Life Sciences 229 (2019) 67–79

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Vardenafil and cilostazol can improve vascular reactivity in rats with diabetes mellitus and rheumatoid arthritis co-morbidity

Mariam Gamal Fahmy Wahba[a], Basim Anwar Shehata Messiha[b,*], Mahmoud El-Sayed El-Daly[c], Ali Ahmed Abo-Saif[a]

[a] Department of Pharmacology and Toxicology, Faculty of Pharmacy, Nahda University, Beni-Suef, Egypt
[b] Department of Pharmacology and Toxicology, Faculty of Pharmacy, Beni-Suef University, Beni-Suef, Egypt
[c] Department of Pharmacology and Toxicology, Faculty of Pharmacy, Minia University, Minia, Egypt

ARTICLE INFO

Keywords:
Vardenafil
Cilostazol
Endothelial dysfunction
Vascular reactivity
Rheumatoid arthritis
Diabetes mellitus

ABSTRACT

Endothelial dysfunction and vascular reactivity defects secondary to metabolic and immunological disorders carry risk of serious cardiovascular complications. Here, the effects of the phosphodiesterase (PDE) inhibitors vardenafil and cilostazol were examined against rheumatoid arthritis (RA)/diabetes mellitus (DM)-co-morbidity-induced endothelial dysfunction and vascular reactivity defects. After setting of RA/DM-co-morbidity model, rats were divided into a normal control group, an RA/DM-co-morbidity group, and two treatment groups receiving oral vardenafil (10 mg/kg/day) and cilostazol (30 mg/kg/day) for 21 days after RA/DM-co-morbidity induction. Aorta was isolated for biochemical estimations of the pro-inflammatory vasoconstrictor molecules angiotensin-II (Ang-II) and endothelin-1 (ET-1), the adhesion molecules P-selectin and vascular cell adhesion molecule-1 (VCAM-1), the energy sensor adenosine-5′-monophosphate-activated protein kinase (AMPK), and the vasodilator anti-inflammatory molecule vasoactive intestinal peptide (VIP) using enzyme-linked immunosorbent assay (ELISA) and western blot analysis. Immunohistochemical estimations of endothelial nitric oxide synthase (eNOS) and matrix metalloproteinase (MMP)-2 were performed coupled with histopathological examination using routine hematoxylin and eosin (H&E) and special Masson trichrome staining. The *in vitro* study was conducted using aortic strips where cumulative concentration response curves were done for the endothelium-dependent relaxing factor acetylcholine and the endothelium-independent relaxing factor sodium nitroprusside after submaximal contraction with phenylephrine. Vardenafil and cilostazol significantly improved endothelial integrity biomarkers *in vivo* supported with histopathological findings in addition to improved vasorelaxation *in vitro*. Apart from their known PDE inhibition, up-regulation of vascular AMPK and eNOS coupled with down-regulation of Ang-II, ET-1, P-selectin, VCAM-1 and MMP-2 may explain vardenafil and cilostazol protective effect against RA/DM-co-morbidity-induced endothelial dysfunction and vascular reactivity defects.

1. Introduction

The vascular bed normally exerts a degree of reactivity to a number of physiologic challenges, which is a necessity to perform its functions. According to Manuck et al. [1], cardiovascular reactivity may be defined as the physiologic changes from a resting or baseline state to some type of physical challenge. Abnormally exaggerated vascular reactivity to stressful conditions may increase risk of cardiovascular complications like hypertension and coronary artery disease [2,3]. On the other side, vascular reactivity may be adversely affected by other pathological conditions including diabetes mellitus [4] and rheumatoid arthritis [5].

Hyperglycemia and insulin resistance observed in type 2 diabetes mellitus (DM) trigger systemic inflammation and damage the vasculature *via* different mechanisms. The elevated free fatty acids availability observed in insulin resistance uncouples mitochondrial function in endothelial cells thus generating reactive oxygen species (ROS) and impairing endothelial nitric oxide synthase (eNOS) activity [6]. The decrease in eNOS expression and the increase in vascular oxidative stress results in increasing the expression of vasoactive factors like endothelin-1 (ET-1) [7] and adhesion molecules like P-selectin [8], which in turn promote the secretion of interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) [9]. Besides, alteration in insulin signaling mediates an increase in the levels of other stressful vasoactive peptides

* Corresponding author.
E-mail address: drbasimanwar2006@yahoo.com (B.A.S. Messiha).

https://doi.org/10.1016/j.lfs.2019.05.024
Received 27 March 2019; Received in revised form 27 April 2019; Accepted 10 May 2019
Available online 11 May 2019
0024-3205/

such as angiotensin II (Ang-II) [10]. As defense mechanism, the cell energy sensor adenosine-5′-monophosphate-activated protein kinase (AMPK) was reported to ameliorate endothelial dysfunction in metabolic syndromes like diabetes [11].

Endothelial dysfunction is generally a hallmark in chronic inflammatory diseases like rheumatoid arthritis (RA) [12]. In RA inflammatory cascade, low density lipoprotein cholesterol (LDL-C) is retained in the endothelium and becomes modified to oxidized form [13]. This causes endothelial activation, leading to an increased expression of adhesion molecules like P-selectin and an increased release of inflammatory cytokines such as IL-1, IL-6 and TNF-α [14]. The pro-inflammatory cytokine TNF-α triggers the releases of other adhesion molecules including vascular cell adhesion molecule-1 (VCAM-1) which is known to help regulate inflammation-associated vascular adhesion and vascular reactivity [15]. Side by side, the gelatinase A or matrix metalloproteinase-2 (MMP-2), together with other gelatinases, is reported to be overproduced in joints in rheumatoid arthritis [16]. Based on its ability to degrade extracellular matrix, the MMP-2 was reported to adversely affect vascular reactivity and enhance vascular injury [17]. Alternatively, the anti-inflammatory molecule vasoactive intestinal peptide (VIP) plays an important modulator role in the pathogenesis of arthritis-induced defects in vascular reactivity [18].

Phosphodiesterases (PDEs) is a group of enzymes that degrade the phosphodiester bonds in cyclic nucleotides [19]. Several isoforms of PDE exist, namely PDE-1, 2, 3, up to 12. Some PDE isoforms are cAMP-specific, others are cGMP-specific and others are non-specific. Inhibition of several isoforms of PDEs was old known to possess clinical significance [20]. Vardenafil is a highly selective and potent inhibitor of PDE-5 [21]. Continuous PDE-5 inhibition was found to reduce expression of vascular inflammatory proteins including inducible nitric oxide synthase (iNOS), cyclo-oxygenase-2 (COX2) and VCAM-1 thus promoting tissue protection [22]. It was also reported that the vasodilator effect of PDE-5 inhibition was mediated in part through inhibition of the ET pathway *via* blocking ET_A/ET_B receptors [23]. Cilostazol is a PDE-3 inhibitor and is a unique antiplatelet agent. It was reported to inhibit platelets activation in hypercholesterolemic rats *via* AMPK and eNOS activation, together with NF-kβ, P-selectin and VCAM-1 suppression [24–26]. Cilostazol was also found to block extracellular calcium influx in mouse smooth muscle cells and arteries, impairing ET-induced vasoconstriction [27,28].

Based on the aforementioned data, the present investigation aims to study the effect of vardenafil and cilostazol against RA/DM co-morbidity-induced defects in vascular reactivity in rats. To fulfill this purpose, the pro-inflammatory vasoconstrictor molecules Ang-II and ET-1, the adhesion molecules P-selectin and VCAM-1, the energy sensor AMPK, and the vasodilator ant-inflammatory molecule VIP were estimated using enzyme-linked immunosorbent assay (ELISA) and western blot analysis. Immunohistochemical estimations of eNOS and MMP-2 were also performed coupled with histopathological examination using routine hematoxylin and eosin (H&E) and special Masson trichrome staining. The *in vitro* study was conducted using rat aortic strips where cumulative concentration response curves were done for the endothelium-dependent relaxing factor acetylcholine (Ach) and the endothelium-independent relaxing factor sodium nitroprusside (SNP) after submaximal contraction with phenylephrine (PE).

2. Material and methods

2.1. Material

2.1.1. Animals

In the present investigation, adult female albino rats, weighing 180–200 g, were used. Animals were obtained from the Animal House of Nahda University, Beni-Suef, Egypt, and were kept in the animal room for 14 days for adaptation before being used in laboratory experiments. Animals were fed on standard forage and tap water *ad libitum* throughout the adaptation and the experimental periods, except for groups receiving high fat diet (HFD) according to the experimental protocol. Animals were kept in a pathogen-free air-conditioned room under stable temperature and relative humidity, with 12-h/12-h dark/light cycles. Handling of animals and animal care were done according to the guidelines of Beni-Suef Animal House approved by the Pharmacology and Toxicology Department, Faculty of Pharmacy, Beni-Suef University, based on the guidelines indicated by the recommendations of the National Institutes of Health (NIH) Guide for Care and Use of Laboratory Animals.

2.1.2. Drugs, chemicals and reagent kits

Complete Freund's adjuvant (CFA; catalog no. MFCD00131105), streptozotocin (STZ; catalog no. MFCD00006607), vardenafil (catalog no. MFCD09027942) and cilostazol (catalog no. MFCD00866780) were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). ELISA kits of ACPA (anti-citrullinated peptide antibody; catalog no. MBS2606863), insulin (catalog no. MBS724709), ET-1 (catalog no. MBS704215), P-selectin (catalog no. MBS012813), Ang II (catalog no. MBS705139), VCAM-1 (catalog no. MBS027532) and VIP (catalog no. MBS025730) were purchased from MyBioSource (San Diego, USA). The primary antibodies for immunohistochemical estimation of eNOS (catalog no. PA1-037) and MMP-2 (catalog no. PA1-16667), and for Western blot analysis of AMPK (catalog no. PA5-36045) were purchased from ThermoFisher Scientific (Rockford, USA). All other used chemicals, solvents and reagents were obtained from official sources and were all of analytical grade.

2.2. Experimental design

Two sets of experiments were performed; in the first one, setting of RA/DM co-morbidity model was performed, while in the second one, the effect of vardenafil and cilostazol on RA/DM co-morbidity-induced defect in vascular reactivity was performed.

In the first set, 24 weight-matched healthy acclimatized rats were randomly divided into 4 groups, each of 6 rats. The first group was kept as a normal control group receiving vehicles only. The second group was kept as RA control group and received CFA only. The third group was kept as DM control group and received STZ only. The fourth group was kept as RA/DM co-morbidity group and received both CFA and STZ in the indicated schedules. At the end of the experiment, fasting blood glucose, fasting serum insulin and serum ACPA were measured to confirm induction of DM and RA, in addition to aorta tissue ET-1, P-selectin and VIP levels as measures of vascular integrity.

In the second set, 24 weight-matched healthy acclimatized rats were divided into 4 groups, namely a normal control group receiving vehicles only, a RA/DM co-morbidity control group, a vardenafil treatment group and a cilostazol treatment group. Test agents were administered once daily for 21 consecutive days starting from day 16 after induction of RA/DM co-morbidity (Fig. 1). Vardenafil was administered in a dose of 10 mg/kg/day, p.o. [29,30]. Cilostazol was administered in a dose of 30 mg/kg/day, p.o. [26,31]. At the end of the experiment, animals were fasted overnight and blood and tissue samples were collected for assessment of all abovementioned biomarkers of vascular integrity, coupled with histopathological and immunohistochemical assays. Rat body weights were also recorded on a weekly basis in both sets.

2.3. Methodology

2.3.1. Induction of DM and RA

2.3.1.1. Induction of DM. Rats were fed for 3 weeks with a high fat diet (HFD) containing 25% raw sheep fat lard prepared as described earlier by Khalifa et al. [32], then injected with a single i.p. dose of STZ (45 mg/kg) dissolved in sodium citrate buffer (0.1 M; pH = 4.5). Rats were then given 20% glucose solution in drinking water for 48 h to prevent hypoglycemia. Blood glucose was measured in the tail vein on

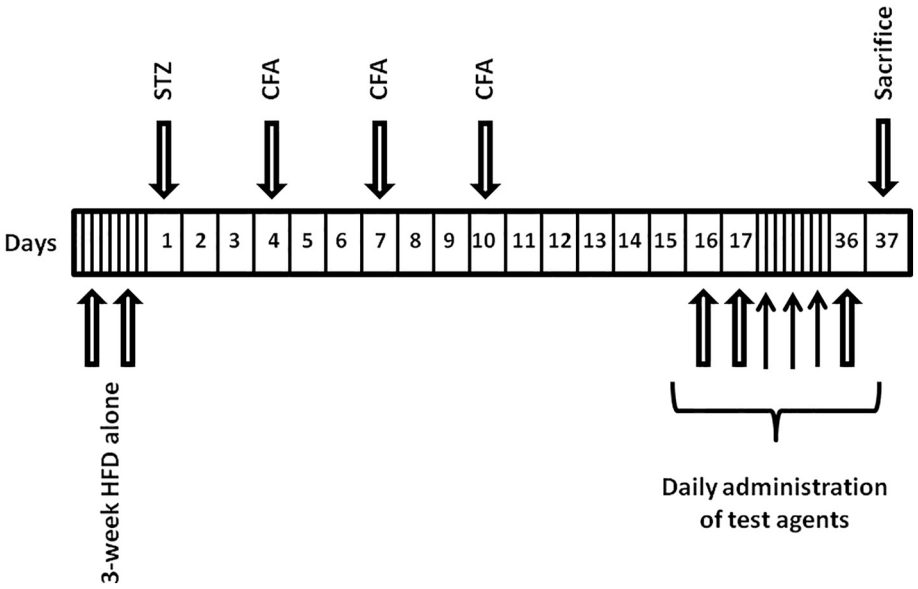

Fig. 1. A diagram showing experimental design of the *in vivo* induction of RA/DM co-morbidity. CFA: complete Freund's adjuvant; DM: diabetes mellitus; HFD: high fat diet; RA: rheumatoid arthritis; STZ: streptozotocin.

the 3rd day after STZ injection using ACCU-CHEK® device (Roche, USA). Rats with a glucose level higher than 300 mg/dL were considered diabetic [33].

2.3.1.2. Induction of RA. Arthritis was induced using three subcutaneous doses of CFA, each of 0.4 ml, injected in three different limbs, with 3 days interval between every two doses [34].

2.3.1.3. Induction of RA/DM co-morbidity. After 3 weeks of HFD feeding, induction of DM was performed using STZ as described above. On day 3 after STZ administration, confirmation of DM induction was performed, then CFA was administered on days 4, 7 and 10. Induction of RA was reported to take place within 10–12 days of the first dose of CFA [35]. Therefore, treatment with test agents started twelve days after the first CFA dose, *i.e.* on day 16, for 21 days then rats were sacrificed (Fig. 1).

2.3.2. Sampling

2.3.2.1. Serum sampling. Blood samples were collected from the retro-orbital plexus using heparinized micro-tubes. Blood was left to coagulate on crushed ice then centrifuged at 3000 rpm for 10 min using a cooling centrifuge (Sigma 3–30 k, USA). The supernatant clear serum layer was withdrawn and stored at −80 °C deep freezer (Als Angelantoni Life Science, Italy) until the time of assay of ACPA and fasting serum insulin.

2.3.2.2. Tissue sampling. After blood withdrawal, animal were killed by cervical dislocation, underwent midline thoracotomy and the thoracic cavity was washed with isotonic saline. The aorta was exposed, carefully dissected free of adjacent tissue, and divided into two parts; the first portion was preserved at −80 °C and kept for the time of assay of Ang-II, ET-1, P-selectin, VCAM-1, AMPK and VIP; the second portion was fixed in 10% isotonic formalin solution in normal saline for 48 h and kept for the time of the histopathological study and immunohistochemical estimation of MMP-2 and eNOS.

2.3.3. Assessment of serum and tissue biomarkers

Serum insulin and ACPA as well as aorta tissue Ang-II, ET-1, P-selectin, VCAM-1 and VIP levels were estimated using ELISA test reagent kits according to the manufacturer's instructions based on the sandwich technique described earlier [36–38] using ELISA Processing System (Model Spectra Max Plus-384 Absorbance Microplate Reader, USA). Tissue levels of total and phosphorylated AMPK (t-AMPK and p-AMPK) were measured by the Western blot technique described earlier [39] using BioRad mini protein electrophoresis separation unit (Model 1658004, Germantown, USA) and presented as p-AMPK/t-AMPK relative expression.

2.3.4. Histopathological study

After tissue hardening, aorta tissues were washed with water then with serial dilutions of alcohol for dehydration. Specimens were then cleared in xylene and embedded in paraffin wax at 56 °C in hot air oven for 24 h. Blocks were then transversely sectioned into 5 μm sections using sledge microtome, then stained with standard Hematoxylin and Eosin (H&E) staining as described earlier by Banchroft and Steven [40]. Alternatively, other sections were stained with the special Masson's trichrome stain using standard techniques for photomicroscopic observations [41].

2.3.5. Immunohistochemical assay

Immunohistochemical assay of eNOS and MMP-2 was performed based on the principle described earlier by Merz et al. [42]. Briefly, paraffin-embedded tissue sections of 5-μm thickness were dehydrated first in xylene and then in graded ethanol solutions. The slides were then blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline (TBS) for 2 h. The sections were then incubated overnight at 4 °C with the primary antibodies against eNOS or MMP-2. After washing the slides with TBS, the sections were incubated with secondary antibody. Sections were then washed with TBS and incubated for 10 min in a solution of 0.02% diaminobenzidine containing 0.01% H_2O_2. Counterstaining was performed using hematoxylin, and the slides were visualized under a light microscope by the aid of a histopathologist blinded to the study.

2.3.6. In vitro study

The *in vitro* study was conducted as previously described by Rhee et al. [43]. Briefly, thoracic aorta was carefully collected at the end of the experiment and rapidly transferred to aerated Krebs-Henseleit buffer solution adjusted at pH 7.4 and temperature 37 ± 0.5 °C. The aorta was carefully dissected from surrounding fat and connective tissue and cut into short transverse segments. Segments of the rat thoracic aorta, excluding the aortic arch region, of 1 mm length was

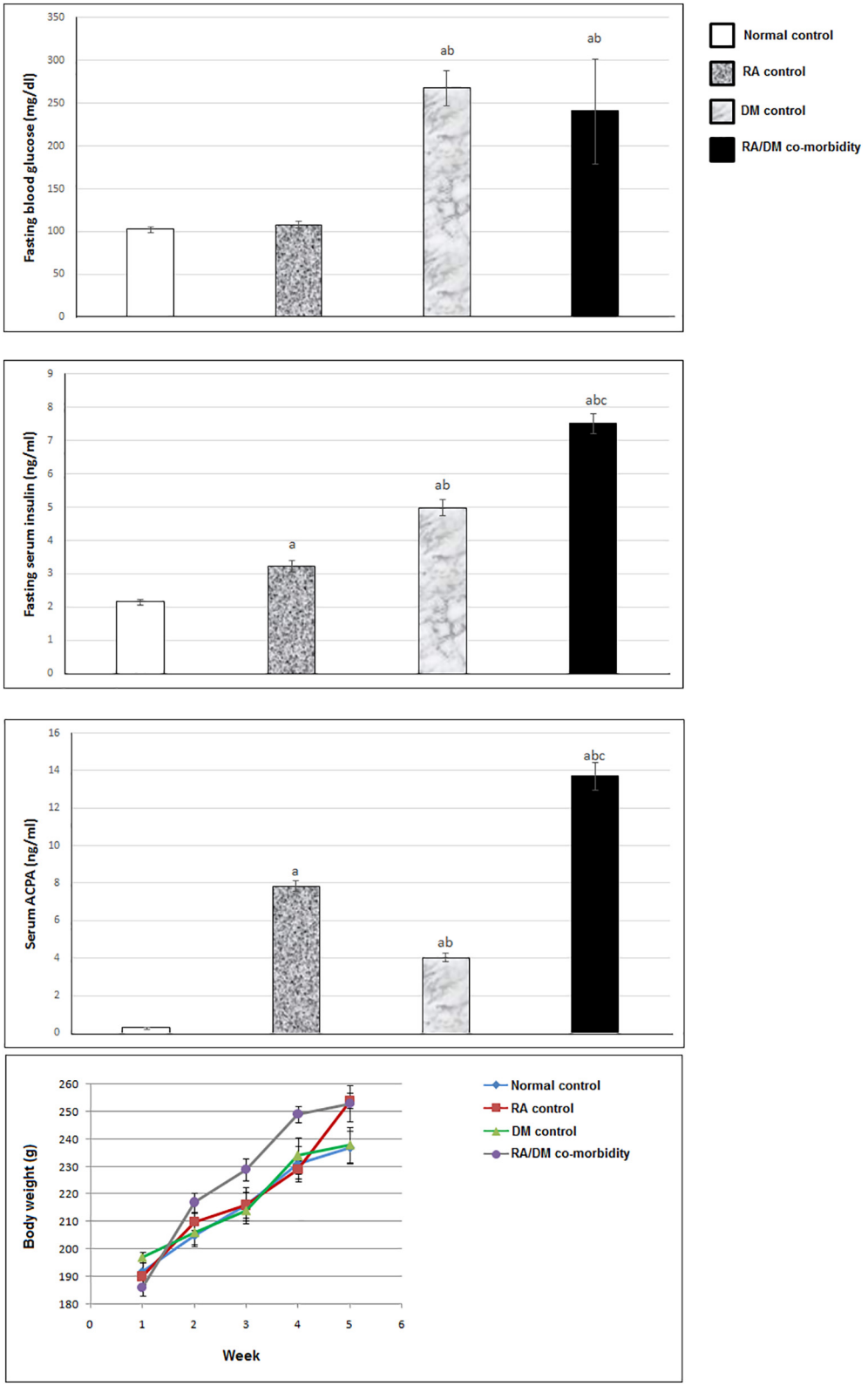

Fig. 2. Fasting blood glucose, fasting serum insulin, serum ACPA and body weight changes in rats with RA, DM and RA/DM co-morbidity. DM: diabetes mellitus; RA: rheumatoid arthritis. [a]Significantly different from the normal control group, [b]Significantly different from the RA control group, [c]Significantly different from the DM control group at $p < 0.05$.

mounted in 10 ml of Krebs-Henseleit buffer and kept at 37 °C in organ baths, continuously aerated, between 2 stainless steel pins, 200 μm in diameter each. A basal tension of approximately 1.5 g was applied gradually to the aortic rings throughout the experiment using routinely calibrated isometric force displacement transducers (MLT0201/D, AD Instruments) connected to a Powerlab/8S data acquisition system and Chart® software that measures changes in circumferential tension and recorded as units of gram (*g*) tension. Tissues were left to equilibrium

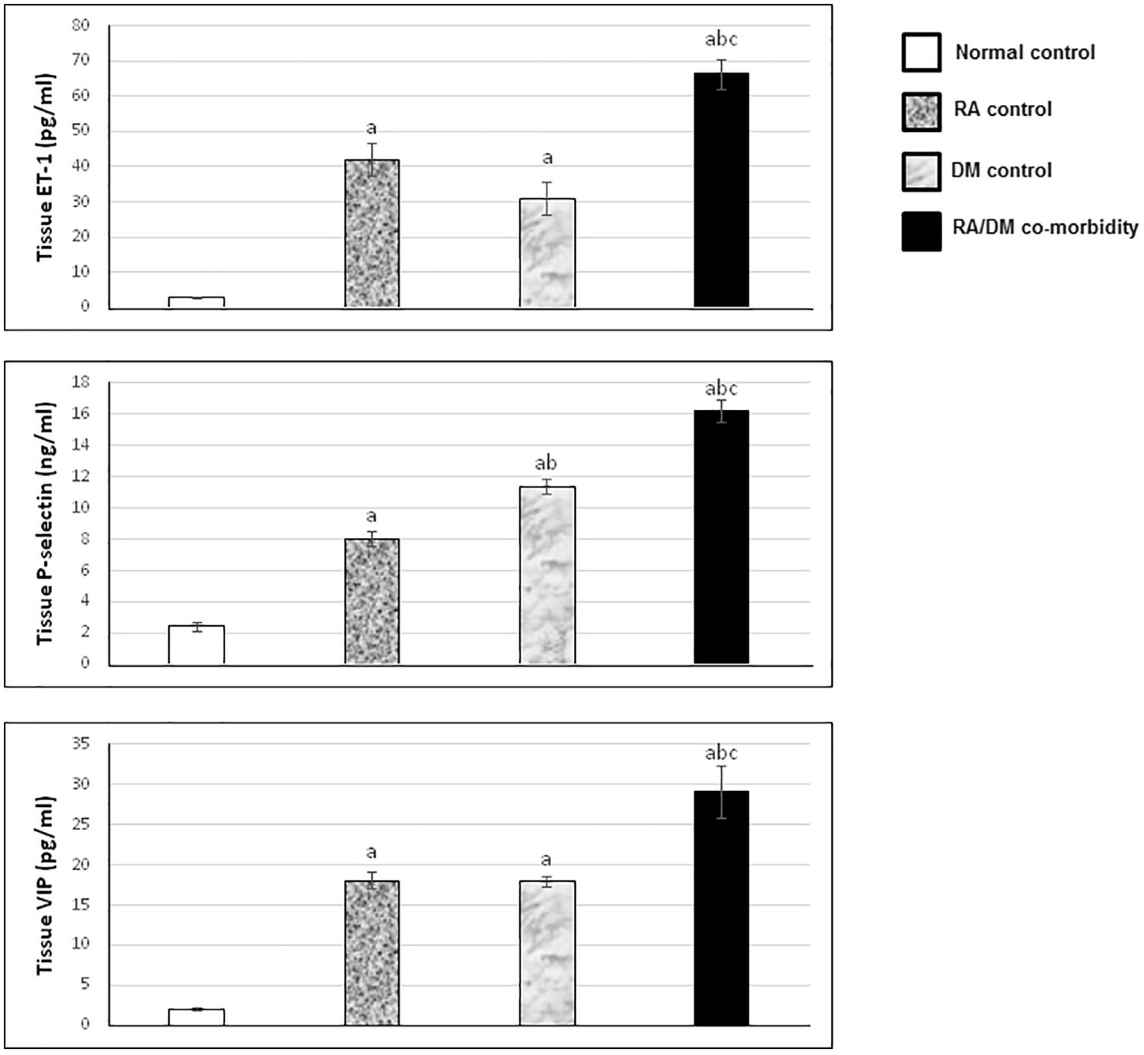

Fig. 3. Tissue ET-1, P-selectin and VIP in rats with RA, DM and RA/DM co-morbidity. DM: diabetes mellitus; ET-1: endothelin-1; RA: rheumatoid arthritis; VIP: vasoactive intestinal peptide. [a]Significantly different from the normal control group, [b]Significantly different from the RA control group, [c]Significantly different from the DM control group at $p < 0.05$.

for a period of 45–60 min while physiological solution was changed every 15 min. Aortic tissue viability was then assessed using KCl and endothelium viability and function were assessed using phenylephrine and acetylcholine. Cumulative concentration response curves were done for the endothelium-dependent relaxing factor Ach and the endothelium-independent relaxing factor SNP after submaximal contraction with PE.

2.4. Statistical analysis

Results were expressed as means ± standard error of the mean (S.E.M). All statistical analyses of biochemical estimations were performed by one-way analysis of variance (ANOVA) test followed by Tukey-Kramer *post hoc* test by the aid of the statistical package for social sciences (SPSS; version 19.0) computer software program (SPSS Inc., Chicago, IL, USA) with a value of $p < 0.05$ considered statistically significant. Quantification of western blot bands was performed using Image J program software (NIH, USA).

3. Results

3.1. Results of the first set; RA/DM co-morbidity induction

3.1.1. Fasting blood glucose, fasting serum insulin, serum ACPA and body weight changes

Normal control rats showed fasting blood glucose level of 102.80 ± 3.74 mg/dl, which did not show significant changes upon induction of RA using CFA. Alternatively, rats with STZ-induced DM and rats with RA/DM co-morbidity showed about 2.6-fold and 2.3-fold increases of fasting blood glucose, respectively, as compared to normal control rats (Fig. 2).

Fasting serum insulin of normal control rats showed a mean value of 2.16 ± 0.28 ng/ml, which showed significant increases in RA rats (1.5 folds), in DM rats (2.3 folds) and in RA/DM co-morbidity rats (3.5 folds) as compared with normal control values (Fig. 2).

Serum ACPA level of normal control rats showed a mean value of 0.28 ± 0.04 ng/ml, which was significantly increased in RA group, DM group and RA/DM co-morbidity group to reach about 28 folds, 14 folds and 49 folds, respectively, as compared with normal control level (Fig. 2).

Weekly estimations of rat body weights revealed no significant difference among animal groups at the end of each week.

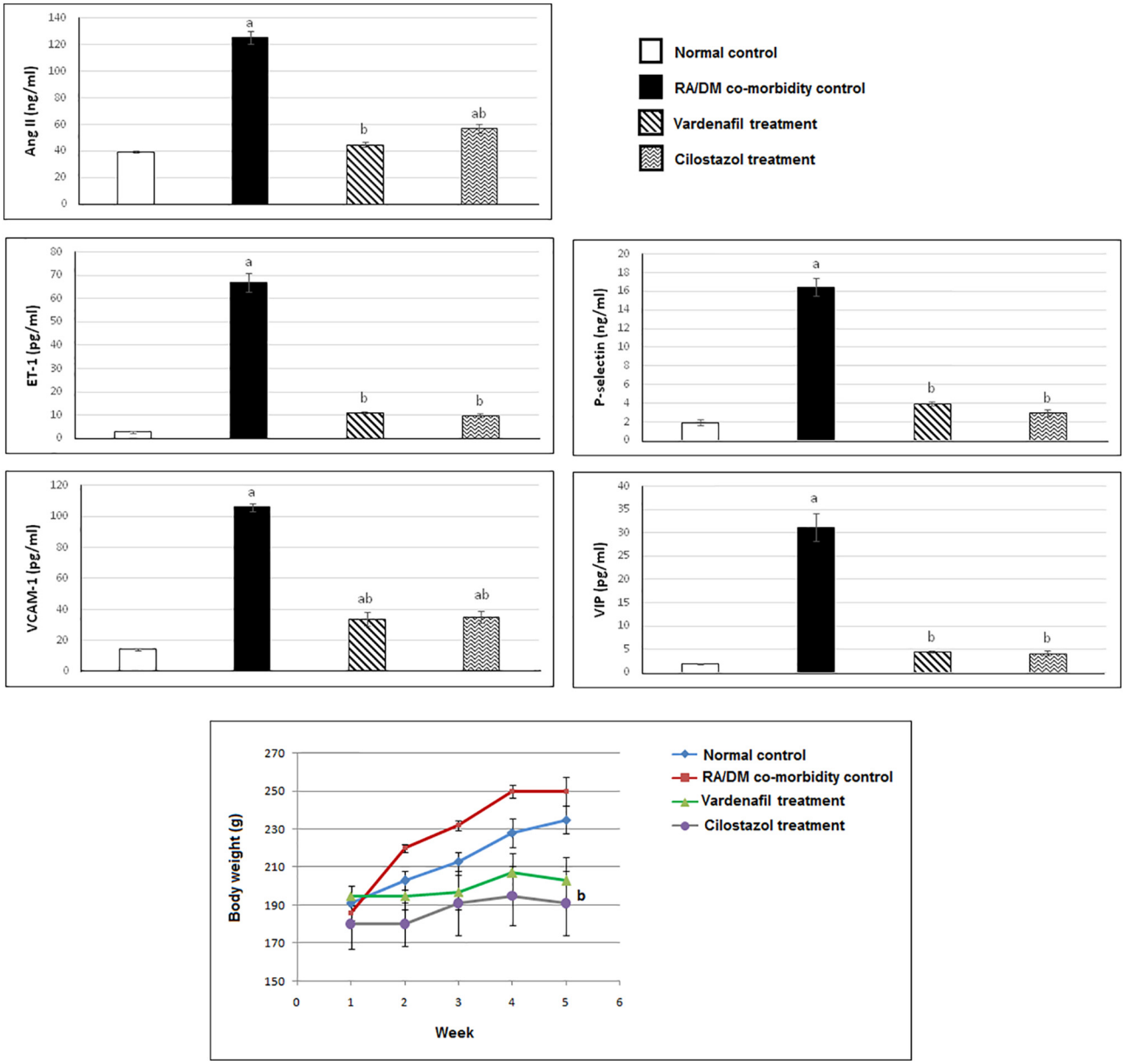

Fig. 4. Effect of vardenafil and cilostazol on aorta tissue Ang-II, ET-1, P-selectin, VCAM-1 and VIP as well as body weight changes in rats with RA/DM co-morbidity. Ang-II: angiotensin-II, DM: diabetes mellitus; ET-1: endothelin-1; VCAM-1: vascular cell adhesion molecule-1; RA: rheumatoid arthritis; VIP: vasoactive intestinal peptide. [a]Significantly different from the normal control group, [b]Significantly different from the RA/DM control group at $p < 0.05$.

3.1.2. Tissue ELISA of ET-1, P-selectin and VIP

Aorta tissue levels of ET-1, P-selectin and VIP in normal control rats were estimated to be 2.79 ± 0.25 pg/ml, 2.46 ± 0.28 ng/ml and 2.09 ± 0.21 pg/ml, respectively. These values were significantly increased in RA rats, DM rats and RA/DM co-morbidity rats regarding ET-1 (about 15 folds, 11 folds and 24 folds, respectively), P-selectin (about 3 folds, 5 folds and 7 folds, respectively) and VIP (about 9 folds, 9 folds and 14 folds, respectively), as compared with normal control levels (Fig. 3).

3.2. Results of the second set; effect of test agents on RA/DM co-morbidity

3.2.1. Tissue ELISA of Ang-II, ET-1, P-selectin, VCAM-1 and VIP as well as body weight changes

Rats with RA/DM co-morbidity showed significantly higher levels of tissue Ang-II, ET-1, P-selectin, VCAM-1 and VIP compared with normal control levels, reaching about 318%, 2409%, 849%, 740% and 1715%, respectively. Treatment of rats with vardenafil or cilostazol significantly decreased the elevated tissue levels of Ang-II, ET-1, P-selectin, VCAM-1 and VIP, reaching about 36%, 16%, 24%, 32% and 14%, respectively, regarding vardenafil and about 46%, 15%, 18%, 33% and 13%, respectively, regarding cilostazol as compared with RA/DM co-morbidity control. Mean body weight of cilostazol-treated rats was significantly lower than the respective mean body weight of RA/DM co-morbidity control (Fig. 4).

3.2.2. Tissue western blot analysis of AMPK

Rats with RA/DM co-morbidity showed significantly lower tissue p-AMPK/t-AMPK relative expression, reaching about 14% of its normal control level. Treatment of rats with vardenafil or cilostazol significantly increased p-AMPK/t-AMPK levels to reach about 3 folds and 2 folds, respectively, as compared with RA/DM co-morbidity control (Fig. 5).

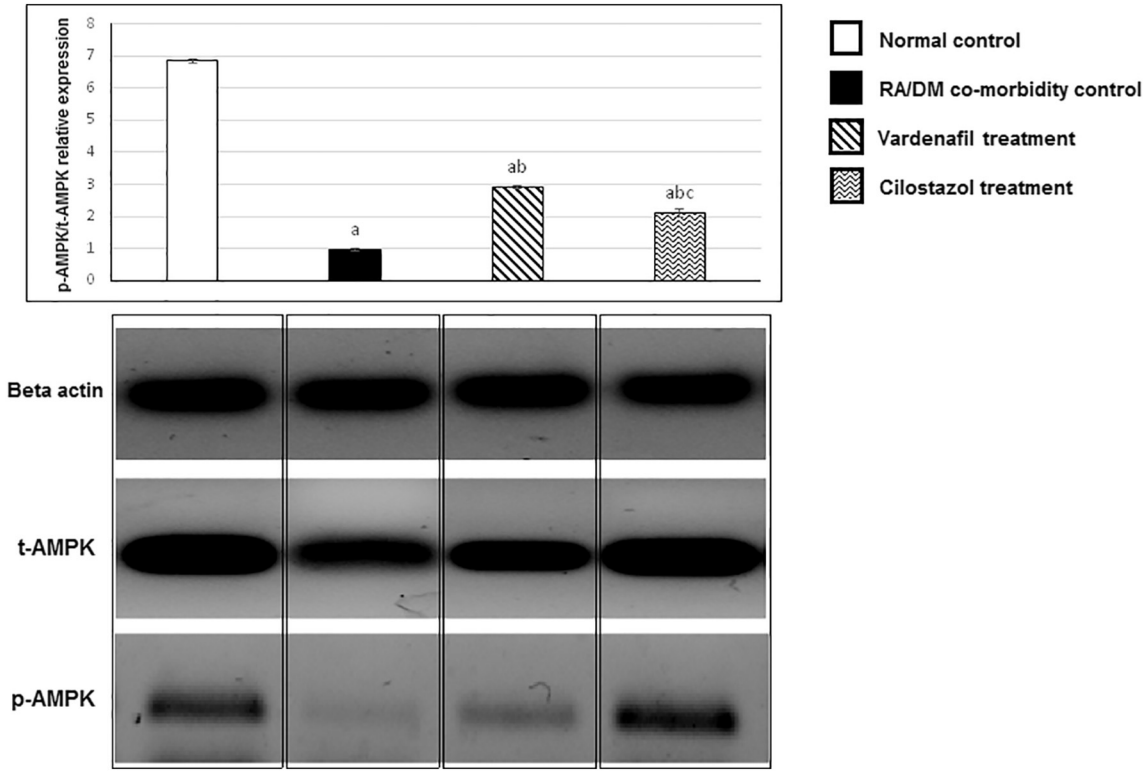

Fig. 5. Effect of vardenafil and cilostazol on aorta tissue p-AMPK/t-AMPK relative expression in rats with RA/DM co-morbidity. AMPK: adenosine-5′-monophosphate-activated protein kinase; t-AMPK: total AMPK; p-AMPK: phosphorylated AMPK; DM: diabetes mellitus; RA: rheumatoid arthritis. [a]Significantly different from the normal control group, [b]Significantly different from the RA/DM control group at $p < 0.05$.

3.2.3. Histopathological study

3.2.3.1. Routine H&E staining. Normal control rat sections showed average intima with average endothelial cells, average media with average smooth muscle cells showing elongated nuclei and eosinophilic cytoplasm, and average elastic tissue with sub-medial clefting. The RA/DM control sections showed aortic wall with intimal destruction, sub-intimal clefting and edema, medial clefting with marked medial destruction and smooth muscle cells showing small pyknotic nuclei and vacuolated cytoplasm, together with marked sub-medial separation. The vardenafil-treated sections showed aortic wall with average intima, minimal medial clefting with average smooth muscle cells and average elastic lamina, and minimal sub-medial separation. The cilostazol-treated sections showed aortic wall with intact intima, minimal medial clefting with average smooth muscle cells and average elastic lamina and sub-medial separation (Fig. 6).

3.2.3.2. Special Masson trichrome staining. Normal control sections showed aortic wall with average collagen distribution in sub-intima and through the media. RA/DM sections showed aortic wall with excess collagen in the media (Fig. 7). Vardenafil-treated sections showed aortic wall with excess collagen in the sub-intima and in the media. Cilostazol-treated sections showed aortic wall with minimal collagen in sub-intima and excess collagen in the media (Fig. 7).

3.2.4. Immunohistochemical assay

3.2.4.1. eNOS. Normal control sections showed aortic wall with strong cytoplasmic reactivity in endothelial cells and smooth muscle cells regarding eNOS. RA/DM sections showed mild eNOS reactivity in endothelial cells and smooth muscle cells. Vardenafil-treated sections showed aortic wall with mild cytoplasmic reactivity in endothelial cells and no reactivity in smooth muscle cells. Cilostazol-treated sections showed aortic wall with mild cytoplasmic reactivity in endothelial cells and no reactivity in smooth muscle cells (Fig. 8).

3.2.4.2. MMP-2. Normal control sections showed aortic wall with no cytoplasmic reactivity in endothelial cells and smooth muscle cells regarding MMP-2. RA/DM sections showed strong MMP-2 reactivity in endothelial cells and smooth muscle cells. Vardenafil-treated sections showed aortic wall showed mild cytoplasmic reactivity in endothelial cells and in smooth muscle cells. Cilostazol-treated sections showed aortic wall with mild cytoplasmic reactivity in endothelial cells and in smooth muscle cells (Fig. 9).

3.2.5. In vitro study

With logarithmic cumulative increase of phenylephrine concentration, aorta tissue contraction (as percentage of KCl contraction) showed gradual increase regarding all groups. Alternatively, acetylcholine and sodium nitroprusside showed gradually increased relaxation of phenylephrine-induced contraction. Regarding the endothelium-dependent relaxing factor acetylcholine, minimal relaxation was evident for RA/DM co-morbidity group while maximal relaxation was evident for normal control group at most acetylcholine concentrations. Vardenafil- and cilostazol-treated rat groups showed significant improvements of relaxation at high concentrations of acetylcholine. Alternatively, regarding the endothelium-independent relaxing factor sodium nitroprusside, cilostazol-treated rat group showed improved relaxation at high sodium nitroprusside concentrations, but maximal relaxation was evident for the vardenafil-treated rat group at high concentrations of sodium nitroprusside (Fig. 10).

4. Discussion

In the present investigation, the effects of the PDE-5 inhibitor vardenafil and the PDE-3 inhibitor cilostazol were examined against RA/DM co-morbidity-induced defects in vascular integrity and reactivity in experimental rats *in vivo* and *in vitro*.

Results of the present investigation showed that induction of RA/

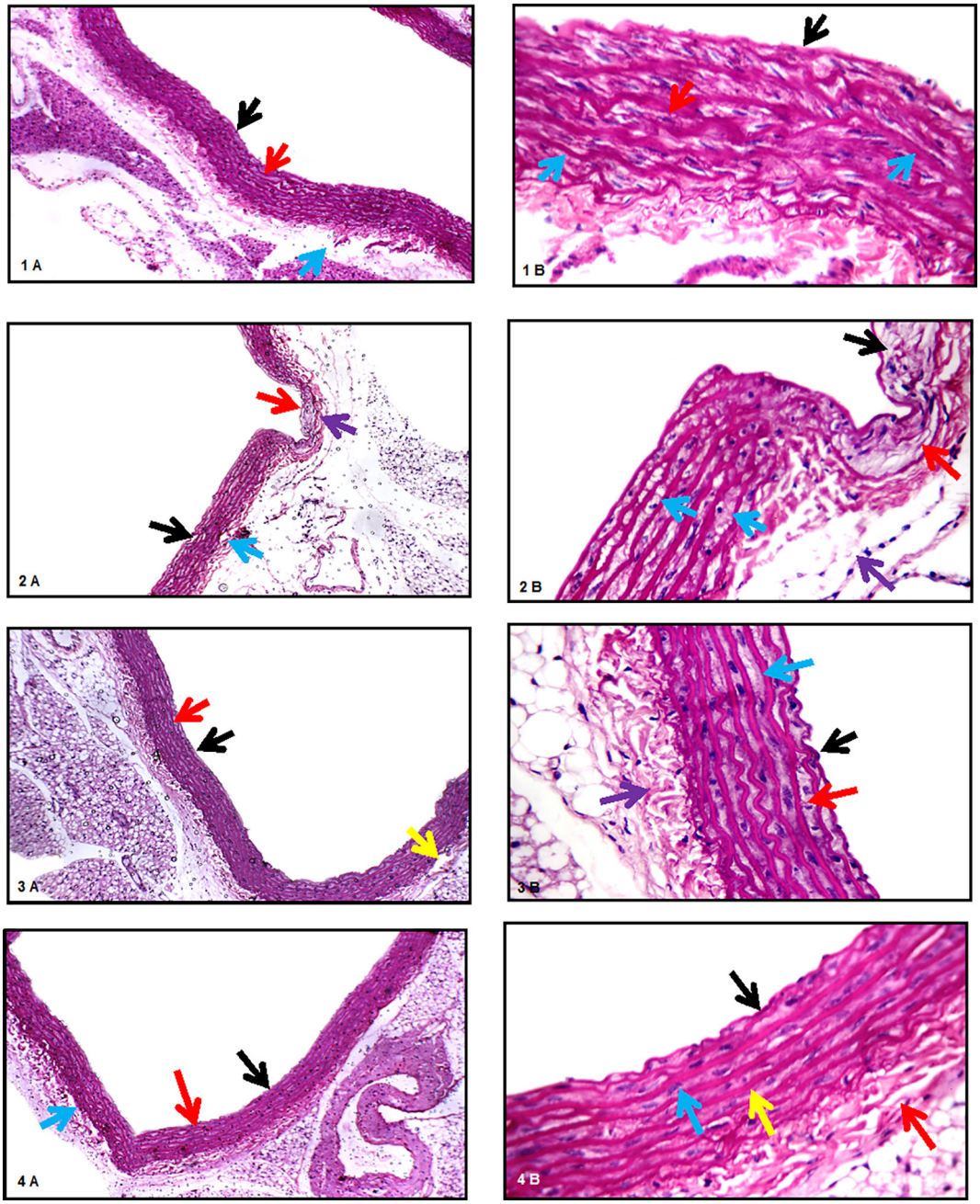

Fig. 6. Photomicrographs of aorta sections stained with routine H&E staining showing the effect of vardenafil and cilostazol administration in rats with RA/DM co-morbidity. 1A: Normal control section (200× magnification) showing blood vessel wall with average intima (black arrow), average media (red arrow), and normal sub-medial clefting (blue arrow); 1B: Normal control section (400× magnification) showing average intima with average endothelial cells (black arrow), average media with average smooth muscle cells showing elongated nuclei with eosinophilic cytoplasm (red arrow) and average elastic tissue (blue arrows); 2A: RA/DM co-morbidity section (200× magnification) showing sub-intimal clefting (black arrow), edema (red arrow) and medial clefting (blue arrow) with marked medial destruction (violet arrow); 2B: RA/DM co-morbidity section (400× magnification) showing sub-intimal edema (black arrow), marked medial destruction (red arrow) with smooth muscle cells showing small pyknotic nuclei and vacuolated cytoplasm (blue arrows) and marked sub-medial separation (violet arrow); 3A: Vardenafil treatment section (200×) showing blood vessel wall with average intima (black arrow), average media (red arrow) and minimal sub-medial separation (yellow arrow); 3B: Vardenafil treatment section (400×) showing average intima (black arrow), average media with average smooth muscle cells (red arrow) and average elastic lamina (blue arrow) with average adventitia (violet arrow); 4A: Cilostazol treatment section (200×) showing blood vessel wall with intact intima (black arrow), average media (red arrow) and minimal sub-medial separation (blue arrow); 4B: Cilostazol treatment section (400×) showing intact intima (black arrow) with minimal medial clefting (red arrow), average smooth muscle cells (blue arrow) and average elastic lamina (yellow arrow). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

DM co-morbidity in rats was associated with vascular reactivity defect as evidenced by ameliorated vasorelaxation of aorta preparation by the endothelium-dependent relaxing factor acetylcholine or the endothelium-independent relaxing factor sodium nitroprusside (Fig. 10). This was coupled with endothelial injury evidenced from histopathological examination (Figs. 6 and 7). In agreement with our findings, RA and DM were long reported to be associated with vascular defects, where Feng et al. [44] reported that microparticles emerging in diabetic serum promote endothelial dysfunction. Similarly, Rahman et al. [4] correlated glycemic markers with impaired vascular reactivity

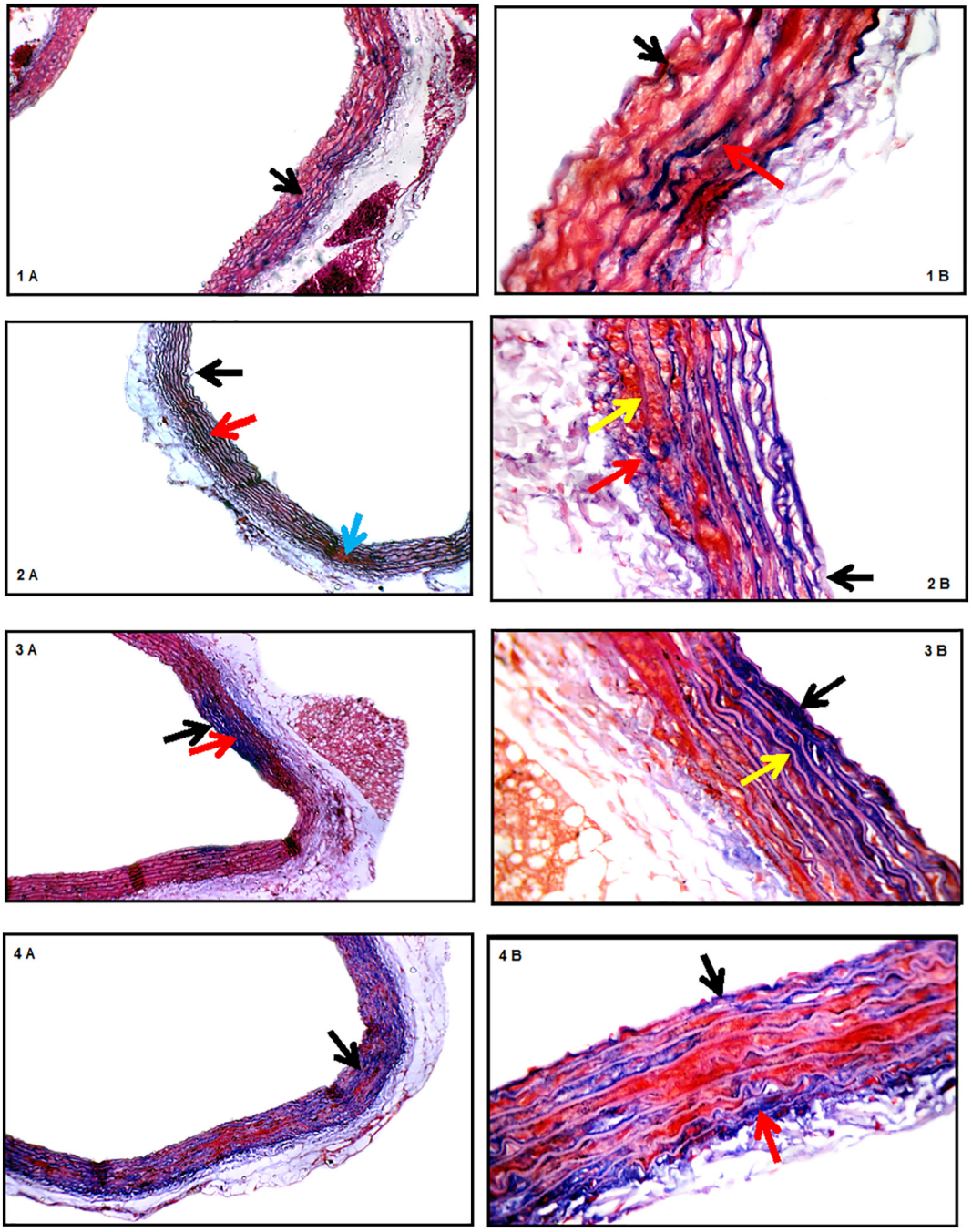

Fig. 7. Photomicrographs of aorta sections stained with special Masson trichrome staining showing the effect of vardenafil and cilostazol administration in rats with RA/DM co-morbidity. 1A: Normal control section (200× magnification) showing average collagen distribution in the media (black arrow); 1B: Normal control section (400× magnification) showing average collagen distribution in sub-intima (black arrow) and through the media (red arrow); 2A: RA/DM co-morbidity section (200× magnification) showing blood vessel wall with intimal destruction (black arrow) and excess collagen in the media (red arrow), with marked medial destruction (yellow arrow); 2B: RA/DM co-morbidity section (400× magnification) showing intimal destruction (black arrow) and excess collagen in the media (red arrow) with marked medial destruction (yellow arrow); 3A: Vardenafil treatment section (200×) showing blood vessel wall with excess collagen in the sub-intima (black arrow) and in the media (red arrow); 3B: Vardenafil treatment section (400×) showing excess collagen in the sub-intima (black arrow) and in the media (yellow arrow); 4A: Cilostazol treatment section (200×) showing vessel wall with excess collagen in the media; 4B: Cilostazol treatment section (400×) showing minimal collagen in sub-intima (black arrow) and excess collagen in the media (red arrow). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

clinically. Alternatively, Bordy et al. [45] and Erre et al. [46] claimed that endothelial dysfunction is a common complication of RA, while Adawi et al. [47] reported that RA-induced endothelial dysfunction is a leading cause to accelerated atherosclerosis.

On the other side, our results showed that vardenafil and cilostazol treatments significantly improved vascular integrity and reactivity by comparable degrees (Figs. 6, 7 and 10). In agreement, Clavé et al. [48] reported the beneficial effects of the PDE-5 inhibitors sildenafil and taladafil on microvascular dysfunction in patients with pulmonary hypertension secondary to congestive heart failure. Similarly, Santi et al. [49] reported that daily treatment with vardenafil improves endothelial inflammation in patients with type 2 DM. Side by side, cilostazol was

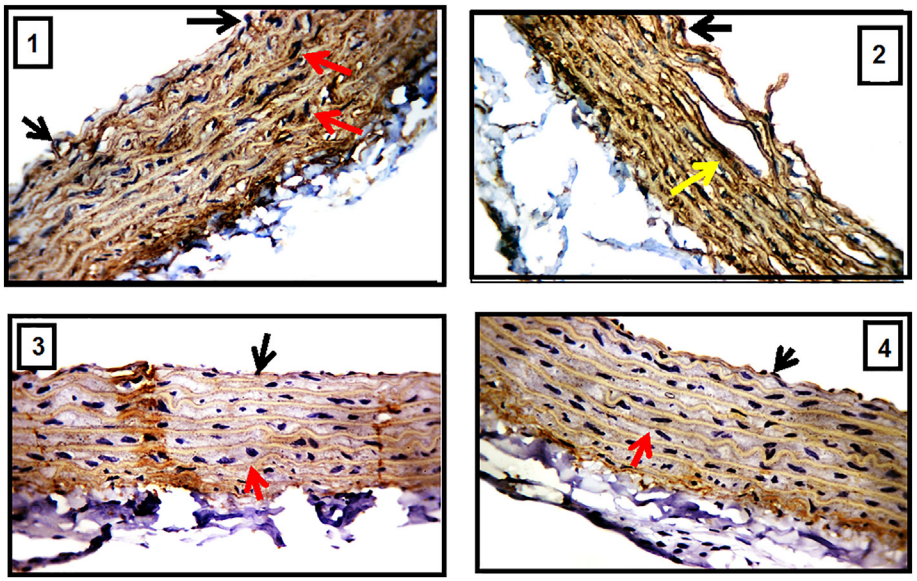

Fig. 8. Photomicrographs of rat aorta sections immunostained for endothelial nitric oxide synthase (eNOS) showing the effect of vardenafil and cilostazol administration on RA/DM co-morbidity. 1: Normal control section (400× magnification) showing blood vessel wall with strong cytoplasmic reactivity to eNOS in endothelial cells (black arrow) and smooth muscle cells (red arrow); 2: RA/DM co-morbidity section (400× magnification) showing blood vessel wall with mild cytoplasmic reactivity to eNOS in endothelial cells (black arrow) and in smooth muscle cells (yellow arrow); 3: Vardenafil treatment section showing mild cytoplasmic reactivity to eNOS in endothelial cells (black arrow) and no reactivity in smooth muscle cells (red arrow); 4: Cilostazol treatment section showing mild cytoplasmic reactivity to eNOS in endothelial cells (black arrow) and no reactivity in smooth muscle cells (red arrow). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

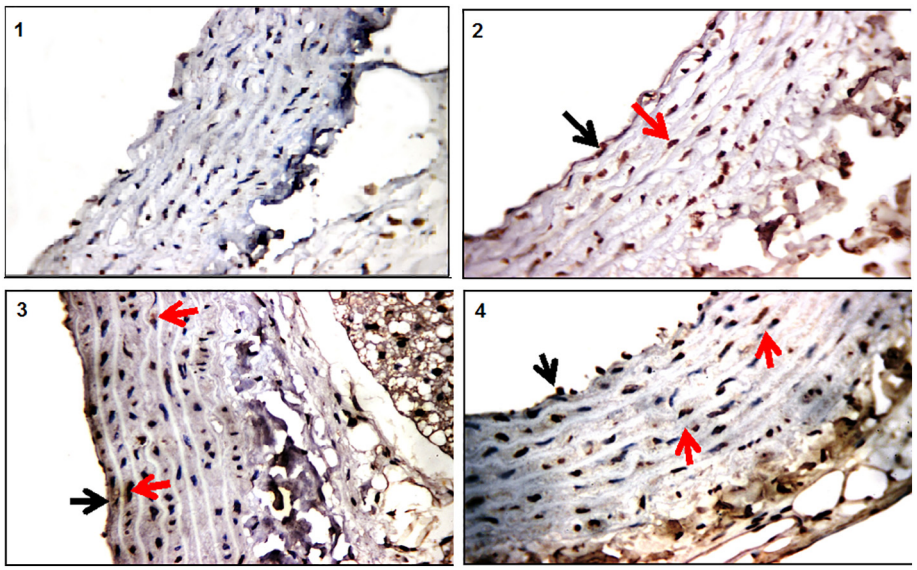

Fig. 9. Photomicrographs of rat aorta sections immunostained for matrix metalloproteinase-2 showing the effect of vardenafil and cilostazol administration on RA/DM co-morbidity. 1: Normal control section (400× magnification) showing vessel wall with no reactivity to MMP-2 in endothelial cells or smooth muscle cells; 2: RA/DM co-morbidity section (400× magnification) showing blood vessel wall showing strong cytoplasmic reactivity to MMP2 in endothelial cells (black arrow) and in smooth muscle cells (red arrow); 3: Vardenafil treatment section showing blood vessel wall with mild cytoplasmic reactivity to MMP-2 in endothelial cells (black arrow) and in smooth muscle cells (red arrows); 4: Cilostazol treatment section showing blood vessel wall with mild cytoplasmic reactivity to MMP-2 in endothelial cells (black arrow) and in smooth muscle cells (red arrows). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

reported to improve endothelial dysfunction in patients with cerebral ischemia [50]. In addition, Asal and Wojciak [51] recorded the beneficial effect of cilostazol against diabetes-induced vascular complications. The beneficial effects of vardenafil and cilostazol regarding endothelial integrity were mostly attributed to PDE inhibition and consequent up-regulation of nitric oxide pathways, but other pathways seem to exist.

In the current study, aorta tissue isolated from rats with RA/DM co-morbidity showed significant suppression of the anti-inflammatory and energy-preserving enzyme AMPK levels compared with normal control level, which were significantly corrected by vardenafil or cilostazol treatments (Fig. 5). In agreement, decline of AMPK levels in different tissues was reported in various experimental models of RA [52,53] and DM [54,55]. Since AMPK plays an essential role in maintaining cellular energy homeostasis, up-regulation of active phosphorylated AMPK seems to explain, at least partly, the beneficial effects of test agents. According to Lu et al. [11], AMPK could play an important role in ameliorating diabetes-induced endothelial dysfunction. In support, AMPK-mediated vasorelaxation was evident in diabetic rats receiving AMPK activator [56]. The effect of vardenafil on AMPK was not investigated earlier, while cilostazol was reported to exert anti-platelet and anti-inflammatory effects in hypercholesterolemic rats through AMPK activation [26].

Rats with RA/DM co-morbidity showed elevated aorta level of the vasoconstrictor pro-inflammatory molecules Ang-II and ET-1, which were significantly improved by vardenafil and cilostazol treatments (Fig. 4). Endothelial production of Ang-II, ET-1 and other inflammatory molecules was reported to be massively up-regulated during the course of RA [57] or DM [58,59] progression. Ang-II was reported to be a potent pro-inflammatory molecule in different experimental models [60,61], while ET-1 production was considered as a predictor and prognostic marker of coronary artery disease [62]. No previous data showed the effect of vardenafil or cilostazol on vascular Ang-II production, while few data were available regarding their effect on ET-1 production, including the work of Santi et al. [49] showing the effect of chronic vardenafil administration on ET-1 production in diabetic patients with erectile dysfunction, and the work of Negrini et al. [63] showing the effect of cilostazol on ET-1 production in systemic sclerosis patients with Raynaud's phenomenon.

Aorta levels of the adhesion molecules P-selectin and VCAM-1 were significantly increased in rats with RA/DM co-morbidity compared with normal control levels, but were significantly improved by vardenafil

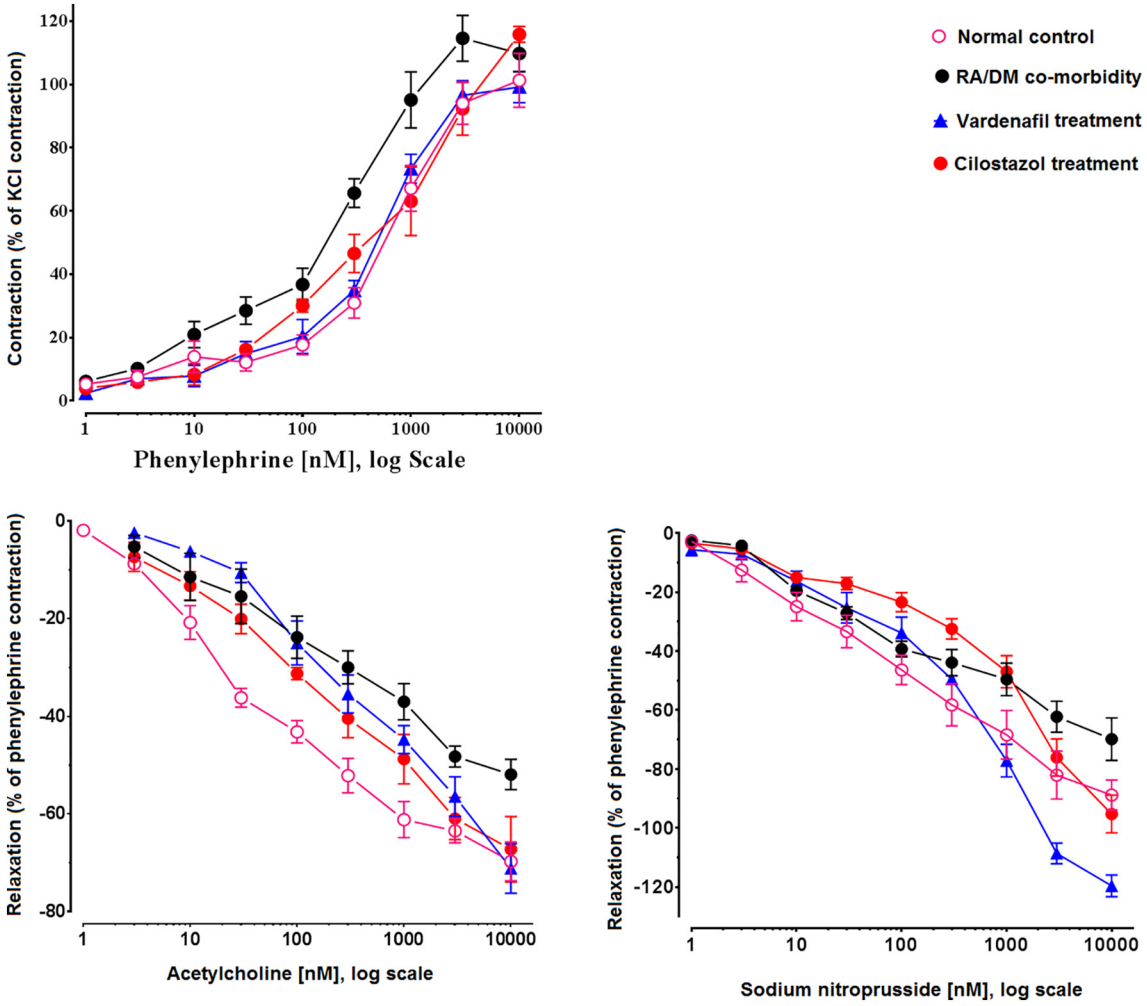

Fig. 10. The *in vitro* contraction and relaxation curves of different treatment groups.

and cilostazol treatments (Fig. 4). In agreement, soluble VCAM-1 was considered a disease marker in several rheumatic diseases [64], while serum and tissue P-selectin levels were increased in rats with diabetic nephropathy [65]. According to Kong et al. [15] and earlier studies, VCAM-1 is closely associated with the progression RA and other immunological disorders. The elevated ACPA level in RA was reported to enhance platelet activation and P-selectin production of [66]. Accumulating evidence showed that P-selectin played a vital role in the progression of immunological endothelial dysfunction, as leukocyte adhesion to vascular endothelium was mediated through binding of endothelium P-selectin with leukocyte P-selectin glycoprotein ligand-1 [67]. Side by side, VCAM-1 was reported to play an important pro-inflammatory role in the progression of systemic atherosclerosis manifestations like peripheral artery disease [68]. We reported no earlier data concerning the effect of vardenafil on vascular P-selectin, but Veres et el. [69] concluded that PDE-5 inhibition by vardenafil could improve vascular graft function through down-regulation of VCAM-1. On the other hand, cilostazol was old known to down-regulate adhesion molecules in different models of endothelial dysfunction. Omi et al. [70] reported that cilostazol could inhibit glucose-induced endothelial-neutrophil adhesion in cultured human endothelial cells through decreasing adhesion molecules like VCAM-1 expression *via* PDE-3 inhibition and NO production. Similarly, Gao et al. [71] reported that PDE-3 inhibition by cilostazol could protect diabetic rats against vascular inflammation through down-regulation of VCAM-1 mediated by inhibition of nuclear factor-*kappa*B.

In the current investigation, induction of RA/DM co-morbidity was associated with elevation of the aorta tissue level of VIP which was decreased again by vardenafil and cilostazol treatments (Fig. 4). Although VIP is known to be a potent anti-inflammatory and vasodilator neuropeptide [18], its expression was reported to increase in some immunopathological conditions [72], probably as an adaptive response.

Concerning our results, RA/DM co-morbid rats showed suppressed aorta tissue level of eNOS, which was significantly restored by test agents (Fig. 8). Previous investigations reported similar results in different models of RA [73,74] and DM [75,76]. The eNOS expression is of great importance in maintaining endothelial integrity and vascular reactivity. The study by Hou et al. [77] showed that eNOS enhancement could reverse homocysteine-induced endothelial dysfunction in human internal mammary arteries. Prolonged administration of PDE-5 inhibitors in male rabbits was reported to have endothelial cytoprotective effect mediated by eNOS up-regulation [78]. Additionally, cilostazol was reported to protect against DM-induced microvascular brain injury in rats *via* up-regulation of eNOS [79].

Results of the current study showed significant aortic MMP-2 up-regulation in rats with RA/DM co-morbidity, which was also corrected by vardenafil and cilostazol treatments (Fig. 9). RA and DM progression are well-known to be associated with vascular oxidative stress [80,81]. Such oxidative stress could enhance induction of metalloproteinases like MMP-2 thus destroying endothelial glycocalyx essential for protecting the endothelium [82]. We reported no earlier data concerning the effect of vardenafil on vascular MMP-2. However, cilostazol was reported to possess vasculoprotective effect on rat coronary arterioles against aldosterone-induced vascular injury *via* down-regulation of

expression of a number of pro-inflammatory genes encoding for MMP-2 and other proteins. In the same manner, cilostazol was reported to ameliorate ethanol-induced endothelial dysfunction in cultured human brain vascular endothelial cells *via* inhibition of ROS-mediated MMP-9 activation [83].

Finally, our results showed that mean body weight of cilostazol-treated rats was significantly lower than the corresponding mean body weight of RA/DM co-morbidity control rats (Fig. 4). This may be explained by the findings of Wada et al. [84] where cilostazol was reported to reduce adipocyte size and improve insulin resistance in obese diabetic mice.

5. Conclusion

In conclusion, vardenafil and cilostazol seem promising agents for correcting vascular integrity and reactivity defects induced by experimental RA/DM co-morbidity. This effect may be mediated by up-regulation of vascular tissue AMPK and eNOS coupled with down-regulation of Ang-II, ET-1, P-selectin, VCAM-1 and MMP-2. Further clinical trials are needed to prove these effects.

Acknowledgements

The authors are thankful to Dr. Sayed Abdel-Raheem, Associate Professor of Histopathology, Faculty of Medicine, Al-Azhar University, Cairo, Egypt, for performing the histopathological examination of the present investigation.

Declaration of Competing Interest

There is no conflict of interest among all authors of the current investigation.

References

[1] S.B. Manuck, A.L. Kasprowicz, S.M. Monroe, K.T. Larkin, J.R. Kaplan, Psychophysiologic reactivity as a dimension of individual differences, in: N. Schneiderman, S.M. Weiss (Eds.), Handbook of Research Methods in Cardiovascular Behavioral Medicine, Kaufmann PG. Plenum, New York, 1989, pp. 365–382.
[2] S. Santana López, C. Perdomo Hernández Mdel, R. Montero Díaz, Cardiovascular hyperreactivity to physical stress predicts high blood pressure in working populations: 4 years follow-up, Clin. Investig. Arterioscler. 26 (6) (2014) 268–273.
[3] F. Schinzari, M. Tesauro, C. Cardillo, Vascular hyperpolarization in human physiology and cardiovascular risk conditions and disease, Acta Physiol. (Oxf.) 219 (1) (2017) 124–137.
[4] A. Rahman, S. Munisamy, N.A. Ghaffar, N.Z. Mahmood, A.H.G. Rasool, Impaired microvascular reactivity in gestational diabetes is associated with altered glycemic parameters, Microcirculation 26 (1) (2019) e12513.
[5] S.S. Nelson, A.K. Khan, To study endothelial dysfunction in rheumatoid arthritis, J. Assoc. Physicians India 63 (10) (2015) 26–30.
[6] S.J. Hamilton, G.F. Watts, Endothelial dysfunction in diabetes: pathogenesis, significance, and treatment, Rev. Diabet. Stud. 10 (2–3) (2013) 133–156.
[7] N. Idris-Khodja, S. Ouerd, M.O.R. Mian, J. Gornitsky, T. Barhoumi, P. Paradis, E.L. Schiffrin, Endothelin-1 overexpression exaggerates diabetes-induced endothelial dysfunction by altering oxidative stress, Am. J. Hypertens. 29 (11) (2016) 1245–1251.
[8] T. Vaisar, E. Couzens, A. Hwang, M. Russell, C.E. Barlow, L.F. DeFina, A.N. Hoofnagle, F. Kim, Type 2 diabetes is associated with loss of HDL endothelium protective functions, PLoS One 13 (3) (2018) e0192616.
[9] A. Janus, E. Szahidewicz-Krupska, G. Mazur, A. Doroszko, Insulin resistance and endothelial dysfunction constitute a common therapeutic target in cardiometabolic disorders, Mediat. Inflamm. 2016 (2016) 3634948.
[10] N. Suganya, E. Bhakkiyalakshmi, D.V. Sarada, K.M. Ramkumar, Reversibility of endothelial dysfunction in diabetes: role of polyphenols, Br. J. Nutr. 116 (2) (2016) 223–246.
[11] Q. Lu, X. Li, J. Liu, X. Sun, T. Rousselle, D. Ren, N. Tong, J. Li, AMPK is associated with the beneficial effects of antidiabetic agents on cardiovascular diseases, Biosci. Rep. 39 (2) (2019) (pii: BSR20181995).
[12] R. Bordy, F. Verhoeven, M. Tournier-Nappey, D. Wendling, C. Demougeot, P. Totoson, Methotrexate did not improve endothelial function in rheumatoid arthritis: a study in rats with adjuvant-induced arthritis, Clin. Exp. Rheumatol. 37 (1) (2019) 81–88.
[13] D. Puttevils, P. De Vusser, P. Geusens, J. Dens, Increased cardiovascular risk in patients with rheumatoid arthritis: an overview, Acta Cardiol. 69 (2) (2014) 111–118.
[14] X. Yang, Y. Chang, W. Wei, Endothelial dysfunction and inflammation: immunity in rheumatoid arthritis, Mediat. Inflamm. 2016 (6813016) (2016).
[15] D.H. Kong, Y.K. Kim, M.R. Kim, J.H. Jang, S. Lee, Emerging roles of vascular cell adhesion molecule-1 (VCAM-1) in immunological disorders and cancer, Int. J. Mol. Sci. 19 (4) (2018) (pii: E1057).
[16] T. Itoh, H. Matsuda, M. Tanioka, K. Kuwabara, S. Itohara, R. Suzuki, The role of matrix metalloproteinase-2 and matrix metalloproteinase-9 in antibody-induced arthritis, J. Immunol. 169 (5) (2002) 2643–2647.
[17] T. Barhoumi, J.C. Fraulob-Aquino, M.O.R. Mian, S. Ouerd, N. Idris-Khodja, K.G. Huo, A. Rehman, A. Caillon, B. Dancose-Giambattisto, T. Ebrahimian, S. Lehoux, P. Paradis, E.L. Schiffrin, Matrix metalloproteinase-2 knockout prevents angiotensin II-induced vascular injury, Cardiovasc. Res. 113 (14) (2017) 1753–1762.
[18] R. Villanueva-Romero, I. Gutiérrez-Cañas, M. Carrión, S. Pérez-García, I.V. Seoane, C. Martínez, R.P. Gomariz, Y. Juarranz, The anti-inflammatory mediator, vasoactive intestinal peptide, modulates the differentiation and function of Th subsets in rheumatoid arthritis, J Immunol Res 2018 (2018) 6043710.
[19] B. Weiss, Differential activation and inhibition of the multiple forms of cyclic nucleotide phosphodiesterase, Adv. Cyclic Nucleotide Res. 5 (1975) 195–211.
[20] B. Weiss, W.N. Hait, Selective cyclic nucleotide phosphodiesterase inhibitors as potential therapeutic agents, Annu. Rev. Pharmacol. Toxicol. 17 (1977) 441–477.
[21] H. Nguyen, A.M. Amanullah, Therapeutic potentials of phosphodiesterase-5 inhibitors in cardiovascular disease, Rev. Cardiovasc. Med. 15 (2) (2014) 158–167.
[22] M.A. Venneri, E. Giannetta, G. Panio, R. De Gaetano, D. Gianfrilli, R. Pofi, S. Masciarelli, F. Fazi, M. Pellegrini, A. Lenzi, F. Naro, A.M. Isidori, Chronic inhibition of PDE5 limits pro-inflammatory monocyte-macrophage polarization in streptozotocin-induced diabetic mice, PLoS One 10 (5) (2015) e0126580.
[23] Z. Zhou, V.J. de Beer, D. de Wijs-Meijler, S.B. Bender, M. Hoekstra, M.H. Laughlin, D.J. Duncker, D. Merkus, Pulmonary vasoconstrictor influence of endothelin in exercising swine depends critically on phosphodiesterase 5 activity, Am. J. Phys. Lung Cell. Mol. Phys. 306 (5) (2014) L442–L452.
[24] S. Lim, R.H. Eckel, Pharmacological treatment and therapeutic perspectives of metabolic syndrome, Rev. Endocr. Metab. Disord. 15 (4) (2014) 329–341.
[25] K.C. Rogers, C.S. Oliphant, S.W. Finks, Clinical efficacy and safety of cilostazol: a critical review of the literature, Drugs 75 (4) (2015) 377–395.
[26] N.A. da Motta, F.C. de Brito, Cilostazol exerts antiplatelet and anti-inflammatory effects through AMPK activation and NF-kB inhibition on hypercholesterolemic rats, Fundam. Clin. Pharmacol. 30 (4) (2016) 327–337.
[27] Y. Kawanabe, M. Takahashi, X. Jin, S. Abdul-Majeed, A.M. Nauli, Y. Sari, S.M. Nauli, Cilostazol prevents endothelin-induced smooth muscle constriction and proliferation, PLoS One 7 (9) (2012) e44476.
[28] N. Shimamura, H. Ohkuma, Phenotypic transformation of smooth muscle in vasospasm after aneurysmal subarachnoid hemorrhage, Transl. Stroke Res. 5 (3) (2014) 357–364.
[29] M. Aziret, O. Irkorucu, E. Reyhan, H. Erdem, K. Das, S. Ozkara, A. Surmelioglu, S. Sozen, I. Bali, S. Cetinkunar, K.C. Deger, The effects of vardenafil and pentoxifylline administration in an animal model of ischemic colitis, Clinics (Sao Paulo) 69 (11) (2014) 763–769.
[30] H.H. Alp, Z. Huyut, S. Yildirim, Y. Başbugan, L. Ediz, M.R. Şekeroğlu, The effect of PDE5 inhibitors on bone and oxidative damage in ovariectomy-induced osteoporosis, Exp. Biol. Med. (Maywood) 242 (10) (2017) 1051–1061.
[31] X.H. Ba, L.P. Cai, W. Han, Effect of cilostazol pretreatment on the PARP/AIF-mediated apoptotic pathway in rat cerebral ischemia-reperfusion models, Exp. Ther. Med. 7 (5) (2014) 1209–1214.
[32] M.M.A. Khalifa, S.A. Mangoura, M.A. El-Moselhy, M.E. El-Daly, The effectiveness of beta adrenoceptor antagonists on progression of insulin resistance and type 2 diabetes in high fat diet-fed rats, Saudi Pharm. J. 17 (1) (2009) 19–28.
[33] M. Kumas, O. Altintas, E. Karatas, A. Kocyigit, Protective effect of ischemic preconditioning on myocardium against remote tissue injury following transient focal cerebral ischemia in diabetic rats, Arq. Bras. Cardiol. 109 (6) (2017) 516–526.
[34] M.G. Fahmy Wahba, B.A. Shehata Messiha, A.A. Abo-Saif, Ramipril and haloperidol as promising approaches in managing rheumatoid arthritis in rats, Eur. J. Pharmacol. 765 (2015) 307–315.
[35] K. Phadke, R. Fouts, J.E. Parrish, Collagen-induced and adjuvant-induced arthritis in rats. Post-immunization treatment with collagen to suppress or abrogate the arthritic response, Arthritis Rheum. 27 (7) (1984) 797–806.
[36] E. Engvall, K. Jonsson, P. Perlmann, Enzyme-linked immunosorbent assay.II. Quantitative assay of protein antigen, immunoglobulin G, by means of enzyme labelled antigen and antibody-coated tubes, Biochim. Biophys. Acta 251 (1971) 427–434.
[37] J. Kekow, K. Ulrichs, W. Müller-Ruchholtz, W.L. Gross, Measurement of rat insulin. Enzyme-linked immunosorbent assay with increased sensitivity, high accuracy, and greater practicability than established radioimmunoassay, Diabetes 37 (3) (1988) 321–326.
[38] R.M. Lequin, Enzyme immunoassay (EIA)/enzyme-linked immunosorbent assay (ELISA), Clin. Chem. 51 (2005) 2415–2418.
[39] W.N. Burnette, Western blotting: electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A, Anal. Biochem. 112 (1981) 195–203.
[40] G.D. Banchroft, A. Steven, Theory and Practice of Histological Technique, fourth ed., Churchil Livingstone Publications, London, 1983.
[41] P. Masson, Some histological methods. Trichrome stainings and their preliminary technique, Bull. Int. Assoc. Med. 12 (1929) 75–90.
[42] H. Merz, R. Malisius, S. Mannweiler, R. Zhou, W. Hartmann, K. Orscheschek,

P. Moubayed, Feller A. ImmunoMax, A maximized immunohistochemical method for the retrieval and enhancement of hidden antigens, Lab. Investig. 73 (1) (1995) 149–156.
[43] H.M. Rhee, B.J. Song, S. Cushman, S.E. Shoaf, Vascular reactivity in alcoholic rat aortas: in vitro interactions between catecholamines and alcohol, Neurotoxicology 16 (1) (1995) 179–185.
[44] Q. Feng, C.J. Stork, S. Xu, D. Yuan, X. Xia, K.B. LaPenna, G. Guo, H. Sun, L.C. Xu, C.A. Siedlecki, K.M. Brundage, N. Sheaffer, T.D. Schell, P. He, Increased circulating microparticles in streptozotocin-induced diabetes propagate inflammation contributing to microvascular dysfunction, J. Physiol. 597 (3) (2019) 781–798.
[45] R. Bordy, P. Totoson, C. Prati, C. Marie, D. Wendling, C. Demougeot, Microvascular endothelial dysfunction in rheumatoid arthritis, Nat. Rev. Rheumatol. 14 (7) (2018) 404–420.
[46] G.L. Erre, M. Piga, A.L. Fedele, S. Mura, A. Piras, M.L. Cadoni, I. Cangemi, M. Dessi, G. Di Sante, B. Tolusso, E. Gremese, A. Cauli, A.A. Mangoni, P.S. Saba, C. Carru, G. Ferraccioli, A. Mathieu, G. Passiu, Prevalence and determinants of peripheral microvascular endothelial dysfunction in rheumatoid arthritis patients: a multicenter cross-sectional study, Mediat. Inflamm. 2018 (2018) 6548715.
[47] M. Adawi, A. Watad, N.L. Bragazzi, H. Amital, G. Saaida, R. Sirchan, A. Blum, Endothelial function in rheumatoid arthritis, QJM 111 (4) (2018) 243–247.
[48] M.M. Clavé, N.Y. Maeda, A.M. Thomaz, S.P. Bydlowski, A.A. Lopes, Phosphodiesterase type 5 inhibitors improve microvascular dysfunction markers in pulmonary arterial hypertension associated with congenital heart disease, Congenit. Heart Dis. (2018), https://doi.org/10.1111/chd.12688 ahead of print.
[49] D. Santi, A.R. Granata, A. Guidi, E. Pignatti, T. Trenti, L. Roli, R. Bozic, S. Zaza, C. Pacchioni, S. Romano, J.R. Nofer, V. Rochira, C. Carani, M. Simoni, Six months of daily treatment with vardenafil improves parameters of endothelial inflammation and of hypogonadism in male patients with type 2 diabetes and erectile dysfunction: a randomized, double-blind, prospective trial, Eur. J. Endocrinol. 174 (4) (2016) 513–522.
[50] S.J. Lee, J.S. Lee, M.H. Choi, S.E. Lee, D.H. Shin, J.M. Hong, Cilostazol improves endothelial function in acute cerebral ischemia patients: a double-blind placebo controlled trial with flow-mediated dilation technique, BMC Neurol. 17 (1) (2017) 169.
[51] N.J. Asal, K.A. Wojciak, Effect of cilostazol in treating diabetes-associated microvascular complications, Endocrine 56 (2) (2017) 240–244.
[52] Y. Wang, Y. Wei, X. Cheng, X. Sun, L. Ma, Y. Song, J. Zhou, F. Wei, H. Liu, 2-deoxyglucose inhibits angiogenesis of rheumatoid arthritis via activating AMPK pathway, Nan Fang Yi Ke Da Xue Xue Bao 38 (8) (2018) 962–968.
[53] Y. Zhang, S. Zhang, Z. Liu, X. Zhao, Y. Yuan, L. Sheng, Y. Li, Resveratrol prevents atrial fibrillation by inhibiting atrial structural and metabolic remodeling in collagen-induced arthritis in rats, Naunyn Schmiedeberg's Arch. Pharmacol. 391 (11) (2018) 1179–1190.
[54] F. Francini, G.R. Schinella, J.L. Ríos, Activation of AMPK by medicinal plants and natural products: its role in type 2 diabetes mellitus, Mini Rev. Med. Chem. (2018), https://doi.org/10.2174/1389557519666181128120726 ahead of print.
[55] Y. Lei, L. Gong, F. Tan, Y. Liu, S. Li, H. Shen, M. Zhu, W. Cai, F. Xu, B. Hou, Y. Zhou, H. Han, L. Qiu, H. Sun, Vaccarin ameliorates insulin resistance and steatosis by activating the AMPK signaling pathway, Eur. J. Pharmacol. 851 (2019) 13–24.
[56] C. Balcilar, I. Ozakca-Gunduz, V.M. Altan, Contributions of Rho-kinase and AMP-related kinase (AMPK) signaling pathways to endothelium-derived contracting factors (EDCF)-mediated responses in diabetic rat aorta, Can. J. Physiol. Pharmacol. (2019), https://doi.org/10.1139/cjpp-2018-0698.
[57] K. Hamilton, L. Dunning, W.R. Ferrell, J.C. Lockhart, A. Mackenzie, Endothelium-derived contraction in a model of rheumatoid arthritis is mediated via angiotensin II type 1 receptors, Vasc. Pharmacol. 100 (2018) 51–57.
[58] R. Xu, R. Yang, H. Hu, Q. Xi, H. Wan, Y. Wu, Diabetes alters the expression of partial vasoactivators in cerebral vascular disease susceptible regions of the diabetic rat, Diabetol. Metab. Syndr. 5 (1) (2013) 63.
[59] S. Jamwal, S. Sharma, Vascular endothelium dysfunction: a conservative target in metabolic disorders, Inflamm. Res. 67 (5) (2018) 391–405.
[60] E.A.M. El-Shoura, B.A.S. Messiha, S.M.Z. Sharkawi, R.A.M. Hemeida, Perindopril ameliorates lipopolysaccharide-induced brain injury through modulation of angiotensin-II/angiotensin-1-7 and related signaling pathways, Eur. J. Pharmacol. 834 (2018) 305–317.
[61] Y.Y. Lu, X.D. Li, H.D. Zhou, S. Shao, S. He, M.N. Hong, J.C. Liu, Y.L. Xu, Y.J. Wu, D.L. Zhu, J.G. Wang, P.J. Gao, Transactivation domain of Krüppel-like factor 15 negatively regulates angiotensin II-induced adventitial inflammation and fibrosis, FASEB J. 33 (5) (2019) 6254–6268 (fj201801809R).
[62] M. Jankowich, G. Choudhary, Endothelin-1 levels and cardiovascular events, Trends Cardiovasc. Med. (2019), https://doi.org/10.1016/j.tcm.2019.01.007 (pii: S1050-1738(18)30233-0).
[63] S. Negrini, F. Spanò, E. Penza, D. Rollando, F. Indiveri, G. Filaci, F. Puppo, Efficacy of cilostazol for the treatment of Raynaud's phenomenon in systemic sclerosis patients, Clin. Exp. Med. 16 (3) (2016) 407–412.
[64] M. Oleszowsky, M.F. Seidel, Serum soluble vascular cell adhesion molecule-1 overexpression is a disease marker in patients with first-time diagnosed antinuclear antibodies: a prospective, observational pilot study, Biomed. Res. Int. 2018 (2018) 8286067.
[65] X.P. Sun, N.S. Wang, Q. Xue, F. Wang, Volatile oil of Magnolia biondii inhibits expressions of P-selectin protein in serum and renal tissue of rats with diabetic nephropathy, Zhong Xi Yi Jie He Xue Bao 6 (5) (2008) 524–529.
[66] K.L. Habets, L.A. Trouw, E.W. Levarht, S.J. Korporaal, P.A. Habets, P. de Groot, T.W. Huizinga, R.E. Toes, Anti-citrullinated protein antibodies contribute to platelet activation in rheumatoid arthritis, Arthritis Res. Ther. 17 (2015) 209.
[67] F.W. Lam, Q. Da, B. Guillory, M.A. Cruz, Recombinant human vimentin binds to P-selectin and blocks neutrophil capture and rolling on platelets and endothelium, J. Immunol. 200 (5) (2018) 1718–1726.
[68] C. Edlinger, M. Lichtenauer, B. Wernly, R. Pistulli, V. Paar, C. Prodinger, F. Krizanic, M. Thieme, J. Kammler, C. Jung, U.C. Hoppe, P.C. Schulze, D. Kretzschmar, Disease-specific characteristics of vascular cell adhesion molecule-1 levels in patients with peripheral artery disease, Heart Vessel. (2018), https://doi.org/10.1007/s00380-018-1315-1 ahead of print.
[69] G. Veres, M. Hagenhoff, H. Schmidt, T. Radovits, S. Loganathan, Y. Bai, S. Korkmaz-Icöz, P. Brlecic, A.A. Sayour, M. Karck, G. Szabó, Targeting phosphodiesterase-5 by vardenafil improves vascular graft function, Eur. J. Vasc. Endovasc. Surg. 56 (2) (2018) 256–263.
[70] H. Omi, N. Okayama, M. Shimizu, T. Fukutomi, A. Nakamura, K. Imaeda, M. Okouchi, M. Itoh, Cilostazol inhibits high glucose-mediated endothelial-neutrophil adhesion by decreasing adhesion molecule expression via NO production, Microvasc. Res. 68 (2) (2004) 119–125.
[71] L. Gao, F. Wang, B. Wang, B. Gong, J. Zhang, X. Zhang, J. Zhao, Cilostazol protects diabetic rats from vascular inflammation via nuclear factor-kappa B-dependent down-regulation of vascular cell adhesion molecule-1 expression, J. Pharmacol. Exp. Ther. 318 (1) (2006) 53–58.
[72] V. Bourlev, C. Moberg, N. Ilyasova, E. Davey, T. KunovacKallak, M. Olovsson, Vasoactive intestinal peptide is upregulated in women with endometriosis and chronic pelvic pain, Am. J. Reprod. Immunol. 80 (3) (2018) e12857.
[73] F. Verhoeven, P. Totoson, C. Marie, A. Prigent-Tessier, D. Wendling, M. Tournier-Nappey, C. Prati, C. Demougeot, Diclofenac but not celecoxib improves endothelial function in rheumatoid arthritis: a study in adjuvant-induced arthritis, Atherosclerosis 266 (2017) 136–144.
[74] A.G.A. Gaafar, B.A.S. Messiha, A.M.L. Abdelkafy, Nicorandil and theophylline can protect experimental rats against complete Freund's adjuvant-induced rheumatoid arthritis through modulation of JAK/STAT/RANKL signaling pathway, Eur. J. Pharmacol. 822 (2018) 177–185.
[75] A. Abbasnezhad, S. Niazmand, M. Mahmoudabady, S.A. Rezaee, M. Soukhtanloo, R. Mosallanejad, P. Hayatdavoudi, Nigella sativa L. seed regulated eNOS, VCAM-1 and LOX-1 genes expression and improved vasoreactivity in aorta of diabetic rat, J. Ethnopharmacol. 228 (2019) 142–147.
[76] N. Fourny, C. Lan, E. Sérée, M. Bernard, M. Desrois, Protective effect of resveratrol against ischemia-reperfusion injury via enhanced high energy compounds and eNOS-SIRT1 expression in type 2 diabetic female rat heart, Nutrients 11 (1) (2019) (pii: E105).
[77] H.T. Hou, J. Wang, X. Zhang, Z.Q. Wang, T.N. Chen, J.L. Zhang, Q. Yang, G.W. He, Endothelial nitric oxide synthase enhancer AVE3085 reverses endothelial dysfunction induced by homocysteine in human internal mammary arteries, Nitric Oxide 81 (2018) 21–27.
[78] M.I. El-Sayed, H.A. Amin, Mechanism of endothelial cyto-protective and thrombo-resistance effects of sildenafil, vardenafil and tadalafil in male rabbit, Arch. Med. Sci. 11 (1) (2015) 190–198.
[79] Y. Tsukamoto, E. Nagata, N. Fukuyama, Y. Itoh, H. Yuzawa, S. Kohara, M. Shimizu, Y. Takahari, S. Takizawa, Cilostazol protects against microvascular brain injury in a rat model of type 2 diabetes, Neurosci. Res. 117 (2017) 48–53.
[80] Q. Li, Y. Lin, S. Wang, L. Zhang, L. Guo, GLP-1 inhibits high-glucose-induced oxidative injury of vascular endothelial cells, Sci. Rep. 7 (1) (2017) 8008.
[81] K.M. Kamel, A.M. Gad, S.M. Mansour, M.M. Safar, H.M. Fawzy, Novel anti-arthritic mechanisms of polydatin in complete Freund's adjuvant-induced arthritis in rats: involvement of IL-6, STAT-3, IL-17, and NF-κB, Inflammation 41 (5) (2018) 1974–1986.
[82] M.M. Ali, A.M. Mahmoud, E. Le Master, I. Levitan, S.A. Phillips, Role of matrix metalloproteinases and histone deacetylase in oxidative stress-induced degradation of the endothelial glycocalyx, Am. J. Physiol. Heart Circ. Physiol. 316 (3) (2019) H647–H663.
[83] T. Takagi, K. Mishiro, M. Shimazawa, S. Yoshimura, T. Iwama, H. Hara, The phosphodiesterase III inhibitor cilostazol ameliorates ethanolinduced endothelial dysfunction, Curr. Neurovasc. Res. 11 (4) (2014) 302–311.
[84] T. Wada, Y. Onogi, Y. Kimura, T. Nakano, H. Fusanobori, Y. Ishii, M. Sasahara, H. Tsuneki, T. Sasaoka, Cilostazol ameliorates systemic insulin resistance in diabetic db/db mice by suppressing chronic inflammation in adipose tissue via modulation of both adipocyte and macrophage functions, Eur. J. Pharmacol. 707 (1–3) (2013) 120–129.