle group while the remaining four groups were rendered diabetic by a single intraperitoneal injection of Streptozotocin (STZ) and being left for 4 weeks to develop DN. Thereafter, the rats were divided into DN group, DN group receiving Telmisartan or Sildenafil or Telmisartan Sildenafil combination. After the specified treatment period, urine samples were collected (using metabolic cages) to measure proteinuria, animals were then euthanized, blood and tissue samples were collected for measurement of Blood glucose,BUN, S.Cr, LDL, NO, TGF-β1, IL-1β, AGEPs, and SOD. The combination therapy showed significant decrease in BUN, S.Cr,LDL, TGF-β1, IL-1β, Proteinuria and AGEPs and significant increase in SOD and NO.

The findings showed that combination therapy was able to ameliorate DN and that the effects were superior to the single drugs alone.

1. Introduction

Diabetic nephropathy (DN) is a principal cause of end-stage renal disease in the world and a major cause of morbidity and mortality from diabetic complications [1]. The major signaling mechanisms involved in the pathogenesis of DN include increasing levels of angiotensin II, formation of advanced glycation end-products (AGE), increasing activation of protein kinase c (PKC), and lipid accumulation [2], Chronic hyperglycemia is a major risk factor that activates all these signaling pathways involved in the development and progression of DN.

Hyperglycemia leads to increased formation of AGE that interact with their receptors (RAGE), induce oxidative stress, and stimulate production/release of cytokines that help lead to increased inflammation and tissue damage. Because oxidative stress and various AGE interact to up-regulate one another, this can lead to matrix accumulation and mesangial cell hypertrophy [3,4].

High glucose levels also help to elevate angiotensin II (Ang II) production [5] that, in turn, mediates pathophysiological changes in the kidney. As a pro-inflammatory peptide, Ang II also contributes to the induction/progression of glomerular inflammation and fibrosis [6,7]. Ang II causes activation of nuclear factor kabba B(NF-KB) that then leads to increased transcription of transforming growth factor (TGF)-β1 and inflammatory cytokines [8]. Ang II also activates NADpH oxidase, the major source of superoxide anions, leading to local oxidative stress. High glucose levels also induce of PKC activation, ultimately leading to generation of ROS, oxidative stress, and stimulated production of TGF β1 and inflammatory cytokines [2].

Hyperglycemia also causes lipid accumulation and increases local/circulating LDL and cholesterol levels that, in turn, contribute to potential lipid peroxidation events [2]. It is also established that the pathogenesis of DN is associated with abnormalities in renal nitric oxide (NO) generation [9,10]. Specifically, it has been shown that NO release and NO-dependent cGMP production declines in glomeruli of diabetic rats [11,12].

Given the multitude of detrimental effects associated with hyperglycemia, and the wide array of mechanisms that could

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contribute to the pathogeneses that lead to DN, development of strategies to treat DN is an urgent issue; however, a clear strategy has not been established. Telmisartan is a known anti-hypertensive drug that is an angiotensin (Ang II) AT1 receptor antagonist. It also acts as a selective agonist of peroxisome proliferator-activated receptor gamma (PPARγ) [13]; accordingly, telmisartan could be used to treat both hemodynamic and biochemical abnormalities [14] and help to reduce blood pressure, resulting in reduced proteinuria, improvement in insulin signaling, and mitigation of both oxidative stress and adipogenesis [8]. Moreover, by activating PPARγ, telmisartan can help improve insulin sensitivity and reduce inflammation [8]. Since the use of angiotensin receptor blockers does not completely prevent progression of nephropathy associated with diabetes, interruption of two or more pathogenic pathways – using combinations of drugs with different mechanism of action – is likely to be more effective than a single drug therapy. Accordingly, this study aimed to examine combinations of telmisartan with other drugs, i.e., sildenafil for its effects on preventing the progression of DN.

Sildenafil (Viagra), a type-5 phosphodiesterase (PDE-5) inhibitor, increases cGMP levels in response to NO and augments relaxation of vascular smooth muscles [15]. Although it was known that PDE-5 is expressed in various tissues, such as vascular and bronchial smooth muscles, as well as platelets [16,17], there is also abundant PDE-5 expression/activity in the kidney, this led to the suggestion that selective inhibition of PDE-5 could be advantageous in treating variable kidney disease [18]. It is for these reasons that sildenafil was selected as the adjuvant drugs for use in the present study.

2. Materials and methods

2.1. Animals

White albino rats (male, 180–200 g, and 8-weeks-of-age) were obtained from the animal house at the College of Veterinary Medicine of Cairo University (Cairo, Egypt). All rats were housed under specific pathogen-free conditions with a 12-h light: dark cycle at constant temperature (25 ± 2 °C) and provided *ad libitum* access to standard rodent chow (El-Nasr, Abuzabal) and filtered water. All rats were acclimatized for 1 week prior to use in experiments.

2.2. Drugs and chemicals

The following were purchased from the indicated suppliers for use in these studies; Streptozotocin (Sigma, St. Louis, MO), Telmisartan (Micardis 80 Tablet, Boehringer Pharmaceutical Co., Ingelheim, Germany.), Sildenafil (Viagra Tablet, Pfizer, USA), and Insulin (Humulin 70/30, Eli Lilly, Indiana, United States). ELISA kits used here included those to measure: Interleukin1β (IL-1β) (Invitrogen,U.S.A), transforming growth factor -Beta 1(TGFβ1) (e Bioscience, Vienna, Austria).Other kits include superoxide dismutase (SOD) (Biodiagnostic, Cairo), Proteinuria (Biodiagnostic, Cairo), fasting blood glucose (Biodiagnostic, Cairo), low density lipoprotein (LDL) (Greiner, Bahlingen, Germany), Cholesterol (Biosystems, Barcelona, Spain), Blood urea nitrogen (BUN) (Greiner), and Serum creatinine (Greiner). All other chemicals and solvents used were of analytical grade.

2.3. Experimental protocol

Rats were rendered diabetic with a single intraperitoneal (IP) injection of streptozotocin (55 mg/kg, stock prepared by dissolving 60 mg in 1 ml citrate buffer (pH 4.5) [19] and pH was measured using a Model HI 110 pH meter (Hanna Instruments, Providence, RI). The animals were examined 48 h later and blood samples were collected from the tail vein and examined for glucose levels using a blood glucose monitoring system BIONIME GM 100 (BIONIME Corporation, Dali, Taiwan). Those with plasma glucose levels ≥300 mg/dl were considered diabetic. These diabetic rats were then treated with insulin to maintain body weight, prevent ketoacidosis, and improve survival. Specifically, 1–4 Units of ultralent insulin (Humulin 70/30) was administered daily to each diabetic rat (S.C) and blood glucose was monitored daily in all rats [19]. Control rats were injected IP with 1 ml sodium citrate buffer/kg body weight. After 4 weeks, diabetic animals were divided into four groups, i.e., a DN group that received no drugs, a DN group treated with telmisartan (10 mg/kg/day; dissolved in carboxy methyl cellulose (CMC) administered orally for 8 weeks [20,21], a DN group treated with sildenafil (3 mg/kg/day;dissolved in filtered water administered orally for 8 weeks [19], and a DN group treated with combination of telmisartan and sildenafil (Tel at 10 mg/kg/day and Sil at 3 mg/kg/day for 8 weeks). The doses used were based upon values reported in the cited papers above. Along with these four groups, there was a normal control group and a vehicle control that received 1% carboxy methylcellulose(CMC) solution. All procedures and experimental protocols complied with the Guide on the Care and Use of Laboratory Animals published by the National Institute of Health and Nutrition and was approved by the Scientific Research Ethics Committee of Tanta University.

2.4. Biochemical analysis

At the end of the treatment period (8 weeks), animals were weighed and after an overnight fasting, urine samples were collected (using metabolic cages) to measure proteinuria, rats were then anaesthetized by diethyl ether and blood was collected via cardiac puncture. The isolated blood was centrifuged at 3000 rpm for 10 min and serum carefully separated and kept at −20 °C until used to asses blood glucose, BUN, S.Cr and LDL cholesterol levels using commercial kits. The rats were then euthanized by cervical dislocation and their kidneys carefully removed and rinsed thoroughly with saline; portions of the kidneys then placed in 10% neutral buffered formalin (pH 7.4) for subsequent histopathological examination. Remaining tissue was placed at −20 °C for use in measurements of SOD, NO, AGEPs, TGFβ1, and IL-1β.

2.4.1. Determination of fasting blood glucose level

Serum glucose was measured by enzymatic colorimetric method according to the principle of [22]. The method was performed using kits from Bio-diagnostic Company, following the instructions indicated in the inscript.

2.4.2. Determination of kidney NO

Kidney NO content was determined according to the method of Miranda et al. [23]. Quantification of nitrite and nitrate provide a reliable and quantitative estimate of NO output and can be detected using Griess reagent. In brief, 0.25 mg of kidney was homogenized in 10 vol ice cold saline (0.9% NaCl, i.e., 2.5 ml) using a PT 3100 polytron homogenizer (Kinematica instruments, Luzerne, Switzerland). One ml absolute ethanol was added to 0.5 ml of the homogenate to precipitate the proteins and the samples were then centrifuged at 3000 rpm for 10 min. To 0.5 ml of the clear supernatant, 0.5 ml VCl_3 (Sigma) was added followed by addition of 0.5 ml freshly prepared Griess reagent (Sigma). The mixture was vortexed, and samples were allowed to incubate at 37 °C for 30 min. Thereafter, the absorbance of each samples was measured at 540 nm using a UV-PC 1601 double-beam spectrophotometer (Shimadzu, Tokyo, Japan). The levels of NO in each sample were calculated by extrapolation from a standard curve prepared in parallel using stock nitrite solutions.