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Article

Vitamin D deficiency in diabetes alters the aortic media thickness but not its functional properties

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This study aimed to determine the effects of a vitamin D deficiency on aortic functional and structural properties in diabetic rats. Diabetic rats were induced with 50 mg/kg streptozotocin and divided into two equal groups: diabetic rats on a normal diet (DR) and diabetic rats on a vitamin D-deficient diet (DRD). Non-diabetic rats that received a normal diet were the controls (CR). At the end of 10 weeks, rats were sacrificed and aortic rings with and without the endothelium were studied in tissue organ baths for isometric force measurements. Histology of aortic tissue was performed to determine the intima-media thickness. Serum levels of 25-hydroxyvitamin D in the DRD group were significantly decreased compared to the CR and DR groups. Acetylcholine-induced endothelium-mediated relaxation was significantly impaired in DR and DRD compared to CR. Endothelium-dependent contraction to calcium ionophore was significantly augmented in DR and DRD aortas compared to CR. The responses to acetylcholine and calcium ionophore were similar in DRD and DR. There were no significant differences in relaxation to sodium nitroprusside or contraction to phenylephrine between aortas of the groups. The intima-media thickness was significantly greater in the DR group compared to the CR group, and this structural change was augmented in aortas of the DRD group. In conclusion, this study showed that endothelial function was impaired with diabetes, and a vitamin D deficiency did not aggravate endothelial dysfunction. However, diabetes with a vitamin D deficiency demonstrated smooth muscle hypertrophy and an increased aortic media thickness.

Keywords: 25-Hydroxyvitamin D. Endothelial dysfunction. Hypertrophy. Streptozotocin.

INTRODUCTION

The endothelium is the main regulator of vascular tone and performs this function by balancing the production of endothelial-derived relaxing and contracting factors. The endothelium also regulates vascular permeability to platelet and leukocyte adhesion and aggregation, and thrombosis (Cahill, Redmond, 2016). A growing body of evidence suggests that endothelial dysfunctions are associated with cardiovascular (CV) events. Endothelial dysfunction is regarded as early pivotal events in atherogenesis and has been shown to precede the development of clinically detectable atherosclerotic plaques. Endothelial dysfunction has also been considered an important event in the development of microvascular complications in diabetes (Cersosimo, DeFronzo, 2006; Polovina, Potpara, 2014). With endothelial dysfunction there is a reduction in the bioavailability of endothelial-derived vasodilators, in particular nitric oxide (NO); however, endothelial-derived contracting factors are increased (Furchgott, Vanhoutte, 1989; Loscalzo, 2013). In addition, aside from causing impaired endothelium-

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dependent relaxation, it also activates a specific state with a pro-inflammatory, proliferative, and procoagulatory environment that favors all stages of atherogenesis. The intima-media thickness is considered a primary marker for CV risk during the early stages of atherosclerosis (Bots *et al*., 2016).

Vitamin D plays a role in CV and metabolic diseases. Vitamin D is synthesized endogenously in the skin after exposure to sunlight. It is then converted in the liver into an inactive precursor, 25-hydroxyvitamin D [25(OH)D]. It has been commonly established that plasma levels of 25(OH)D less than 12 ng/mL indicate a vitamin D deficiency. Subsequently, 25(OH)D is then converted to the active form calcitriol [1,25$(OH)_2$D] in the kidneys (Thacher, Clarke, 2011). In addition to its well-known roles in calcium and phosphate homeostasis and in bone metabolism, calcitriol has been reported to be involved in vascular homeostasis and in the modulation of endothelial function (Melaku, Mossie, 2018). The majority of calcitriol action is mediated through its action on vitamin D receptors (VDRs). VDRs are expressed in more than 30 different tissues, including the vasculature (Andress, 2006).

A low vitamin D status has been shown to have a strong association with arterial disease and the severity of atherosclerosis in patients with symptomatic peripheral arterial disease and/or an aortic aneurysm (Van de Luijtgaarden *et al*., 2012). Vitamin D insufficiency has also been shown to reduce endotheliumdependent" to "endothelium-dependent vasodilation in rat mesenteric arteries (Tare *et al*., 2011). Similarly, in type 2 diabetic patients, a vitamin D deficiency is associated with endothelial dysfunction (Yiu *et al*., 2011). However, there seems to be a lack of data reporting the effects of a vitamin D deficiency on the atherosclerotic risks in the aortas of diabetic rats. Therefore, the aim of this study was to evaluate the effects of a vitamin D deficiency on atherosclerotic risk parameters in diabetes by analyzing the changes in the functional and morphological properties of the aorta.

MATERIAL AND METHODS

Material

A standard chow diet (AIN93G) and a modified vitamin D-deficient diet (SF03009) were purchased from Specialty Feeds Pty Ltd. (Glen Forrest, Western Australia). Acetylcholine hydrochloride, calcium ionophore, phenylephrine hydrochloride, streptozotocin, and sodium nitroprusside were purchased from the Sigma Chemical Co. (St. Louis, MO, USA). Ketamine hydrochloride was purchased from Troy Laboratories Pty Ltd. (Smithfield, NSW, Australia). Deionized distilled water was used to prepare the drug solutions.

Animals and experimental protocols

The experimental protocols used in this study were approved by the Animal Ethics Committee of Universiti Sains Malaysia (AECUSM) [No. USM/Animal Ethics Approval/2016/(98)(711)]. Male Sprague-Dawley rats aged 10 weeks and weighing 250–350 g were used. Type 1 diabetes was induced with a single intraperitoneal injection of streptozotocin (50 mg/kg) dissolved in a citrate buffer (pH 4.5). After 72 hours and an overnight fast, blood samples were obtained from the tail vein and the glucose concentration was measured using a one-touch glucometer. The induction of diabetes was considered successful when the glucose level was higher than 16.0 mM. Diabetic rats were then divided into two groups as follows: diabetic rats that received a standard chow diet (DR group) and diabetic rats that received a modified vitamin D-deficient diet (DRD group) throughout the study period. Rats that were induced with a citrate buffer alone were used as controls (CR group) and were given a standard chow diet throughout the study period. Rats were placed in individual cages and maintained at 25 ºC with a 12-hour light/dark cycle. All rats had access to tap water *ad libitum* throughout the experiments. Ten weeks later, the rats were anesthetized with intraperitoneal ketamine hydrochloride (100 mg/kg) and exsanguinated. Serum 25(OH)D and blood glucose levels were measured from the blood samples taken.

Functional study

After the rats were sacrificed, aortic tissues were isolated and immediately placed in an ice-cold Krebs solution (118 mM NaCl, 4.7 mM KCl, 1.18 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 5.5 mM D-glucose, and 2 mM $CaCl_2$). Aortas were cleared of surrounding adhering tissue and cut into 3-mm-long ring segments. Care was taken during the dissection to prevent damaging the endothelial layer. In some preparations, the endothelial cells were removed by rubbing the intimal surface of the arteries with

blunt forceps tips. The ring segments were mounted between two stainless steel hooks in a 10-mL organ bath filled with Krebs solution. One hook was fixed to the bottom of the bath and the other was connected to a force-displacement transducer for isometric tension measurements. The bathing solution was maintained at 37 °C and continuously bubbled with carbogen (95% O_2 and 5% CO_2). The aortic rings were equilibrated for 60 minutes at a baseline tone of 1 gram. Afterwards, the rings were exposed twice to potassium chloride (60 mM) followed by phenylephrine (1 x 10^{-6} M). When a steady contraction to phenylephrine was obtained, acetylcholine (1 x 10^{-6} M) was added to assess the presence, or absence, of functional endothelial cells.

Endothelium-dependent relaxation and contraction were studied in rings with endothelium. For endothelium-dependent relaxation, rings were contracted with phenylephrine (1 x 10^{-6} M) followed by a cumulative addition of acetylcholine (1 x 10^{-9} to 1 x 10^{-5} M). Endothelium-dependent contraction was studied using cumulative concentrations of calcium ionophore (1 x 10^{-8} to 1 x 10^{-5} M) in the quiescent rings.

Endothelium-independent relaxation was determined in rings without endothelium. Rings were contracted with phenylephrine (1 x 10^{-6} M) followed by a cumulative addition of sodium nitroprusside (1 x 10^{-9} to 10^{-5} M). Endothelium-independent contraction was determined by using cumulative concentrations of phenylephrine (1 x 10^{-9} to 1 x 10^{-5} M) in the quiescent rings.

Histological analysis and morphometric parameters

The thoracic aortas were fixed in 10% formalin for 12 hours and then embedded into paraffin. Specimens were cut with a microtome in 3-µm-thick sections and stained with hematoxylin-eosin for the evaluation of morphometric parameters. The sections were examined at a magnification of 100x under an Olympus microscope with a digital camera attached (Model BX41TF, Olympus Corporation, Tokyo, Japan). The thickness of the media layer of the aorta was determined using AnalySIS software on 10 consecutive measurements in a systemic manner to evaluate all segments of the circumference of the aorta (Salum *et al.*, 2012).

Statistical analysis

Continuous variables are expressed as means (standard deviation) or medians (interquartile range) for non-Gaussian distributions. Continuous variables were compared using a one-way analysis of variance (ANOVA) or Kruskal-Wallis test, as appropriate. All analyses were performed using GraphPad Prism 6.0 software. A p value of < 0.05 was considered significant.

RESULTS

Basic and laboratory parameters

The results are presented in Table I. The initial body weights were similar in all study groups. Blood glucose levels were significantly higher in DR and DRD compared to CR. The serum 25(OH)D level was significantly lower in DRD compared to CR and DR.

Table I - Body weight, blood glucose and 25(OH)D levels for experimental rats

Parameters	CR	DR	DRD
Body weight baseline, g	237.00 (27.30)	244.86 (18.33)	253.75 (25.92)
Body weight (week 10th), g	451.00 (73.43)	271.86 (41.35)	332.38 (46.06)
Blood glucose baseline, mmol/L	5.99 (0.45)	26.00 (5.64)[a]	26.43 (4.47)[a]
Blood glucose (week 10th), mmol/L	5.83 (0.60)	30.57 (2.66)[a]	28.84 (5.41)[a]
Serum 25(OH) D level (ng/mL)	35.85 (7.70)	40.87 (9.21)	3.11 (2.16)[b]

Data are presented as mean (standard deviation) of 7-9 rats, [a]$p < 0.05$ vs CR, [b]$p < 0.05$ vs CR and DR. [25(OH)D, 25-hydroxyvitamin D; CR, control rats; DR, diabetic rats; DRD, diabetic rats with vitamin D-deficiency].

Functional study of rat aorta

Maximal relaxation of acetylcholine-mediated endothelium-dependent relaxation was significantly reduced in aortas of DR and DRD compared to CR. Endothelium-dependent contraction mediated by calcium ionophore was significantly augmented in aortas

of both DR and DRD compared to CR. A vitamin D deficiency for 10 weeks in the DRD group did not cause any changes in aortic endothelium-dependent relaxation or contraction compared to DR (Figure 1; Table II). There were no significant differences in the responses of aortic smooth muscle cells to sodium nitroprusside and phenylephrine in any of the experimental groups (Figure 2; Table II).

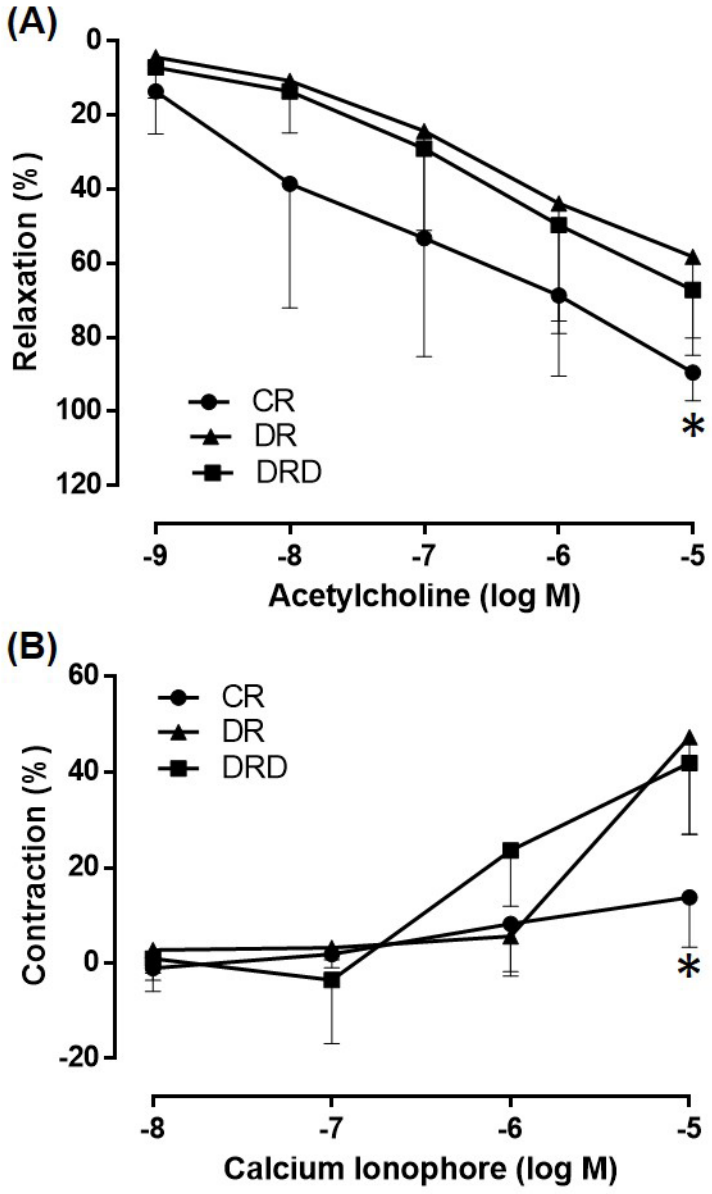

FIGURE 1 - (A) Endothelium-dependent relaxation and (B) contraction in aorta of experimental animals. *Indicates a statistically significant difference in the maximal relaxation between the study groups. Values are presented as means (standard deviation). [CR, control rats; DR, diabetic rats; DRD, diabetic rats with vitamin D-deficiency].

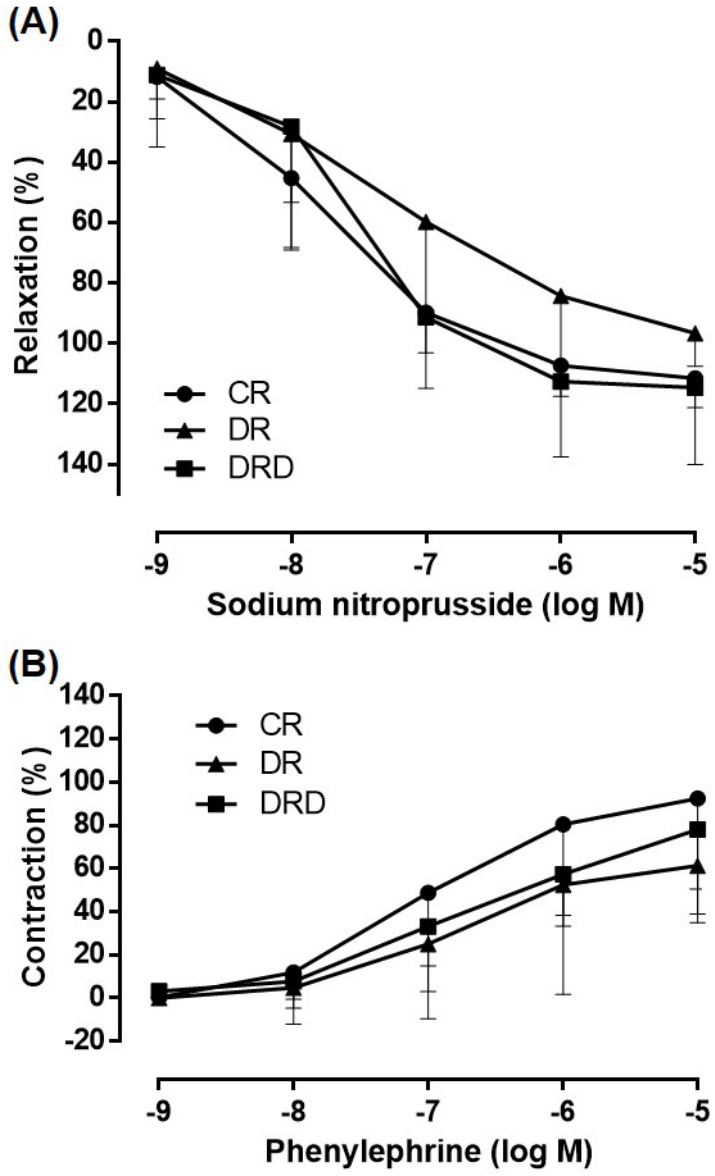

FIGURE 2 - (A) Endothelium-independent relaxation and (B) contraction in aorta of experimental animals. Values are presented as means (standard deviation). [CR, control rats; DR, diabetic rats; DRD, diabetic rats with vitamin D-deficiency].

Table II - Percentage of maximal relaxation (R_{max}) and area under the curve (AUC) to acetylcholine and sodium nitroprusside and maximal contraction (C_{max}) and AUC to calcium ionophore and phenylephrine in rat aorta

Groups		Endothelium-mediated function		Smooth muscle-mediated function	
		Acetylcholine	Calcium ionophore	Sodium nitroprusside	Phenylephrine
CR	R_{max}/C_{max}	86.85 (13.39)	10.10 (13.70)	111.60 (15.90)	78.15 (34.90)
	AUC	150.3 (57.2)	9.80 (27.16)	321.90 (76.00)	137.80 (113.4)
DR	R_{max}/C_{max}	53.46 (48.91)[a]	44.60 (33.35)[a]	96.88 (24.34)	65.65 (38.85)
	AUC	79.84 (106.14)	33.23 (29.00)	210.30 (149.10)	121.40 (52.74)
DRD	R_{max}/C_{max}	57.01 (26.81)[a]	39.90 (21.47)[a]	113.50 (30.40)	59.60 (96.50)
	AUC	121.30 (60.27)	52.42 (11.55)[b]	299.80 (126.30)	206.0 (278.8)

Data are presented as median (interquartile ranges) of 7-9 rats, [a]$p < 0.05$ vs R_{max}/C_{max} CR, [b]$p < 0.05$ vs AUC CR [CR, control rats; DR, diabetic rats; DRD, diabetic rats with vitamin D-deficiency].

Histological analysis and morphometric parameters

Figure 3 shows the typical histology images following hematoxylin-eosin staining of the aortic media from all study groups. The vascular smooth muscle cells were aligned in the CR group (Figure 3A). In contrast, the aortic media showed several alterations and vascular smooth muscle cells appeared disordered in the DR and DRD groups (Figure 3B and 3C). The cross section of the aorta shows a thicker layer of tunica media (smooth muscle) in the DR and DRD groups compared to the CR group. The aortic intima-media thickness in DR was significantly greater than that observed in CR. In DRD, the media thickness of the aorta was significantly greater compared to DR, indicating that a vitamin D deficiency in the presence of diabetes exacerbated the hypertrophy of the smooth muscle cells (Figure 3D).

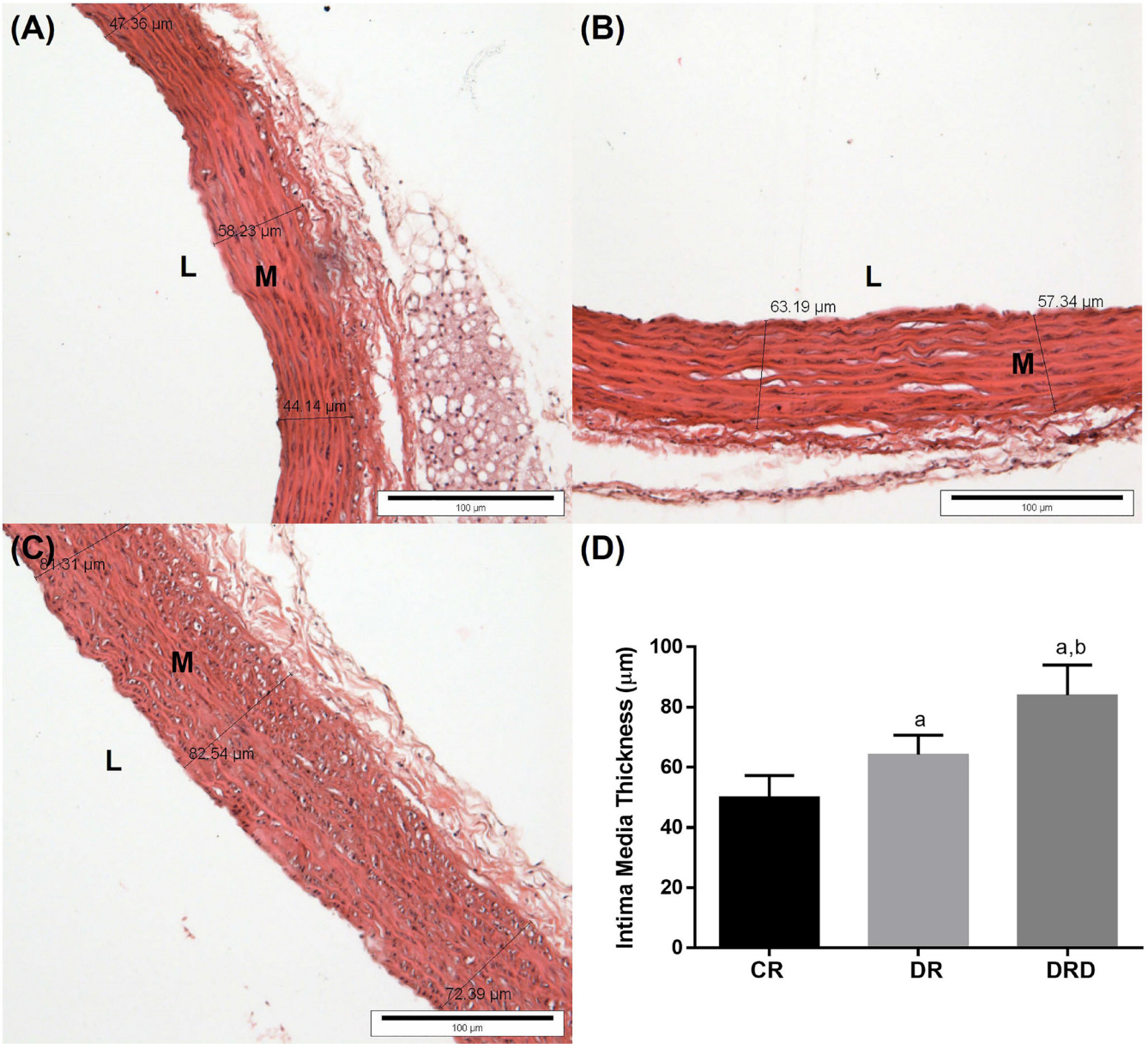

FIGURE 3 - Hematoxylin-eosin staining of the aortic tissues from (A) CR, (B) DR and (C) DRD. The cross section of the aorta shows a thicker layer of smooth muscle cells (intima-media thickness) in DR and DRD compared to CR. Magnification at x100. Values are presented as means (standard deviation). [a]$p<0.05$ compared with CR and [b]$p<0.05$ compared with DR. [CR, control rats; DR, diabetic rats; DRD, diabetic rats with vitamin D-deficiency; L, lumen; M, Media).

DISCUSSION

The present study showed that a) endothelium-dependent relaxation was significantly decreased, whereas contraction was increased, in aortas of diabetic rats compared to control rats; b) vitamin D deficiency in diabetic rats did not alter endothelium-dependent relaxation and contraction; and c) intima-media thickness was significantly increased in diabetic rats and was exacerbated by a vitamin D deficiency.

Impaired endothelium-dependent relaxation has been consistently reported in micro, and macrovessels of diabetic animals (Absi *et al.*, 2013; Joshi, Woodman, 2012; Mokhtar *et al.*, 2016). Endothelium-dependent relaxation occurs due to the release of vasodilator substances, termed endothelium-derived relaxing

factors (EDRF), which include NO, prostacyclin, and endothelium-dependent hyperpolarization. NO is the predominant EDRF in large conduit arteries, including the aorta (Mokhtar, Rasool, 2015; Shimokawa, Godo, 2016). In the aortas of diabetic rats, hyperglycemia may impair NO release from endothelial cells and lead to a reduction in the relaxation response (De Vriese *et al*., 2000). Under certain conditions, such as aging, hypertension, and diabetes, the withdrawal of NO production can initiate a contraction of vascular smooth muscle cells (Vanhoutte *et al*., 2005); as demonstrated by the increased endothelium-dependent contraction in aortas of diabetic rats in the present study.

This study showed that a vitamin D deficiency (low levels of 25[OH]D) in diabetes did not affect the aortic endothelial function when compared to diabetic rats with normal vitamin D levels. Diabetic rats in this study may have had normal levels of 25(OH)D, but there may have been an impairment in the vitamin D signaling mechanisms associated with the disease itself, which may have prevented the action of vitamin D in diabetic animals. The impairment in vitamin D signaling may be due to a reduction in calcitriol levels (the active form of vitamin D) or a reduced expression of intracellular VDR.

Although the diabetic rats in this study presented with normal levels of serum 25(OH)D, this may not fully represent the true value of calcitriol in the blood. Serum 25(OH)D is indeed the best indicator of vitamin D status, however, it may only be applicable in subjects without kidney disease. Diabetic kidney disease develops in approximately 30% of patients with type 1 diabetes (Alicic *et al*., 2017). In the kidney, 25(OH)D is converted to the active form calcitriol. Thus, diabetic rats in this study may have had impaired kidney function, which would have reduced the conversion of 25(OH) D to calcitriol. Unfortunately, calcitriol levels were not measured in this study because the measurement of serum calcitriol is difficult due to its short half-life (4-6 hours) (Wootton, 2005). Furthermore, calcitriol circulates at picomolar levels compared to 25(OH) D, which has one less hydroxyl residue and circulates at a 1,000-fold higher concentration (Souberbielle *et al*., 2015). In addition, the assay is difficult due to the lipophilic nature of calcitriol (Wootton, 2005).

The VDR is an absolute determinant for the biological activity of calcitriol. The VDR expression in cells is a requirement for a vitamin D response, and the receptor concentration itself is a key component of sensitivity to the hormone. While little is known of the molecular determinants of the basal expression of VDRs in cells, the VDR gene is regulated by a variety of hormones, including the parathyroid hormone, retinoid acid, and glucocorticoid hormones. Interestingly, calcitriol by itself can also increase the level of VDR gene expression (Costa *et al*., 1985). Local intracellular concentrations of the VDR agonist calcitriol, but not that of serum 25(OH)D, may be directly associated with its beneficial effects. A few studies have suggested an associative link between impaired signaling of VDR ligands and endothelial dysfunction (Andrukhova *et al*., 2014; Ni *et al*., 2014). In the endothelial-specific VDR knockout mice model (a mice model with an absence of endothelial VDR), acetylcholine-induced aortic relaxation was significantly impaired compared to control mice. This suggests that endothelial VDR plays an important role in the regulation of vascular tone (Ni *et al*., 2014).

It is accepted that increased aortic media thickness reflects an atherosclerotic burden and is a predictor for subsequent CV events (O'Leary, Polak, 2002). The current study demonstrated that in diabetic rats there were significant increases in the aortic media thickness compared to normal rats. This is in addition to the impairment of vascular endothelial function. These results are in accordance with a previous study that reported an increased tunica media thickness in diabetic rats (Elbe *et al*., 2014). An increased intima-media thickness with diabetes might be due to the hyperglycemic state occurring with diabetes. Hyperglycemia creates oxidative stress and nitrosative stress, which act on the arterial wall to initiate the thickening process (Ceriello, 2005). It has been reported that the blood glucose level is a major independent determinant of carotid intima-media thickness in patients with hypertension and hyperglycemia (Tropeano *et al*., 2004). Recently, fasting blood glucose levels were shown to be linked to carotid intima-media thickness in low-income populations in China (Liu *et al*., 2017).

In this study, the aortas of diabetic rats with a vitamin D deficiency presented with structural changes, although the function of the endothelium appears to be intact when compared to diabetic rats with normal levels of vitamin D. Low levels of serum 25(OH)D in diabetes may be a reason for the increased aortic media thickness. Low 25(OH)D levels have been shown to be negatively correlated with intima-media thickness and the incidence of diabetic plaques in patients with type

2 diabetes (Chen *et al*., 2017; Liu *et al*., 2012; Targher *et al*., 2006; Wang, Zhang, 2017). However, the present study was not able to demonstrate the importance of 25(OH)D in the regulation of vascular tone.

The present experiments have some limitations. First, this study did not provide data on the level of serum NO. Moreover, the contribution of individual NO in endothelium-mediated relaxation in rat aortas was also not determined. The contribution of NO in endothelium-mediated relaxation is usually measured by inhibiting the production of NO using N omega-nitro-L-arginine methyl ester (L-NAME, an endothelial NO synthase inhibitor). However, decreased endothelium-dependent relaxation in rat aortas has been suggested to be due to the reduced synthesis of NO, as it is the major endothelium-derived relaxing factor in large conduit vessels (Mokhtar, Rasool, 2015; Shimokawa, Godo, 2016). Second, biomarkers associated with inflammation, such as the intracellular adhesion molecule (ICAM-1) and vascular cell adhesion molecule (VCAM), should have been measured to support the data for intima-media thickness. Lastly, VDR levels should have been measured in the experimental animals in this study as these play an important role in the regulation of vascular tone.

In conclusion, this study showed that endothelial function was impaired in diabetes. The impairment was not exacerbated in the presence of low serum levels of 25(OH)D. However, diabetes with a vitamin D deficiency demonstrated smooth muscle hypertrophy and increased aortic media thickness.

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

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