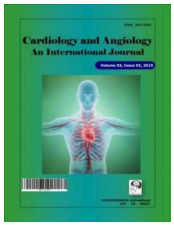

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C-peptide Attenuates Progression of Atherosclerosis in Late Stages of Type 2 Diabetes in Male Albino Rats

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Authors' contributions

This work was carried out in collaboration between all authors. Author AEDRAR designed the study, wrote the protocol and wrote the first draft of the manuscript. Author HMI managed the literature searches and analyses of the study performed the spectroscopy analysis. Author AHA managed the experimental process. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Study the role of C-peptide in development of atherosclerosis in late stages of type 2 diabetes in rats.
Methodology: Late stages of type II diabetes were induced by administeration of high fat diet and intraperitoneal injection of a single dose of streptozotocin 35 mg/kg, then rats were divided into 5 groups: 1) *control, 2) Diabetic (DM), 3) Diabetic + C-peptide, 4) Diabetic + L-NAME, and 5) Diabetic + C-peptide + L-NAME*.
Measurements: At the end of the experiment blood samples were taken for measurement of serum glucose and insulin levels. The arch of the aorta was taken for: Histopathological study and measurement of tissue lipid peroxides (MDA), Bcl-2 (B-cell lymphoma protein 2, apoptotic factor), nitric oxide (NO) and tumor necrosis factor-α (TNF-α).
Results: Aorta of diabetic rats showed severe atherosclerotic changes, and there were significant increase in serum glucose, tissue MDA, and TNF-α alongside there were significant decrease in

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serum insulin, tissue NO and Bcl-2 as compared to control group. Significant improvement in atherosclerotic changes were observed in *Diabetic + C-peptide group* which was accompanied with significant decrease in serum glucose, tissue MDA, and TNF-α. There were also significant increase in serum insulin and tissue NO and Bcl-2 as compared to DM group. In *DM + L-NAME* group, there were severe deterioration of atherosclerotic changes, and there were significant increase in serum glucose, tissue MDA, and TNF-α alongside significant decrease in serum insulin, tissue NO and Bcl-2 as compared to DM group. Although adding L-NAME to DM + *C-peptide* group abolished increasing in serum insulin level but improvement in atherosclerotic changes and other parameters were still present.

Conclusion: C-peptide has a protective effect against development of atherosclerosis through many mechanisms including hypoglycemic, antioxidant, antiapoptotic, and anti-inflammatory effects. So administration of C-peptide as an adjuvant therapy in late stages of type II diabetes can significantly decrease incidence of vasculopathy.

Keywords: C-peptide; diabetes; atherosclerosis; Bcl-2.

1. INTRODUCTION

Diabetes mellitus is still an increasing health problem in both developing and developed countries. Diabetes is associated with several complications such as retinopathy, nephropathy, neuropathy and cardiovascular diseases [1].

Human C-peptide is a 31-amino acid peptide cleaved from pro-insulin and secreted in equimolar concentrations with insulin by pancreatic β -cells into the circulation. Deficiency of C-peptide, along with insulin, is a typical feature of type 1 diabetes mellitus (DM) and the later stages of type 2 DM [2].

For years, C-peptide has been considered to be biologically inert, until recent works has demonstrated that C-peptide can activate intracellular signalling pathways in various cell types [3].

Vasic et al. [4] supported the hypothesis that C-peptide may have an active role in atherogenesis in patients with diabetes and insulin resistance. On the contrary C-peptide has been shown to protect against diabetic complications including neuropathy, nephropathy, vascular dysfunctions, and inflammation in animal models of diabetes and in type 1 DM patients [5].

Apoptosis also plays a crucial role in DM, especially mitochondria-associated signaling. For example, the B-cell lymphoma protein 2 (Bcl-2) protein family members are intrinsic pathway operators of apoptosis and control the release of cytochrome c and other intermembrane mitochondrial proteins to the cytosol [6].

Therefore, the aim of this work is to investigate the role and underlying mechanisms of C-peptide in the development of atherosclerosis in adult male albino rats in late stages of experimentally induced type 2 iabetes.

2. MATERIALS AND METHODS

2.1 Chemicals

C-peptide was obtained from Biorbyte, United Kingdom. L-NAME and Streptozotocin were obtained from (Sigma, USA). Glucose Kit (G) (Spinreact SPAIN). Lipid peroxide (MDA) and nitrite kits (Biodiagnostic EGYPT). Insulin (I) and Bcl-2 ELISA kits (Calbiotech USA). TNF-α ELISA Kit (Glory Science Co., Ltd, USA).

2.2 Animals

This prospective study was conducted at the Physiology department of Minia University, Faculty of Medicine, Egypt during the period from January 2016 to April 2016. Fifty adult male albino rats from local strain weighing 150 - 200gw were purchased from National Research Center (Giza, Egypt). Rats were freely allowed tap water and standard diet of commercial rat chow ad libitum and were left to accommodate for one week under natural light/dark regular cycles in partially humid and well-aerated room. From the beginning of the experiment, rats were housed individually. The use of animals Ethical issues were addressed according to the guidelines of the Animal Care and Use Committee of Faculty of Medicine, Minia University, Minia, Egypt.

2.3 Experimental Protocol

Rats were randomly divided into 5 groups (10 rats each) as follows:

- **Control group:** in which rats were fed standard pellet chow.

- **DM group:** in which the rats were fed high fat diet (58% fat, 25% protein and 17% carbohydrate, as a percentage of total kcal) for 2 weeks followed by a single intraperitoneal injection of low dose of streptozotocin 35 mg/kg body weight in 0.1 M citric acid buffer, pH (4.5). Diabetes was verified 7 days later (after 3 weeks of dietary manipulation in rats), by evaluating blood glucose levels with the use of glucose-oxidase reagent strips (Accu-Check, Roche Inc. Indianpolis. Rats having blood glucose level of 200mg/dI or greater were considered to be diabetic and selected for the study. These rats were maintained on high fat diet for an additional 4 weeks during which the treatment was given [7].
- **DM + C-peptide group (DM + CP):** in which rats were given HFD and STZ as above. One week later these rats received C-peptide 50 nmol/kg/day by intraperitoneal injection for 4 weeks [8].
- **DM + L-NAME:** in which ras were given HFD and STZ as above. One week later these rats received L-NAME 20 mg/kg/day in the drinking water for 4 weeks [9].
- **DM + C-peptide + L-NAME (DM + CP + L-NAME):** in which rats were given HFD and STZ then received C-peptide and L-NAME as above for 4 weeks [8,10].

2.4 Biochemical Analysis

2.4.1 Collection of blood samples

At the end of the experiment, all groups were fasted for overnight and then decapitated. After collection of blood, the samples were left to clot at room temperature, and then centrifuged at 3000 rpm for 15 min in a cooling centrifuge (Hettich centrifuge). The serum layer was then withdrawn into identified eppendorf tubes and stored at - 20℃ till the time of assay [11]. Biochemical assay were done according to manufacturer's instructions.

The arch of the aorta was rapidly removed, blotted dry, and divided into two parts one part was weighed and kept at -80℃ until analysis. The other part was kept in 10% formalin for histopathological examination.

2.4.2 Aortic tissue analysis

Specimens from the aorta were weighed and homogenized in potassium phosphate buffer 10 mM pH (7.4). The ratio of tissue weight to homogenization buffer was 1:10. The homogenates were centrifuged at 5000 rpm for 10 min at 4℃. The resultant supernatant was used for determination of MDA, Bcl2, NO, and TNF-α according to the manufacturer's instructions.

2.5 Statistical Analysis

Data were represented as means ± standard errors of the mean (SEM). Statistical analysis was performed using Prism computer program (Graph pad Prism 6, software Inc., San Diego, CA, USA). Significant difference between groups was done by one-way ANOVA followed by Tukey-Kramar post hoc test for multiple comparisons with a value of $P \leq 0.05$ considered statistically significant.

3. RESULTS

3.1 Effect of DM with and without Treatment on Serum Glucose Level

As shown in Fig. 1 DM group showed significantly higher serum level of glucose than the control group. DM + CP and DM + CP + L-NAME groups showed a significantly lower serum level of glucose than the DM group. The level of glucose was significantly higher in DM + L-NAME group than the DM group.

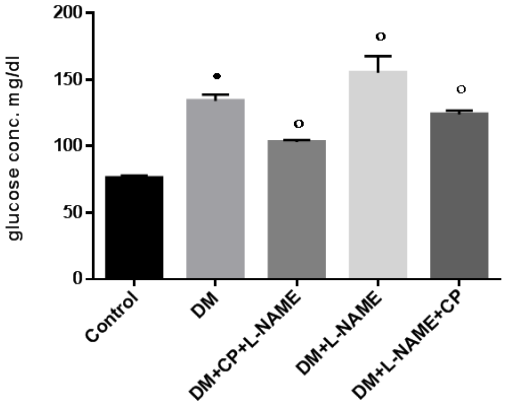

Fig. 1. Serum glucose level in diabetic rats with and without treatment

Data are expressed as M±SEM of 10 rats in each group; ●: significant from control group; o: significant from DM group P ≤0.05; DM: diabetes mellitus group; CP: C-peptide

3.2 Effect of DM with and without Treatment on Serum Insulin Level

As shown in Fig. 2 DM group showed significantly lower serum level of insulin than the

control group. DM + CP group showed a significantly higher serum level of insulin than the DM group. The levels of insulin were significantly lower in DM + L-NAME and DM + CP + L-NAME groups than the DM.

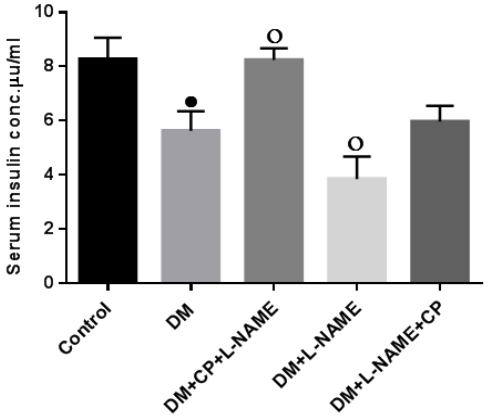

Fig. 2. Serum insulin level in diabetic rats with and without treatment

Data are expressed as M±SEM of 10 rats in each group; ●: significant from control group; o: significant from DM group, P ≤0.05; DM: diabetes mellitus group; CP: C-peptide

3.3 Effect of DM with and without Treatment on Aortic Tissue MDA, Bcl-2, NO, and TNF-α Levels

As shown in Table 1, DM group showed significantly higher MDA and TNF-α and significantly lower NO (nitrite) and Bcl-2 than the control group. DM + CP and DM + CP + L-NAME groups showed significantly lower MDA and TNF-α and significantly higher NO (nitrite) and Bcl-2 than the DM group. DM + L-NAME group showed significantly higher MDA and TNF-α and significantly lower NO (nitrite) and Bcl-2 than the DM group.

4. DISCUSSION

Vasculopathy is a major complication of diabetes; however, molecular mechanisms mediating the development of vasculopathy and potential strategies for prevention have not been identified [12].

C-peptide is considered as a physiologically active peptide for the amelioration of diabetes-induced complications. Although hyperglycaemia alone can cause alterations in body homeostasis during the diabetic state, the deficiency or absence of circulating insulin as well as C-peptide, could play an important role in the development and progression of hyperglycaemia-induced complications [13].

In diabetic group there were significant increase in blood glucose, tissue MDA, and TNF-α. On the other hand, there were significant decrease in blood insulin level and tissue Bcl-2 and NO levels as compared with the control group. And there were severe atherosclerotic changes in histological study as compared with the control group.

These data were in agreement with other researchers who reported that HFD lead to expansion of adipose tissue and accumulation of lipid metabolites which produces a systemic inflammation process in the form of increased TNF-α, interleukin-6 and C-reactive protein synthesis. Simultaneously, this fat excess promotes the appearance of insulin resistance. All this contributes to the development of atherosclerosis and increases the risk of cardiovascular diseases [14].

Table 1. Effect of type II DM with and without treatment on aortic tissue MDA, Bcl-2, NO (nitrite), and TNF-α levels

Groups parameter	Control	DM	DM + CP	DM +L-NAME	DM + CP +L-NAME
MDA (nmol/gm)	14.23±0.57	51.05±1.29•	35.13±1.02^{o}	68.09±2.49^{o}	43.46±1.09^{o}
Bcl-2 (nanog/gm)	81.70±1.84	62.01±1.22•	80.48±1.70^{o}	52.79±1.83^{o}	70.23±1.09^{o}
Nitrite (nM/gm)	151±2.87	124.4±2.5•	147.4±1.40^{o}	83.62±2.49^{o}	135.3±1.85^{o}
TNF-α (pg/mg tissue protein)	17.55±1.07	45.11±1.98•	27.9±0.99^{o}	54.02±2.47^{o}	37.25±2.15^{o}

Data are expressed as M±SEM of 10 rats in each group; ●: significant from control group; o: significant from DM group, P ≤0.05; DM: diabetes mellitus group; CP: C-peptide

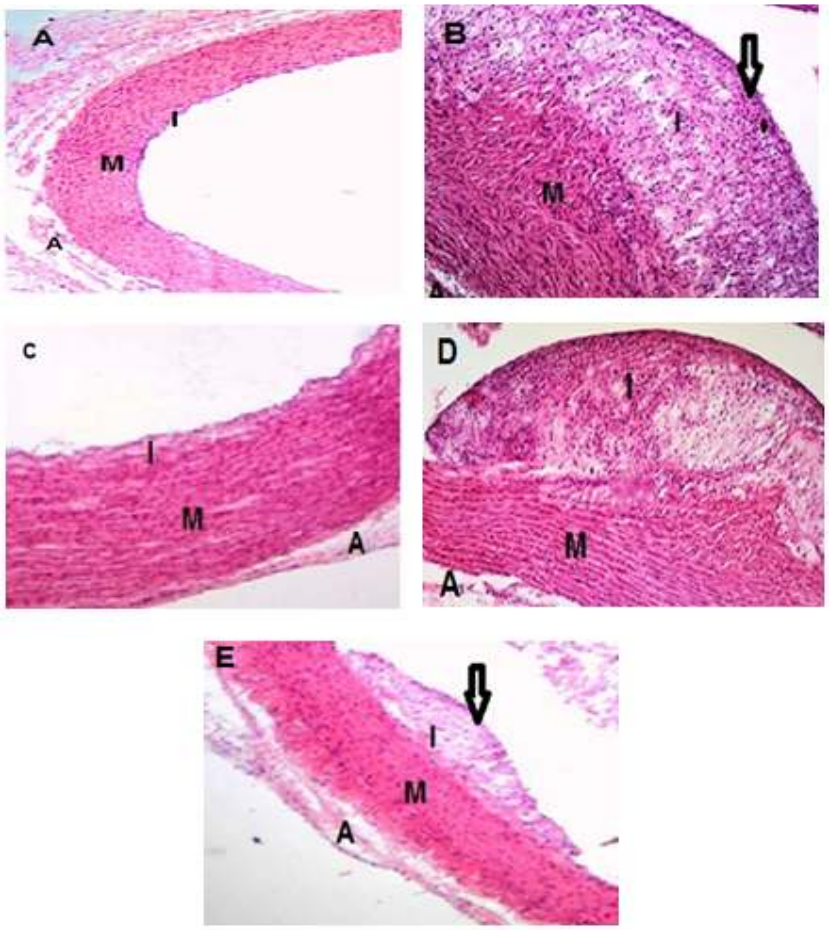

Fig. 3. Representative micrographs of the intimal lesions in rats of the: (A) control group; (B) diabetic group showing large intimal plaque (arrow); (C) Diabetic + CP group showing a marked reduction in the size of the plaque. (D): Diabetic + L-NAME group showing an increase in the size of the plaque (arrow) (E) diabetic + CP + L-NAME group, where the size of the plaque was moderately reduced (arrow). (H&E ×100) (I): intima, (M): media, (A): adventitia

Hyperglycemia has also been recognized to play a crucial role in the development of endothelial dysfunction leading to vascular complications. At the cellular level, hyperglycemia causes an increased vascular tone, and permeability of the endothelial cell monolayer. This is mainly a consequence of impaired production and bioavailability of vasodilators such as nitric oxide, vasoconstrictors such as endothelin-1 and permeability factors such as vascular endothelial growth factor [15]. In addition, exposure of endothelial cells to high glucose increases the production of reactive oxygen species (ROS), mainly through the mitochondrial electron transport chain and by upregulating antioxidant enzymes. The excessive production of ROS caused by hyperglycemia leads to alterations in endothelial cell proliferation and adhesion properties [12]. These alterations contribute to acceleration of the apoptotic process in endothelial cells [6]. High glucose also stimulates the secretion of the chemokines IL-8 and monocyte chemotactic protein type 1 (MCP-1) by endothelial cells. These inflammatory mediators are crucial in recruiting monocytes to the vessel wall. Simultaneously, the adipocyte itself is recognized to secrete circulating cytokines (adipokines) such as interleukin-6, tumor necrosis factor-α, leptin, and PAI-1, exacerbating the ongoing vascular inflammation in the vessel wall of diabetic patients [16].

NO is synthesized in 2 steps from the amino acid L-arginine (L-Arg) along with L-citrulline. This reaction is catalyzed by nitric oxide synthase (NOS) [17]. The expression of iNOS is stimulated

by inflammatory cytokines, and, once activated; iNOS can produce up to 1000-fold more NO than eNOS [18]. High glucose concentrations have been shown to increase cytokine-induced protein and gene expression levels of iNOS [19].

In our research the decrease in NO level in DM group can be explained by increase of advanced glycation end products, excessive generation of superoxide which associated with hyperglycemia and its interaction with NO results in the formation of peroxynitrites. These oxidize tetrahydrobiopterin, an important cofactor involved in normal eNOS activity [19].

Lipid peroxidation products; (MDA) serves as a marker of cellular oxidative stress and had long been recognized as a major causative factor of oxidative damage in chronic diseases [20]. In our work there were signs of oxidative stress as evidenced by the significant elevation in oxidative markers, namely MDA along with depletion of NO contents. Significant increase of tissue MDA in diabetic group can be explained by marked increase in oxygen free radicals and peroxynitrites.

DM group showed a decrease in tissue Bcl-2 level which demonstrate that the degree of apoptotic activity is higher under diabetic conditions than normal conditions. This can be explained by high glucose-induced cell death which has several important stages. It begins with the activation of key enzymes in the polyol pathway that are probably linked to glucose transporters at the cell membrane. The generation of reactive nitrogen species, in combination with ROS, rapidly induces apoptotic and necrotic cell death via mitochondria dependent and mitochondria-independent pathways. So high glucose level affects several stages of apoptotic signaling, resulting in cell death by increasing oxidative and nitrosative stress [6].

In DM + C-peptide group there was a marked improvement in atherosclerotic changes in the aorta with significant decrease in blood glucose, tissue MDA and TNF-α. There were significant increase in blood insulin and tissue Bcl-2 levels and NO levels as compared to DM group.

The glucose lowering effect of C-peptide was investigated in human by Johansson and coworkers [21] who reported that infusion of physiological concentrations of C-peptide to patients with T1D augments whole body glucose utilization by approximately 25%. They explained this augmentation by increasing muscle glucose uptake rather than inhibition of hepatic gluconeogenesis [22]. Also hypoglycemic effect of C-peptide protein in our study was in agreement with experimental studies on diabetic rats which showed that C-peptide prolongs the hypoglycemic effect of insulin and increases whole-body glucose utilization [23]. Bhatt et al. [12] added another explanation of hypoglycemic effect of C-peptide which was through inhibition of protein tyrosine phosphatase activity and autophosphorylation and activation of insulin receptor substrate-1, without direct binding to insulin receptor.

Hyperinsulinemia and IR are important risk factors for coronary heart disease (CHD). Animal models have demonstrated that supranormal serum insulin level is able to induce experimental atherosclerosis [24]. Additional studies show that super-physiologic concentrations of insulin can promote the proliferation and migration of cultured aortic smooth muscle cells [25]. Insulin could also stimulate accumulation of cholesterol in vascular endothelial cells as well as the binding of low-density lipoprotein (LDL) to arterial smooth muscle cells and monocyte-derived macrophages [26]. Furthermore, longterm administration of exogenous insulin was found to cause dyslipidemia and thickening of arterial walls and result in accelerated atherosclerosis in the arteries receiving insulin infusion. Therefore, insulin-induced atherosclerosis is one of the major causes of coronary artery disease [24]. So potentiation of hypoglycemic effect of insulin through C-peptide is a very important advantage because it helps to decrease the dose of insulin and so avoid complications of hyperinsulinemia.

In the present work, administration of C-peptide produced significant increase in serum insulin level which can be explained by significant increase in NO secretion. Our results are in agreement with Nakata and Yada [27] who found that increase in NO production resulted in potentiation of glucose-induced insulin release, in which a Ca2+-mediated pathway could be involved. Also endothelial NOS-derived NO dilates all types of blood vessels by stimulating soluble guanylyl cyclase and increasing cyclic GMP in smooth muscle cells [28]. These two beneficial effects of NO can explain the significant increase in insulin secretion in our work.

Inhibition of cellular oxidative stress in the form of a significant decrease in MDA and significant increase in NO was in agreement with Bhatt

et al. [12] who found that C-peptide (1 nmol/L) prevented endothelial cell death by inhibiting protein kinase C and NADPH oxidase–dependent intracellular ROS generation in streptozotocin diabetic mice.

Nitric oxide released towards the vascular lumen is a potent inhibitor of platelet aggregation and adhesion to the vascular wall [29]. Besides protection from thrombosis, NO also prevents the release of platelet-derived growth factors that stimulate smooth muscle proliferation and its production of matrix molecules. Nitric oxide decreases the expression of chemoattractant protein MCP-1 [30]. Nitric oxide can also inhibit leucocyte adhesion to the vessel wall by either interfering with the ability of the leucocyte adhesion molecule CD11/CD18 to bind to the endothelial cell surface or by suppressing CD11/CD18 expression on leucocytes [31]. Leucocyte adherence is an early event in the development of atherosclerosis, and therefore, NO may protect against the onset of atherogenesis. A disturbed integrity of the endothelial monolayer barrier can initiate proinflammatory events. Endothelium-derived NO prevents endothelial cell apoptosis induced by proinflammatory cytokines and proatherosclerotic factors including reactive oxygen species (ROS) and angiotensin II (AT). The suppression of apoptosis may also contribute to the antiinflammatory and anti-atherosclerotic effects of endothelium-derived NO [28]. So significant elevation of NO in DM + C-peptide group may participate greatlly in improvement of atherosclerotic changes and significant elevation of Bcl-2.

The inhibitory effect of C-peptide on TNF-α is in agreement with *in vivo* data showing that survival rates of mice following endotoxic shock is improved after C-peptide administration. In these mice, plasma levels of the pro-inflammatory cytokines TNF-α and MCP-1 were also decreased, suggesting a decreased generalised inflammatory response [32].

In our work, significant increase of Bcl-2 after C-peptide administration can be explained by its hypoglycemic effect alongside with improvement in oxidative stress and inflammation.

Addition of L-NAME to DM group ameriolated atherosclerotic changes in the aorta which was accompanied with a significant increase in blood glucose, tissue MDA and TNF-α levels. On the other hand, there were significant decrease in blood insulin, tissue Bcl-2 and NO level as compared to DM group.

In the present work addition of C-peptide to L-NAME in DM rats produced partial improvement of atherosclerotic changes. Also there were significant decrease in blood glucose, tissue MDA and TNF-α alongside with significant increase in tissue Bcl-2 and NO levels as compared to the DM group. On the other hand, blood insulin level wasn't significantly changed as compared to the DM group. These data support that NO is essential for insulin secretion and approve that the protective effect of c-peptide on pathogenesis of atherosclerosis could be achieved through its hypoglycemic, antioxidant, and anti-inflammatory effects independent on NO production.

5. CONCLUSION

Our results support the idea of administration of physiological quantities of C-peptide to late stage of type 2 diabetes patients as a new trial to lessen endothelial dysfunction and complications that may potentially occur during the course of the disease and give a chance to dercrease the insulin dose to avoid complications of hyperinsulinemia.

CONSENT

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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