**Research article** 



# Effect of Cilostazol in Alleviating Cardiovascular Complications through Regulation of Type 1 Plasminogen Activator Inhibitor and Transforming Growth Factor-β<sub>1</sub> Overexpression in Experimental Rats

**Rasha Hussieny MOHAMED** 

Department of Biochemistry, Faculty of Pharmacy, Zagazig University, Zip code 44511, Zagazig, Egypt.

E-mail: rashahussieny@yahoo.com

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## Abstract

Cilostazol is a potent phosphodiesterase inhibitor; its major effects are prevention of platelet aggregation and dilation of blood vessels via an increase in tissue cAMP levels. This study examined the effect of cilostazol on serum cAMP, type 1 plasminogen activator inhibitor and transforming growth factor- $\beta_1$ in relation to alleviating cardiovascular complications. This was achieved in rats through administration of L-NAME (0.1 mg/ml) for two weeks, and then followed by i.p. single dose of streptozotocin (65mg/kg). Rats were classified to three groups; normal rats, control diabetic hypertensive rats and the third group was treated with cilostazol (1.8 mg daily, orally) for six weeks. Cilostazol improved serum cAMP level and increased plasma NO concentration leading to dilation of blood vessels. In addition, cilostazol has beneficial lipoprotein-modifying effect. Cilostazol treatment confirmed the positive correlation between plasma PAI-1 activity and serum TGF- $\beta_1$  which is beneficial in reducing the hazards of cardiovascular complications. Thus, cilostazol therapy provides a broad spectrum of effects in alleviating cardiovascular complications induced in experimental animals.

# Keywords

Cilostazol. phosphodiesterase inhibitor • Cyclic AMP • Plasminogen activator inhibitor-1 • Transforming growth factor- $\beta_1$  • Cardiovascular complications.

# Introduction

Endothelial dysfunction plays a key regulatory role in initiation of cardiovascular complications (CVC) [1]. Epidemiologic data has confirmed an increased likelihood of developing CVC when hypertension and diabetes mellitus (DM) are combined [2]. The relationship among endothelial function, diabetes, and hypertension is complex whereas hypertensive-diabetic rats show more severe coronary microvascular abnormalities [3]. Moreover, there is an increased prevalence of hypertension in diabetic patients and *vice versa*, suggesting that both conditions commonly coexist [4]. Hypertension exacerbates diabetic cardiomyopathy, indeed, hypertension is well documented as an important risk factor for myocardial infarction and stroke. These complications are predominantly occurring due to thrombosis of arterioles [5].

Plasminogen activator inhibitor-1 (PAI-1) is well-established marker of endothelial dysfunction in several pathological conditions [6]. Disregulation of PAI-1 expression plays a potential role not only in thrombosis [7] but also in the formation of tissue fibrosis [8]. Nevertheless, PAI-1 is significantly elevated in diabetic patient before the onset of clinical vascular complication. Interestingly, PAI-1 is markedly upregulated by transforming growth factor- $\beta_1$  (TGF- $\beta_1$ ), which could directly increase PAI-1 mRNA levels of endothelial cells by promoting gene transcription [9]. Consequently, both PAI-1 and TGF- $\beta_1$  may be taken as meaningful biomarkers for incidence and propagation of cardiovascular complications.

Endothelial dysfunction in hypertensive diabetic rats involves a decreased production or bioavailability of nitric oxide (NO) [10]. Definitely, chronic NO blockade enhances thrombus formation as demonstrated by increased plasma levels of PAI-1. This NO decrement is concomitant with a decrease in arterial wall content of cyclic adenosine monophosphate (cAMP) [11]. Cyclic AMP is the second messenger which can regulate the fibroblasts in many tissues. Several studies reported that cAMP analogues, which increase intracellular cAMP concentration, inhibit platelet aggregation. Cyclic nucleotide phosphodiesterases (PDEs) are the major enzymes involved in metabolic processing of cyclic AMP and cyclic GMP [12, 13].

Specifically, Type III phosphodiesterase inhibitor (PDE3) is the only cyclic AMP-regulating isozyme expressed in both vascular smooth muscle cells (VSMC) and platelets, so, its inhibition has been shown to block platelet aggregation and proliferation of VSMC *in vitro* [14].

Cilostazol {6-[4-(1-cyclohexyl-1*H*-tetrazol-5-yl)butoxy]-3,4-dihydroquinolin-2(1*H*)-one} is a specific inhibitor of PDE3. Its major effects are prevention of platelet aggregation and a dilation of blood vessels via an increase in tissue cAMP levels through blocking its hydrolysis by type III phosphodiesterase [15, 16]. It also improved postprandial lipemia in diabetic patients [17].

# **Materials and Methods**

### Experimental animals

Male Wistar rats with an initial body weight of 200 to 230 g were supplied by the Egyptian Organization for Biological Products and Vaccines. Rats were subjected to controlled conditions of temperature  $(25 \pm 2^{\circ}C)$  and illumination (12h-light/dark). Animals were allowed free access to normal rat chow diet and water *ad libitum*. This protocol was approved by the Animal Care and Use Committee of the Biochemistry Department, Faculty of Pharmacy, Zagazig University.

#### Experimental work design

One week after acclimatization, rats were randomly divided into two groups: normal controls, n = 10 and N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME), n = 40. L-NAME was given in the drinking water (0.1 mg/ml) as inhibitor to nitric oxide synthesis which leads to systemic hypertension for two weeks before induction of diabetes.

Diabetes was induced by a single i.p. injection of streptozotocin, STZ, at a dose of 65 mg/kg prepared in 0.1 M citrate buffer, pH 4.2, as described previously [18]. Seven days after STZ injection, blood glucose (BG) was measured enzymatically by a glucose oxidase method using Spinreact Diagnostics Kits (Spain), and animals were considered to be diabetic when the concentration of BG was equal to or higher than 200 mg/dl. Diabetic hypertensive rats were subdivided into two groups: the first one was treated with cilostazol (n = 12, 1.8 mg daily, orally) for 6 weeks [19] while the other group was left as a control (n=15). Cilostazol was kindly provided by Otsuka Pharmaceutical Co. (Egypt) and other chemicals were purchased from Sigma Chemical, St. Louis, USA. At the end of the study, rats from each group were anaesthetized with urethane (1.3 mg/ kg). Blood samples were collected for plasma and serum separation and then stored at -80 °C as aliquots for further analysis.

## The method of recording of the arterial blood pressure and electrocardiogram (ECG)

Animals were anaesthetized with ethyl carbamate (urethane) at a dose of 1.75-2.0 gm/kg i.p. as freshly prepared aqueous solution. The blood pressure and ECG were determined employing the reported method [20] through introduction of polyethylene arterial cannula with 3-way valve (filled with heparinized saline solution 16 I.U./ml to inhibit blood clotting) in the common carotid artery. The cannula was connected to PT 400 blood pressure transducer, which was connected to FC137 strain gauge coupler which was fixed to one of the 4-channel oscillograph MD<sub>4</sub> (Bioscience, U.K.). The blood pressure was recorded on chart paper. The ECG limb cable was connected to FC123 coupler which switched on lead II and connected to another channel of the 4-channel oscillograph MD<sub>4</sub>.

Mean arterial pressure = Diastolic BP+1/3 (Systolic BP-Diastolic BP)

## Induction of venous thrombosis

At the end of treatment period and twelve hours before induction of venous thrombosis, four rats from each group were deprived of food but had free access to water. The venous thrombosis was induced as previously described [21]. The abdomen was opened under thiobarbital anesthesia (40 mg/ kg body weight i.p.); the vena cava was carefully separated from the surrounding tissues and then ligated tightly with a cotton thread just below the left renal vein. Subsequently, the abdomen was closed with a double layer of sutures (peritoneum with muscles and skin separately). After 4h the animals were anesthetized again and the abdomen was then re-opened, the vena cava was carefully dissected and inspected for the presence of a thrombus. The thrombus was air-dried at 37°C and after 24h, its weight was determined.

#### Measurement of serum cyclic AMP and plasma nitric oxide content

The Adenosine 3',5'-cyclic monophosphate (cAMP) levels were evaluated by a Parameter<sup>™</sup> ELISA kit (R&D Systems) according to the kit's instructions. Plasma nitric oxide (NO) was measured as nitrite concentration after the reduction of nitrate to nitrite using Griess reagent. The nitrite concentration was measured at 546 nm using standard sodium nitrite [22].

## Transforming growth factor- $\beta_1$ and plasminogen activator inhibitor-1 Assay

Serum TGF- $\beta_1$  [23] and plasma PAI-1 [24] were evaluated by ELISA technique using kits purchased from Biosource (USA) and Hyphen Bio Med (France), respectively.

The samples were acidified using 4 mM HCl to activate a latent form of TGF- $\beta_1$ , making it accessible for measurement in the immunoassay.

#### Determination of serum lipogram pattern

Serum total cholesterol (TC), triacylglycerols (TAG), high density lipoprotein cholesterol (HDL-C) and low density lipoprotein cholesterol (LDL-C) were estimated enzymatically [25], using Spinreact diagnostics kits (Spain).

#### Statistical analyses

Results were expressed as means  $\pm$  S.D. Comparisons between groups were conducted by one-way ANOVA followed by the multiple comparison tests (least significant different, LSD) (SPSS program, Chicago, IL). The statistical associations between functional parameters were assayed using Spearman's nonparametric correlation analysis. *P* < 0.05 was the established level of significance.

# Results

## Effect of cilostazol treatment on hyperglycemia

STZ-induced diabetes in Wistar rats recapitulates many aspects of type 1 diabetes in humans and is the most commonly used animal model for the study of diabetes-induced complications [18, 26]. Hyperglycemia was detected in diabetic hypertensive animals and was not altered in cilostazol- treated groups compared to nontreated animals (Table 1).

Parameters	Control	Control Disbetio	Cilostazol	р
	Normai	Diabelic	liealineill	
	rats	hypertensive rats		
S-Glu (mg/dl)	93±9	230±21*	229±15*	NS
SBP(mmHg)	120±3.2	170±5.9*	$140\pm4.1^{\#}$	0.0001
DBP(mmHg)	90±2.1	120±3.4*	$100\pm2.9^{\#}$	0.0001
MAP	100.0±1.28	136.7±2.13*	113.3±2.9* <sup>#</sup>	0.0001
HR(beat/min)	240±15	320±13*	270±19 <sup>#</sup>	0.008

**Tab. 1.** Values of various parameters in normal rats, cilostazol-treated and -untreated diabetic hypertensive rats

Key: S-Glu=serum glucose concentration; SBP= systolic blood pressure; DBP= diastolic blood pressure; MAP= mean arterial pressure; HR= heart rate. Values were expressed as means ± SD., \*P<0.001 v.s normal rats, <sup>#</sup>P v.s diabetic hypertensive control. NS, non significant difference from diabetic hypertensive control.

#### Effect of cilostazol on blood pressure and heart rate

As shown in Table1, the systolic, diastolic blood pressure, mean arterial pressure, and heart rate were significantly higher in diabetic hypertensive rats as compared with normal rats (P<0.001). Treatment with cilostazol significantly decreased these parameters (21.42%), (20.0%), (20.65%) and (14.8%), respectively in our established diabetic hypertensive rats.

#### Effect of cilostazol on serum cyclic AMP and plasma nitric oxide content



Fig. 1. Effect of cilostazol treatment on serum cAMP (pmol/ml) and plasma NO (μmol/ml) in diabetic hypertensive rats. All values were expressed as means ± S.D., \*P <0.001 vs. normal rats, <sup>#</sup>P<0.001 vs. diabetic hypertensive control. N (normal rats), DH (diabetic hypertensive rats), Cil (cilostazol treatment).</p>

The effect of L-NAME and STZ combination on serum cyclic AMP and plasma NO levels were summarized in Fig. (1), which showed significant decrease in both cyclic AMP (from 2.9 $\pm$ 0.24 to 0.4 $\pm$ 0.06) and plasma NO levels (from 10.4 $\pm$ 1.3 to 3.6 $\pm$ 0.8) as compared to normal group. Treatment with cilostazol for 6 weeks led to significant increase in serum cyclic AMP from 0.4 $\pm$ 0.06 to 1.72 $\pm$ 0.13 (*P*<0.001) and plasma NO level from 3.6 $\pm$ 0.8 to 8.9 $\pm$ 0.9 (*P*<0.001).

## Effect of cilostazol treatment on PAI-1 activity and dry thrombus weight

Figure (2) showed that plasma PAI-1 activity and dry thrombus weight were significantly increased in diabetic hypertensive rats at P<0.001. Treatment with cilostazol for 6 weeks significantly decreased both plasma PAI-1 activity from 3.6±0.5 to 0.85±0.07 (P<0.001) and venous thrombus weight from 2.7±0.21 to 1.2±0.14 (P<0.001).



**Fig. 2.** Effect of cilostazol treatment on plasma PAI-1 (ng/ml), dry thrombus weight (mg), and serum TGF- $\beta_1$  (pg/ml) in diabetic hypertensive rats. All values were expressed as means  $\pm$  S.D., \*P<0.001 vs. normal rats, <sup>#</sup>P<0.001 vs. diabetic hypertensive control.

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N (normal rats), DH (diabetic hypertensive rats), Cil (cilostazol treatment).

## Effect of cilostazol treatment on TGF-β<sub>1</sub>

The transforming growth factor- $\beta_1$  is remarkably increased in diabetic hypertensive animals by 70.08% *vs.* normal rats. Cilostazol-treatment caused 81.11% significant reduction in this parameter as compared to nontreated group (Fig. 2).

#### Effect of cilostazol treatment on serum lipoproteins

Table 2 revealed that TC, TAG, LDLC and atherogenic indexes (TC/HDLC and LDLC/HDLC) were markedly increased while HDLC was significantly decreased at P>0.001 in diabetic hypertensive group as compared to normal rats. After 6 weeks of cilostazol treatment, there was a significant decrease in total cholesterol (22.3%), serum triglycerides (35.46%) and LDLC (30.5%). Moreover, the two atherogenic indexes (TC/HDLC and LDLC/HDLC) were significantly decreased by 50.0% and 52.0%, respectively. In addition, significant increase in HDL-C level was observed upon comparison the treated group with nontreated one (Table 2).

Parameters	Control Normal rats	Control Diabetic hypertensive rats	Cilostazol treatment	Ρ
TC (mg/dl)	97±10	170±13*	139±9.4 <sup>#</sup>	0.013
TAG(mg/dl)	84±7	123±7.4*	$90.8 \pm 9^{\#}$	0.002
LDLC(mg/dl)	50±9	92±5*	70.5±6 <sup>#</sup>	0.009
HDLC(mg/dl)	33±2.6	24±1.1*	29±1.4 <sup>#</sup>	0.015
TC/HDLC	2.9±0.32	7.2±1.13*	$4.8\pm0.23^{\#}$	0.005
LDLC/HDLC	1.52±0.25	3.8±0.13*	2.5±0.31 <sup>#</sup>	0.001

Tab. 2. Effect of cilostazol treatment on lipogram pattern in diabetic hypertensive rats.

Key: TC= total cholesterol; TAG= triaceylglycerol; LDLC= low density lipoprotein cholesterol; HDLC= high density lipoprotein cholesterol. Values were expressed as means ± SD., \*P<0.001 v.s normal rats, <sup>#</sup>P v.s diabetic hypertensive control.

# Discussion

The present study have shown that coexist of diabetes and hypertension in experimental animals has resulted in a decreased levels of NO and cAMP. In fact, the decreased level of such parameters may be attributable to endothelial dysfunction which, in turn led to cardiovascular disorders.

The previous study reported that powerful inhibition of NO biosynthesis causes severe cardiovascular disorders in rats treated with high dose of L-NAME [27]. These disorders are due to decrease in production or bioavailability of nitric oxide that associated with a decrease in cyclic GMP and cyclic AMP content [28] leading to hypertension and consequently accelerate the progression of CVC [29].

In addition, other reports documented that endothelial dysfunction represents a common patho-physiological pathway of diabetic complications which play a central role in cardiovascular complications [30]. It was also observed that endothelial dysfunction led to vascular dysfunction as evidenced by reduced endothelial nitric oxide-mediated vasorelaxation which causes increase in arterial pressure [31].

PAI-1 is plasmatic marker that reflects endothelial cell damage and its elevated level in cardiovascular disorders confirmed this phenomenon [32]. Moreover, PAI-1 is a prominent causative factor in several progressive and chronic fibrotic disorders, particularly in the context of elevated tissue TGF- $\beta_1$  levels, as well as an important contributor to the pathophysiology of vascular sclerosis [33]. TGF- $\beta$  is a key player in the fibrotic response, whereas it activates TGF- $\beta$  receptors causing activation of Smad proteins especially Smad3/4 which acts as transcription factors [34, 35]. This activation led to enhancement of transcription of numerous genes, including connective tissue growth factor, plasminogen activator inhibitor-1 and various collagen genes [36].

The results presented in this study clearly showed the presence of higher levels of PAI-1 and TGF- $\beta_1$  in diabetic hypertensive rats when compared with normal rats. Moreover, a direct correlation between PAI-1 and TGF- $\beta_1$  (r=0.92, P<0.001) was also observed. Thus, blocking TGF- $\beta_1$  signal transduction should reduce PAI-1 production, blunt fibrosis, maintain normal endothelium function, and finally suppress progression of cardiovascular complications.

Our findings revealed that administration of cilostazol for 6 weeks improved endothelial functions, in part, through an increasing in serum cAMP and plasma NO levels, besides it decreased the arterial blood pressure in diabetic hypertensive animals due to its vasodilatation effect.

Several reports documented our results whereas one study demonstrated that cilostazol caused an increase in serum cAMP presumably by reducing its hydrolysis due to inhibition of phosphodiesterase type III [37]. In addition, others illustrated that cilostazol stimulates NO production via the activation of cAMP-dependent protein kinase (protein kinase A; PKA) [38] owing to inducing phosphorylation of eNOS at Ser-1177 [39].

Furthermore, another study explained the role of cilostazol in increasing cyclic AMP via an increase in the nitric oxide production which causes activation of soluble guanylate cyclase leading to an increase in the cGMP level. The increased level of cyclic GMP could inactivate PDE3, thereby increasing the cAMP concentration which causing further dilatation, increasing peripheral blood flow and inhibiting the proliferation of vascular smooth muscle cells [40].

This work demonstrated that supplementary daily doses of cilostazol 1.8 mg induced a significant reduction of plasma PAI-1, serum TGF- $\beta_1$ , and also inhibiting the venous thrombus formation due to its well known antithrombotic effect. Moreover, there is a strong negative correlation between PAI-1 and cAMP (r=0.90, P<0.001) as well as TGF- $\beta_1$  and cAMP (r=0.98, P<0.0001). Thus, cilostazol can protect diabetic hypertensive animals from the progression of cardiovascular disorders through several ways: firstly, by improvement of endothelial dysfunction; secondly, by its direct action in reducing overexpression of TGF- $\beta_1$  which, in turn inhibits PAI-1 production via interaction with the TGF- $\beta$  signaling

pathway; thirdly, by inhibiting the venous thrombus formation; and finally through modifying lipoproteins.

Previous study presented definitive evidence that cAMP-elevating agents block Smad3/4specific transcription in response to TGF- $\beta$ , as well as PAI-1 gene expression at the transcriptional level [41]. Another work suggested that activation of the cAMP/PKA signaling pathway inhibits Smad-mediated transcription by abolishing Smad interaction with key transcriptional activators [42].

Moreover, cilostazol also favorably modifies lipoproteins through elevation of cAMP [43]. There are several possible mechanisms by which the increased cAMP might led to decreasing in serum triglycerides. One possible mechanism is via reducing hepatic triglyceride (ie, VLDL) secretion [44] and the other one depends on promoting the release of lipoprotein lipase from rat adipocytes [45] which could reduce serum triglycerides.

# Conclusions

It can be concluded that the findings of current study indicate that the drug interfering with the cAMP pathway offer therapeutic opportunity to alleviate cardiovascular complications. The phosphodiesterase type III inhibitor cilostazol fulfills this idea whereas it improves cAMP level leading to increase in NO concentration causing a decrease in arterial blood pressure. Moreover, it decreases overexpression of TGF- $\beta_1$  and also prevents thrombosis via inhibition of plasma PAI-1 activity. Consequently, cilostazol therapy provides a broad spectrum of effects in alleviating cardiovascular complications induced in experimental animals.

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# Author's Statements

#### Competing Interests

The author declares no conflict of interest.

## Animal Rights

The institutional and (inter)national guide for the care and use of laboratory animals was followed. See the material and methods part for details.

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