Original Article

Calcitriol and *Punica Granatum* Extract Concomitantly Attenuate Cardiomyopathy of Diabetic Mother Rats and Their Neonates via Activation of Raf/MEK/ERK Signalling and Mitigation of Apoptotic Pathways

(calcitriol / *Punica granatum* / diabetes / cardiomyopathy / MAPK pathway / apoptosis)

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Abstract. We investigated the detrimental effects of diabetes on myocardium of pregestational streptozotocin (STZ)-diabetic mother rats and their neonates via evaluations of oxidative redox, inflammatory and apoptotic pathways, also aiming to characterize whether calcitriol and/or pomegranate peel extract confer myocardial protection in hyperglycaemic dams and their foetuses via modulation of the Raf/MEK/ERK cascade. Sixty Sprague-Dawley female rats were randomized into five groups (N = 12): control, diabetic, diabetic treated with calcitriol and/or pomegranate peel extract (PPE), and mated with non-diabetic healthy males. After confirmation of pregnancy, treatments were kept until gestational day (E-18). Serum and cardiac tissues of mothers and foetuses were collected and processed for biochemical, histopathological, and molecular assessments. We observed that, compared to the control, diabetic mothers showed dramatically increased hyperglycaemia and hyperlipidaemia associated with decreased myocardial functions and disrupted maternal performance. Also, diabetic mothers and their neonates exhibited elevated levels of myocardial injury (troponin I, endothelin 1, creatine kinase-MB, lactate dehydrogenase), with increased pro-inflammatory cytokines (interleukin 1, interleukin 1β, transforming growth factor β) and oxidative redox. Concurrently, the MAPK pathway was significantly down-regulated with increased myocardial apoptotic activity. Furthermore, mRNA expression of angiogenic and fibrotic markers was significantly increased. Paradoxically, calcitriol and/or pomegranate peel extract alleviated these diabetic myocardial insults and normalized the aforementioned assayed parameters. Our findings hypothesized that calcitriol and/or pomegranate peel extract exerted cardioameliorative impacts due to their unique anti-oxidative and anti-inflammatory properties, and thus may be a promising treatment that directly targets the secondary myocardial complications of diabetes in dams and their offspring.

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Abbreviations: CAT – catalase, CK – creatine kinase, CK-MB – myocardial bound creatine kinase, DCM – diabetic cardiomyopathy, FBG – fasting blood glucose, FPG – fasting plasma glucose, GPx – glutathione peroxidase, HDL – high-density lipoproteins, HR – heart rate, LDH – lactate dehydrogenase, LDL – low-density lipoproteins, MDA – malondialdehyde, MMP – matrix metalloproteinase, PPAR – peroxisome-proliferator-activated receptor, PPE – pomegranate peel extract, ROS – reactive oxygen species, SOD – superoxide dismutase, STZ – streptozotocin, TC – total cholesterol, TG – triglyceride, TNF-α – tumour necrosis factor α.

Introduction

Diabetic cardiomyopathy (DCM), one of the most common cardiovascular complications, represents the main culprit and a leading cause for morbidity and mortality (Kobayashi and Liang, 2015; van Diepen et al., 2016).

Accumulating evidence demonstrated that hyperglycaemia plays a crucial role in promoting various cellular and molecular cardiac remodelling events via triggering pathophysiological alterations (Al-Rasheed et al., 2016), hypertrophy (Lorenzo-Almorós et al., 2017) and fibrosis (Shang et al., 2017). Furthermore, it has been well documented that hyperglycaemia and dyslipidaemia usually coexist (Martín-Timón, 2014) resulting in accumulation of free fatty acids in myocardial cells, entailing contractile dysfunctions and fibrosis (Kenchaiah et al., 2002).

A previous study of Ilkun and Boudina (2013) showed that increased oxidative redox induced mitochondrial apoptotic pathways and as a consequence led to cardiac remodelling in diabetic heart. In this setting, another explanation for diabetic myocardial damage was mainly attributed to increased lipid peroxidation and production of reactive aldehydes, which represent an essential predictor for pathogenesis of DCM (McIntyre and Hazen, 2010; Xu et al., 2017). Also, hyperglycaemia mechanistically induced caspase-3 apoptotic signalling that may be triggered by reactive oxygen species (ROS) production (Sari et al., 2010). Noteworthy, it was postulated that inflammatory cytokines, including tumour necrosis factor (TNF)-α, interleukin (IL)-6, IL-1β (Rajesh et al., 2010; Mittal et al., 2014) and transforming growth factor β (Yue et al., 2017), displayed increased expression, as early signs of myocardial damage, in patients with DCM.

During pregestational diabetes, the increased prevalence of embryonic intra-uterine growth retardation (Kanguru et al., 2014), perinatal mortality and birth defects is a direct consequence of hyperglycaemia (Bequer et al., 2018). Furthermore, epidemiologic studies have reported dramatically increased rates (3-fold) of miscarriage in diabetic women (Boulot al., 2003; Eidem et al., 2011) associated with decreased foetal body weights, macrosomia (Damasceno et al., 2013a), and congenital anomalies (Rosenstein et al., 2012).

Importantly, it was postulated that the mitogen-activated protein kinase (MAPK) family mainly regulated intra-cellular signal transduction in cardiomyocytes via four major pathways: c-Jun N-terminal kinases (JNK1, 2, and 3), p38 kinase (α, β, γ, δ), MAP kinase (BMK or ERK5), and extracellular signal-regulated kinases (ERK1/2) (Wang, 2007; Xu et al., 2016). Although several studies illustrated the mechanistic pathways of the MAP kinase, here, we characterized the molecular and functional ERK-mediated myocardial protection in our diabetic dams and their foetuses. The intracellular activation of the ERK1/2 cascade, Ras/Raf/MEK/ERK, is responsible for cell growth and proliferation (Mutlak and Kehat, 2015). In parallel, several studies have implicated the ERK pathway in mitigation of cardiac deterioration, inflammation and fibrotic remodelling through enhancing its phosphorylation (Peake et al., 2013; Lips et al., 2004; Diwan and Dorn, 2007).

Recent evidence indicated that calcitriol administration had a pivotal role in prevention of cardiovascular complications in diabetic rats (Pilz et al., 2013; Lee et al., 2014; Wei et al., 2017). Also, it was demonstrated that vitamin D deficiency is a key factor in the development of cardiovascular complications in diabetic patients (Pittas et al., 2007; Herrmann et al., 2015). A study of Rahman et al. (2007) reported altered matrix metalloproteinase expression in vitamin D receptor knocked-out mice; anticipating that vitamin D is a key regulatory factor in the extracellular matrix metabolism. In addition, it was suggested that vitamin D displayed anti-hypertrophic potential in neonatal ventricular cardiomyocytes (Wu et al., 1996; Chen et al., 2011). Furthermore, other approaches hypothesized that calcitriol could modulate adipogenesis in diabetic cardiomyopathy by activation of peroxisome-proliferator-activated receptor (PPAR) α and down-regulation of PPARγ (Dunlop et al., 2005; Ding et al., 2012).

To address the respective role of phytotherapy in alleviation of cardiovascular complications in diabetic cardiomyopathy of mother rats and their neonates, we characterized the efficacy of pomegranate (*Punica granatum*) peel extract (PPE) in the treatment. Pomegranate peel chemical constituents are remarkable for their high content of phenolic compounds such as proanthocyanidins and ellagitannins (Ismail et al., 2012), as well as flavonoids (Li et al., 2006; Prakash et al., 2013), which exert cardiovascular protection via anti-oxidative and anti-atherogenic effects (Aviram et al., 2002). However, the effective therapeutic potential of pomegranate including anti-inflammatory (Lee et al., 2010), antioxidant (Les et al., 2015) and antidiabetic (Aqil et al., 2012) impacts have been well investigated. The mechanism whereby pomegranate mediates its anti-diabetic effects is still debated and controversial. In addition, it was shown that pomegranate juice notably improved the lipid profile of hyperlipidaemic diabetic patients (Esmaillzadeh et al., 2004).

Despite the fact that several rationales were hypothesized highlighting impacts of hyperglycaemia on the cardiac functions, the precise understanding of cardiovascular complication development is still elusive. Our present study was designed to gain insights into the MAPK pathway that mechanistically displayed cardioprotective effects in streptozotocin (STZ)-diabetic rats and their neonates. In addition to figuring out the efficacy of calcitriol and pomegranate peel extract in attenuation of these changes, we highlighted its modulatory role in the Raf/MEK/ERK cascade.

Material and Methods

Animals

Sixty healthy Sprague-Dawley virgin female rats weighing about 170 ± 30 g and 20 fertile healthy male rats were obtained from our animal facility of the College of Science at King Khalid University, Saudi Arabia. Animals were housed in their specific cages under 12:12 h light-dark cycle of illumination in ambient temperature (24 ± 2 °C). Animals were fed a semi-purified control diet (AIN-93 G diet) and water *ad libitum*. All experimental procedures and animal handling were performed in accordance with the guidelines of the Research Ethics Committee of King Khalid University, which follow the guidelines established by the US National Institutes of Health (NIH publication No. 85–23, revised 1996).

Punica granatum peel extract (PPE) preparation and FTIR analysis

Fresh ripe pomegranate (*Punica granatum*) fruits were gathered from Abha, Aseer, Kingdom of Saudi Arabia in the month of May 2018. Pomegranate was kindly identified by a taxonomist at the Biology Department, King Khalid University, Saudi Arabia. Then, the peel was removed and shadow dried for one week before milling. The crushed powder (50 g) was vibrated in absolute methanol (500 ml) for one day at room temperature. The filtrate was centrifuged at 5000 × *g* for 15 min, supernatant was collected, and methanol was evaporated at 45 °C under low pressure in a rotary evaporator. Finally, we kept the crude extract (23.5%, w/w) at 20 °C until use.

Then, we performed Fourier transform infrared (FTIR) spectroscopy (Perkin- Elmer Spectrum 2000, Shelton, CT) within the range 500–4000 cm^{-1} at a rate of 16 times and the clarity of 4 cm^{-1}. Functional groups in pomegranate extract were explored according to Ibrahim et al. (2018).

Generating type-2 diabetic animal model

To establish our diabetic model, rats were injected intraperitoneally with a single dose of streptozotocin (STZ) (40 mg/kg dissolved in 0.1 165 mol/l sodium citrate buffer, pH 6.5; Sigma, Mannheim, Germany) (Damasceno et al., 2011). The rats in the normal control group received an equivalent amount of citrate buffer. After one week of STZ administration, we evaluated the fasting blood glucose level (FBG) using two consecutive analyses within two days, and animals were considered diabetic with FBG ≥ 16.7 mmol/l.

Mating procedures

After confirmation of diabetes establishment, we defined the oestrous cycle of virgin female rats by performing a vaginal smear daily as described previously by Kiss et al. (2009). Then, all female rats were mated with non-diabetic healthy males (1 male/3 females) in separate cages (Fig. 1). On the next morning, approximately at 6:00 am, mating was checked and confirmed by examining the vaginal outer surface for the presence of vaginal plug and, hence, it was designated as gestational day 0 (E0).

Experimental design and treatments

Later, pregnant rats were randomized into five groups (N = 12/group) as follows: *i*) control (receiving citrate buffer (C)), *ii*) diabetic group (40 mg/kg dissolved in 0.1 165 mol/l sodium citrate buffer, as described previously (D)), *iii*) diabetic group treated with vitamin D (VD)) (rats injected subcutaneously with $25(OH)_2D_3$, 150 ng/kg/daily dissolved in 1,2-propanediol, Sigma, St Louis, MO), *vi*) diabetic group treated with pomegranate peel extract PPE (DP)) (rats received 150 mg/kg/daily by gavage), *v*) diabetic group treated with both vitamin D and pomegranate peel extract (rats received a dual treatment with the same doses as in groups *iii* and *vi* through the same route (DPV)). All treatments were kept during the gestational period until day 18 (E-18) (Fig. 1). Pregnant rats were separated into individual cages and maternal blood glucose levels and body weights were recorded at E-0, E-7, E-14, and E-18.

On day 18 of pregnancy (E-18), at the end of the treatment, pregnant rats were fasted overnight before sacrifice and their blood was collected after cardiac puncture. Then, the blood was centrifuged at 3000 × *g* for 15 min and the serum was separated and stored at –80 °C to be used for biochemical analysis. The uterine horns were separated after laparotomy and reproductive outcomes of mothers were recorded. Also, the corpora lutea were quantified using a stereomicroscope. In addition, we determined the foetal and placental weights, placental index, number of implantations, resorptions, pre-$\left(\frac{\text{No of corpora lutea} - \text{No of implantaions}}{\text{No of corpora lutea}} \times 100\right)$ and post-implantation loss $\left(\frac{\text{No of implantaions} - \text{No of live foetuses}}{\text{No of implantaions}} \times 100\right)$ percentage according to Saito et al. (2010). Then we assayed the following parameters:

General features and biochemical profile of mother rats

Heart rate (HR) was measured using a non-invasive tail-cuff plethysmography system (BP2000, Visitech Systems, Apex, NC) according to Wang et al. (2016). To determine the fasting plasma glucose level (FPG), we used the glucose oxidase method, whereas serum insulin levels were estimated using a radioimmunoassay technique with a multi-well gamma counter. Serum total

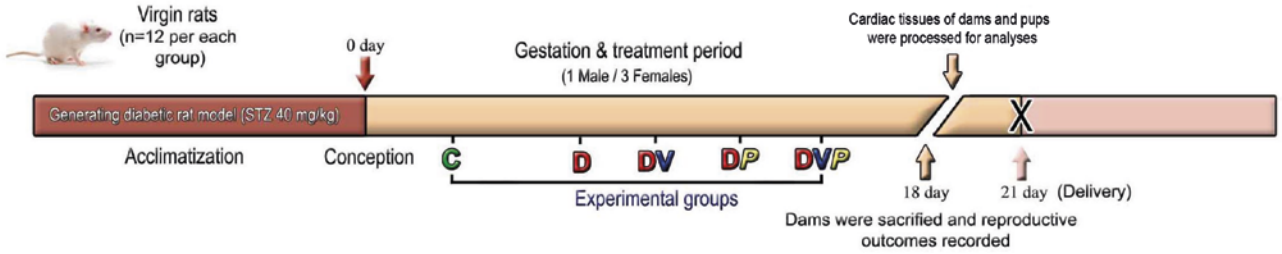

Fig. 1. Chronological chart for our experimental design in days
Abbreviations: C – control; D – diabetic group; DP – diabetic group treated with pomegranate peel extract; DV –diabetic group treated with vitamin D; DVP – diabetic group treated with both vitamin D and pomegranate peel extract.

cholesterol (TC), triglyceride (TG), high-density lipoproteins (HDL), and total creatine kinase (CK) were evaluated using a biochemical auto-analyser (AU–640 Medical System, Olympus, Japan). Then, we calculated the low-density lipoproteins (LDL) from the total cholesterol, TG and HDL values. To further assess albumin and myoglobin contents, we used commercial ELISA kits (Abcam, Cambridge, UK, cat. No. ab108789 and ab157739, respectively).

Histopathological investigation

Hearts of dams and their neonates after sacrifice were separated, bisected transversely at the mid-ventricular level and immediately fixed in 10% phosphate-buffered formalin (pH 7.4), dehydrated in ascending grades of ethanol, cleared in xylene and embedded in molten paraplast at 58–62 °C. Then, tissue samples were cut into 5 µm sections and stained with haematoxylin and eosin. To calculate the cross-sectional area, we assessed the circumferential length of the cardiomyocytes using Image J v1.41 software (http://rsbweb.nih.gov/ij/index.html) according to Chen et al. (2018).

Maternal and foetal oxidative stress and redox status

To explore the oxidative activity of the maternal and foetal cardiac tissues in experimental groups, we colorimetrically estimated the catalase (CAT) enzymatic activity according to Block et al. (1980). We further determined the cardiac enzymatic activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx) using Abcam assay kits (cat. Nos. ab65354, ab102530). Additionally, we estimated malondialdehyde (MDA), lipid peroxidation activity marker, using an Abcam assay kit (cat. No. Ab118970) according to manufacturer's instructions.

Quantitative real-time RT-PCR

After cardiac tissue homogenization, total RNA was prepared with the TRIzol reagent (Invitrogen, Carlsbad, CA). Later, RNA was treated with RNase-free DNase (Ambion, Austin, TX) to remove traces of genomic DNA. RT-PCR was carried out using the listed primers as follows:

Gene	Forward primer	Reverse primer
β-actin	TGCACCACCAACTGCTTAG	GGATGCAGGGATGATGTTC
ANG-2	TTGGACAATTATTCAGCGACGTG	GCTGGTCGGATCATCATGGTTG
VEGF	GTCCTCACTTGGATCCCGATA	CCTGGCAGGCAAACAGACTTA
α-SMA	GGAGTGATGGTTGGAATGG	ATGATGCCGTGTTCTATCG
MMP-9	TCTTCTGGCGTGTGAGTTTCC	CGGTTGAAGCAAAGAAGGAGC
COL-I	TGGCCTTGGAGGAAACTTTG	CTTGGAAACCTTGTGGACCAG
COL-III	TTGGAGGTGAAAAGTCTGGCGGCT	TGCAGCCTTGGTTAGGATCAACCC

Maternal and foetal markers of cardiac injury

Serum troponin I (Tp-I) was measured using appropriate kits in a RA-50 semi-automated analyser. To determine endothelin 1 (ET-1), we used a commercial ELISA Kit (cat. No. ab133030, Abcam), whereas lactate dehydrogenase (LDH) and myocardial bound creatine kinase (CK-MB) levels were estimated using a biochemical analyser (ADVIA-1200, Siemens, Uetersen, Germany).

Maternal and foetal cardiac tissue cytokine assay

To prepare the cardiac tissue homogenate, we homogenized maternal and embryonic frozen tissue samples in ice-cold PBS (pH 7.4) and stored them at –80 °C. Then, the activity of transforming growth factor β (TGF-β) (cat. No. ab119558, Abcam), interleukin (IL)-6) (cat. No. ab100772, Abcam) and IL-1β (cat. No. ab100768, Abcam) was estimated using rat ELISA kits according to manufacturer's instructions.

Then, the reactions were performed in a real-time PCR thermocycler (IQ5 Real-Time PCR cycler; Bio-Rad, Hercules, CA). Expression of the target genes was normalized to the internal reference gene *(β-actin)*. Finally, relative expression for the assayed genes was determined using the $2^{-\Delta\Delta CT}$ method as described by Livak and Schmittgen (2001).

Western blot analysis

Whole protein was extracted from maternal and foetal cardiac tissues, and its concentration was measured using the Bradford assay kit. Immunoblotting was carried as described previously by Sun et al. (2007). We used antibodies against Raf-1 (cat. No. E-10: sc-7267), MEK-1/2 (cat. No. 9G3: sc-81504), p-MEK-1/2 (cat. No. 7E10: sc-81503), ERK-1 (cat. No. G-8: sc-271269), p-ERK 1/2 (cat. No. 12D4: sc-81492), Akt1 (cat. No. B-1: sc-5298), p-Akt1 (cat. No. 104A282: sc-52940), PPARP-γ (cat. No. E-8: sc-7273), Glut4 (cat. No. IF8: sc-53566), MYH (cat. No. B-5: sc-376157), GSK-3β (cat. No. 11B9: sc-81462) and β-Actin (cat. No. C4: sc-47778). All antibodies were purchased from Santa Cruz Biotechnology, Dallas, TX. After washing, blots were incubated with a peroxidase-conjugated anti-IgG secondary detection antibody for 2 h at room temperature.

Immunoreactivity of bands was visualized with an ECL detection system and subsequently quantified by scanning with a Fusion Image Dock Station (Fusion FX5, Vilber Lourmat, France). Then, protein expression was normalized to the reference protein β-actin.

Comet assay

Maternal and foetal cardiac tissue samples were separated and immediately stored at –80 °C. To obtain 10% tissue solution, we processed homogenization in chilled homogenizer buffer (containing 75 mM NaCl & 24 mM Na_2EDTA). Then, the alkaline-comet assay was performed as described previously by Wu et al. (2011).

Flow cytometric analysis of CASP-3

Maternal and foetal caspase-3 activity was analysed and quantified by flow cytometry using a caspase-3 detection kit (Calbiochem, Merck, Darmstadt, Germany) in accordance to manufacturer's instructions. In brief, 2×10^5 myocardial cells for all experimental groups were harvested and incubated with 1 µl fluorescein-labelled caspase inhibitor (FITC-DEVD-FMK for caspase-3) for one hour at 37 °C. Then, after incubation, cells were centrifuged and pellets were washed with buffer and analysed by a FACS Calibur Flow Cytometer (Becton Dickinson, San Jose, CA). Fluorescence was detected with a FL1 detector. Data acquisition and histogram analyses were carried out using the CellQuest software.

Statistical analysis

Data were presented as mean ± standard error (SE). To analyse the body weight gain of mother rats during the gestational period, we used univariate two-way ANOVA followed by Dunnett's post-hoc analysis. The mean cardiomyocyte cross-sectional area was calculated using the paired sample *t*-test. For other statistical analyses, one-way ANOVA followed by Tukey's post-hoc test were performed using the SPSS (version 18) software package for Windows. Differences between groups were considered significant at * $P < 0.05$, ** $P < 0.01$ and ***$P < 0.001$.

Results

Phytochemical analyses and functional constituents of Punica granatum

In our study we generally highlighted the main functional groups of pomegranate. As depicted in Fig. 2, a strong stretching vibrational band appeared at 3754.12 cm^{-1} assigned to the OH group corresponding to alcohols. A medium stretching band at 2933.7 cm^{-1} was assigned to the C-H group due to the presence of alkanes. A strong stretching peak at 1730.27 cm^{-1} assigned to the CO group related to α,β-unsaturated ester and aldehyde. The observed strong vibrational stretch-

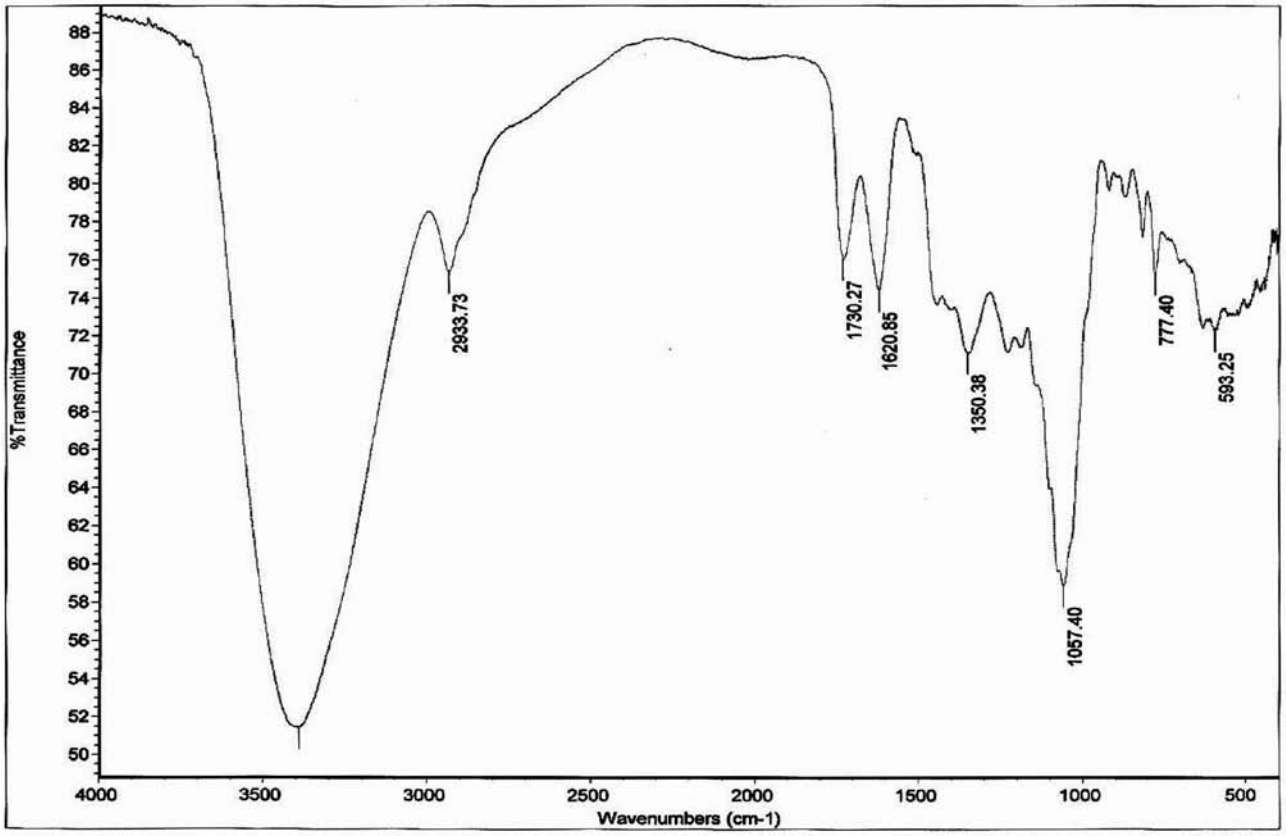

Fig. 2. Fourier transform infrared (FTIR) spectrum of *Punica granatum* peel extract

ing peak at 1620.85 cm^{-1} can be attributed to the C=C group, which indicates the presence of α,β-unsaturated ketone and alkene. A bending band appearing at 1350.38 cm^{-1} assigned to the CH group may be due to the presence of alkanes. A strong stretching vibrational band at 1067.40 cm^{-1} due to the presence of a CO group may be related to a primary alcohol. Weak bands occurring at 777.40 and 593.25 cm^{-1} are due to the presence of bonded C–Cl and C–Br stretching vibration in chloro-alkane and bromo-alkane, respectively (Nakamoto, 2009; Larkin, 2011).

General body features and biochemical profile of pregnant rats

As shown in Fig. 3, the body weight gain of pregnant rats significantly increased in all experimental groups from the 7th gestational day (E-7) until the term of pregnancy (E-18). Diabetic mothers showed a significant decrease of their body weights and relative increase in heart weights ($P < 0.01$) compared with the control. However, treatments with calcitriol and/or pomegranate revealed a noticeable improvement in the mean body weights ($P < 0.05$), with a slight variation from that of the control (Fig. 3a, b). In addition, the heart rate was significantly decreased in diabetic rats (Fig. 3c).

Also, diabetic mothers had a higher level of circulating plasma glucose (21.36 ± 2.04, $P = 0.0007$) and decreased insulin (159.52 ± 4.37, $P = 0.0009$) compared to the control (5.36 ± 0.47 and 311.76 ± 5.54, respectively) (Fig. 3d, e). Our results showed that sera of hyperglycaemic pregnant mothers displayed a significant increase in circulating TG, total cholesterol, and LDL levels compared to the control; while there were decreased

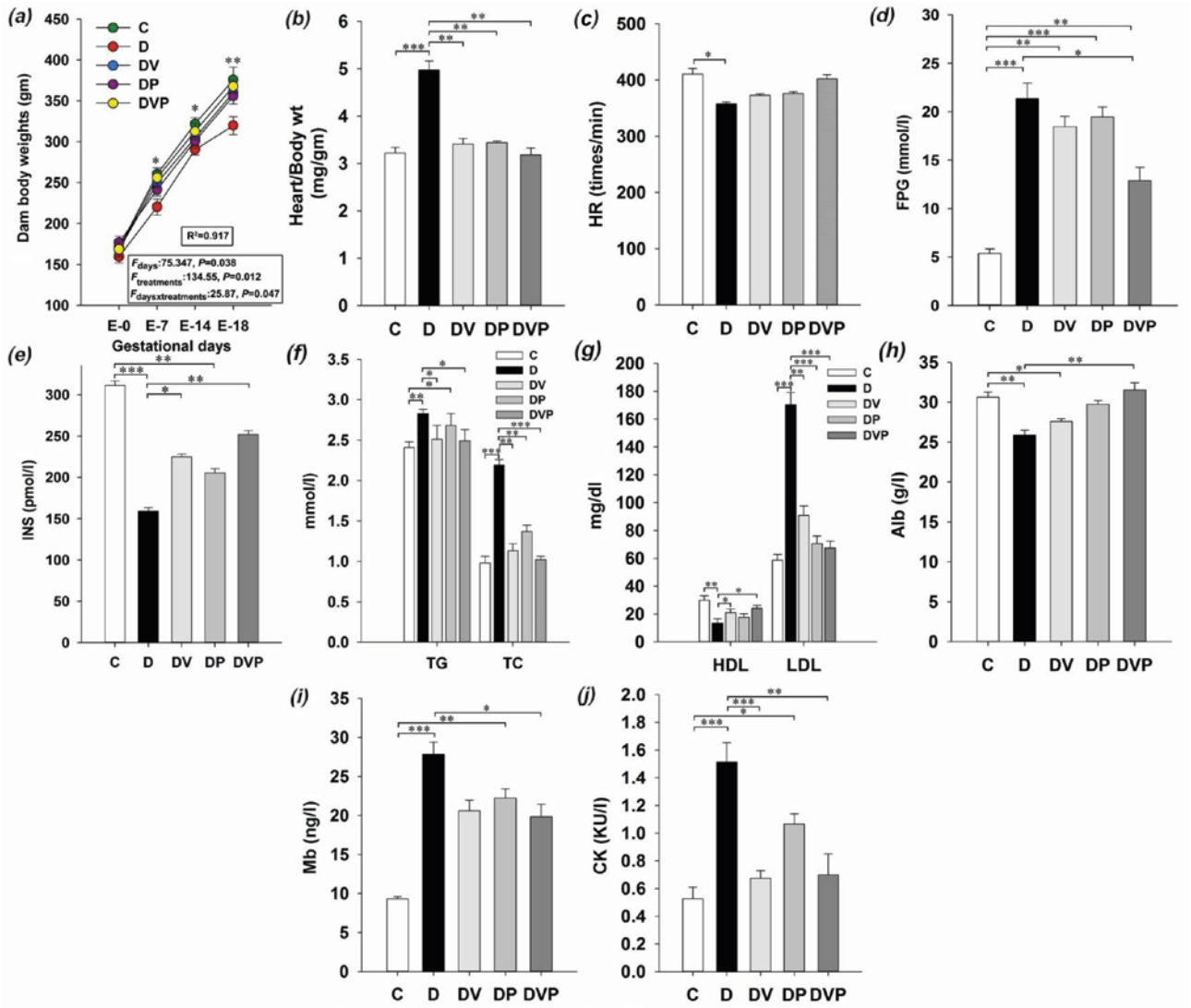

Fig. 3. General features and biochemical characters of pregnant rats on day E-18. Abbreviations: Alb – albumin; C – control; D – diabetic group; DP – diabetic group treated with pomegranate peel extract; DV – diabetic group treated with vitamin D; DVP – diabetic group treated with both vitamin D and pomegranate peel extract, FPG – fasting plasma glucose; HR – heart rate; INS – fasting plasma insulin; Mb – myoglobin.
Data were presented as mean ± SE (N = 5); * significant at $P < 0.05$, ** at $P < 0.01$ and *** at $P < 0.001$.

levels of HDL (Fig. 3f, g). Additionally, we explored the plasma indicators of myocardial function including albumin, myoglobin and CK levels. There was a significant decrease in albumin levels (25.88 ± 2.74, P = 0.0007) coinciding with increased myoglobin and CT levels (27.84 ± 1.57, P = 0.001; 1.51 ± 0.17, P = 0.0009) in diabetic mothers compared with those of the controls (30.65 ± 2.68, 9.29 ± 1.24, 0.53 ± 0.12, respectively). Interestingly, we recorded a noticeable amelioration of the assayed biochemical parameters in experimentally treated groups administered with calcitriol and/or pomegranate compared to the diabetic groups (Fig. 3h, i, j).

Calcitriol and/or pomegranate enhanced reproductive outcomes and performance indices in STZ-diabetic mothers

To evaluate the impacts of gestational diabetes on maternal neonates and the efficacy of vitamin D and PPE in the treatments, we calculated the mean number of implantations, corpora lutea, live foetuses and their corresponding weights, and crown rump length. As shown in Table 1, our data displayed a significant reduction in the aforementioned records in the diabetic groups. Furthermore, diabetic rats showed increased numbers of reabsorptions, percentage of both pre- and post-implantations and placental weight compared to the control. Conversely, vitamin D and/or pomegranate supplementation exhibited remarkable improvements of maternal outcomes (Table 1).

Calcitriol and/or pomegranate improved cardiac histological architecture in STZ-diabetic mothers and their foetuses

To elucidate the beneficial effect of vitamin D and/or pomegranate, we evaluated the cardiac pathological changes in experimental groups. In the control maternal and embryonic myocardium, photomicrographs showed a patterned structure and normally organized cardiac muscle cells (Fig. 4a A and A1). In the diabetic mother group, there were noticeable myocardial histological changes manifested by irregular arrangement of myocardial fibres, degenerated and eosinophilic myofibrils with internal haemorrhage (Fig. 4a B). In addition, diabetic embryos revealed significant histological damage evidenced by increased inflammatory cells, pyknotic nuclei and vacuolar degeneration of myocardial cells (Fig. 4a B1). In the diabetic groups administered with vitamin D and/or pomegranate, for both mothers and embryos, our observations revealed a notable mild improvement of myocardial cell proliferation and organization. Moreover, the myocardial cells showed normal cellularity and architecture with mitigated leukocytic activity and myofibril vacuolation (Fig. 4a C-E1).

Then, we further quantified the mean cardiomyocyte cross-sectional area of mothers and their neonates in different experimental groups. Histological observations of the diabetic group displayed a significantly increased cross-sectional area in both mothers and their neonates (282.78 ± 6.74; 145.67 ± 4.29, P < 0.05) compared to the control (223.37 ± 5.46, 96.61 ± 4.14; respectively). In contrast, treatments with vitamin D, pomegranate, or combination of both to diabetic mothers and their neo-

Table 1. Reproductive outcomes and maternal performance indices of experimental groups

	C	D	DV	DP	DVP
No. of rats used	12	12	12	12	12
No. of pregnant rats at term	12	8	11	10	11
No. of implantations	145 (12.08 ± 2.77)^{a}	56 (7.00 ± 1.58)^{b}	98 (8.91 ± 2.14)^{c}	87 (8.7 ± 2.63)^{c,d}	127 (11.55 ± 2.09)^{a,e}
No. of corpora lutea	165 (13.75 ± 2.12)^{a}	106 (13.25 ± 2.78)^{a}	135 (12.27 ± 2.47)^{b}	125 (12.5 ± 2.05)^{b}	157 (14.27 ± 2.87)^{c}
No. of live fetuses	140 (11.6 ± 1.87)^{a}	48 (6.00 ± 1.14)^{b}	93 (8.45 ± 2.54)^{c}	81 (8.10 ± 1.98)^{c,d}	117 (10.63 ± 2.57)^{a,e}
Reabsorptions	4	15	6	7	5
Pre-implantation loss (%)	12.12 %	47.16 %	33.33 %	32.00 %	20.38 %
Post-implantation loss (%)	3.45 %	14.29 %	5.10 %	6.89 %	7.87 %
Foetal body weight (g)	5.62 ± 0.51^{a}	4.21 ± 0.35^{b}	5.14 ± 0.45^{a,c}	5.21 ± 0.48^{a,c}	5.44 ± 0.55^{a,c}
Crown-rump length (cm)	4.57 ± 0.36^{a}	3.89 ± 0.18^{b}	4.32 ± 0.21^{a,c}	4.19 ± 0.24^{a,c}	4.46 ± 0.26^{a,c}
Placental weight (gm)	0.41 ± 0.07^{a}	0.63 ± 0.08^{b}	0.53 ± 0.07^{c}	0.57 ± 0.06^{c}	0.48 ± 0.06^{a,d}
Placental index (%)	7.89 ± 1.21^{a}	14.96 ± 1.98^{b}	10.31 ± 1.37^{c}	10.94 ± 1.44^{c,d}	8.42 ± 1.57^{a,e}

Data were expressed as mean ± SE (N = 8), * significant at P < 0.05. $^{a\text{-}e}$ means in a row without a common superscript letter significantly differed (P < 0.05) as analysed by one-way ANOVA.

Abbreviations: C – control; D – diabetic group; DP – diabetic group treated with pomegranate peel extract; DV – diabetic group treated with vitamin D; DVP – diabetic group treated with both vitamin D and pomegranate peel extract.

nates promoted a significant decrease in the cross-sectional area (Fig. 4b). Indeed, these observations suggested that vitamin D and pomegranate attenuated the myocardial destruction in diabetic cardiomyopathy.

Calcitriol and/or pomegranate improved myocardial functions in STZ-diabetic mothers and their foetuses

To estimate myocardial injury, we characterized Tp-I, ET-1, LDH, and CK-MB levels in the sera of mothers and their neonates in different experimental groups. As shown in Table 2, gestational diabetes induced a significant increase in these parameters compared to the control. Conversely, vitamin D and/or pomegranate treatments normalized the assayed markers and attenuated signs of myocardial injuries.

Cardiac tissue cytokine assay

Consistent with the myocardial injury findings, we evaluated inflammatory indicators within maternal and foetal cardiac tissues. We detected significantly elevated

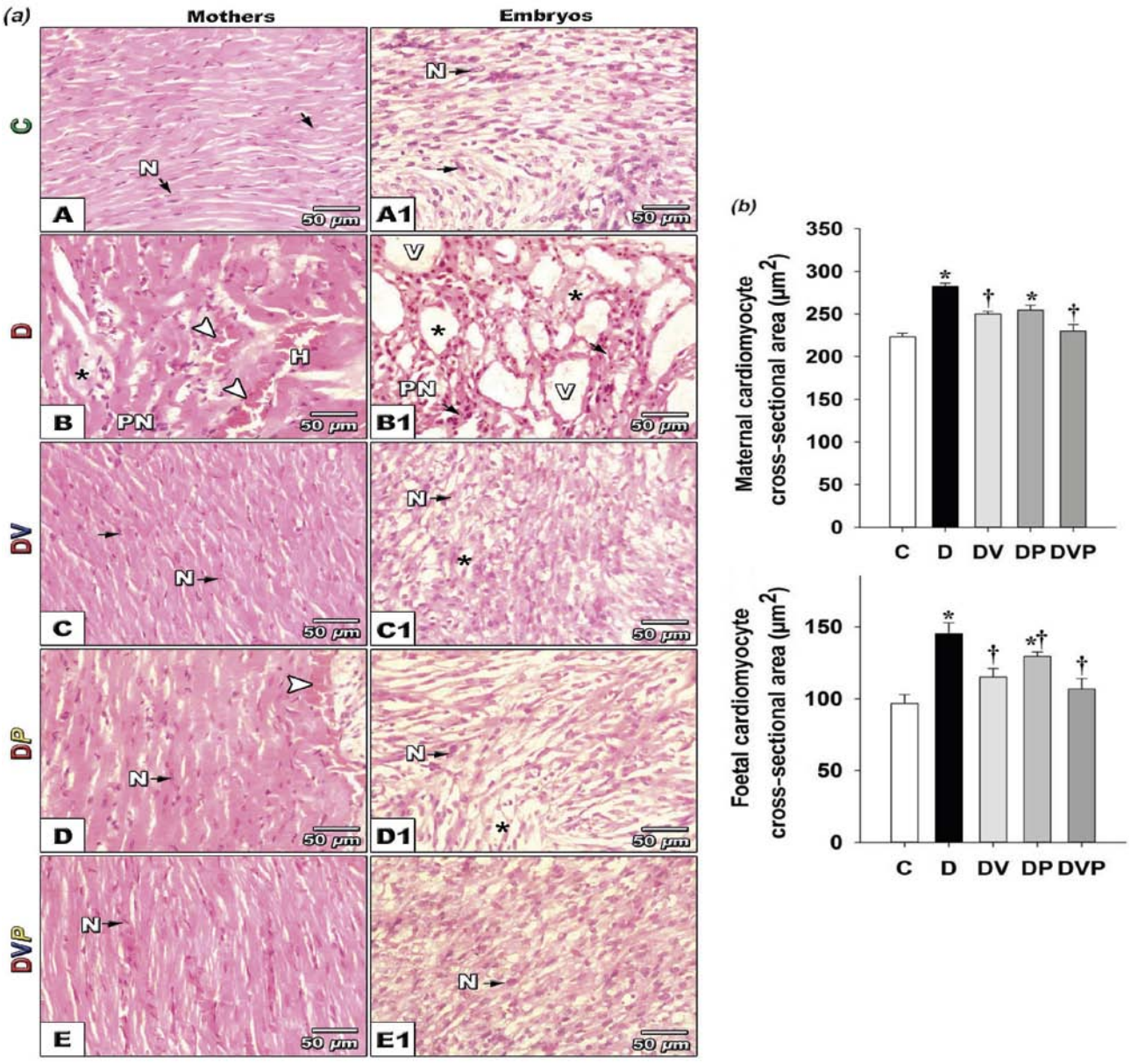

Fig. 4. (*a*): Representative histopathological micrographs of maternal (A-E) and foetal (A1-E1) myocardial sections. HX-E. (*b*): Maternal and foetal cardiomyocyte cross-sectional area. Abbreviations:
C – control; D – diabetic group; DP – diabetic group treated with pomegranate peel extract; DV – diabetic group treated with vitamin D; DVP – diabetic group treated with both vitamin D and pomegranate peel extract; H – haemorrhage; N – nucleus; PN – pyknotic nucleus; V – vacuole (asterisks refer to disorganized myofibrils).
Data were presented as mean ± SE (N = 8); *P < 0.05, as compared to the control group, and †P < 0.05, as compared to the diabetic group.

Table 2. Maternal and foetal markers of cardiac injury in different experimental groups

		C	D	DV	DP	DVP
Tp-I (*ng/ml*)	**M**	0.75±0.04^{a}	1.56±0.12^{b}	0.93±0.06^{c}	1.34±0.09^{b,d}	0.82±0.08^{a}
	E	0.48±0.35^{a}	1.12±0.32^{b}	0.71±0.40^{c}	0.93±0.32^{c,d}	0.53±0.39^{a,c}
ET-1 (*pg/ml*)	**M**	3.76±0.21^{a}	8.87±0.68^{b}	5.08±0.31^{c}	6.87±0.41^{c}	4.55±0.38^{a,c}
	E	2.64±0.19^{a}	5.16±0.37^{b}	3.10±0.26^{c}	3.59±0.27^{c,d}	2.88±0.22^{a}
LDH (*U/l*)	**M**	548.74±7.54^{a}	1045.32±12.45^{b}	746.22±8.88^{c}	759.10±8.47^{c,d}	605.37±7.55^{e}
	E	181.12±5.29^{a}	312.54±7.88^{b}	270.55±6.23^{c}	230.24±6.08^{c}	193.74±6.78^{a,d}
CK-MB (*U/l*)	**M**	170.32±5.45^{a}	507.84±9.41^{b}	231.54±6.10^{c}	275.80±6.38^{d}	186.77±5.91^{a,e}
	E	42.31±4.31^{a}	260.70±6.74^{b}	106.23±5.58^{c}	115.64±5.67^{c}	67.88±4.20^{d}
TGF-β (*pg/ml*)	**M**	10.82±2.12^{a}	25.41±3.45^{b}	15.58±2.78^{c}	19.81±2.45^{d}	14.98±2.04^{c}
	E	5.12±1.24^{a}	11.23±2.71^{b}	8.14±1.98^{c}	8.87±1.88^{c}	6.14±1.56^{a,c}
IL-6 (*pg/ml*)	**M**	2.21±0.98^{a}	6.44±1.98^{b}	3.54±1.12^{c}	3.74±1.08^{c}	2.42±1.57^{a,d}
	E	0.87±0.07^{a}	2.18±0.29^{b}	1.18±0.17^{c}	1.30±0.15^{c}	0.98±0.08^{a,c,d}
IL-1β (*pg/ml*)	**M**	3.78±0.89^{a}	7.99±1.23^{b}	3.97±1.17^{a,c}	5.12±1.24^{d}	4.16±1.04^{c,e}
	E	1.18±0.12^{a}	4.12±0.57^{b}	2.47±0.28^{c}	2.78±0.48^{c}	1.45±0.37^{a,d}

Each result represents the mean ± SE (N = 5), $^{a\text{-}e}$ means in a row without a common superscript letter significantly differed ($P < 0.05$) as analysed by one-way ANOVA.
Abbreviations: C – control; D – diabetic group; DP – diabetic group treated with pomegranate peel extract; DV – diabetic group treated with vitamin D; DVP – diabetic group treated with both vitamin D and pomegranate peel extract; E – embryos; M – mothers.

levels of pro-inflammatory cytokines, including TGF-β (25.41 ± 3.45, P = 0.04; 11.23 ± 2.71, P = 0.03; VS 10.82 ± 2.12, 5.12 ± 1.24), IL-6 (6.44 ± 1.98, P = 0.05; 1.18 ± 0.17, P = 0.008; VS 2.21 ± 0.98, 0.87 ± 0.07), and IL-1β (7.99 ± 1.23, P = 0.03; 4.12 ± 0.57, P = 0.004; VS 4.12 ± 0.57, 1.18 ± 0.12) in the diabetic group versus control (Table 2). After vitamin D and/or PPE treatments, we observed significantly decreased assayed cytokines in the myocardium of diabetic rats (Table 2). Our data demonstrated the efficacy of calcitriol and/or pomegranate phytochemicals in mitigation of myocardial inflammatory activities.

Calcitriol and/or pomegranate prevents oxidative stress of maternal and foetal myocardium

Next, we determined whether vitamin D and/or pomegranate exerted a protective antioxidant defence in the context of experimental diabetic groups. We evaluated the enzymatic antioxidant activity of catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), and malondialdehyde (MDA). Our observations showed that the mothers and their embryos had significantly decreased levels of CAT (5.68 ± 1.58, P = 0.04; 2.76 ± 0.64, P = 0.0008, respectively) and SOD (2.63 ± 0.66, P = 0.04; 0.51 ± 0.08; P = 0.0008, respectively), and increased GPx (12.36 ± 2.12, P = 0.0006; 2.86 ± 0.34, P = 0.0003, respectively) and MDA (7.36 ± 1.38, P = 0.0005; 1.98 ± 0.27, P = 0.0007, respectively) compared to controls (Fig. 5a, b). However, administration of vitamin D and/or pomegranate to diabetic mothers significantly restrained MDA and GPx activities with remarkably reversed levels of CAT and SOD contents, retrieving their antioxidant capacity (Fig. 5a, b).

Calcitriol and/or pomegranate attenuate angiogenesis and fibrosis in maternal and embryonic cardiac tissues

To ascertain whether calcitriol and/or vitamin D modulated angiogenic and fibrotic activities in myocardial tissues, we characterized the relative mRNA expression of ANG-2 and VEGF as indicators of angiogenesis. Also, we determined the activities of fibrotic markers, including α-SMA, MMP-9, COL-I, and COL-III. Significant increases in fold changes of relative mRNA expression were scored in the diabetic maternal (Fig. 6a) and foetal (Fig. 6b) myocardium. Intriguingly, the abundance of expression of these mRNA was dramatically down-regulated by administration of vitamin D or pomegranate alone, and to a greater extent by a combination of both (Fig. 6a, b).

Calcitriol and/or pomegranate up-regulated Raf-1/MEK1/2-ERK1/2 in STZ-diabetic mothers and their foetuses

To better understand the molecular mechanisms involved in attenuation of myocardial dysfunction in STZ-induced diabetic rats via calcitriol and pomegranate treatments, we evaluated MAPK protein activities in mothers and their foetuses. Significantly, we found decreased levels of Raf-1, MEK1/2, p-MEK1/2, ERK1/2, p-ERK1/2, Akt and p-Akt in myocardial cells of diabetic mothers and their neonates, compared to the control.

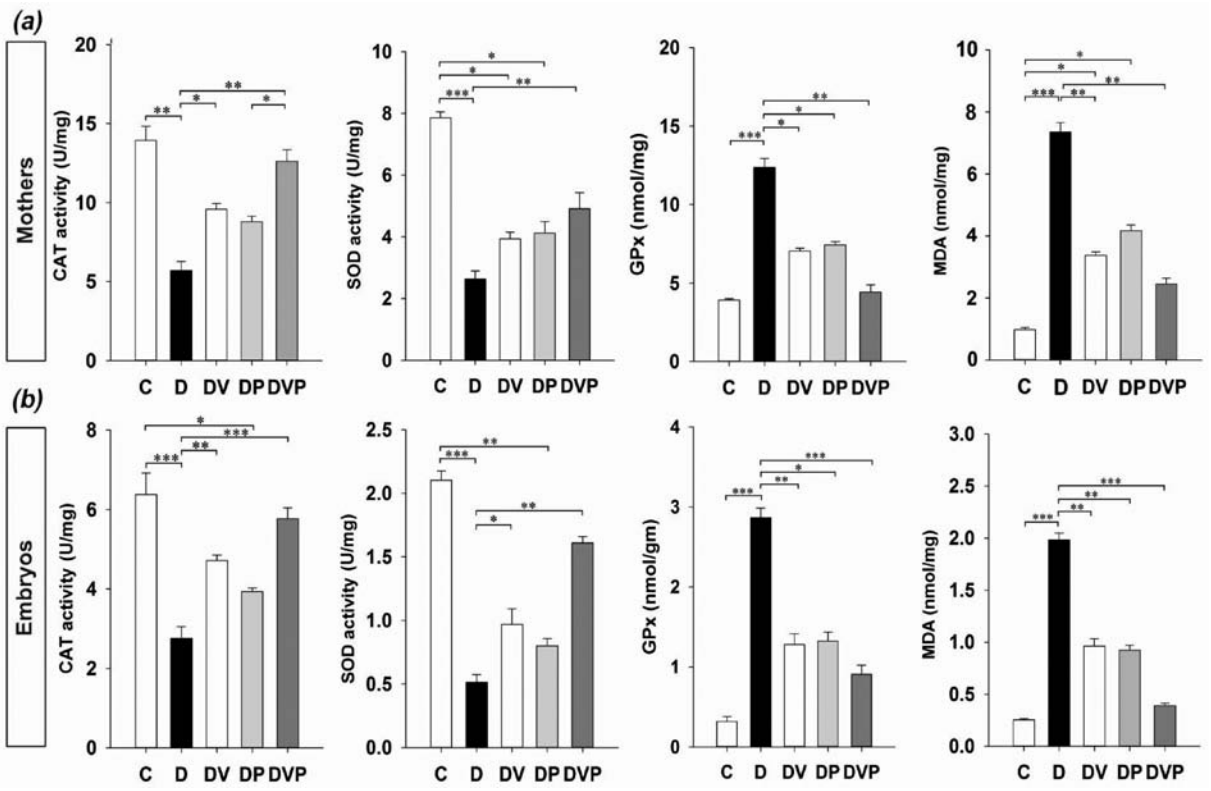

Fig. 5. Effects of vitamin D and/or pomegranate on maternal (***a***) and foetal (***b***) myocardial antioxidant enzymatic activities of catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and malondialdehyde (MDH) in different experimental groups

Abbreviations: C – control; D – diabetic group; DP – diabetic group treated with pomegranate peel extract; DV – diabetic group treated with vitamin D; DVP – diabetic group treated with both vitamin D and pomegranate peel extract.

Data were presented as mean ± SE (N = 5); * significant at $P < 0.05$, ** at $P < 0.01$ and *** at $P < 0.001$.

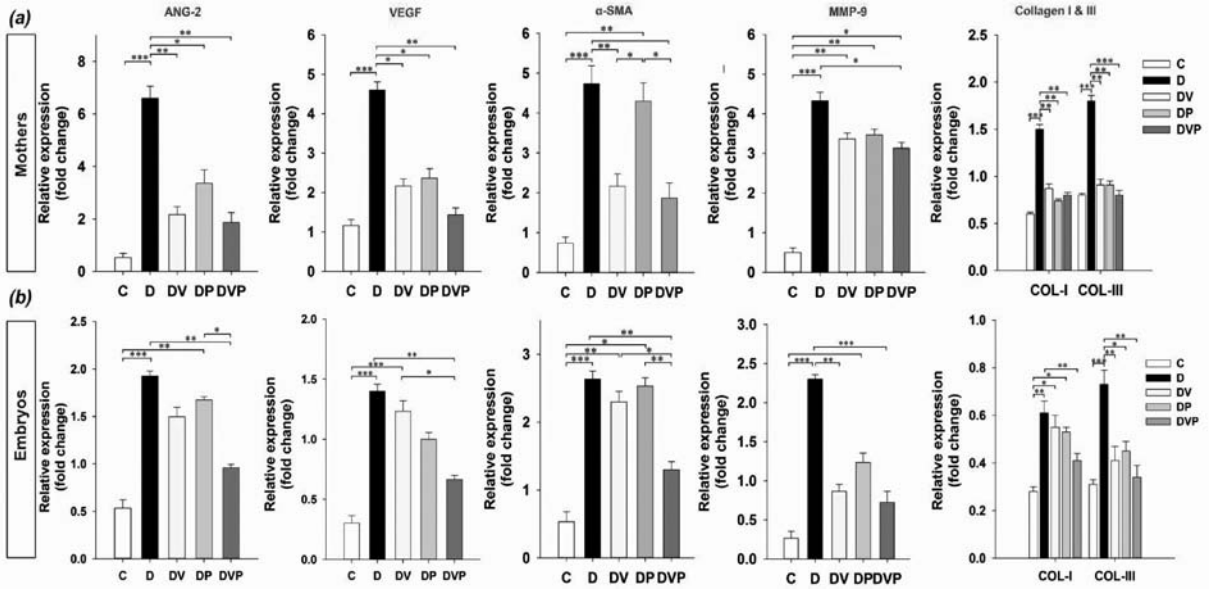

Fig. 6. Effects of vitamin D and/or pomegranate on maternal (***a***) and foetal (***b***) relative mRNA expression of myocardial angiogenic and fibrotic markers

Abbreviations: α-SMA – α-smooth muscle actin; ANG-2 – angiopoietin-2; C – control; COL-I, -III – collagen type-I, III; D – diabetic group; DP – diabetic group treated with pomegranate peel extract; DV – diabetic group treated with vitamin D; DVP – diabetic group treated with both vitamin D and pomegranate peel extract, MMP-9 – matrix metallopeptidase 9; VEGF – vascular endothelial growth factor.

Data were presented as mean ± SE (N = 5); * significant at $P < 0.05$, ** at $P < 0.01$ and *** at $P < 0.001$.

In addition, we observed suppressed expression of GLUT-4 and PPAR-γ associated with increased GSK-3β and MYH. In contrast, calcitriol and/or pomegranate treatments exhibited enhanced expression of the assayed MAPK cascade, PPAR-γ, and GLUT-4 and mitigated GSK-3β and MYH activities (Figs. 7, 8, 9, and 10).

Comet assay

Figure 11 depicted a significantly increased DNA damage in both maternal and foetal cardiomyocytes, in the diabetic groups, manifested by increased tail length and DNA percentage (~3-fold) ($P< 0.001$) compared to the control. However, comet tails produced in cardiomyocytes treated with vitamin D and/or pomegranate showed little or no migration of damaged DNA from the nuclei and substantially alleviated apoptotic activity within the cardiomyocytes.

Flow cytometric analysis of CASP-3

To explore the apoptotic activity in experimental groups, we analysed the activity of caspase-3 of maternal and foetal cardiomyocytes. Importantly, we observed dramatically activated caspase-3 expression in the myocardium of diabetic mothers and their embryos compared to the control ($P < 0.001$). On the other hand, vitamin D and/or pomegranate-treated groups showed remarkably suppressed activity of caspase-3 (Fig. 12).

Discussion

The main goals of our study were to characterize the detrimental effects of diabetes on mother rats and their developing foetuses, in addition to applying phytotherapeutic treatment using the pomegranate peel extract separately or in combination with vitamin D. Our findings show that diabetic mothers exhibited body weight loss and elevated fasting plasma glucose associated with decreased insulin levels. In parallel, we recorded increased circulating triglycerides, total cholesterol, low-density lipoproteins in STZ-induced diabetic mothers compared to the control (Lee et al., 2003; Guimaraes et al., 2015). These altered maternal sera profiles are associated with deteriorated cardiac contractility and increased myocardial injury markers including albumin, myoglobin and creatine kinase (Li et al., 2018). In contrast, treatment of STZ-diabetic mothers with vitamin D (Wei et al., 2017) and/or pomegranate (Salwe et al., 2015) restrained the assayed parameters.

Importantly, we observed reduced numbers of corpora lutea, implantation sites, live embryos, and litter weights of STZ-diabetic mothers. Additionally, we recorded significantly increased pre- and post-implantation loss percentages. Our results were corroborated by recent studies (Damasceno et al., 2013b) demonstrating that the altered intrauterine milieu fundamentally decreased the fecundity potential of diabetic mothers

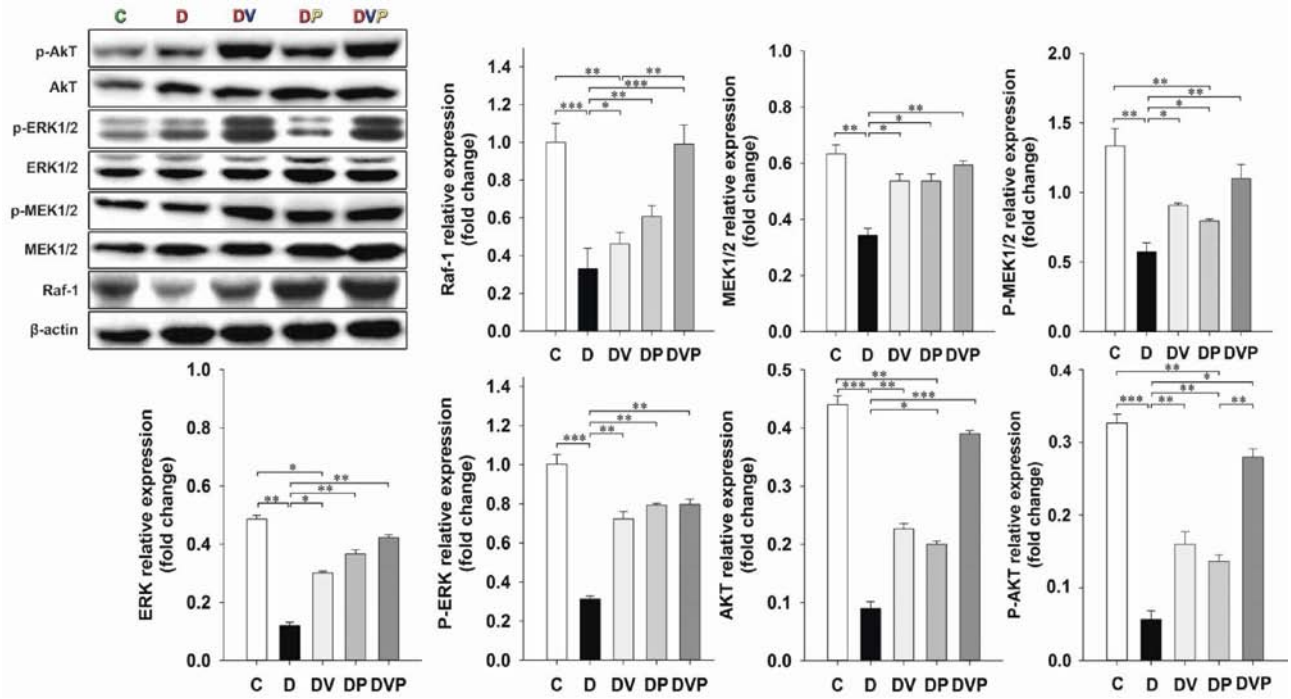

Fig. 7. Protein levels of maternal Raf-1, MEK, phospho-MEK, ERK, phospho-ERK, Akt and phospho-Akt in all experimental groups relative to the expression of the reference protein, β actin

Abbreviations: Akt – serine-threonine protein kinase; C – control; D – diabetic group; DP – diabetic group treated with pomegranate peel extract; DV – diabetic group treated with vitamin D; DVP – diabetic group treated with both vitamin D and pomegranate peel extract; ERK – extracellular signal–regulated kinase; MEK – mitogen-activated protein kinase; Raf – rapidly accelerated fibrosarcoma.

Values were expressed as mean ± SE (N = 5); * significant at $P < 0.05$, ** at $P < 0.01$ and *** at $P < 0.001$.

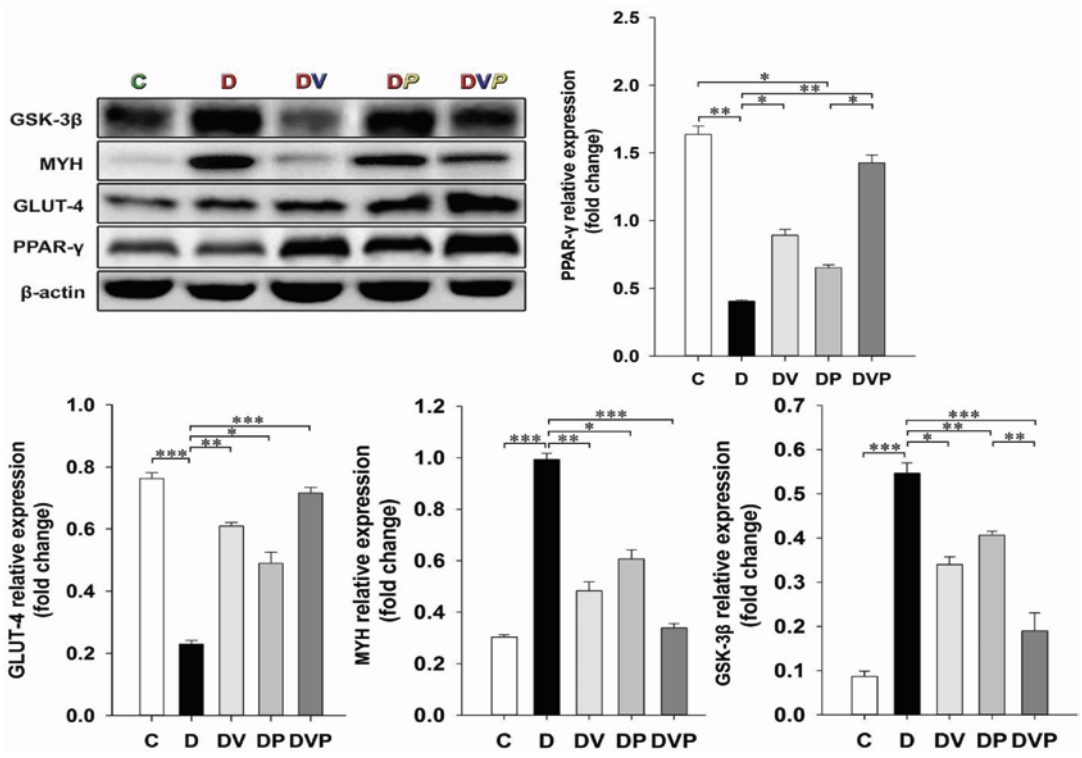

Fig. 8. Protein levels of maternal PPAR-γ, GLUT-4, MYH, GSK-3β in all experimental groups relative to the expression of the reference protein, β actin

Abbreviations: C – control; D – diabetic group; DP – diabetic group treated with pomegranate peel extract; DV – diabetic group treated with vitamin D; DVP – diabetic group treated with both vitamin D and pomegranate peel extract; GSK-3β – glycogen synthase kinase-3; MYH – myosin heavy chain.

Values were expressed as mean ± SE (N = 5); * significant at $P < 0.05$, ** at $P < 0.01$ and *** at $P < 0.001$.

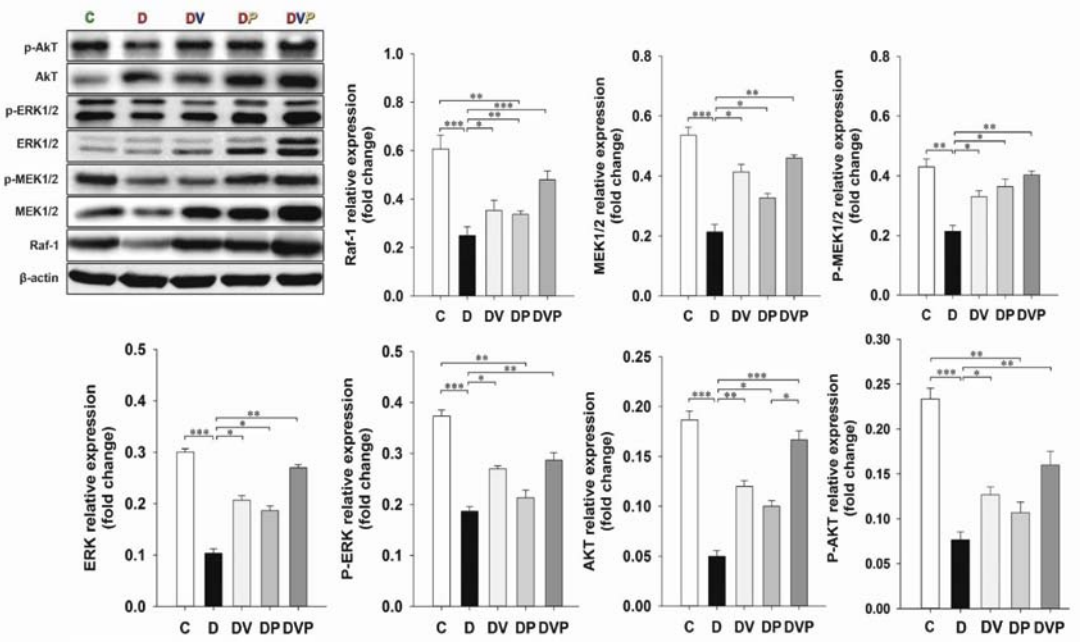

Fig. 9. Protein levels of fetal Raf-1, MEK, phospho-MEK, ERK, phospho-ERK, Akt and phospho-Akt in all experimental groups relative to the expression of the reference protein, β actin

Abbreviations: Akt – serine-threonine protein kinase; C – control; D – diabetic group; DP – diabetic group treated with pomegranate peel extract; DV – diabetic group treated with vitamin D; DVP – diabetic group treated with both vitamin D and pomegranate peel extract; ERK – extracellular signal-regulated kinase; MEK – mitogen-activated protein kinase; Raf – rapidly accelerated fibrosarcoma.

Values were expressed as mean ± SE (N = 5); * significant at $P < 0.05$, ** at $P < 0.01$ and *** at $P < 0.001$.

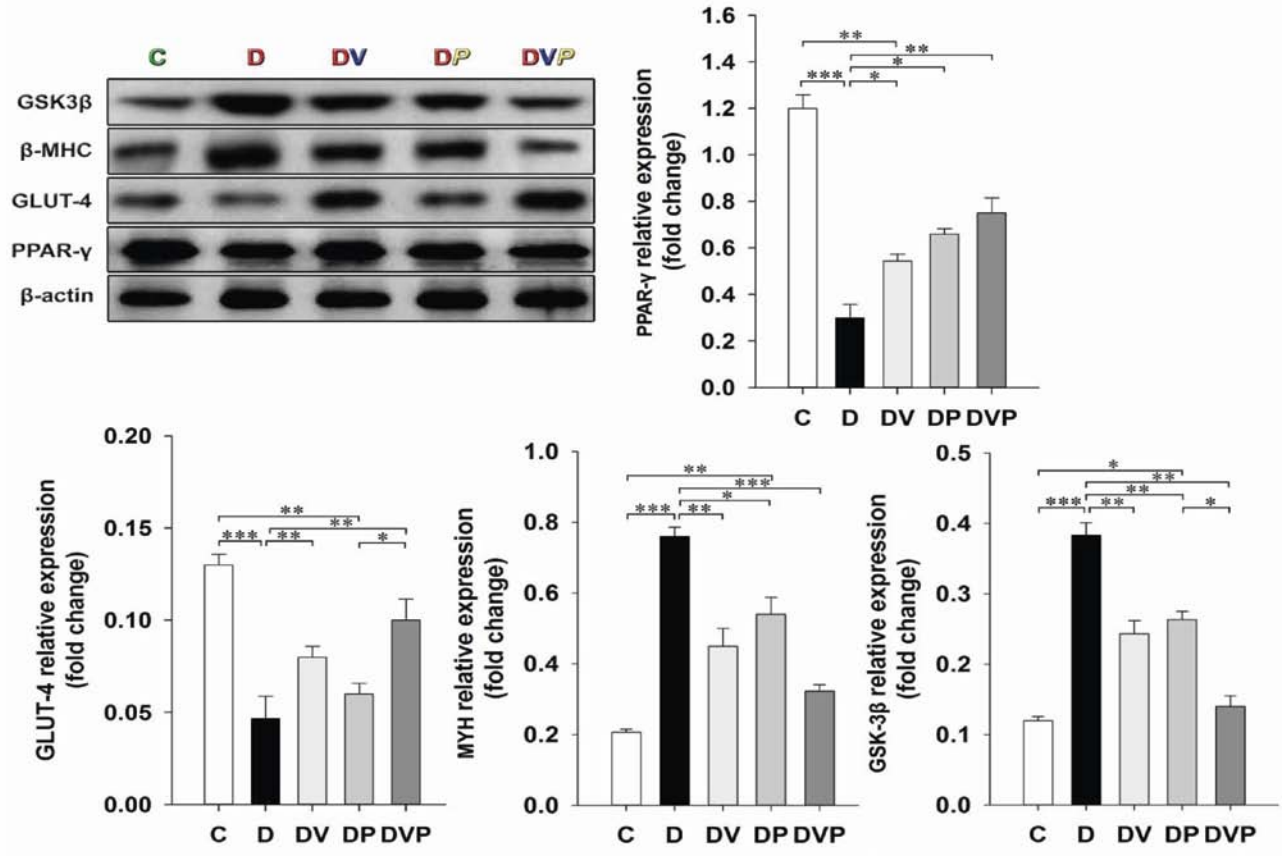

Fig. 10. Protein levels of foetal PPAR-γ, GLUT-4, MYH, GSK-3β in all experimental groups relative to the expression of the reference protein, β actin

Abbreviations: C – control; D – diabetic group; DP – diabetic group treated with pomegranate peel extract; DV – diabetic group treated with vitamin D; DVP – diabetic group treated with both vitamin D and pomegranate peel extract; GSK-3β – glycogen synthase kinase-3; MYH – myosin heavy chain.

Values were expressed as mean ± SE (N = 5); * significant at $P < 0.05$, ** at $P < 0.01$ and *** at $P < 0.001$.

coinciding with cardiac defects (Zhao et al., 2017). Accordingly, we observed decreased activities for both PPAR-γ and GLUT-4 (Asghar et al., 2009). Consistent with previous reports (Wang et al., 2009), our study revealed significant activation of GSK-3β and MHC and suppression of Akt and phosphorylated Akt in the hearts of experimentally STZ-induced diabetic rats, rising strong evidence for disrupted myocardial contractility.

In our histopathological findings, diabetic mothers revealed noticeable hypertrophy manifested by an increased myocardial cross-sectional area, irregular arrangement of myocardial fibres with internal haemorrhage (Al-Rasheed et al., 2016). However, embryos of diabetic mothers showed increased levels of inflammatory leukocytes, pyknosis, and vacuolar degeneration of myocardial cells (Lin et al., 2017). Notably, all of these histopathological alterations were prevented by treatment with calcitriol and/or pomegranate restoring the normal histological architectures. These histopathological alterations were directly linked to elevated levels of circulating Tp-I, ET-1, LDH, and CK-MB in diabetic mothers and their neonates (Kain et al., 2010). This myocardial injury represents the main culprit for the myocardial disintegration and increased vascular permeability (Howard-Alpe et al., 2006); however, ET-1 was implicated in exacerbation of endothelial damage and increased oxidative stress (Idris-Khodja et al., 2016). Also, we recorded increased levels of cardiac pro-inflammatory cytokines for both mothers and their neonates, including TGF-β, IL-6 and IL-1β. Previous reports have postulated that increased inflammatory responses triggered the signalling pathways of apoptosis (Rajesh et al., 2010; Suzuki et al., 2015), as evidenced in our study by increased capase-3 and comet DNA tailing (Kain et al., 2010; Rajesh et al., 2010).

Consistent with the aforementioned histopathological and inflammatory activities in the myocardium of diabetic mothers and their neonates, our results showed a significant decrease of CAT and SOD activities associated with increased GPx and MDA levels (Rajesh et al., 2010; Brouwers et al., 2013; Al-Rasheed et al., 2016). These findings, in the context of our study, represent clear evidence that hyperglycaemia is directly linked to liberation of reactive oxygen species (ROS) in myocardial cells (Damasceno et al., 2014). Accordingly, Kinalski et al. (2001) recorded increased levels of MDA

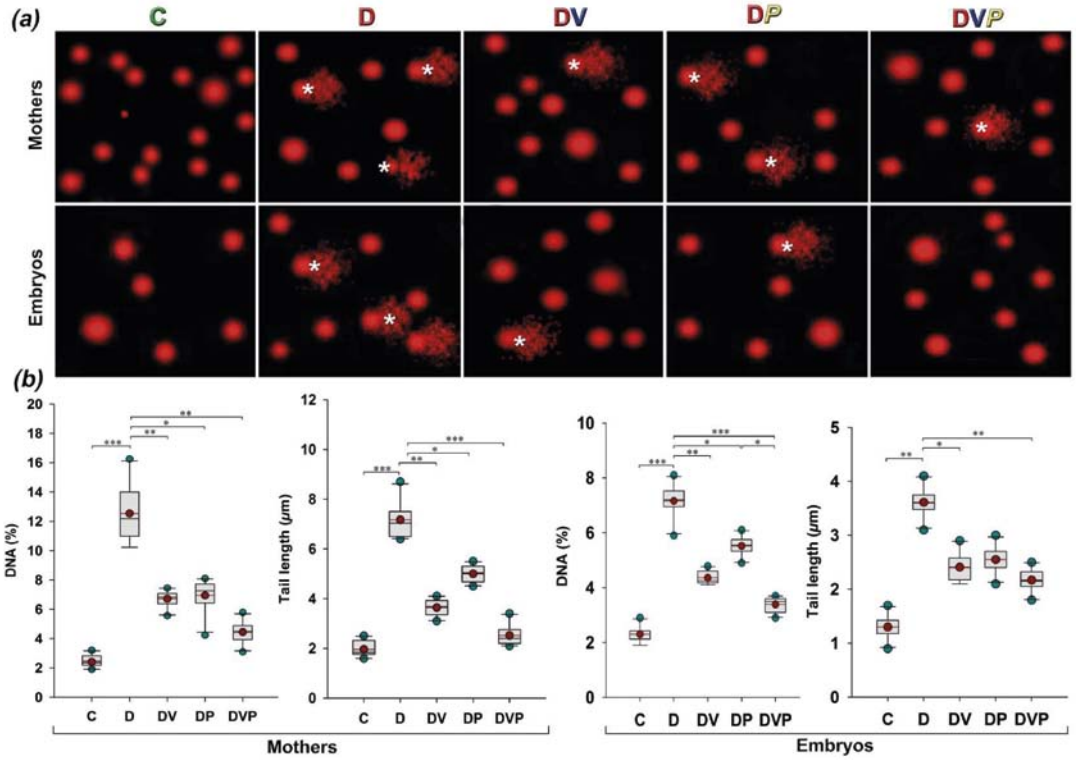

Fig. 11. (***a***) Representative images of maternal and foetal alkaline comet assay showed that vitamin D and/or pomegranate could attenuate myocardial DNA damage (asterisks refer to stretched myocardial cells with DNA damage). (***b***) Maternal and foetal DNA percentage and tail length quantification in different experimental groups. Abbreviations: C – control; D – diabetic group; DP – diabetic group treated with pomegranate peel extract; DV – diabetic group treated with vitamin D; DVP – diabetic group treated with both vitamin D and pomegranate peel extract.
Data were expressed as mean ± SE (N = 5); * significant at $P < 0.05$, ** at $P < 0.01$ and *** at $P < 0.001$.

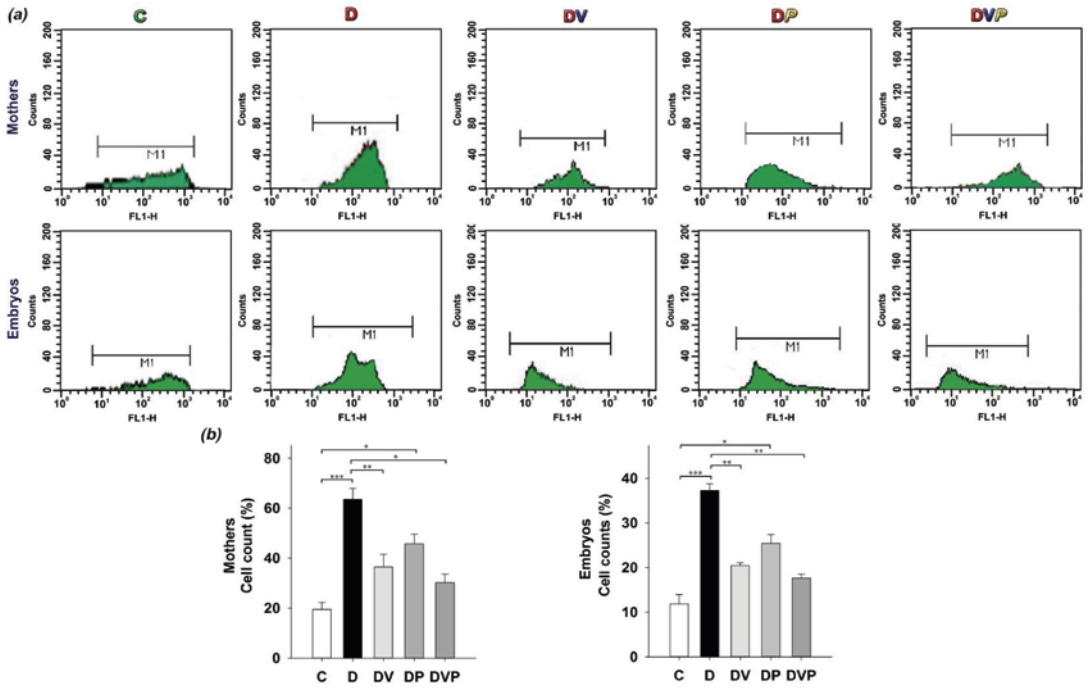

Fig. 12. (***a***) Representative flow cytometric graphs of maternal and foetal caspase-3 with FITC/PI double staining in different experimental groups. (***b***) Quantitative analysis of myocardial cell counts.
Abbreviations: C – control; D – diabetic group; DP – diabetic group treated with pomegranate peel extract; DV – diabetic group treated with vitamin D; DVP – diabetic group treated with both vitamin D and pomegranate peel extract.
Data were expressed as mean ± SE (N = 5); * significant at $P < 0.05$, ** at $P < 0.01$ and *** at $P < 0.001$.

and GSH in infants of pregestational diabetic dams, which gives strong evidence for foetal distress as a consequence of maternal hyperglycaemia. Concomitantly, there was increased expression of angiogenic (ANG-2 and VEGF) and fibrotic markers (α-SMA, MMP-9), as well as massive deposition of collagens (COL-I, COL-III) in the myocardium of diabetic mothers and their neonates. A recent study of Chen et al. (2012) emphasized that the increased ANG-2 and VEGF expression causes vascular inflammation via production of endothelial adhesion molecules ICAM and VCAM.

To the best of our knowledge, here we were the first to report that ERK1/2 and its regulatory cascade, Raf-1, MEK1/2 and phospho-MEK1/2, were remarkably decreased in cardiomyocytes of the neonates of pregestational diabetic mothers. Paradoxically, calcitriol and/or pomegranate administration notably enhanced both all assayed kinases and their upstream regulatory enzymes. These findings were emphasized by many recent studies interpreting the salutary function of ERK1/2 as a cardioprotective prosurvival key factor in diabetic cases (Lips et al., 2004; Kehat et al., 2011). In this setting, Cox and Der (2003) reported that ERK1/2 mitigated pro-apoptotic pathways, as demonstrated by decreased caspase-3 expression and comet tail lengths in our calcitriol and/or pomegranate-treated diabetic models, through blocking of the down-stream pathways of caspases, p53 and PKCζ. Moreover, it was demonstrated that specific deletion of ERK2 in the mouse model increased apoptosis (Sari et al., 2010; Ulm et al., 2014), and myocardial infarction and DNA laddering (Lips et al., 2004).

Here, we described the role of calcitriol and/or pomegranate in alleviation of these effects. In STZ-diabetic mothers and their embryos supplemented with calcitriol and/or pomegranate peel extract, our results showed considerable refinement and amelioration of myocardial cells, as well as diminished vacuolation, pyknosis and leukocytic infiltration. Moreover, the oxidative capacity was dramatically improved, associated with decreased inflammatory and fibrotic activities. Also, the maternal performance and offspring outcomes were remarkably normalized. Significantly, we observed the activated Raf/MEK/ERK cascade, specifically by dual administration, which enhanced the synergistic effects in the treatments.

Importantly, it has been reported that calcitriol is a key regulatory factor in cardiac differentiation and proliferation in embryonic and adult cells (Hlaing et al., 2014), in addition to ensuring availability of energy to the developing foetuses via regulation of glucose and insulin metabolism (Pittas et al., 2007). It has been well documented that calcitriol exerted hypoglycaemic effects through decreasing plasma glucose and improved myocardial functions in diabetic animal models, as demonstrated by decreased LDH and CK levels (Wei et al., 2017; Zeng et al., 2017). Also, calcitriol had been shown to antagonize production of inflammatory cytokines, causing noticeable reduction of matrix metalloproteinase (MMP) (Andress, 2006). Together, these fibrotic activities induce increased expression of collagen I and III, and TGF-β1 (Artaza and Norris, 2009).

Pomegranate fruit is rich in phenolic compounds, including punicalagin or ellagic acid; however, the predominant constituents of its peel are tannins (Lee et al., 2010; Ismail et al., 2012). Recent studies demonstrated that punicalagin, ellagic acid and ellagitannins exert anti-oxidative and anti-inflammatory effects (Lee et al., 2010; Cao et al., 2015). Also, in the context of our findings, large evidence showed that pomegranate supplementation reduced myocardial cell death via diminishing Tp-I, LDH, and CK-MB levels and quenching ROS coinciding with decreased lipid peroxidation in diabetics (Mollazadeh et al., 2016), exerting hypoglycaemic potentials (Parmar and Kar, 2007).

To sum up, the anti-oxidative, anti-inflammatory and anti-apoptotic properties of calcitriol and/or pomegranate played crucial roles in the cardio-protective potential in STZ-diabetic rats. Our records thus brought novel insights into the mechanism of action of calcitriol and/or PPE, as an anti-diabetic therapy, and identified new targets for the therapeutic treatment of diabetes via regulation of the Raf/MEK/ ERK pathways.

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Disclosure statement

The authors report no conflicts of interest in this work.

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