Applied Physiology, Nutrition, and Metabolism

Physiologie appliquée, nutrition et métabolisme

Protective effect of C-peptide on experimentally induced diabetic nephropathy and the possible link between C-peptide and Nitric Oxide

Journal:	<i>Applied Physiology, Nutrition, and Metabolism</i>
Manuscript ID	apnm-2017-0617.R1
Manuscript Type:	Article
Date Submitted by the Author:	10-Dec-2017
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Keyword:	C-peptide, diabetic nephropathy, Nitric Oxide
Is the invited manuscript for consideration in a Special Issue? :	N/A

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- **Title:** Protective effect of C-peptide on experimentally induced diabetic nephropathy and the possible link between C-peptide and Nitric Oxide

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Abstract

Diabetic nephropathy one of the major microvascular diabetic complications. Besides hyperglycemia, other factors contribute to the development of diabetic complications as the proinsulin connecting peptide, C-peptide. We described the role of C-peptide replacement therapy on experimentally induced diabetic nephropathy, and its potential mechanisms of action by studying the role of Nitric oxide (NO) as a mediator of C-peptide effects by in vivo modulating its production by NG-nitro-L-arginine methyl ester (L-NAME). Renal injury markers measured were serum urea, creatinine, TNF- α and Angiotensin II and renal tissue malondialdehyde, total antioxidant, Bcl-2 and NO. Conclusion, diabetic induction resulted in islet degenerations and decreased insulin secretion with its metabolic consequences, and subsequent renal complications. C-peptide deficiencies in diabetes might contributed to the metabolic and renal error, since C-peptide treatment to the diabetic rats corrected completely these errors. The beneficial effects of C-peptide partially antagonized by L-NAME co-administration indicating that NO partially mediates C-peptide effects.

Keywords

C-peptide, diabetic nephropathy, Nitric Oxide

Introduction

Diabetic nephropathy, as one of the microvascular diabetic complications, is clinically defined as the progressive development of renal insufficiency with hyperglycemia. This disease is now the major single cause of end stage renal failure in various countries (Afkarian et al. 2013).

There are several theories for the pathogenic mechanisms that result in the development of diabetes-induced renal dysfunction. Early in the course of type 1 diabetes, specific organs' function deteriorates and tissue abnormalities arise, while hyperglycemia results in abnormal homeostasis in blood flow and vascular permeability in the glomerulus. The increased blood flow and intra-capillary pressure is assumed to reveal hyperglycemia-induced decreased nitric oxide production on the efferent side of renal capillaries and eventually an increased sensitivity to angiotensin II. As a consequence of increased intra-capillary pressure and endothelial cell dysfunction, glomerular capillaries have a higher albumin excretion rate. By this early stage, the increased permeability is still reversible, but below the continuing triggering effect of hyperglycemia, the lesions become irreversible (Papadopoulou-Marketou et al. 2017).

Besides hyperglycemia, other causal factors appear to contribute to the development of diabetic complications. One such factor is the proinsulin connecting peptide, C-peptide. C-peptide is a cleavage product of insulin production formed in the pancreas as part of insulin production, and is released into the circulation with insulin. When insulin synthesis is impaired, as in type 1 diabetes and late type 2 diabetes, synthesis of C-peptide is also impaired (Samnegard and Brundin 2001). Moreover, (Luppi and Drain 2014; Luppi and Drain 2017) reported that C-peptide has an anti-oxidant effect on the beta cells producing it, that limit beta cell dysfunction and loss contributing to diabetes and suggesting a positive feed-back that might potentiate the endogenous and exogenous C-peptide benefits on the kidney.

Beside the two major factors (insulin deficiency and subsequent hyperglycemia) that contribute to the development of diabetic complications, C-peptide deficiency is suggested to be the third major factor because of beneficial effects of C-peptide against diabetic complications (Wahren et al. 2012). Thus, therapeutic approaches to hyperglycemic control only have been ineffective in preventing diabetic complications, and alternative therapeutic approaches are necessary to target both hyperglycemia and diabetic complications.

Numerous clinical and experimental studies demonstrated that C-peptide treatment alone or in combination with insulin has physiological functions and might be beneficial in preventing diabetic complications (Lachin et al. 2014; Yosten et al. 2014).

In this work, we describe the role of C-peptide replacement therapy in diabetic nephropathy as one of the most serious diabetic complications, and its potential mechanisms of action.

To elucidate this concept, this study was planned to: 1) induce experimental type I diabetes mellitus by streptozotocin (STZ) in adult male albino rats, 2) study the effects of C-peptide treatment on the developed renal injury, and 3) study the role of Nitric oxide (NO) as a mediator of C-peptide effects by *in vivo* modulating its production by blocking its synthesis using the nitric oxide synthase (NOS) inhibitor; NG-nitro-L-arginine methyl ester (L-NAME).

Materials and methods:

I- Animals

Fifty adult male albino rats (Sprague-Dawley strain), of average weight 150-200 g, about 4 months old were used in the present study. They were purchased from the National Research Center, Cairo; Egypt. They were housed in groups of six in stainless steel cages that offered adequate space for free movement and wandering (40 cm x 40 cm x 25 cm) at room temperature with natural dark/light cycles, and allowed free access to water and commercial rat's diet (Nile

Company, Egypt) for two weeks for acclimatization. All experimental protocols were approved by the animal care committee of Minia University which coincides with international guidelines. Rats were classified randomly into the following groups (ten rats each):

- 1- Control group (C): in which 10 rats were fed a commercial rat's diet and received citrate buffer only.
- 2- STZ treated group (T1DM): in which 10 rats were injected by a single intra-peritoneal injection (ip) of streptozotocin (Sigma aldrich, Egypt) at a dose level of 55 mg/kg of body weight (Al-Trad et al. 2015) and was left for 4 weeks untreated to induce early diabetic nephropathy (Honore et al. 2012).
- 3- STZ+ C-peptide treated group (T1DM+ CP): in which 10 rats were injected by a single ip injection of streptozotocin as above. Three days later (Diabetes was verified) these rats received C-peptide (Biorbyte, United Kingdom) 50 nmol/kg/day by ip injection for 4 weeks (Samnegard et al. 2005).
- 4- STZ + L-NAME treated group (T1DM+ L-NAME): in which 10 rats were injected by a single ip injection of streptozotocin as above. Three days later (Diabetes was verified) these rats received L-NAME (Sigma, USA) 20 mg/kg/day given in the drinking water for 4 weeks (Regadas et al. 2014).
- 5- STZ + L-NAME + C-peptide treated group (T1DM+ L-NAME+ CP): in which 10 rats were injected by streptozotocin then received C-peptide and L-NAME as above for 4 weeks.

II- Induction of type 1 diabetes mellitus (T1DM):

To induce experimental type 1 diabetes (T1DM), rats were injected by a single ip injection of freshly prepared streptozotocin 55 mg/kg, dissolved in 0.1 M citric acid buffer, pH (4.5) after an overnight fast (Al-Trad et al. 2015). The STZ-treated animals were allowed to drink 20% glucose solution for 24h to overcome initial drug-induced hypoglycemic mortality. Diabetes was verified

3 days later by evaluating blood glucose levels with the use of glucose-oxidase reagent strips (Accu-Chek, Roche Inc., Indianapolis, IN). Rats having blood glucose level of 200 mg/dl or greater were considered to be diabetic and selected for the study. At the end of study, histological examination of pancreas will confirm our finding.

III- Experimental protocol

By the termination of experimental procedure, rats were sacrificed by decapitation under light halothane anesthesia and blood samples were collected from the jugular vein, allowed to clot, and centrifuged and supernatant serum was collected in eppendorf tubes and stored at -20°C till the time for biochemical assay. The pancreas and kidneys were rapidly removed, weighed, and were divided; some specimens were fixed for paraffin embedding and some renal specimens were stored at -80°C for renal tissue assay of malondialdehyde (MDA), total antioxidant capacities (TAC), Bcl-2 and NO levels.

IV- Biochemical analyses

Sera were used for estimation of urea by Berthelot enzymatic colorimetric method, creatinine by Jaffé calorimetric-end point method (20), TNF- α by enzyme-linked immunosorbent assay kit (ELISA) (ALPCO Diagnostic) and Angiotensin II (AGGII) by enzyme-linked immunosorbent assay kit (ELISA) (ALPCO Diagnostic) according to the manufacturer's instructions.

V- Preparation of tissue homogenates

Kidney specimens were weighed and homogenized separately 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°C. The resulting supernatant was used for determination of malondialdehyde (MDA) according to the method of (Ohkawa et al. 1979), total antioxidant capacities (TAC) using colorimetric assay kit, Nitric oxide (NO) by

enzymatic colorimetric methods using commercial kits (Biodiagnostic, Egypt) and B-Cell Leukemia/Lymphoma-2 (Bcl-2) by enzyme-linked immunosorbent assay kit (ELISA) (Calbiotech, USA) according to the manufacturer's instructions.

VI- Histological Examination:

Pancreases (the tail of pancreas) and kidney specimens from all groups were fixed in 10% neutral-buffered formalin, dehydrated in a graded ethanol series, cleared in xylene embedded in paraffin wax, and sectioned into 6-7 micrometer sections. Sections were stained with hematoxylin and eosin (H&E).

Image capture:

Tissue sections were examined and images were digitally captured using a digital camera mounted on an Olympus microscope and connected to a computer.

Morphometry

For histological evaluation of the severity of renal lesions, a semi-quantitative analysis of sections were done (Houghton et al. 1978): Score - : assigned normal, Score + : in between normal and mild level, Score ++: mild level; <25% of the total fields examined revealed alterations, Score +++: moderate level; <50% of the total fields examined revealed alterations, and Score ++++: severe level; < 75% of the total fields examined revealed histopathological alterations.

VII- Statistical analysis

Data were represented as means $\pm$ standard errors of the mean (SEM). Statistical analysis was performed using Graph pad Prism 5 software and significant difference between groups was done by one-way ANOVA followed by Tukey-Kramer post hoc test for multiple comparisons with a value of $P \leq 0.05$ considered statistically significant.

Results

A- Metabolic and oxidative Parameters:

As shown in table 1, the diabetic non treated group showed significantly higher fasting serum glucose and lower insulin levels as compared to rats of the control group. These effects were completely reversed with C-peptide treatment. Administration of L-NAME to diabetic rats significantly decrease the insulin level as compared with the diabetic group, and the hyperglycemia of diabetes was worsened as indicated by a significantly higher blood glucose level. When L-NAME was co-administered with C-peptide, it partially, but significantly antagonized the hypoglycemic and the insulin increasing effects of C-peptide.

In table 2, the levels of serum urea, serum creatinine and renal MDA significantly increased with significantly decreased in renal TAC level (renal injury parameters) in T1DM group as compared with control group. However, compared with T1DM group, these levels significantly decreased in T1DM+CP group. Administration of L-NAME was injurious as it markedly increased the renal injury parameters as compared with T1DM group. On the other hand, treatment of the diabetic group by both L-NAME and C-peptide significantly antagonize the positive renal effect of C-peptide.

B- Serum TNF- α and Angiotensin II (ANGII) levels:

Both serum levels of TNF- α (figure 1) and ANGI (figure 2) were found to be significantly increased in T1DM group as compared with control group. In addition, administration of L-NAME significantly produced higher serum levels of TNF- α (figure 1) and ANGI as compared with T1DM group. On the other hand, C-peptide administration significantly lowered these levels as compared with T1DM group, while its co-administration with L-NAME significantly increased these levels as compared with T1DM+CP group but still significantly lower than T1DM+L-NAME group.

C- Kidney Bcl-2 and NO levels:

As shown in table 3, *T1DM+ CP* group recorded the highest renal NO level amongst all studied groups, while *T1DM+ L-NAME* group recorded the lowest renal NO level. On the other hand, treatment of the diabetic group by both L-NAME and C-peptide produced a significant lower in the renal NO level as compared to *T1DM+ CP* group. In addition, renal NO level was found to be lower in the *T1DM* group as compared with control group.

As regard renal *Bcl-2* level, the lowest mean value was recorded in the *T1DM+ L-NAME* group. However, C-peptide treatment alone significantly elevated the renal *Bcl-2* level as compared to all studied group but failed to produce any significant change when compared with control group. While treatment of the diabetic group by both L-NAME and C-peptide significantly lowered the renal *Bcl-2* level as compared to *T1DM+CP* group and still insignificant from *T1DM* group.

D- Histological study of kidney and pancreas using H&E:

H&E stained sections of pancreas from control group showed a normal lobular architecture with delicate interlobular connective tissue. Numerous islets of Langerhans surrounded by the pancreatic acini. The islets appeared lightly stained and consisted of cords of polygonal cells separated by blood capillaries. The acinar cells were characterized by its basal basophilia and apical acidophilia (Figure 3 a & b). The pancreatic sections of the T1DM showed marked morphological changes. Some islets were completely destroyed leaving empty spaces. Other islets showed degenerations as some cells appeared with nuclear pyknosis and fragmentation and dense acidophilic cytoplasm; apoptotic figures, while other islet's cells showed vacuolated cytoplasm and ghosts of nuclei. The degenerated cells surrounded by empty spaces which filled with amyloid-like material (Figure 3c&d).

In T1DM+CP group, C-peptide administration showed improvement in the previous morphological changes. Many islets were observed and showed with an increase in the cellular density. Appearance of small newly formed islets was also observed (Figure 3 e & f).

In T1DM+L-NAME group, L-NAME administration worsens the condition compared to T1DM group, where more degenerations were observed with aggregation of lymphocyte infiltration (Figure 3g&h). While treatment of the diabetic group by L-NAME and C-peptide in T1DM+L-NAME+CP group decreased this picture (Figure 3 i&j) where islets of variable sizes were observed with few degenerated cells compared to T1DM+L-NAME group.

H&E stained sections of the rat renal cortical tissue of control group showed normal architecture; numerous renal corpuscles (RC), proximal (PCTs) and distal (DCTs) convoluted tubules. The RC contained the glomeruli which surrounded by the Bowman's capsules with urinary spaces in between. The parietal layers were lined by simple squamous epithelium. The PCTs were lined with thick large cubical epithelium with acidophilic cytoplasm. The DCTs showed considerably lower cubical epithelium surrounding relatively larger regular distinct lumens (Figure 4a).

The histological changes in T1DM group were variable and patchy, it showed slightly congested vascular glomerulus with few cytoplasmic vacuulations of some PCT and DCT cells (Figure 4b).

In T1DM+CP group, apparent normal renal cortical tissues were observed where apparent normal corpuscles, PCTs and DCTs were seen (Figure 4c).

In T1DM+L-NAME group, marked distortion of the cortical architecture were observed in the form of markedly congested glomerulus with obliterated Bowman's space, severe tubular dilatation and disturbed morphology of the convoluted tubules with marked cytoplasmic vacuulations, peritubular capillaries dilatation and congestion, and appearance of epithelial cast in the tubular lumen (Figure 4d).

While treatment of the diabetic group by both L-NAME and C-peptide in T1DM+L-NAME+CP group ameliorated the damaging effects. Less tubular distortion and few cytoplasmic vacillations of some tubular cells compared to group T1DM+L-NAME group with minimal congestion. Some tubules showed casts in the lumen (Figure 3E).

Morphometric Results:

The severity of the morphological changes was assessed semi-quantitatively; L-NAME exposed groups showed increase in the glomerular and tubular morphological changes at the light microscopic levels when compared with the diabetic group. These changes were suppressed by the administration of C-peptide (Table 4).

Discussion:

STZ is broadly used as a chemical inducer to set up the model of type 1 diabetes mellitus (Kasono et al. 2004). The results of the present study showed that STZ injection resulted in a significant increase in fasting serum glucose to the diabetic levels, with a significant decrease in serum insulin level. The histological finding of this study showed that STZ selectively destroys pancreatic β cells which could explain high fasting serum glucose levels in T1DM group. This was in agreement with (Al-Trad et al. 2015). These effects were completely reversed with C-peptide treatment. While, administration of L-NAME to diabetic rats worsened the condition more as indicated by the more increase in blood glucose level and the more decrease in blood insulin level. When L-NAME was co-administered with C-peptide, it partially, but significantly antagonized the hypoglycemic effects of C-peptide.

These results are compatible with previous studies which had shown that C-peptide is much more than a byproduct of insulin synthesis and has biological role in metabolism and its deficiency with insulin in DM predisposes to the metabolic error. Through binding to insulin by charge interaction, C-peptide prevents insulin aggregation to form polymeric inactive forms,

therefore keeping the monomeric biologically active insulin (Ghorbani and Shafiee-Nick 2015). C-peptide increases muscles and peripheral tissues glucose utilization by increasing translocation of glucose transporter 4 (GLUT₄) to cell membranes to facilitate glucose uptake. Moreover, by activating tyrosine kinase and phosphorylating insulin receptor substrate (ISR), C-peptide can activate insulin signaling pathways, thus, increasing its sensitivity. These effects can explain the normalizing effects of C-peptide on blood glucose level seen in the present study that agrees with (Wu et al. 2012).

The partially antagonizing effect of L-NAME on the positive metabolic effect induced by C-peptide come in line with (Wu et al. 2012) who reported that L-NAME has the ability to block about 85% of the C-peptide-induced increase in glucose disposal rates, suggesting the mediating role of NO in C-peptide stimulation of glucose utilization. A G-protein coupled C-peptide receptor has been recognized and its stimulation induces nitric oxide synthase (NOS) activation, C-peptide can produce vasodilatation of pancreatic vessels with enhanced function of β -cells and increased insulin secretion through increased NO production. In the present study, the higher levels of insulin in the diabetic group treated with C-peptide and the lower levels found with L-NAME treatment supports this role of NO according to (Bhatt et al. 2014).

In the present study, diabetes induction of nephrotoxicity was confirmed by renal histopathological changes that showed morphological damage of the renal tissue. This was in agreement with (Patschan and Muller 2016)

Serum urea and creatinine levels considered as diagnostic markers of renal cell injury (Zuo et al. 2008). The results of the present study show that the levels of both serum urea and creatinine significantly increased in diabetic group as compared with control group, and these results are compatible with previous studies as (Maheshwari et al. 2017) which was confirmed by the histological changes observed in the T1DM group that owed to the evidenced occurrence

of oxidative stress and release of ROS. L-arginine (L-Arg), the main source for the generation of NO *via* NOS, the metabolism and the enzymes that participate in its synthesis are down-regulated in DM thus contributing to the resulting NO deficiency (Ortiz et al. 2014). Moreover, diabetic animals have increased hepatic Arg degradation leading to decrease in plasma Arg levels, that limit the renal NO levels (Palm et al. 2008). On the other hand, serum levels of asymmetric dimethylarginine (ADMA), an endogenous inhibitor of NOS, are increased in both type 1 and type 2 DM, leading to a decrease in NO production, as proved in our study, and therefore contributes to diabetic complications as nephropathy through renal hypoxia induction and retinopathy (Altinova et al. 2007).

Oxidative stress plays a significant role in the pathogenesis and progression of renal disease (Jha et al. 2016). The data presented in this study showed increased lipid peroxidation expressed by increasing level of MDA, and decreased TAC in renal tissue with diabetic induction, and these results come in line with previous experimental (Huang et al. 2012), and clinical (Chang et al. 2012) (Whaley-Connell and Sowers 2012) studies.

In DM, oxidative stress is present because the stimulatory effect of glucose on mitochondrial nicotinamide adenine dinucleotide phosphate (NADP) oxidase leading to generation of excess superoxide ions (Palm et al. 2003). In addition, in endothelial cells, hyperglycemia inhibits endothelial NOS (eNOS) activity leading to reduce NO and increased ROS production (Boger 2003). On the other hand, inducible NOS is stimulated by inflammatory cytokines like interleukins; -1 and -6 and TNF- α resulting in excess NO formation which reacts with superoxide ions to form the highly toxic peroxynitrite, and thus decrease the bioavailability of NO. Reactive oxygen and nitrogen species (ROS/RNS) further destroy metabolic enzymes, membrane phospholipids, and insulin receptors decreasing both insulin release and sensitivity. ROS/RNS could also oxidize and nitrosylate NOS leading to decrease its activity and producing

a vicious cycle of reduced NO, reduced blood flow, ischemia and more ROS generation. This can contribute to both diabetic pathogenesis and complications (Palm et al. 2005).

Many studies suggested the role of inflammatory cytokines in the development of diabetic nephropathy. In this study, serum TNF- α as one of the pro-inflammatory cytokines was significantly increased with diabetic induction. This results come in line with previous studies (Donate-Correa et al. 2015). They added that increased oxidative stress, as we found in this study, can increase the production of inflammatory cytokines and stimulate the production of free radicals.

Regarding serum angiotensin II level; we found that induction of diabetes produced significant increase in its level. There have been contradictory reports on the activity of the renin-angiotensin system (RAS) in diabetes mellitus and numerous abnormalities have been described (Chawla et al. 2010). These studies strongly concerned the RAS as a mediator of diabetic nephropathy. These results may be because during inflammation, lymphocytes and macrophages can generate reactive oxygen species and Ang II. Diabetic nephropathy being an inflammatory condition, Ang II levels have been found to be elevated. This rise activates immune cells and causes production of chemokines which lead to further renal damage (Ruiz-Ortega et al. 2001).

Apoptosis, a form of programmed cell death, can be induced by various stimuli. The anti-apoptotic protein Bcl-2 plays a main role in apoptosis regulation, both in physiological and pathological conditions (Zhang et al. 2008) which explained increased apoptotic like features observed in the islets of the T1DM group and epithelial degeneration and epithelial casts in the tubular lumen. (Bugliani et al. 2007) reported that exposure to C-peptide reduced human islet cell apoptosis, which was accompanied by increased expression (both at the gene and protein levels) of the anti-apoptotic molecule Bcl-2. In our study we found significant decrease in renal

Bcl-2 level in diabetic group compared to control group. This result come in line with previous studies as (Verzola et al. 2004) which found that high glucose concentration promotes apoptosis in variety of cell types including proximal tubular epithelial cells. The mechanism by which hyperglycemia leads to apoptosis is not completely understood but it may be because of increase oxidative and nitrosative stress. It was also found a decrease in Bcl- 2 gene expression with diabetes (da Silva Faria et al. 2015).

As reported before exogenous C-peptide administration in type 1 diabetes has been shown to exert beneficial effects in many tissues affected by microvascular complications (Nordquist and Wahren 2009). In this study, C-peptide administration to the diabetic rats enhanced both morphological changes occurred in the islets and renal structures.

The renal injury parameters, either in the blood or the kidney, was improved; serum urea, creatinine, TNF- α and ANG II decreased, and renal tissue level of TAC, NO and Bcl-2 increased with decreasing renal MDA. In addition, the pancreatic and renal histopathological changes induced by diabetes was improved by C-peptide treatment. These results were in agreement with (Samnegard and Brundin 2001) and (Sun et al. 2010) who detected a protective effect of C-peptide on diabetes induced nephrotoxicity in rats. However, other studies as (Bhatt et al. 2014) have reported C-peptide hazardous effects in DM, with increased recruitment of inflammatory cells in sub-endothelial layer of blood vessels and stimulation of smooth muscle proliferation that predispose to atherosclerosis. Differences in doses, duration of treatment, animal species and experimental protocol can explain such variations in responses.

C-peptide can affect regional blood flow of the kidney by stimulating eNOS and eNOS gene transcription with increasing NO and vasodilatation (Bhatt et al. 2014). It also decrease intracellular ROS generation by improving mitochondrial respiration or by reducing or inhibiting activity of the plasma membrane NAD(P)H oxidase enzyme or by lowering ROS sources in the

cell (Cifarelli et al. 2011). However, until now the mechanism underlying C-peptide-mediated inhibition of intracellular ROS production and subsequent apoptosis is not clear. NF- κ B is a protein complex which controls cytokine production, transcription of DNA, and cell survival. In renal cells, C-peptide protects against TNF- α -mediated apoptosis through an increasing NF- κ B mediated mechanism. (Cifarelli et al. 2008).

From our results, the mechanism underlying the beneficial effect of C-peptide on renal function in diabetes is clear to some extent. C-peptide, through its hypoglycemic effect, may indirectly improve renal function due to the injurious effect of hyperglycemia on the renal tissue as discussed before. However, it is possible also that C-peptide may have exerted a direct effect on the renal tissue.

Trying to investigate the role of NO in mediating C-peptide renal effect, L-NAME, the non-selective blocker of NOS, was given either alone or combined with C-peptide to diabetic rats. Administration of L-NAME alone to the diabetic rats was injurious as it worsened the renal error produced by diabetes. On the other hand, when combined with C-peptide, L-NAME partially antagonize its renal correcting effect. Our renal histopathological and morphometrical showed increased severity of tissue injury induced by L-NAME, either alone or in combination with C-peptide, on the kidney. Also L-NAME increased inflammatory cell infiltration and vascular congestion. These results were in agreement with (Choi et al. 1999), (Komers et al. 2000), and (Komers and Anderson 2003).

These results confirmed the role of NO in mediating C-peptide hypoglycemic and renal effect. The injurious renal effect of L-NAME either alone or in combination with C-peptide because of the concept we discussed above about the significant role of NO in regulating the main functions in all tissues including the kidney.

In conclusion, STZ injection to rats induced diabetic picture similar to type I diabetes in human; resulting in islet degenerations and decreased insulin secretion with its metabolic consequences, and subsequent renal complications. C-peptide deficiencies in diabetes might contributed to the metabolic and renal error, since C-peptide treatment to the diabetic rats corrected completely these errors. The beneficial effects of C-peptide were partially antagonized by L-NAME co-administration indicating that NO partially mediates the effects of C-peptide. These results open the way for trials of C-peptide with insulin in treating type 1 diabetes with absolute insulin and C-peptide loss, and this should be a subject of future research as it represents a potential therapeutic option to protect renal tissue from detrimental effects of diabetes.

Compliance with Ethical Standards:

Funding:

Authors declare that this study was not funded by any one, and it was done on their own cost.

Conflict of Interest:

All authors declare that they have no conflict of interest.

Ethical approval:

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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Table 1 Changes in fasting serum glucose, and insulin in the different studied groups:

<div>Groups</div> <div>Parameters</div>	<i>C</i>	<i>T1DM</i>	<i>T1DM+ CP</i>	<i>T1DM+ L-NAME</i>	<i>T1DM+ L-NAME+ CP</i>
<i>Glucose (mg/dL)</i>	80.6 ± 1.2	224.6 ± 3.5^a	85.7 ± 1.4^b	242.5 ± 4.7^{abc}	161.3 ± 2.4^{acd}
<i>Insulin (μIU/ml)</i>	7.9 ± 0.4	5.5 ± 0.3^a	7.3 ± 0.5^b	4.2 ± 0.4^{abc}	6.9 ± 0.7^{acd}

Data are expressed as mean $\pm$ S.E.M. of 10 rats in each group. ^a: Significant from control group; ^b: Significant from STZ treated group; ^c: Significant from STZ+ C-peptide treated group; ^d: Significant from STZ + L-NAME treated group; $P \leq 0.05$. T1DM: STZ treated group; T1DM+CP: STZ+ C-peptide treated group; T1DM+L-NAME: STZ + L-NAME treated group; T1DM+L-NAME +CP: STZ + L-NAME + C-peptide treated group.

Table 2 Change in the renal injury parameters in the different studied groups:

Groups			<i>C</i>	<i>T1DM</i>	<i>T1DM+ CP</i>	<i>T1DM+ L-NAME</i>	<i>T1DM+ L-NAME+ CP</i>
Parameters							
Renal injury parameters	In blood	Urea (mmol/l)	23.4 ± 1.2	87.6 ± 4.7 ^a	26.4 ± 1.85 ^b	99.9 ± 1.7 ^{abc}	76.4 ± 3.1 ^{acd}
		Creatinine (µmol/l)	0.5 ± 0.09	1.7 ± 0.09 ^a	0.6 ± 0.02 ^b	0.1 ^{abc}	± 0.1 ^{acd}
	In renal tissue	Renal MDA (pg/mg tissue)	41.3 ± 1.8	79.09 ± 3.4 ^a	40 ± 2.7 ^b	89.5 ± 2.1 ^{abc}	69.5 ± 3.5 ^{acd}
		Renal TAC (µM/mg tissue)	79.5 ± 3.2	54.8 ± 2.9 ^a	± 4.5 ^b	± 2.2 ^{abc}	± 2.1 ^{acd}

Data are expressed as mean ± S.E.M. of 10 rats in each group. ^a: Significant from control group; ^b: Significant from STZ treated group; ^c: Significant from STZ+ C-peptide treated group; ^d: Significant from STZ + L-NAME treated group; $P \leq 0.05$. T1DM: STZ treated group; T1DM+CP: STZ+ C-peptide treated group; T1DM+L-NAME: STZ + L-NAME treated group; T1DM+L-NAME +CP: STZ + L-NAME + C-peptide treated group; MDA: Malondialdehyde; TAC: total antioxidant capacity.

Table 3 kidney Nitric oxide (NO) and Bcl-2 in the different studied groups:

<div>Groups</div> <div>Parameters</div>	<i>C</i>	<i>T1DM</i>	<i>T1DM+</i> <i>CP</i>	<i>T1DM+ L-</i> <i>NAME</i>	<i>T1DM+ L-</i> <i>NAME+ CP</i>
NO (pmol/mg wet tissue)	8.5±0.4	4.2±0.1 ^a	14.6±0.7 ^{ab}	2.4±0.09 abc	3.9±0.12 ^{acd}
Bcl-2 (ng/mg wet tissue)	1.5±0.09	0.7±0.05 ^a	1.4±0.04 ^b	0.5±0.02 abc	0.8±0.07 ^{acd}

Data are expressed as mean ± S.E.M. of 10 rats in each group. ^a: Significant from control group; ^b: Significant from STZ treated group; ^c: Significant from STZ+ C-peptide treated group; ^d: Significant from STZ + L-NAME treated group; P ≤ 0.05. T1DM: STZ treated group; T1DM+CP: STZ+ C-peptide treated group; T1DM+L-NAME: STZ + L-NAME treated group; T1DM+L-NAME +CP: STZ + L-NAME + C-peptide treated group; NO: Nitric oxide.

Table 4 Scoring of morphological changes observed in kidneys of the studied groups by lightmicroscope (n=6):

Findings	C	T1DM	T1DM+CP	T1DM+L- NAME	T1DM+L- NAME+CP
<u>Renal corpuscles:</u>					
-Glomerular vaculation	-	-	+	+++	-
-Enlarged congested renal corpuscles	-	+++	+	++++	++
<u>PCTs:</u>					
-Tubular cells vaculation	-	+	+	+++	-
-Lumen widening and Distortion	-	++	-	++++	+
-Casts	-	-	-	++	-
<u>DCTs:</u>					
-Tubular cells vaculation	-	+	+	+++	++
-Lumen widening and Distortion	-	++	-	++++	+
-Casts	-	-	-	++	+
<u>Interstitial:</u>					
-Peritubular capillary dilatation and congestion	-	-	-	+++	+
-Inflammatory cells	-	-	-	++++	-

Normal (-), in-between normal and mild (+), mild (++) , moderate (+++) and sever (++++).T1DM: STZ treated group; T1DM+CP: STZ+ C-peptide treated group; T1DM+L-

NAME: STZ + L-NAME treated group; T1DM+L-NAME +CP: STZ + L-NAME + C-peptide treated group.

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Figure 1: TNF- α levels in serum samples of the different studied groups.

Data are expressed as mean $\pm$ S.E.M. of 10 rats in each group. ^a: Significant from control group; ^b: Significant from STZ treated group; ^c: Significant from STZ+ C-peptide treated group; ^d: Significant from STZ + L-NAME treated group; $P \leq 0.05$. T1DM: STZ treated group; T1DM+CP: STZ+ C-peptide treated group; T1DM+L-NAME: STZ + L-NAME treated group; T1DM+L-NAME +CP: STZ + L-NAME + C-peptide treated group.

Figure 2: ANGII levels in serum samples of the different studied groups.

Data are expressed as mean $\pm$ S.E.M. of 10 rats in each group. ^a: Significant from control group; ^b: Significant from STZ treated group; ^c: Significant from STZ+ C-peptide treated group; ^d: Significant from STZ + L-NAME treated group; $P \leq 0.05$. T1DM: STZ treated group; T1DM+CP: STZ+ C-peptide treated group; T1DM+L-NAME: STZ + L-NAME treated group; T1DM+L-NAME +CP: STZ + L-NAME + C-peptide treated group; ANGII: Angiotensin II.

Figure 3: Photomicrographs of rat pancreatic tissue:**H&E a, c, e, g, i x 100 & b, d, f, h, j, Insets x1000**

a) Control group showing normal lobular architecture with delicate interlobular connective tissue (CT). Islets of Langerhans (stars) surrounded by the pancreatic acini (PA). b) Islet of Langerhans consisting of cords of cells (arrows) separated by blood capillaries (red arrows). Notice acinar cells with an apical cytoplasm packed with acidophilic cytoplasmic granules (*) and basal nuclei (arrowheads).

c) The T1DM group showing completely destroyed islets leaving empty spaces (stars), and widening of interlobular spaces (arrow). d) Degenerated islet with vacuolated cells and pale nuclei (black arrows), others with deep acidophilic cytoplasm and nuclear pyknosis (white

arrow). Notice the spaces leaved empty (stars) after cell degeneration or filled with amyloid-like material (A).

e) Group T1DM+CP showing many islets (black arrows). f) Increase islet cells (arrows) with vesicular nuclei resembling normal. The inset shows small newly formed (star) islet.

g) Group T1DM+L-NAME showing degenerated islet (IS). Notice aggregation of lymphocyte infiltration (arrow). The inset showing congested blood vessel (BV) and lymphocytic infiltration (arrow). h) An islet with degenerated cells (arrows). Notice the empty spaces leaved after cell degeneration (stars). Inset showing a completely destroyed islet leaving remnants of degenerated cells (circle).

i) Group T1DM+L-NAME+CP showing islets of variable sizes (arrows). j) Islet (IS) with few degenerated cells (arrows) compared to T1DM+L-NAME+CP group.

Figure 4: photomicrographs of renal cortex of:

H&E x400

- a. Control group showing renal corpuscles (RC), PCTs (p) and DCTs (d). The convoluted tubules have a relatively regular distinct lumen.
- b. Group T1DM showing slightly congested vascular glomerulus (g). Notice few cytoplasmic vacuations (arrows) of some PCT (p) and DCT (d) cells.
- c. Group T1DM+CP showing apparent normal renal corpuscles (RC), PCTs (p) and DCTs (d).
- d. Group T1DM+L-NAME showing marked distortion of the cortical architecture; markedly congested glomerulus (g) with obliterated Bowman's space (white arrow), severe tubular dilatation (d) with disturbed morphology of the convoluted tubules, marked cytoplasmic vacuations (black arrows), peri-tubular capillaries dilatation and congestion (circle). Notice

the nuclei of desquamated tubular cells (green arrow) in the tubular lumen and the epithelial cast (stars).

- e. Group T1DM+L-NAME+CP showing less tubular distortion and less cytoplasmic vacuulations (arrows) of the renal PCT (p) and DCT cells (d) than in T1DM+L-NAME group with minimal congestion (circle). Some tubules show casts in the lumen (star, inset).

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Figure 1

Figure 2

Figure 3

Figure 4