

Concise Review of Natural Remedies: the Impact of CAPE Effects on Induced Autoimmune Type 1 Diabetes in Vivo

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Abstract: *Diabetes mellitus (DM) is a chronic metabolic disorder that is characterized by hyperglycemia due to lack of or resistance to insulin. Patients with DM are frequently afflicted with ischemic vascular disease or impaired wound healing. Type 2 DM is well-known to accelerate the atherosclerotic process, endothelial cell dysfunction, glycosylation of extracellular matrix proteins, and vascular denervation. Naturally occurring products play an essential role in treating and managing diabetes, especially in developing countries, due to healthcare costs. Therefore, for a long time, natural treatments have been used worldwide to treat DM. Among many medications and alternative medicines, several herbs and natural medicinal plants have been recognized to cure and control diabetes with no side effects. Caffeic acid phenethyl ester (CAPE) is a phenolic antioxidant which has been shown to have anti-inflammatory properties. This study investigates the effects of administering caffeic acid phenethyl ester (CAPE) on mice-induced type 1 diabetes by measuring its effects on angiogenic parameters and on the anti-angiogenic factor. Additional details and analyses on cytokine, nitric oxide levels and histopathological examination are also presented. The introductory section of this work reviews the perplexing features of aberrant angiogenesis, abnormalities in growth factors, cytokines, oxidative stress and metabolic derangements relevant to diabetes.*

I. Introduction

Diabetes mellitus (hyperglycemia) is a metabolic disorder caused by either of the following situations: when cells discharge less insulin or when insulin binds less effectively to cell outward receptors thus leading to high blood glucose level. According to the investigation in low- and middle income countries, there are 366 million people living with diabetes and the count is estimated to increase to 552 million by 2030 [1]. In developing countries, for example, the number of people living with diabetes has multiplied greatly within the last decade. It has been predicted that, this ailment is likely to become a novel epidemic in the Middle East, Sub-Saharan Africa, Latin America, India, and the rest of Asia in this 21st century [2].

Extreme weight loss, polyuria, polydipsia and polyphagia among others, are the major symptoms of diabetes [3]. Diabetes affects the physical, psychological and social health of an individual and is becoming the third-largest “killer” of mankind’s health alongside micro- and macrovascular diseases, such as neuropathy, nephropathy, and cerebrovascular and cardiovascular diseases [4]. The knowledge regarding diabetes mellitus dates back to ancient Egypt and Greece. The word “diabetes” is derived from the Greek word “Diab” (which means to pass through and refers to the cycle of intense thirst and frequent urination); “mellitus” is the Latin word for “sweetened with honey” (which refers to the presence of sugar in the urine) [5].

Both type 1 and type 2 diabetes cause hyperglycemia, which in turn causes endothelial cell dysfunction (ECD) [6] by its different glycooxidative products. Type 2 diabetes causes insulin resistance, which is also responsible for endothelial dysfunction. Obesity is individually a risk factor for ECD and is related to type 2 diabetes [7]. Both obesity and insulin resistance have been found to accelerate ECD more frequently.

Presently, there is a growing interest in the use of herbal remedies for the treatment of diabetes mellitus because of the side effects associated with oral hypoglycemic agents (therapeutic agents). Traditional herbal medicines, which are primarily obtained from plants, play an important role in managing diabetes mellitus. In recent years, herbal medicines have gained importance as a source of hypoglycemic agents [8,9]. The World Health Organization (WHO) listed 21,000 plants, which are used for medicinal purposes around the world [10, 11]. The study briefly reviews the role of traditional therapeutics and natural medicines from the traditional medicinal system in the treatment of diabetes mellitus. Additionally, this research study investigates the effects of administering caffeic acid phenethyl ester (CAPE) on mice-induced type 1 diabetes, as well as angiogenic parameters, such as matrix metalloproteinase-9, angiopoietin and the anti-angiogenic factor, endostatin, which is known to be associated with diabetes. Also, the study provides additional details and analyses on cytokine, nitric oxide levels and histopathological examination, which are necessary to investigate CAPE’s mode of action.

Angiogenic stimulating condition in diabetes

The formation of a new capillary network (microvascular) in response to hypoxia or other stimuli is termed angiogenesis. The process involves the local secretion of angiogenic factors from both hypoxic endothelium and supporting pericytes that induce endothelial proliferation and neovessel sprouting [12]. Angiogenesis differs from arteriogenesis, which is a process of growth and expansion of existing arteriole networks in response to acute arterial occlusion (e.g., arterial occlusion) or physical forces (e.g., exercise-induced shear stress). Abnormal blood vessels in glomeruli were first observed in patients with long-term type 1 diabetes [13], and these symptoms were later observed in patients with type 2 diabetes [14]. Initially, the concept of angiogenesis referred to the sprouting of ECs from pre-existing vessels into a previous vascular tissue. Recently, the concept has been expanded to include the growth and remodeling process by which an initial vascular system is modified to form a complex branching network, characteristic of the mature vasculature [15]. Angiogenesis is, therefore, a complex multistep process involving extracellular matrix (ECM) degradation, proliferation, survival, migration and morphological changes of EC and their anastomosis to assemble into a vascular structure [16].

Mechanisms of angiogenesis in diabetes

For angiogenesis to occur, many receptors need to be activated by special ligands. These receptors include: placental growth factor (PlGF), acidic and basic fibroblast growth factors (aFGF and bFGF, respectively), angiopoietins, hepatocyte growth factor (HGF), platelet derived growth factor (PDGF), and platelet derived-endothelial cell growth factor (PD-ECGF) [17]. For more than a decade, the role of vascular endothelial growth factor (VEGF) in the regulation of angiogenesis was the focus of intense investigation. Recent evidence indicate that the growth and maturation of new vessels are highly complex and coordinated processes, requiring the sequential activation of a series of receptors by numerous ligands; however, VEGF signaling often represents a critical rate-limiting step in physiological angiogenesis [18]. VEGF engages and activates its tyrosine kinase receptors VEGFR-1 and VEGFR-2 on ECs.

Although VEGF binds to both receptors, the majority of its biological functions are mediated by VEGFR-2 signaling, and VEGFR-2 is involved in several steps of the angiogenic process [19]. In addition, VEGF is the major factor involved in the mobilization of endothelial progenitor cell (EPC) from the bone marrow to the peripheral circulation and to the angiogenic sites, where they differentiate and integrate the neovasculature [20]. The majority of these receptors present in vascular wall cells act by triggering signaling cascades of phosphorylating kinases. Nonetheless, for these cellular transduction pathways to properly function, a balance between phosphorylating kinases and dephosphorylating phosphatases must exist. Protein tyrosine phosphatases (PTP) comprise a very large family of enzymes that catalyze dephosphorylation of tyrosine residues [21].

Chronic inflammation in diabetes

Inflammatory mediators, such as cytokines, growth factors and free radicals are characteristic features of diabetes mellitus. Free radicals may be cytotoxic to β -cells and may accelerate the incidence and development of type 1 diabetic phase. Among the free radicals, nitric oxide (NO) has attracted special attention [22]. It has been observed that the inducible form of nitric oxide synthase (iNOS) is expressed in different autoimmune diseases. Islet cells and invading macrophages express iNOS in the insulinitis present in diabetes-prone biobreeding rats and NOD mice [23].

Also, iNOS mRNA expression has been detected in both rodent and human pancreatic islets exposed in vitro to cytokines [24]. The transgenic expression of iNOS in cells induces cell destruction and diabetes, whereas the lack of iNOS expression prevents diabetes induced by multiple subdiabetogenic doses of streptozotocin and the in vitro inhibitory effects of interleukin (IL)-1 on mouse cell function. Additionally, based on available data, researchers are not agreed on whether blocking iNOS activity by pharmacological agents prevents cytokine-induced cell dysfunction and death in both rodent and human islets [25, 26]. It is unclear whether the radical NO contributes to cytokine-induced cell necrosis and apoptosis [27]. These conflicting observations may be attributed to the use of non-specific pharmacological blockers of iNOS activity [28]. For example, the pancreatic β -cells express a constitutive isoform of NOS (nNOS), which may participate in cell physiology. Thus, it is conceivable that pharmacological NO synthase inhibitors will affect both isoforms of the enzyme [29]. In addition, several analogs used to inhibit iNOS activity, such as aminoguanidine, may directly interfere with cell function, making it difficult to evaluate their potential impact on cytokine-induced β -cell dysfunction [30]. When pancreatic cells are exposed to cytokines, they express several genes and proteins that may contribute to cell dysfunction, death or cell repair [31]. Among these genes are the putative protective agents, heat shock protein (hsp) 70 [32], manganese superoxide dismutase (MnSOD) [33, 34], and the proapoptotic gene Fas [35-37].

Oxidative stress in diabetes

The presence of oxidative and nitrosative stress is another pathophysiological condition in diabetes. Several lines of evidence indicate that ROS activate signaling pathways that promote angiogenesis [38, 39]. ROS can be formed in many distinct ways in the human organism. NAD(P)H oxidases (Nox) are a relevant source of ROS formation from molecular oxygen and they are present in vascular EC and smooth muscle cells (SMC). A wide variety of angiogenic stimulators can be up-regulated by Nox [40]. EC present the endothelial isoform of nitric oxide synthase (eNOS), which contributes to ROS generation and catalyzes the synthesis of NO [41]. NO is an established scavenger of superoxide anion (O_2^-) and is also a potent vasodilator and angiogenic stimulator. Recently, NO has been reported to promote ROS production [42].

Mechanism of action of anti-diabetic agent

Diabetes therapy is aimed at inducing hypoglycemic activity in the body [58], and there are different ways through which anti-diabetic agents can act. These include stimulating the β -cell of pancreatic islet to release insulin, resisting the hormones that increase blood glucose, increasing the number and sensitivity of insulin receptors, increasing the glycogen content, enhancing the use of organ glucose in the tissue and free radical scavenging, resisting lipid peroxidation, correcting the metabolic disorders of lipid and protein, and promoting microcirculation. However, if free radicals overwhelm the body's ability to regulate them, oxidative stress ensues. Free radicals adversely alter lipids, proteins, and DNA and trigger a number of human diseases. Hence, applying an external source of antioxidants can assist in managing oxidative stress [44].

Herbal medicines and treatment of diabetes

The chemical composition of plant products used as alternative medicines for treating diabetes influences their biological action. These plant products are usually rich in phenolic compounds, flavonoids, terpenoids, coumarins, and other constituents that can cause reductions in blood glucose levels [47, 48]. Many species of plants have been described in scientific and popular literature as having anti-diabetic activity [49]. However, some of these plants have been used as dietary adjuvant for treating numerous diseases without adequate knowledge of their functions and constituents. This practice may have been caused by the cost and side effects of synthetic hypoglycemic agents [50, 51]. Another reason may be that the effectiveness of hypoglycemic agents is lost in a significant percentage of patients.

Effect of caffeic acid phenethyl ester on induced type 1 diabetic model

Caffeic acid phenethyl ester (CAPE) is a phenolic antioxidant and is an active anti-inflammatory natural resinous product of honeybees' propolis (bee glue). Studies have shown that CAPE may be used to control hyperglycemia and to modulate glucose in vivo. In addition to being a powerful antioxidant, CAPE was proposed to influence and restrain the transcription nuclear factor-kB (NF-kB), which may be considered for its anti-inflammatory action. Moreover, notable evidence suggests that angiogenesis and chronic inflammation are co-dependent; therefore, obstructing angiogenesis results in an anti-inflammatory effect [52, 53]. The animal models of diabetes are important in understanding the pathophysiology and in deciding the therapeutic strategy for diabetes. Diabetes mellitus can be induced by pharmacological, surgical or genetic manipulations in several animal species. The majority of the experiments in diabetes are conducted in rodents, although several studies on diabetes are performed in a large number of new animal models, including knock-in, generalized knock-out and tissue-specific knockout mice. A previous study [54] of CAPE on mice-induced immune-regulated type 1 diabetes has provided valuable information in understanding the CAPE's mode of action and potential anti-diabetic effects. Additional research on CAPE could establish CAPE as a leading compound for future use in anti-diabetic medication. Furthermore, histopathological examination on different organs were investigated in this study for assurance of CAPE effects.

II. Materials And Methods

Experimental animals

Laboratory-bred adult male Swiss mice, 8 weeks of age and weighing 22 ± 2 g, were purchased from King Abdel Aziz University. The mice were housed, Faculty of Medicine, Albaha University, Saudi Arabia Kingdom, under stable conditions (12-h light/dark cycle, regimen consisting of oriental chow pellet food and water ad libitum). All animal procedures were performed in accordance with the standards of the institution's guidelines for the care and use of experimental animals [55].

Chemicals and medications

Cyclosporin A (CsA), Sandimmune® injection, and streptozotocin (STZ) were obtained from Sigma Chemicals Co. (St. Louis, MO, USA). IL-1 β and IFN- γ detection kits were obtained from Life Technologies™,

Biosource, Belgium. Insulin kits were purchased from Abbott Laboratories, USA. All other chemicals used in the present study were purchased from Sigma Chemicals Co.

Caffeic acid phenethyl ester

Caffeic acid phenethyl ester was dissolved in DMSO to create a 100-mM stock solution and stored at -20°C . The serial concentrations in PBS solution were prepared as needed during the mouse treatments.

Lethality study in Swiss albino mice

The male Swiss albino mice were randomly distributed into several groups of 6 mice each. The mice were treated using different doses (50–300 mg/kg) of caffeic acid phenethyl ester and the number of surviving mice was recorded daily. This process was continued for 30 days. The mice were subjected to experiments in accordance with ethical standards [56].

Induction of autoimmune type 1 DM

The mice were weighed and ear-notched. STZ was dissolved in cold 0.01 M citrate buffer, pH 4.5. The buffer was always freshly prepared for immediate use. STZ was injected intraperitoneally (i.p.), and the doses were determined according to the animals' body weights. CsA (20 mg/kg/day, s.c.) were administered daily to fifty mice for 10 days before STZ treatment. The mice also received, simultaneously, multiple low doses of streptozotocin (MLDSTZ) (20 mg/kg/day, 40 mg/kg/day and 60 mg/kg/day, i.p.) for 5 consecutive days (10 mice/group) [57]. Non-fasting blood samples were collected twice per week by tail bleeding into heparinized tubes. The glucose concentrations in the plasma samples were determined using the enzymatic colorimetric method [58]. The mice were defined as diabetic when their non-fasting blood glucose level (BGL) measured more than 200 mg/dl in two consecutive readings [59].

Study design

Fourteen days after the last dose of CsA/MLDSTZ co-administration, the mice that exhibited signs of diabetes were selected. The diabetic mice were divided into two equal groups ($n = 10$ for each group), either untreated (group 1, diabetic mice) or treated with $5\ \mu\text{M}/\text{kg}$ CAPE (group 2, diabetic CAPE). Simultaneously, twenty normal mice were divided into two equal groups ($n = 10$ for each group) and were treated with either saline (group 3, normal mice) or $5\ \mu\text{M}/\text{kg}$ CAPE (group 4, normal CAPE). All of the treatments were administered i.p., and 10 injections were administered over a 21-day period. At the end of the treatment period, the mice were anesthetized with ether, and the blood samples were collected by heart puncture and centrifuged at 2000 rpm for 5 min. The serum samples were separated to determine the serum insulin levels and other parameters. The pancreas was removed from each mouse and bisected longitudinally. One half was dried carefully on filter paper and homogenized in PBS to prepare a 10% homogenate. The supernatant was removed to determine IL- 1β , IFN- γ and nitric oxide (NO) levels. The remaining half of each excised pancreas and the other excited organs (kidney, liver and ocular) were immersion-fixed in 10% neutral formalin for 24 h. The sections were embedded in paraffin wax, were serially sectioned ($5\ \mu\text{m}$), and were stained using hematoxylin and eosin (H&E) for histopathological examination [60].

Determination of nitric oxide content in pancreatic islet cells

The nitric oxide levels in the pancreatic islet cells were measured as nitrite and nitrate according to the method of Miranda, which is based on the Griess reaction with a prior reduction step using vanadium chloride to convert nitrates to nitrite ions [61]. The absorbance of the samples was measured at 540 nm using a double-beam spectrophotometer (Shimadzu UVPC 1601, Japan).

Determination of serum insulin levels

The insulin levels in the serum samples were determined using the micro particle enzyme immunoassay (MEIA) and the Abbott AxSYM® system [62].

Determination of IL- 1β and IFN- γ content in pancreatic islet cells

The pancreatic islet cells were obtained from different mice groups and the levels of IL- 1β and IFN- γ were determined using ELISA kits with monoclonal antibodies specific for IL- 1β and IFN- γ . The samples were run in duplicate according to the manufacturer's instructions. The cytokine concentrations were determined from standard curves using the purified recombinant cytokines provided with the kits.

Determination of serum levels of matrix metalloproteinase (MMP-9) and endostatin (sE)

Serum levels of MMP-9 and endostatin were assessed by collecting blood samples from the punctured hearts of each treated and untreated mouse. The samples were delivered into plastic tubes that did not contain

any anticoagulant and were left to clot. The samples were subsequently centrifuged to obtain serum, which was stored at -70°C . For the quantitative determination of MMP-9 and endostatin, competitive enzyme-linked immunosorbent assay (ELISA) was used. The technique measures the natural and recombinant forms of the cytokine (Cytoimmune Science Inc., MD). For each sample, 100 μl of serum sample was added to the designated wells. This assay employs the quantitative sandwich enzyme immunosorbent assay technique. A monoclonal antibody specific for cytokine was precoated onto a microplate. The standards and samples were pipetted into the wells, and cytokine was bound by the immobilized antibody. After washing away the unbound substances, an enzyme-linked polyclonal antibody specific for cytokine was added to the wells. After a wash to remove any unbound antibody, an enzyme reagent and a substrate solution were added to the wells, and a color was developed in proportion to the amount of total cytokine (pro and active) bound in the initial step. The color development was ceased and the intensity of the color was measured [63].

Determination of the plasma levels of angiotensin 1 (Ang-1) and angiotensin 2 (Ang-2)

The plasma samples were collected from each group of mice in a sterile tube containing EDTA. The plasma samples were separated by centrifugation at 2500 rpm for 10 min in a refrigerated centrifuge. The plasma samples were stored at -70°C and thawed before the assay. The enzyme-linked immunosorbent assay (ELISA) was performed using commercially available kits from R&D Systems (Minneapolis, MN, USA) according to the manufacturer's instructions. The samples were collected using the anticoagulant ethylene diamine tetra acetic acid (EDTA) and stored at -70°C . The plasma samples were added to separate microplates, each containing a specific antibody for Ang-1 and Ang-2. The mixtures were incubated at room temperature for 2 h. The plates were washed four times to remove unbound antigen. Enzyme-linked polyclonal antibodies specific for each angiogenic factor were added, and the mixture was incubated for 2 h, followed by another washing. Subsequently, the substrate solution was added to the wells. Color development ceased, and the intensity of the color was measured and compared using a standard curve. The optical density of each well was determined at 570 nm [64].

Histopathology

The kidney, liver, ocular tissue and pancreas of the normal, diabetic and the diabetic group treated with CAPE were periodically removed and fixed in 10% formaldehyde. The tissues were washed with water, dehydrated using a gradient of increasing ethanol concentrations and embedded in paraffin. Five-micrometer thick sections were prepared using a microtome and placed onto glass microscope slides. The sections were stained with hematoxylin and eosin and were subjected to a histopathological examination [65].

Statistical analysis

The data are expressed as the mean \pm standard deviation (SD) values. The comparisons between two different groups were performed using Mann-Whitney U test and between more than two groups using Kruskal-Wallis one-way analysis of variance (ANOVA) followed by the Tukey-Kramer test. The GraphPad Software Instat (version 9) was used to perform the statistical analysis.

III. Results

Autoimmune type 1 DM model

Streptozotocin-induced diabetes is a well-documented model of experimental diabetes. Co-treatment of mice with CsA/MLDSTZ resulted in autoimmune type 1 DM. This model is confirmed and validated by the time-dependent change in the BGL of 75% of the mice (30 of 40 mice). In the present study, the experimental group demonstrated that a single CsA/MLDSTZ i.p. injection (20 mg/kg) caused no significant changes in BGL (128.7 ± 6.1 mg/dl) compared with normal mice from the first day of injection through the 5th day. However, CsA/MLDSTZ (40 mg/kg) induced a gradual and progressive increase in BGL, producing mice with type 1 DM or insulin-dependent diabetes (229.1 ± 12.6 mg/dl) and an accompanying marked reduction in serum insulin levels from the second day of administration. Moreover, CsA/MLDSTZ (60 mg/kg) induced hyperglycemia from the first day of injection (368.5 ± 14.4 mg/dl), which is significant in type 2 or non-insulin-dependent diabetes, with complete damage to the islet cells (Fig. 1).

Figure 1. Represent mice normal BGL value and mice treated with STZ (20, 40 and 60 mg/kg) respectively versus BGL mg/dl expressed as means \pm SD which obtained from each group of mice in four separate experiments

Effect of caffeic acid phenethyl ester on blood glucose levels

Treating diabetic mice with CAPE 5 μM i.p./every 2 days resulted in a time-dependent decrease in the BGL. This reduction became significantly effective by day 12 of treatment compared with the levels in the

untreated mice. By day 21, the BGL was reduced to 137.1 ± 7.2 mg/dl (Fig. 2), and this value was significantly different from the values observed in the diabetic control mice ($P < 0.005$, ANOVA). The BGL of control diabetic mice, which did not receive treatment, increased throughout the treatment period in a time-dependent manner. The normal mice treated with CAPE $5 \mu\text{M}/\text{kg}$ i.p./every 2 days for 21 days showed non-significant changes in the BGL compared with the normal saline group ($P > 0.05$, ANOVA). Sores and increased irritability have been previously observed in mice that were administered CAPE treatment with $10 \mu\text{M}/\text{kg}$; hence, this dosage was avoided in the experimental study.

Figure 2. CAPE effects on the blood glucose level in diabetic mice. Mice were treated with CAPE ($5\mu\text{M}/\text{kg}$ i.p./every 2 days) for 21 days. Serum glucose levels were determined at the end of the treatment period.

Effect of CAPE on serum insulin

The untreated diabetic mice showed a significant decrease (88.7%) in serum insulin levels compared with the normal saline group. The treatment of diabetic mice with CAPE $5 \mu\text{M}/\text{kg}$ i.p./every 2 days for 21 days resulted in a significant increase (by 93.8%) in the serum insulin levels. Moreover, the normal mice treated with CAPE $5 \mu\text{M}/\text{kg}$ i.p./every 2 days showed a non-significant change in serum insulin levels compared with the normal saline group.

Effect of CAPE on IL-1 β , IFN- γ and NO content in pancreatic islet cells

The untreated diabetic group showed a significant increase by 81.6%, 53.7% and 92.8% in the pancreatic islets levels of IL-1 β , IFN- γ and NO, respectively, compared with the normal saline group. The treatment of diabetic mice with CAPE $5 \mu\text{M}/\text{kg}$ i.p./every 2 days for 21 days resulted in significant decreases in IL-1 β and IFN- γ levels ($P < 0.01$, ANOVA) and a highly significant decrease in NO levels ($P < 0.001$, ANOVA) (43.2%, 26.3% and 58.3%, respectively) compared with the diabetic untreated group. However, there was a non-significant change compared with the normal saline group. The normal mice treated with CAPE revealed a non-significant change in the pancreatic islets levels of IL-1 β , IFN- γ and NO content compared with the control saline group (Fig. 3).

Figure 3: CAPE effects on the IL-1 β , IFN- γ and NO content in pancreatic tissue. Mice were treated with CAPE ($5\mu\text{M}/\text{kg}$ ip) compared with diabetic group.

In vivo evaluation of serum metalloproteinase-9 and endostatin (sE)

Untreated diabetic mice elicited a highly significant increase in serum MMP-9 levels with the administration of 197.3 ng/ml, which was reduced to 139.1 ng/ml in mice treated with CAPE ($P < 0.01$) and approximated the serum levels of the normal mice. However, in the untreated diabetic mice, the endostatin (sE) levels were significantly reduced to 1.2 ng/ml compared with the normal mice ($P < 0.01$). Additionally, the serum of the CAPE-treated diabetic mice, endostatin levels were significantly higher (1.8 ng/ml) ($P < 0.001$) than the serum levels in the diabetic control group (Table 1). There was a significant correlation between sMMP-9 and the BGL ($r = 0.254$, $P < 0.030$) and negative correlations between sMMP-9 and sE, total white blood cells (WBCs), hemoglobin (HB) and platelet counts in the mice (Table 2). Furthermore, MMP-9 is strongly correlated with Ang-2 ($r = 0.334$, $P = 0.004$) and the calculated Ang-2/Ang-1 ratio ($r = 0.321$, $P < 0.004$) but not with Ang-1.

Table 1. Levels of MMP-9 and Endostatin serum in normal mice group, diabetic mice group and treated group.

Group Parameters	Normal Group	Diabetic Group	Treated Group	P1	P2
MMP-9(ng/ml)	133.4	197.3	139.1	P< 0.01	P< 0.001
Range	79.6 – 166.4	88.2 – 216.1	84.5 – 196.3		
Endostatin(ng/ml)	1.9	1.2	1.8	P<0.001	P<0.01
Range	1.1-6.3	1.00-5.4	1.3-9.3		

P1: Diabetic group Vs Normal group

P2: Treated group Vs Diabetic group

Table 2. Correlation between investigated angiogenic factors and blood glucose level (BGL), hemoglobin (HB), White blood cells count (WBCs) and platelet counts in the treated mice group with CAPE.

Group Parameters	BGL	HB	WBCs Count	Platelets Count
MMP-9	r=0.254 P <0.030*	r= 0.13,P >0.05	r= 0.16, P>0.05	r= 0.18,P >0.05
Endostatin	r=0.19, P>0.05	r= 0.11, P>0.05	r= 0.11 ,P >0.05	r= 0.08,P >0.05

Plasma levels of Ang-1 and Ang-2 in treated diabetic mice and diabetic controls

The median and range of the plasma levels of Ang-1 and Ang-2 in normal saline-treated, normal CAPE-treated, diabetic control mice and CAPE-treated diabetic mice are presented in Table 3. The circulating levels of Ang-1, Ang-2 and the calculated Ang-2/Ang-1 ratio are significantly higher in diabetic mice compared with the normal saline mice ($P < 0.05$, $P < 0.01$ and $P < 0.001$). The Ang-2 and Ang-2/Ang-1 ratio are significantly reduced in the treated diabetic mice compared with the untreated mice ($P < 0.01$). Moreover, there is a significant correlation between Ang-2 and BGL and between the calculated Ang-2/Ang-1 ratio and BGL ($P < 0.01$, $P < 0.01$). There is no correlation with Ang-1 ($P > 0.05$).

Table 3: Levels of Ang- 1 and Ang- 2 plasma in normal mice group, diabetic mice group and treated group.

Group Parameters	Normal Group	Diabetic Group	Treated Group	P1	P2
Ang-1 (ng/ml)	1.63	3.31	2.61	P< 0.05	P< 0.01
Range	0.669 -2.1	1.92 – 4.63	1.30– 3.37		
Ang-2 (ng/ml)	2.31	1.2	4.91	P < 0.01	P < 0.01
Range	0.91-3.6	1.00-5.4	3.42– 5		
Ratio Ang-2/Ang-1	1.42	3.0	1.9*	P < 0.001	P < 0.01

P1: Diabetic group Vs Normal group

P2: Treated group Vs Diabetic group

IV. Discussion

Diabetes mellitus type 1 results from the autoimmune destruction of insulin-producing beta cells in the pancreas. Genetic and currently undefined environmental factors participate in the onset of this disease. In the present study, experimental animals were employed as models for type 1 diabetes induced by CsA/MLDSTZ co-treatment. The mice involved in this study were normal and did not have any underlying immune abnormalities that could complicate the study [66]. For centuries, propolis obtained from honeybee hives has been used in folk medicine as an anti-carcinogenic, anti-inflammatory, anti-bacterial and immunomodulatory agent. CAPE is an active component of propolis that has attracted the attention of investigators and researchers because of its potential effects in the treatment of numerous disorders and illnesses [67–69].

In the present study, to gain more insight into the effects and mechanisms of action of CAPE, we performed an in vivo study using a diabetes-induced mouse model. Our results showed that CAPE, administered at 5 µM/kg i.p./every 2 days for 21 days, proved to be beneficial for the host in two ways. First, CAPE induced a marked anti-diabetic activity in type 1 DM, as manifested by the significant decrease in the elevated BGL, which reached nearly normal values at the end of the treatment period. Second, the reduction in the BGL appeared to be parallel to a significant increase in the circulating level of insulin after treatment. This finding seems to be consistent with similar findings reported by Jung et al., who found that caffeic acid induced a significant reduction in the blood glucose and glycosylated hemoglobin levels in treated C57BL/KsJ-db/db mice compared with the control group [70]. Moreover, the beneficial effect of CAPE on autoimmune type 1 diabetic mice group was confirmed by the histopathological findings. The kidney shows no alteration in glomerular architecture except some hydropic changes in the glomerular cells in addition to scanty mononuclear glomerular

and interstitial infiltrates. The liver variable morphology of hepatocytes as majority of hepatocytes show area of reversible cell injury in the form of hydropic changes as marked by cytoplasmic vacuolization minority show restoration of eosinophilia cytoplasmic appearance as a part of healing process, dilated congested sinusoids are seen. The ocular tissue appears with normal structures. Finally, for the pancreas; islets of Langerhans's has a pleomorphic pattern as area is being heavily infiltrated by mononuclear cells while another area is being less denoting some regeneration occurred in this area (Fig. 6)

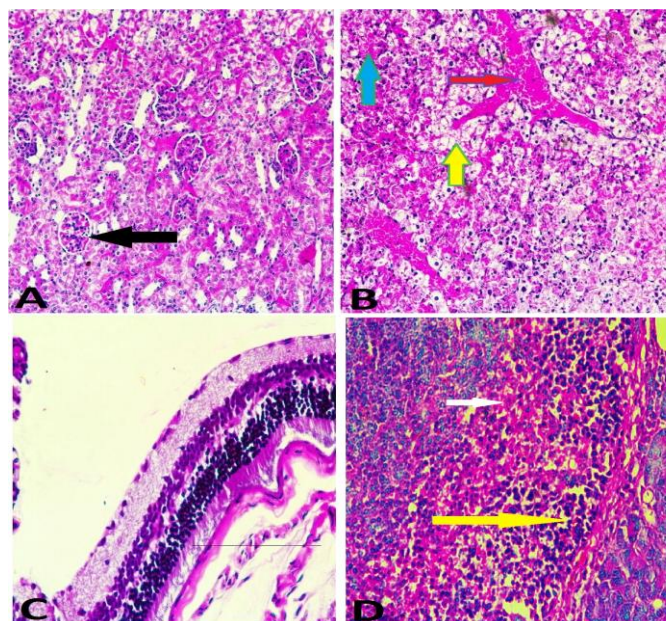


Figure 6: A. kidney; no alteration in glomerular architecture except some hydropic changes in the glomerular cells (black arrow) in addition to scanty mononuclear glomerular and interstitial infiltrates(x200) B. liver variable morphology of hepatocytes as majority of hepatocytes show area of reversible cell injury in the form of hydropic changes as marked by cytoplasmic vacuolization (yellow arrow) minority show restoration of eosinophilia cytoplasmic appearance as a part of healing process (blue arrow), dilated congested sinusoids are seen (red arrow) (x200) C. Ocular tissue with normal structures (x200) D. Pancreas; islets of Langerhans's has a pleomorphic pattern as area is being heavily infiltrated by mononuclear cells(yellow arrow) while another area is being less denoting some regeneration occurred in this area (x400).

Inflammation is a well-recognized localized tissue reaction to infection, irritation, or other injury. However, when inflammation persists or control mechanisms are dysregulated, diseases can develop, such as type 1 diabetes. Although inflammatory cytokines and oxidative stress play a central role in the pathogenesis of acute pancreatitis, CAPE administration of 5 μ M/kg to Swiss mice for 21 days led to the inhibition of IL-1 β , IFN- γ and NO concentrations (43.2%, 26.3% and 58.3%, respectively) compared with untreated diabetic mice. This finding demonstrates the anti-inflammatory and immunomodulatory activity of CAPE. These results are in agreement with the findings of Durmus et al., who reported that CAPE treatment inhibits lipid peroxidation and NO overproduction and regulates SOD enzyme activities in diabetic rats [71]. The results are also supported by Wang et al., who documented that a CAPE-treated diabetic group exhibited protective effects against oxidative damage compared with an untreated diabetic group in the sciatic nerve tissues of the diabetic rats [72].

Because type 1 DM is characterized by the infiltration of pancreatic islets with activated macrophages and activated T lymphocytes [73], our data indicate that CAPE can inhibit the expression of inflammatory mediators IL-1 β , IFN- γ and NO, which participate in the pathogenesis of type 1 DM. Furthermore, the level of MMP-9 in the serum of untreated diabetic mice was significantly higher (197.3 ng/ml, $P < 0.001$) compared with the serum of normal mice (133.4 ng/ml). Moreover, we found a highly significant reduction in serum MMP-9 (139.1 ng/ml, $P < 0.01$) in the treated group compared with the untreated group. This finding seems to be consistent with Kowluru, who illustrated the potential role of MMP-9 in diabetic retinopathy [74]. Additionally, the data from our study indicate that the endogenous inhibitor of the angiogenic serum (endostatin) has been demonstrated to be overexpressed (1.8 ng/ml) at significantly higher levels in the treated mice compared with the untreated mice (1.2 ng/ml) and to approximate the values in the serum of normal mice (Table 1). Thus, strategies for neutralizing MMP-9 should be considered as supplements to the anti-angiogenic therapeutic strategies that are currently employed in clinical settings. This suggestion is consistent with the findings of Rishi and Bhende and Smith and Steel because these authors have mentioned using anti-vascular endothelial growth factor for

preventing post-operative vitreous cavity hemorrhage after vitrectomy for proliferative diabetic retinopathy [75, 76].

Over the last several years, attention has been directed towards the prognostic impact of angiogenic growth factors in diabetes. The results of the present study demonstrate that the levels of plasma Ang-2 (but not Ang-1), similar to the levels of MMP-9, are selectively elevated in diabetic mice and are associated with indexes of endothelial damage/dysfunction, regardless of vascular disease. Our results showed that Ang-2 and the calculated Ang-2/Ang-1 ratio were reduced in the treated diabetic mice and may be considered as independent prognostic parameters that could predict the efficacy of diabetic treatment strategies. Additionally, the levels of Ang-2 and the calculated Ang-2/Ang-1 ratio in the untreated diabetic group were significantly elevated compared with the normal controls. This finding is, in part, consistent with the results of Calderari et al. [77], who reported similar outcomes in which Ang-2 was elevated in diabetic mice and inhibited using Ang-2 inhibitor IL1-10, with a concomitant increase in beta-cell density in IL1-10-treated hyperglycemic SCID mice.

Based on the findings of this study, we suggest that CAPE is a potent anti-inflammatory agent that has anti-diabetic and anti-angiogenic properties. Our experimental data confirm that CAPE could potentially be useful for controlling the BGL in experimental models. The action of CAPE is accompanied by the shift and elevation of angiostatic and angiogenesis-inhibiting factors.

V. conclusions

In conclusion, our study has shown that CAPE may be effective in treating autoimmune type 1 DM. The anti-diabetic effect of CAPE seems to be a secondary consequence of its anti-inflammatory and immunomodulatory properties because using CAPE resulted in a significant decrease in the elevated levels of IL-1 β , IFN- γ and NO. Furthermore, Angi-2 in the circulation may represent an attractive target when introducing CAPE as an anti-angiogenic strategy for the treatment of diabetes as an independent predictor of a favorable prognosis and treatment for diabetes. These findings have demonstrated that endostatin itself may be used as an effective therapeutic agent. Finally, CAPE has many biological and pharmacological properties with potential future applications in human clinical experimentation.

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