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Article

Effects of *Cinnamomum cassia* extract on oxidative stress, immunreactivity of iNOS and impaired thoracic aortic reactivity induced by type II diabetes in rats

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Type II diabetes is known to cause neuropathy, nephropathy and retinopathy. However, cardiovascular disorders associated with diabetes have been ignored. In traditional medicine, cinnamon (*Cinnamomum cassia*) bark has been used for its abilities to relieve fever, inflammation and chronic bronchitis. In the present study, the effect of *Cinnamomum cassia* extract (CN) on the thoracic aorta in an experimental type II diabetes model was investigated. In rats administered with nicotinamide + streptozotocin, significant endothelial dysfunction and oxidative stress were characterised by increased inducible nitric oxide synthase (iNOS) and decreased insulin/proinsulin levels. This impairment was prevented by administering 1000 mg/kg metformin or 500-1000-1500 mg/kg CN. CN administration attenuated the inflammatory response by decreasing the levels of malondialdehyde (MDA), Nitric oxide (NO) and increasing Glutathione peroxidase (GPx), glutathione (GSH). In addition, CN administration was shown to cause down-regulating effects on iNOS in thoracic aorta. These findings reveal that CN could prevent chronic complications of experimentally induced type II diabetes by attenuating inflammation, oxidant/antioxidant imbalance, and normalised contraction and relaxion responses in the thoracic aorta.

Keywords: Type II diabetes. Oxidative stress. iNOS. Thoracic aorta reactivity. *Cinnamomum cassia*/ effects.

INTRODUCTION

Diabetes mellitus is associated with numerous complications that are considered as major health problems in the developed world. For instance, it increases susceptibility to many diseases including cardiovascular issues. It is estimated that more than 75% of deaths in diabetes patients are caused by cardiovascular diseases (Xu, Zou, 2009). Diabetic patients are at an increased risk of three major macrovascular diseases, namely peripheral vascular disease, coronary heart disease, and stroke (Zou, Cohen, Ullrich, 2004; Scirica *et al.,* 2013). Endothelial cells are thought to potentially regulate basal vascular tone and reactivity in physiological and pathological conditions (Furchgott, Vanhoutte, 1989; Nitenberg *et al.,* 1993) and prolonged exposure to hyperglycemia in diabetic patients increases the risk of endothelial damage (Macías *et al.,* 2014).

Endothelial damage and its mechanism in type II diabetes remains to be understood. However, it has been suggested that endothelial damage may be due to decreased endothelium-derived relaxing factor (EDRF) production and hence diminished smooth muscle response to EDRF (De Vriese *et al.,* 2000). Alternative mechanisms that have been suggested include: Deterioration of the vasodilating mechanism of bradykinin (Kiff *et al.,* 1991), abnormalities at the G-protein level and impaired nitric oxide mediated vasodilating mechanism (Williams *et al.,* 1996), and overproduction of endothelium-derived contracting factor (Mayhan, Simmins, Sharpe, 1991).

Cinnamon bark and twigs have long been used in traditional Chinese herbal medicine for their therapeutic abilities, lowering fever, inflammation, chronic bronchitis, and for improving blood circulation (Barceloux, 2009). Cinnamon consumption has also been shown to

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increase glucose uptake and glycogen synthesis (Imparl-Radosevich *et al.,* 1998) and have insulin-mimetic effects (Jarvill-Taylor, Anderson, Graves, 2001). In the current study, effects of *Cinnamon cassia* extract on oxidative stress markers and thoracic aorta in experimentally type II diabetes mellitus induced rat models were investigated.

MATERIAL AND METHODS

Sixty female Sprague–Dawley rats weighing 200–250 g were used in this study. Animals were housed under a 12:12 h light–dark cycle in a controlled environment temperature (23 °C ± 2 °C) and humidity (55% ± 5%). Standard rat feed and water were provided *ad libitum* throughout the experimental period. All animal procedures were conducted in accordance with the guidelines of Kafkas University Animal Ethics Committee (2014/003). The rats were equally divided into six groups, namely Normoglycemic Control (NC), Diabetes (DC), diabetes plus 500 mg/kg *Cinnamomum cassia* extract (CN 500), diabetes plus 1000 mg/kg CN (CN 1000), diabetes plus 1500 mg/kg CN (CN 1500), and Diabetes plus metformin (M).

Induction of type II diabetes

Type II diabetes was induced using a previously documented method (Masiello *et al.,* 1998). Rats received an intraperitoneal injection of a single dose nicotinamide (NAD, 230 mg/kg) followed by an intravenous injection of streptozotocin (STZ, 65 mg/kg) (Masiello *et al.,* 1998). Rats were considered diabetic if their fasting blood glucose levels were >200 mg/dL 72 h after NAD plus STZ administration.

Experimental design

NC rats were given 500 µL dimethyl sulfoxide (DMSO) orally for 35 days. In the DC group, diabetes was induced using the aforementioned method, and DMSO was administered as in the NC group. While the CN 500, CN 1000, and CN 1500 groups were administered 500, 1000, and 1500 mg/kg *Cinnamomum cassia* extract (CN), respectively, via orogastric tubes for 35 days after hyperglycemia had stabilised 7 days post-injections.. The M group, received oral metformin (1000 mg/kg) for 35 days.

Preparation of cinnamon extract

Cinnamomum cassia barks were supplied by a local market and were grounded into fine powder. A sample of 50 g was used, and the extraction was accomplished in Soxhlet apparatus for 6 hours. The remaining liquid was left to evaporate and a 9.5 g dry extract was obtained. The dry extract was dissolved in DMSO and the daily doses were calculated from the stock solution.

Biochemical analysis

At the end of the experimental period, the rats were sacrificed by 0.4 mL/kg intramuscular pentobarbital sodium injections followed by cervical dislocation. Thoracic aorta of rats were excised, washed with physiological saline and homogenised in phosphate buffered saline pH 7.4. Serum levels of nitric oxide (NO), malondialdehyde (MDA), reduced glutathione (GSH), and glutathione peroxidase (GPx) were determined spectrophotometrically according to previously described methods (Miranda, Espey, Wink, 2001; Placer, Cushman, Johnson, 1966; Sedlak, Lindsay, 1968; Matkovics, Szabo, Varga, 1988).

Preparation of isolated rat thoracic aortic rings

Thoracic aorta samples collected at necropsy were immediately removed to be cleaned of the adhering connective tissue and fat. The samples were cut into rings approximately 3-4 mm in length. The aortic rings were immersed in a 10 ml chamber bath which contained Krebs solution (composition, mM: mM: NaCl 119, KCl 4.75, KH_2PO_4 1.2, $NaHCO_3$ 25, $MgSO_4$ 1.5, $CaCl_2$ 2,5, glucose 11, pH 7.3), maintained at a 37 °C, mounted on steel and oxygen (95%) and CO (5%) was continuously bubbled through it. A resting tension of 1 g was applied to each tissue and equilibrated at least 1 h. During the equilibrium period, the Krebs solution was changed every 15 min. After equilibration, endothelial integrity was verified with a sub-maximal pre-contraction of phenylephrine hydrochloride (PH) (10^{-9}–10^{-5} M) and KCI (20-80 mM). After the tension was stabilised, ACh (10^{-8}–10^{-5} M) was directly added into the chamber bath. Relaxation of the rings was considered indicative of an endothelium-intact ring. Relaxation was calculated as a percentage of the maximal contraction induced by PH. Changes in tension were detected using isometric force transducers (ELJ-S045C-EMKA-R04003 ve R04004).

Histopathology

Tissue samples of the pancreas and thoracic aorta were collected at necropsy, and fixed in 10% phosphate buffered formaldehyde solution, and then embedded in paraffin. Tissue sections at 5 µ thickness were cut

and stained routinely with hematoxylin and eosin for microscopic examination.

Immunohistochemistry

Insulin/proinsulin expression in pancreas and inducible nitric oxide synthase (iNOS) activity in thoracic aorta tissues were investigated using the streptavidin biotin immunoperoxidase complex method with 3.3'-diaminobenzidine tetrahydrochloride as the chromogen. Anti-insulin/proinsulin antibody (Thermofisher Scientific, Catalog No: MA1-16710) and anti-iNOS polyclonal antibody (Lab Vision, Catalog No: RB9242P) were used in 1:500 dilutions with 1 h incubation times. Antigen retrieval was provided by microwave treatment in 0.1 M sodium citrate solution (pH 6.0). Negative controls were provided by omitting the primer antibodies. All tissue sections were examined under a light microscope. A semi-quantitative (negative, weak, moderate, or strong immunoreactivity) grading system was used to score the degree of cell immunoreactivity.

RESULTS AND DISCUSSION

Normal pancreas histomorphology was observed in the NC group (Figure 1A a). Islets of Langerhans and acini were also structurally unremarkable. While in rats administered STZ+NAD (DC group), fewer and smaller islets were noted, with some cases revealing a complete absence. Occasional hydropic and granular degeneration in islets and no involvement of acini were noted in this group (Figure 1A b). The CN 500, CN 1000, CN 1500 groups all revealed varying degrees of pancreatic degeneration (Figure 1A c-e). Finally, the M group

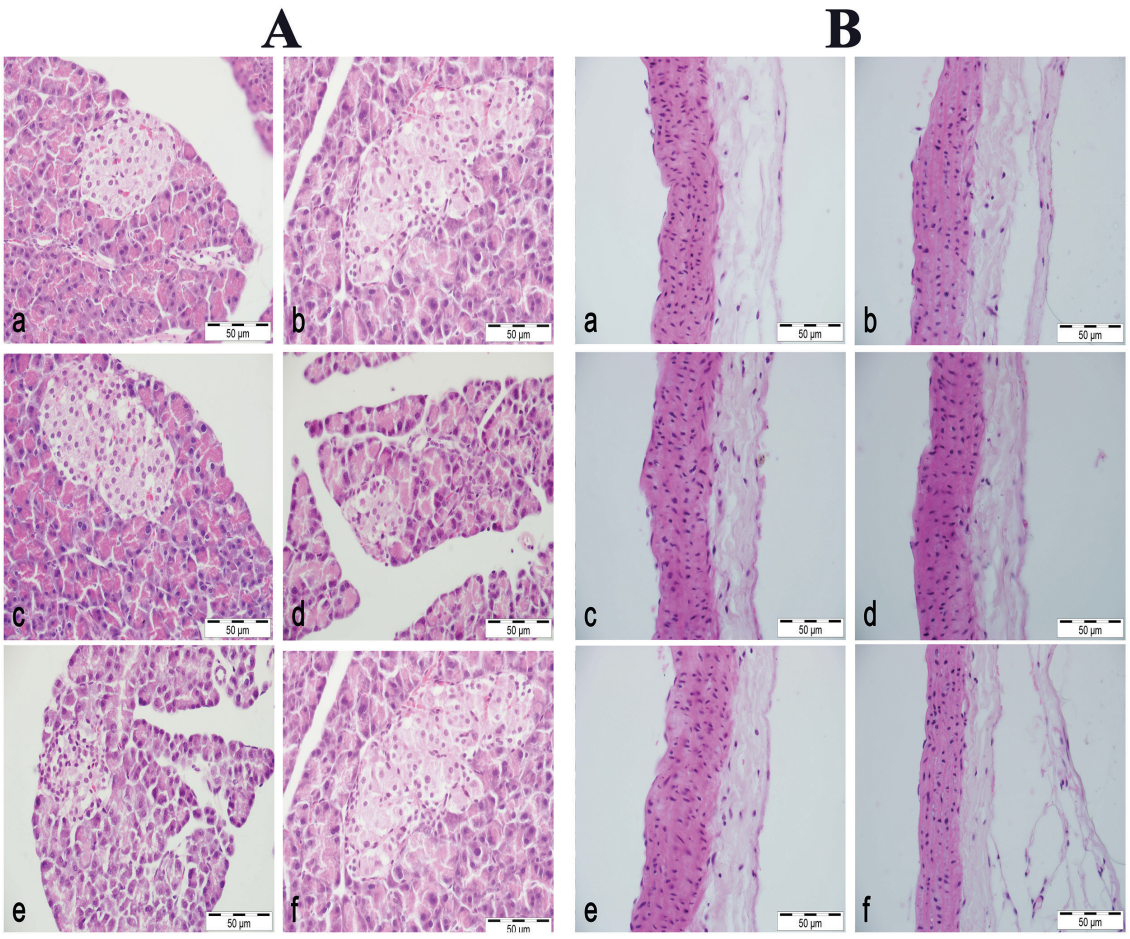

FIGURE 1 - A: Pancreas a) Normoglycemic Control group: Normal pancreas histomorphology, b) Diabetes control group: Significant amount of hydropic degeneration in the islet of Langerhans with some necrosis, c-e) Diabetes + Extract groups; 500, 1000 and 1500 mg, respectively: Some hydropic degeneration in the islet of Langerhans, and f) Metformin group: Weak degeneration. **B:** Thoracic aorta a) Normoglycemic Control group: Normal aorta histomorphology, b) Diabetes control group: No evident histopathological changes, c-e) Diabetes + Extract groups; 500, 1000 and 1500 mg, respectively: No histopathological changes and f) Metformin group: No pathology.

revealed histopathological changes in the pancreas similar to the DC group (Figure 1A f). Microscopic examination of the tissue sections of thoracic aorta from all groups revealed no histopathological changes(Figure 1B a-f).

In the NC group, insulin/proinsulin immunoreactivity in pancreas tissue samples was high, illustrated by the heavy immunostaining of the islets of Langerhans (Figure 2A a). In DC group, few cells in the islets demonstrated immunoreactivity (Figure 2A b). Insulin/proinsulin immunoreactivity of the CN 500, CN 1000 and CN 1500 generally resembled that of the DC group, presenting mostly weak immunostaining(Figure2A c-e). There were no differences between the groups in terms of the immunoreactivity. Immunostaining in the M group was also no different than the DC group (Figure 2A f). Weak iNOS immunoreactivity in the tunica adventitia and occasionally in the tunica media was also observed in the NC group. Little immunoreactivity was noted in the tunica intima of the thoracic aorta samples in the NC group (Figure 2B a). While the DC group revealed strong immunoreactivity in almost all cells of the tunica intima and tunica media. Most of the cells in the tunica media also had strong immunoreactivity in this group (Figure 2B b). In the diabetes + extract groups (CN 500, CN 1000, CN 1500), iNOS immunoreactivity pattern in the thoracic aorta sections were similar to those observed in the DC group. However, immunostaining was generally moderate to strong, and comparably fewer cells showed immunoreactivity in these groups (Figure 2B c-e). There seemed to be no significant difference among the treatment

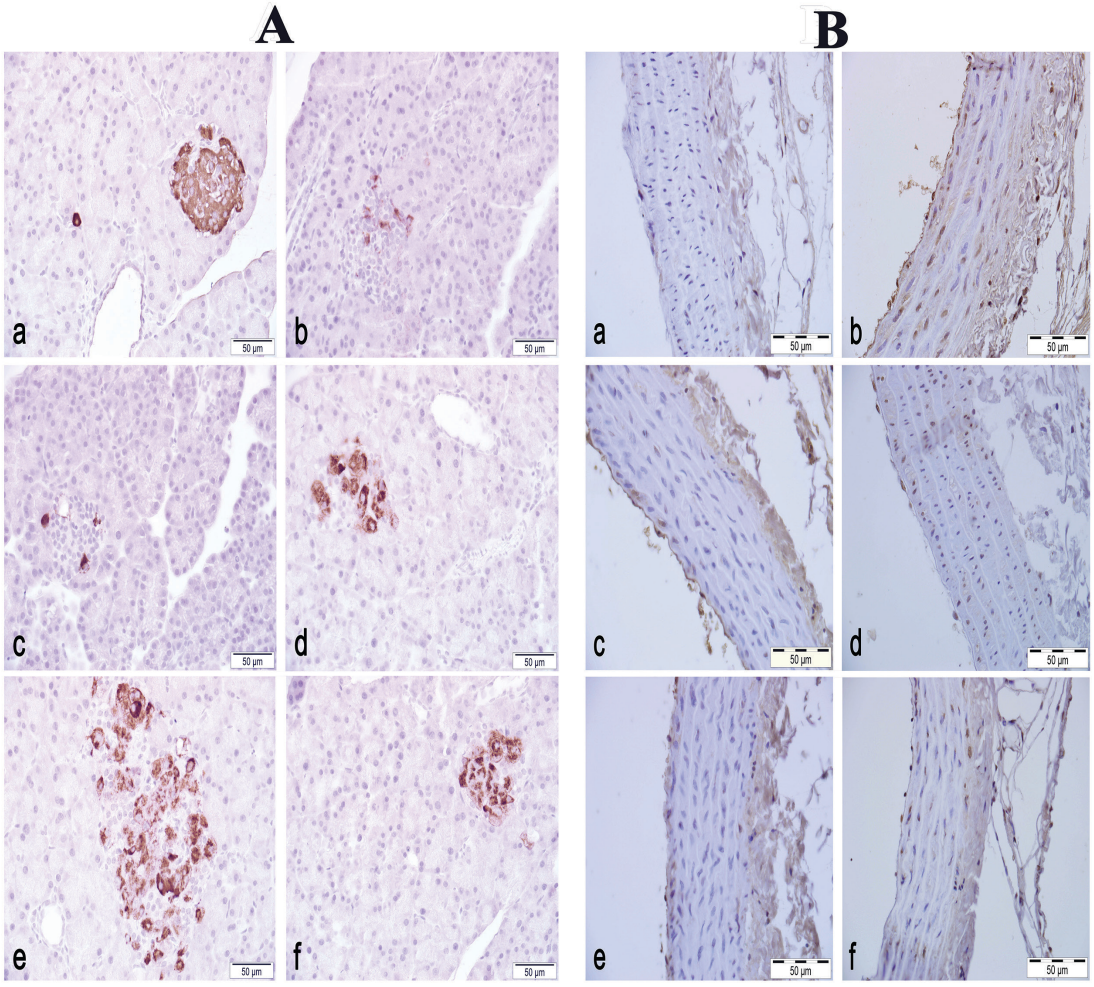

FIGURE 2 - A: Pancreas, insulin/proinsulin immunohistochemistry a) Normoglycemic Control group: strong immunoreactivity in the islet of Langerhans, e) Diabetes group: Weak immunoreactivity in few cells, c-e) Diabetes + Extract groups; 500, 1000 and 1500 mg, respectively: Weak immunoreactivity in few cells in the islet, and f) Metformin group: Weak immunoreactivity in few cells **B:** Thoracic aorta, inducible nitric oxide synthase (iNOS) immunohistochemistry a) Normoglycemic Control group: Mostly weak immunoreactivity located only in the tunica adventitia, b) Diabetes control group: Strong immunoreactivity in all three layers c-e) Diabetes + Extract groups; 500, 1000 and 1500 mg, respectively: moderate to strong immunoreactivity in all three layers of the thoracic aorta, and f) Metformin group: Strong immunoreactivity.

groups as well. Thoracic aorta iNOS activity in the M group resembled to those of the DC group (Figure 2B f).

The contractile responses of aortic rings to cumulative concentrations of PH (10^{-9} to 10^{-5} M) are shown in Figure 3. The Cinnamomum cassia extract (CN500, CN1000 and CN 1500) and M significantly decreased the contractile responses to higher concentrations of PH (10^{-7} 10^{-6} and-10^{-5} M) compared to DC ($p<0.05$) (Figure 3A). In aortic rings pre-contracted with PH, ACh relaxation was impaired in the DC group The relaxation response to ACh 10^{-8} - 10^{-5} M, was increased in CN 1000, CN 1500, and M groups as compared to group DC ($p<0.05$), (Figure 3B).

A significant increase in the MDA level was observed in group DC when compared to the NC($p<0.05$). Both the CN 500 and M groups revealed significantly lower aorta MDA levels compared to group DC, $p<0.05$ (Figure 4A). Aorta GSH levels were also significantly lower in group DC as compared to the NC($p<0.01$). Aorta GSH levels were significantly higher in all CN treatment groups and M group compared to group DC ($p<0.05$, CN 500 ($p<0.01$)) (Figure 4B). The levels of GPx in the aorta were significantly increased in CN 500, CN 1000, CN 1500 and M groups as compared to group DC ($p<0.05$) (Figure 4C). The level of aorta NO was significantly higher in group DC as compared to those of the NC group ($p<0.001$) and remaining groups ($p<0.05$). Aorta NO levels were significantly decreased in the CN 500, CN 1000, CN 1500 and M groups compared to DC ($p<0.01$) (Figure 4D).

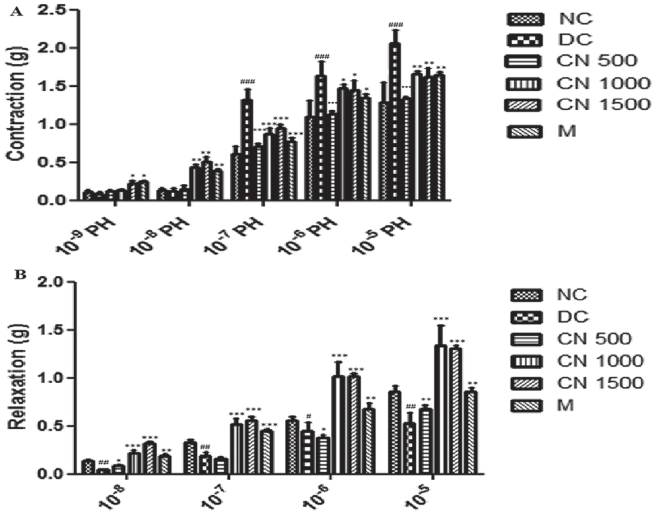

FIGURE 3 - (A) The contractile response of aortic rings to cumulative concentrations of phenylepherine (10-9 - 10-5 M) and (B) relaxation response of aortic rings to cumulative concentrations of Ach (10-8 - 10-5 M) in Normoglycemic Control (NC), Diabetes control group (DC), Cinnamomum cassia extract (CN 500, CN 1000, and CN 1500), and metformin (M) treated groups. Values are presented as means±SEM, * and # =p<0.05, ** and ## =p<0.01, *** and ### =p<0.001, (n = 10).

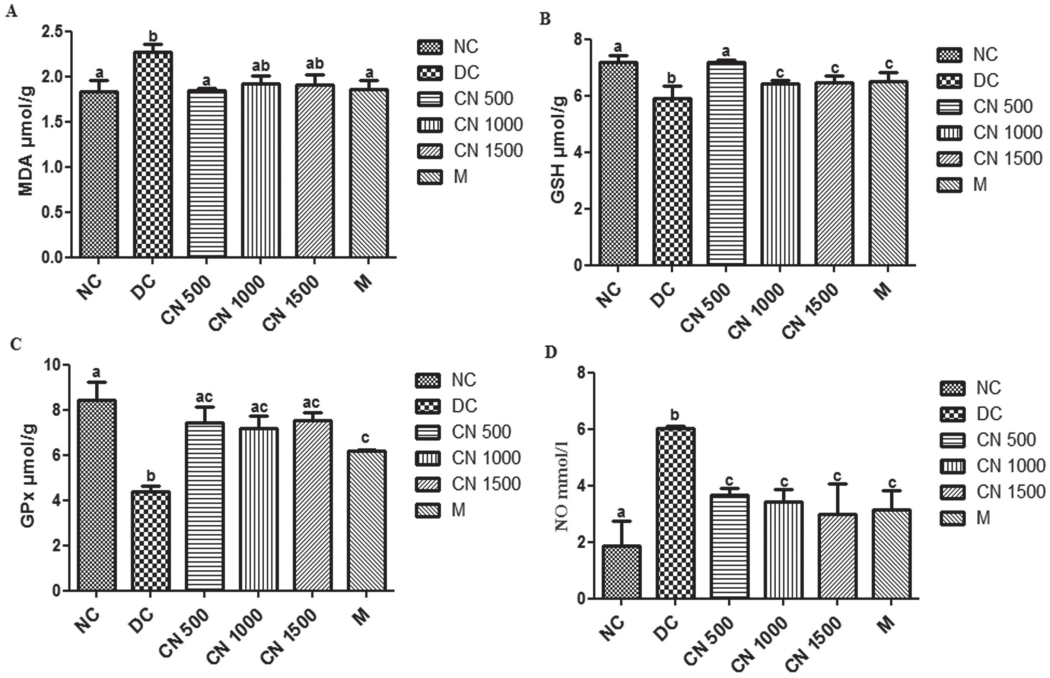

FIGURE 4 - The aorta MDA (A), GSH (B), GPx (C) levels and NO (D) activitiy in experimental groups (a-b-c=p<0.05 (A), a-b=p<0.01, a-c=p<0.05, b-c= p<0.05 (B and C), a-b=p<0.001, b-c=p<0.01, a-c= p<0.05 (D) n= 10 there is no statistical difference among the groups expressed the with same letters).

Increased free radicals in hyperglycemic conditions have been reported to react with NO resulting in the formation strong oxidants like peroxynitrite and nitrotyrosine. These strong oxidants may cause cytotoxic effects in the heart and vessels (Flesch *et al.,* 1999; Nagareddy *et al.,* 2005). Although decreased endothelial NO expression due to endothelial dysfunction has been noted, increased NO in diabetes may be consequent to iNOS and neuronal nitric oxide synthase (nNOS) (Nagareddy *et al.,* 2005). Previous studies have shown that iNOS expression is increased in cardiomyocytes of diabetic rats (Smith, Paulson, Romano, 1997) and in platelets of type I-type II diabetic patients (Tannous *et al.,* 1999). Therefore, it is stated that the investigation of iNOS activity is very important in understanding the mechanism of endothelial and vascular dysfunction associated with long-term hyperglycemia in recent years (Bardell, MacLeod, 2001). In the present study, strong iNOS immunoreactivity was detected in all three layers (tunica intima, tunica media, tunica adventitia) in DC group. Goswami *et al*. (2014) indicated that the *C. cassia* extract inhibits arginase activity and enhances sexual function. However, studies have already shown that increased expression of iNOS during inflammation suppresses arginase II expression (Carraway *et al.,* 1998). In addition, other studies have reported that aldose reductase inhibitors (ferulic acid, cinnamaldehyde) or arginase inhibitors (citrulline, norvaline, ornithine) have no effect on blood sugar levels and lack hypoglycaemic effects (Waleed Barakat, Askar, Fahmy, 1998). In this study, we believe that the *C. cassia* extract decreased NO levels by reducing hyperglycemia and consequently iNOS activity.

However, moderate immunoreactivity was detected in CN extract-treated groups. Type II diabetes is characterised by insulin resistance and is associated by improved insulin secretion disorders and decreased beta cell mass due to prolonged hyperglycemia (Butler *et al.,* 2003). It is also stated that amylin accumulates in islets in type 2 diabetes, and cause apoptosis in beta cells (Lorenzo *et al.,* 1994). CN extract did not alter insulin/proinsulin levels in the pancreas.

In the study, it was determined that decreased antioxidant levels (GSH, GPx) due to diabetes on the thoracic aorta were significantly increased in the CN extract group (CN 500, CN 1000, CN 1500), and NO levels were decreased significantly. Although it is expected that decreased endothelial production of NO is due to endothelial damage, iNOS and nNOS are thought to be the source of increasing NO. Nagareddy *et al*. (2005) suggested that impaired endothelial function in rats with Type I diabetes is associated with increased iNOS and eNOS expression in cardiovascular tissues. Noori, Azmat and Mahboob (2012) determined that garlic and cinnamon significantly reduced MDA levels in the heart. Badalzadeh *et al*. (2014) showed a significant decrease in serum MDA in the cinnamon extract-administered groups. Further, cinnamaldehyde, one of the key components of *C. cassia*, has been reported to have antioxidant and anti-inflammatory effects.

The results of this study show that administration of *C. cassia* extract increases aortic reactivity in response to vasoconstrictor and vasodilator agents in rats with type II diabetes. Cinnamaldehyde has been shown to cause relaxation of noradrenaline, potassium and prostaglandin F2a-induced aortic contraction (Xu *et al.,* 2006; Xue *et al.,* 2011). It has been determined by Yanaga *et al*. (2006) that cinnamaldehyde stimulates endothelial NO synthesis in the isolated aorta. Abebe *et al* (Abebe, Harris, Macleod, 1990) have shown that the vascular response to α-1 adrenoceptor agonists differs from that of diabetic animals. The increased kinking responses may be due to increased myofilament Ca^{2+} sensitivity (Kizub *et al.,* 2010), increased susceptibility to oxidative stress (Tabit *et al.,* 2010), and increased calcium flux with voltage-sensitive L-type Ca^{2+} channels (Pinho *et al.,* 2010), resulting in impaired endothelial function (Potenza *et al.,* 2009). The adrenoreceptor agonist, PH, causes Ca^{2+} flow through receptor-mediated Ca^{2+} channels and contraction in the aortic area by Ca^{2+} release from the sarcoplasmic reticulum (Thorneloe, Nelson, 2005; Mccarron *et al.,* 2003). Thorneloe and Nelson (2005) stated that PH stimulation of phospholipase C with the production of diacylglycerol and 1,4,5 triphosphate inositol and then has been activated the light chain of myosin with DG protein kinase C activation. Thus, contraction is occurred which with Ca^{2+} release from the sarcoplasmic reticulum after induction of IP3 receptors. Moreover, voltage-dependent Ca^{2+} channels are responsible for KCl-dependent contractions. Zhang *et al.* (2011) demonstrated that the CN effect on PH and KCl-induced vasoconstriction of diabetic rat aortic rings may be due to these effects. These effects of CN were reported that the formation and release of EDRFs including endothelial-derived hyperpolarising factor, NO and prostacyclin in the endothelial cells of the vascular bed by ACh in the vascular smooth muscle may causes an endothelium-dependent relaxation.

CONCLUSION

Neuropathy, nephropathy and retinopathy are the first to come to mind when chronic complications of type II diabetes are mentioned. Cardiovascular disorders and

endothelial damage mechanisms are generally ignored. This study indicates that 35 days of oral intake of varying doses of CN extract, reduced iNOS immunreactivity, oxidative stress and inflammation in the thoracic aorta of rats. Further, CN treatment was shown to have a positive effect on the impaired contraction mechanism in the thoracic aorta of rats with type II diabetes mellitus. However, insulin/proinsulin immunoreactivity did not change.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest and are responsible for the contents of this research.

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