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Original Article

Progesterone eliminates 17β-estradiol-Mediated cardioprotection against diabetic cardiovascular dysfunction in ovariectomized rats

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ABSTRACT

Background: Type2 Diabetes (T2D) remains one of the most important causes of cardiovascular diseases (CVD). Menopause leads to an increase in CVD and metabolic syndrome, which indicates the role of sex steroids as a protective factor. In the present study, we surveyed the effects of 17β-estradiol (E2) alone and in combination with progesterone (P4) on cardiovascular dysfunction in T2D.

Methods: Female ovariectomized (OVX) diabetic rats were divided into eight groups: Sham-Control, Diabetes (Dia), OVX + Dia, OVX + Dia + Vehicle, OVX + Dia + E2, OVX + Dia + P4, OVX + Dia + E2+P4, and OVX + Dia + E2+Vehicle. T2D was induced by a high-fat diet and streptozotocin. E2 and P4 were administrated every four days for four weeks. The heart cytokines and angiotensin II, lipid profile, insulin, water, and food intake and cardiovascular indices were measured.

Results: Results showed that single treatment with E2 decreased fasting blood glucose, water, and food intake, atherogenic and cardiac risk indices, and blood pressure. Also, P4 led to a decrease in atherogenic and cardiac risk indices. TNFα and IL-6 levels were increased and IL-10 was decreased in the Dia group, while E2 alone was able to inhibit these changes. The combined use of E2 and P4 eliminated the beneficial effects of E2 on these indices. Although diabetes results in an increment of cholesterol, LDL and triglyceride, hormone therapy with E2 was associated with improved dyslipidemia.

Conclusion: The use of E2 alone, and not the individual use of P4, and its combination with E2 improved cardiovascular function in OVX diabetic animals, possibly by reducing the amount of inflammatory cytokines and improving metabolic parameters.

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At a glance commentary

Scientific background on the subject

Post-menopausal diabetic women have more cardiovascular diseases than healthy women, which indicates the role of sex hormones as mediators. Ovarian hormones play a key role in the regulation of metabolism that may influence diabetic cardiomyopathy; but, which hormone or combination of hormones, or how it or they work, isn't yet apparent.

What this study adds to the field

This study adds new knowledge that the increase in cardiometabolic disorders observed in a postmenopausal diabetes is related to increased inflammation, but only estrogen therapy improved cardiometabolic dysfunction through a diminution in the cardiac inflammatory cytokines. However, combination therapy with estrogen-progesterone eliminated the cardiometabolic protective effects of estrogen in postmenopausal diabetes.

Cardiovascular diseases (CVD) are the main cause of death in postmenopausal women (PMW). Although it is beyond doubt that CVD are multifactorial and several factors come into play, such a difference has been attributed to the cardioprotective effects of female sex hormones, especially 17β-estradiol (E2) and progesterone (P4), before menopause [1].

Type 2 diabetes (T2D) is a major risk factor for myocardial damage and CVD [2]. Growing numbers of clinical and basic studies have confirmed that hyperglycemia is the leading cause of the progression of diabetic cardiomyopathy. An acute increase in circulating glucose level induces structural and functional changes in the cardiomyocytes through the activation of several signal pathways including cardiac hypertrophy, inflammation, and fibrosis. Chronic inflammation is a common feature of T2D and many inflammatory indicators produced by adipocytes are associated with the prevalence of diabetes. Chronic inflammation, directly and indirectly, results in increased myocardial fibrosis, necrosis, and cardiac failure [3]. This is reinforced by increased expression of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin 6 (IL-6) in myocardium. Excessive pro-inflammatory cytokines level results in left ventricular dysfunction and cardiomyopathy [4].

Steroid sex hormones are a regulator of inflammation, exerting a number of known anti-inflammatory effects through a variety of distinct genomic and non-genomic pathways. Their anti-inflammatory roles range from regulating leukocyte recruitment to reducing oxidative stress and promoting cell survival. These effects contribute to dampening inflammation in the cardiovascular system [5].

Sex hormones play a key role in the regulation of food intake, energy expenditure and sugars and lipids homeostasis [6]. There is considerable evidence that the risk conferred by T2D is higher in women than in men. The elevated risk of CVD in PMW seems related, in part, to the loss of the protection offered by endogenous steroid hormones. However, despite this epidemiological evidence, the mechanisms by which T2D impairs the cardiovascular function have not yet completely manifested [7].

E2 is known as an anti-inflammatory agent that inhibits the production of inflammatory cytokines such as IL-6, TNFα, and interleukin 1β (IL-1β), and P4 therapy also reduces the expression of inflammatory cytokines [8]. In ovariectomized (OVX) animals, E2 deficiency has been reported to increase the expression of Angiotensin-converting enzyme (ACE), and thereby increasing angiotensin II (Ang II) [9]. It has been shown that P4 stimulates α and β-pancreatic cells to increase insulin secretion and improve insulin's liver response [10].

Considering the above evidence, postmenopausal diabetic women have more CVD than postmenopausal non-diabetic women, and this increase in CVD can be due to the lack of female sex steroids. Therefore, in the present study, the effects of female sex steroids (E2 and P4), alone and combined were investigated on cardiovascular indices in OVX (menopause model) diabetic animals. Moreover, in order to determine the possible cardiovascular protective effects of these steroids, the metabolic and cardiovascular indices, Ang II, changes in cytokine production, and inflammatory balance were also investigated in this study.

Materials and methods

Animals

Female Wistar rats (aged 3–4 months and body weight of 200–250 g) were used in this study, in accordance with the national guidelines for the Care and Use of Laboratory Animals. All procedures were approved by the ethical committee (Permission No: 95/105 KA). Care was taken to avoid stressful conditions, and all procedures were performed between 9 and 11 a.m.

Experimental protocol

The schematic representation of the experimental protocol is illustrated in Fig. 1. All of the animals were OVX two weeks before the experiments as previously described [11]. In order to induce diabetes, rats were placed on a modified high fat diet (HFD) [12] containing powdered normal pellet diet (365 g/kg), sheep fat (310 g/kg), casein (250 g/kg), cholesterol (10 g/kg), vitamin and mineral mix (60 g/kg), DL-methionine (3 g/kg), yeast powder (1 g/kg), and NaCl (1 g/kg) for 8 weeks. Afterward, animals were kept on an overnight fast and injected a single dose of streptozotocin (STZ, 30 mg/kg). After 3 days, blood sample was collected from the tail vein and rats with a fasting blood glucose (FBG) range ≥300 mg/dL (11.1 mM) were considered diabetic and included in the study. Diabetic rats were maintained on the HFD for an additional 8 weeks. Animals were randomly divided into 8 groups of 6 animals in each group. Group 1: Sham-control; 2: Diabetes (Dia); 3: OVX + Dia; 4: OVX + Dia + Vehicle (Veh); 5: OVX + Dia + E2 (1 mg/kg) [13]; 6: OVX + Dia + P4 (8 mg/kg) [13]; 7: OVX + Dia + E2+Veh; 8: OVX + Dia + E2+P4. Vehicle (Dimethyl sulfoxide [DMSO]), E2 and P4 were administered through intraperitoneal (i.p.) injection every four days (in order to mimic the natural estrous

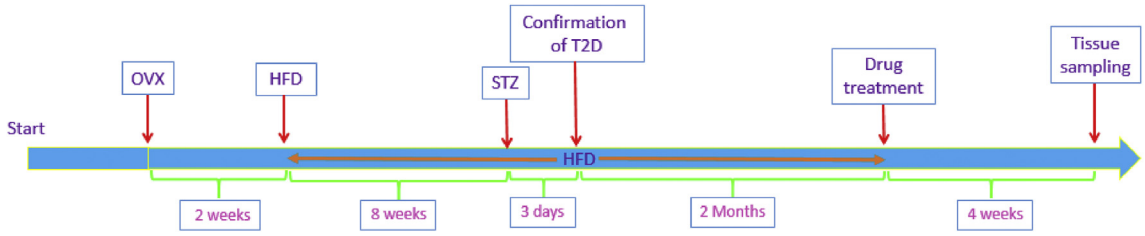

Fig. 1 Schematic representation of the experimental protocol. Abbreviations: HFD: High fat diet; OVX: Ovariectomy; STZ: Streptozotocin; T2D: Type 2 diabetes.

cycle) for four weeks after two months of induction of diabetes. Body weight was recorded at the end of the study while food and water intake were recorded each day. Mean blood pressure (MBP), heart rate (HR) recording were performed as described previously [14]. At the end, blood samples were collected from the left ventricle under deep anesthesia. The hearts were removed and rinsed with cold saline and then weighed. The degree of cardiac hypertrophy was assessed by the ratio of heart weight to body weight (mg/g), called cardiac weight index (CWI) [14].

Drugs

STZ and DMSO were purchased from Sigma (St. Louis, MO, USA). Ketamine and xylazine were purchased from Alfasan Inc., Utrecht, Netherlands. E2 and P4 were obtained from Aburaihan Pharmaceutical (Tehran, Iran). STZ was dissolved in 0.1 M citrate buffer (pH 4.4). E2 and P4 were dissolved in DMSO before administration.

Estimation of anthropometric parameters

Body weight, food, and water consumption were recorded in each group. Body weight was recorded at the end of the study while food and water consumption were recorded each day.

Blood biochemistry, cardiovascular, and atherogenic indices

Total cholesterol (TC), triglycerides (TG), HDL-cholesterol (HDL-C) and FBG were determined spectrophotometrically using commercial kits (Pars Azmoon, Iran). Also, plasma insulin was assayed by ELISA Kit (Hangzhou, Eastbiopharm, China). The low-density lipoprotein (LDL) cholesterol, atherogenic index, and the cardiovascular risk indices were calculated using the following formulas:

$$\text{LDL cholesterol} = \text{total cholesterol} - [\text{HDL cholesterol} + (\text{triglyceride}/5)]$$ [15].

$$\text{Atherogenic index} = [(\text{total cholesterol} - \text{HDL cholesterol})/\text{HDL cholesterol}]$$ [15].

$$\text{Cardiovascular risk index} = \text{Total cholesterol}/\text{HDL cholesterol}$$ [14].

Evaluation of HOMA-IR, HOMA-β, and QUICKI

We used the homeostasis model assessment (HOMA) to assess insulin resistance {HOMA-IR = [fasting glucose (mmol/L) × fasting insulin (μU/mL))/22.5]} [16] and pancreatic β-cell function {HOMA β-cell = [20 × fasting insulin (μU/mL)]/[fasting glucose (mmol/L)−3.5]} [16]. Also, Quantitative Insulin Sensitivity Check Index (QUICKI) was calculated using fasting insulin and glucose levels according to the following formula: {QUICKI = 1/[log(fasting insulin)+ 1/log (fasting glucose)]} [17].

Measurement of cardiac inflammatory markers and Ang II

The levels of TNF-α, IL-6, IL-10, and Ang II were measured from the left ventricle (LV) homogenate by enzyme-linked immunosorbent assay (ELISA). Cytokines TNF-α, IL-6, IL-10, and Ang II in myocardium were assayed using Hangzhou, Eastbiopharm (China) kits. The ELISA procedure was performed according to the manufacturer's protocol. The inflammatory balance was calculated from the ratio of the pro-inflammatory cytokines TNF-α and IL-6 levels to the anti-inflammatory cytokine IL-10 level, which provides important information about the state of cardiac inflammation.

Statistical analyses

All statistical analyses were performed by the GraphPad Prism 6.0 software (GraphPad Software, San Diego, CA, USA). Statistical differences were assessed using a one-way analysis of variance, followed by the Tukey–Kramer as a post hoc test. Results are presented as mean ± S.E.M and $p < 0.05$ was considered as statistically significant.

Results

P4 therapy eliminated the effects of E2 on body weight, water, food intake and lipid profile

Changes in body weight and water and food intake are shown in Table 1. Diabetes reduced the body weight and increased

Table 1 The effects of diabetes, ovariectomy, E2 and P4 on the physiological and biochemical parameter.

Parameters	Sham-Con	Dia	OVX + Dia	OVX + Dia + Veh	OVX + Dia + E2	OVX + Dia + P4	OVX + Dia + E2+Veh	OVX + Dia + E2+P4
BW (% change body)	7.9 ± 1.8	−6.05 ± 1.2***	−13.9 ± 0.5$^{\#\#\#}$	−16.2 ± 0.8	−7.2 ± 1†††	−10.3 ± 1.1††	−5.2 ± 1	−4.5 ± 0.7
UW (g)	0.63 ± 0.02	0.42 ± 0.02***	0.3 ± 0.03	0.31 ± 0.01	0.55 ± 0.03†††	0.34 ± 0.01	0.58 ± 0.04	0.41 ± 0.04‡‡
FI (g/day/rat)	22 ± 0.6	57 ± 2.7***	60 ± 1.4	62 ± 1.1	51 ± 2.6†	57 ± 2	52 ± 1.6	57 ± 2.2
WI (ml/day/rat)	24.5 ± 1.3	61.7 ± 3.1***	84.7 ± 2.6$^{\#\#}$	86 ± 3.1	72.6 ± 1.7†	78 ± 2.7	68 ± 1.7	65 ± 2.9
TG (mg/dl)	38 ± 1	61 ± 2***	84 ± 2$^{\#\#\#}$	83 ± 2	62 ± 2†††	78 ± 2	68 ± 5	59 ± 1‡‡‡
TC (mg/dl)	52 ± 2	106 ± 4***	128 ± 3$^{\#}$	126 ± 2	101 ± 4††	117 ± 5	99 ± 4	101 ± 3
HDL (mg/dl)	52 ± 1	39 ± 1***	30 ± 1$^{\#}$	29 ± 1	38 ± 1†	36 ± 2	38 ± 1.3	41 ± 1.5
LDL (mg/dl)	7 ± 1	53 ± 4***	81 ± 4$^{\#\#}$	79 ± 3	50 ± 4†††	65 ± 4.9	45 ± 4.7	48 ± 4.2

Data are expressed as mean ± S.E.M., n = 6 rats/group. ***$p < 0.001$ vs. Sham + Con. #$p < 0.05$, ##$p < 0.01$ and ###$p < 0.001$ vs. Dia. †$p < 0.05$, ††$p < 0.01$ and †††$p < 0.001$ vs. OVX + Dia + Veh. ‡‡$p < 0.01$ and ‡‡‡$p < 0.001$ vs. OVX + Dia + E2+Veh. Abbreviations: BW: body weight; Dia: diabetes; E2: 17β-estradiol; FI: food intake; HDL: high density lipoprotein; LDL: low density lipoprotein; OVX: ovariectomized rats; P4: progesterone; Sham-Con: Sham-control; TC: total cholesterol; TG: triglyceride; UW: uterus weight; Veh: vehicle; WI: water intake.

water and food intake compared to the sham-control group ($p < 0.001$) and the ovariectomy increased the effects of diabetes on body weight (OVX + Dia vs. Dia, $p < 0.001$) and water intake (OVX + Dia vs. Dia, $p < 0.01$). While E2 therapy eliminated the effects of diabetes and ovariectomy in the three aforementioned indices, P4 therapy only prevented weight loss (OVX + Dia + P4 vs. OVX + Dia + Veh, $p < 0.01$). On the other hand, E2 in combination with P4 did not have any effect on body weight, water and food intake as compared to the E2+Veh group.

As shown in Table 1, the induction of diabetes increased the serum levels of TG, TC, and LDL while reducing HDL (Dia vs. Sham-Con, $p < 0.001$). In addition, the ovariectomy in diabetic animals exacerbated these changes. Unlike treatment with P4, E2 therapy led to a decrease in TG, LDL ($p < 0/001$) and TC ($p < 0/01$) and an increase in HDL (OVX + Dia + E2 vs. OVX + Dia + Veh, $p < 0/05$) levels.

E2 therapy in combination with P4 reversed the effects of E2 on uterine weight

Table 1 shows the changes in uterine weights in different groups. As shown, T2D reduced the uterine weight compared to the sham-control group ($p < 0.001$). While E2 prevented the uterine weight loss due to the ovariectomy (OVX + Dia + E2 vs. OVX + Dia + Veh, $p < 0.001$), P4 did not change this index. On the other hand, E2 therapy in combination with P4 reversed the effects of E2 on uterine weight, so that in this group the weight of the uterus reduced compared to the E2+Veh group ($p < 0.01$).

E2 therapy in combination with P4 had no effect on the pancreatic status

As shown in Table 2, this method of inducing T2D, on the one hand, led to decreased insulin level ($p < 0.05$), HOMA-β and QUICKI ($p < 0.001$), and on the other hand, increased FBG and HOMA-IR ($p < 0.001$) compared to the sham-control group. When diabetes is combined with the ovariectomy, it leads to a greater increase in FBG and HOMA-IR (OVX + Dia vs. Dia, $p < 0.001$ and $p < 0.01$, respectively). Contrary to E2 treatment, which reduced FBG and HOMA-IR compared to the Veh group ($p < 0.001$ and $p < 0.01$, respectively), P4, either alone or in combination with E2 did not change the two variables. Also, combination therapy, similar to single P4 therapy, has no effect on HOMA-β and QUICKI.

E2 therapy in combination with P4 had no effect on cardiovascular indices and Ang II

Table 3 shows the changes in cardiovascular function. Diabetes increased MBP, cardiac risk index ($p < 0.001$), CWI, and atherogenic index ($p < 0.05$) compared to the sham-control group. On the other hand, when the ovaries were removed, the effects of diabetes on the expressed indices were intensified. Treatment with E2 reduced MBP, CWI ($p < 0.01$), cardiac

Table 2 The effects of diabetes, ovariectomy, E2 and P4 on pancreatic status.

Parameters	Sham-Con	Dia	OVX + Dia	OVX + Dia + Veh	OVX + Dia + E2	OVX + Dia + P4	OVX + Dia + E2+Veh	OVX + Dia + E2+P4
Ins (mIU/L)	22.5 ± 1.6	16 ± 0.7*	14.2 ± 1.2	13.6 ± 1.1	14.7 ± 1.3	15.7 ± 1.2	14.5 ± 1.1	16.9 ± 0.9
FBG (mg/dl)	106 ± 3	469 ± 30***	677 ± 28$^{\#\#\#}$	670 ± 19	497 ± 21†††	584 ± 35	508 ± 16	475 ± 25
HOMA-IR	5.7 ± 0.3	18.2 ± 0.5***	24.1 ± 1$^{\#\#}$	23.1 ± 1.1	17.4 ± 0.9††	22.9 ± 2.5	16.9 ± 0.9	19.8 ± 1.3
HOMA-β	211 ± 26	13.39 ± 0.64***	8.5 ± 0.88	8 ± 0.53	13.95 ± 1.3	11 ± 0.9	12.6 ± 1	15.2 ± 0.7
QUICKI	0.296 ± 0.001	0.258 ± 0.0009***	0.251 ± 0.001	0.253 ± 0.001	0.259 ± 0.001	0.253 ± 0.003	0.259 ± 0.001	0.256 ± 0.001

Data are expressed as mean ± S.E.M., n = 6 rats/group. *$p < 0.05$ and ***$p < 0.001$ vs. Sham-Con. ##$p < 0.01$ and ###$p < 0.001$ vs. Dia.††$p < 0.01$ and †††$p < 0.001$ vs. OVX + Dia + Veh. Abbreviations: Dia: diabetes; E2: 17β-estradiol; FBG: Fasting Blood Glucose; HOMA-IR: Homeostasis Model Assessment of Insulin Resistance; HOMA-β: Homeostasis Model Assessment of β-cell function; Ins: insulin; OVX: ovariectomized rats; P4: progesterone; QUICKI: Quantitative insulin sensitivity check index; Sham-Con: Sham-control; Veh: vehicle.

Table 3 The effects of diabetes, ovariectomy, E2 and P4 on cardiovascular function.

Parameters	Sham-Con	Dia	OVX + Dia	OVX + Dia + Veh	OVX + Dia + E2	OVX + Dia + P4	OVX + Dia + E2+Veh	OVX + Dia + E2+P4
MBP (mmHg)	97 ± 2	***119 ± 1	130 ± 2$^{\#}$	132 ± 2	118 ± 1††	127 ± 2	117 ± 2	115 ± 2
HR (bpm)	383 ± 10	406 ± 6	396 ± 12	388 ± 16	402 ± 4	366 ± 12	381 ± 14	350 ± 15
HW (mg)	683 ± 30	750 ± 34	716 ± 30	700 ± 25	700 ± 25	716 ± 33	700 ± 25	733 ± 33
CWI (mg/g)	3.4 ± 0.1	4 ± 0.09*	4.8 ± 0.09$^{\#\#}$	5 ± 0.1	4.2 ± 0.1††	5.2 ± 0.1	4.2 ± 0.1	4.6 ± 0.2
AI (units)	0.99 ± 0.02	1.8 ± 0.13*	3.3 ± 0.24$^{\#\#\#}$	3.2 ± 0.19	1.6 ± 0.14†††	2.2 ± 0.1††	1.5 ± 0.09	1.4 ± 0.1
CRI	0.99 ± 0.02	2.8 ± 0.13***	4.3 ± 0.24$^{\#\#\#}$	4.2 ± 0.19	2.6 ± 0.14†††	3.2 ± 0.1††	2.5 ± 0.09	2.4 ± 0.1

Data are expressed as mean ± S.E.M., n = 6 rats/group. *$p < 0.05$ and ***$p < 0.001$ vs. Sham-Con. #$p < 0.05$, ##$p < 0.01$ and ###$p < 0.001$ vs. Dia. ††$p < 0.01$ and †††$p < 0.001$ vs. OVX + Dia + Veh. Abbreviations: AI: Atherogenic index; CWI: cardiac weight index; CRI: Cardiovascular risk index; Dia: diabetes; E2: 17β-estradiol; HR: heart rate; HW: heart weight; MBP: mean blood pressure; OVX: ovariectomized rats; P4: progesterone; Sham-Con: Sham-control; Veh: vehicle.

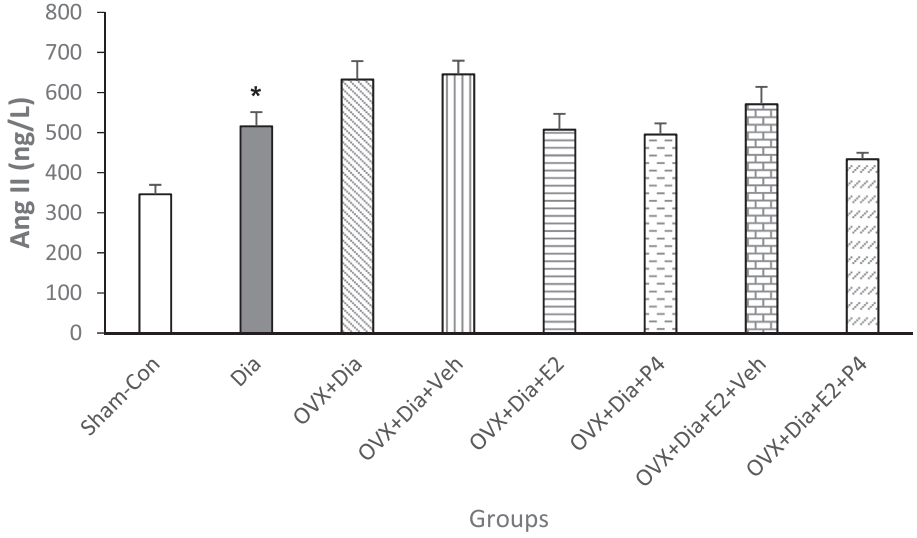

Fig. 2 The effect of diabetes, ovariectomy, E2 and P4 on the level of Ang II in the heart. Data are expressed as mean ± S.E.M., n = 6 rats/group. *$p < 0.05$ vs. Sham-Con. Abbreviations: Dia: diabetes; E2: 17β-estradiol; OVX: ovariectomized rats; P4: progesterone; Sham-Con: Sham-control; Veh: vehicle.

risk and atherogenic indices ($p < 0.001$) in comparison to the Veh group. Similar to E2, P4 therapy also reduced the atherogenic and cardiac risk indices compared to the Veh group ($p < 0.01$). Unlike single therapy, E2 therapy in combination with P4 had no effect on cardiovascular indices. Moreover, as shown in Fig. 2, T2D increased cardiac Ang II level (Dia vs. Sham-Con, $p < 0.05$), and ovariectomy failed to alter the level of Ang II in the heart. In addition, both single and combination therapy with E2 and P4 led to a decrease in elevated Ang II level caused by diabetes, but this reduction was not significant.

E2 and P4 therapy alone or in combination have no effect on cardiac cytokines

Fig. 3 shows the changes in cardiac TNF-α concentration in the various groups. The induction of diabetes increased the level of this cytokine compared to the sham-control group ($p < 0.001$), and induction of menopause model did not change the cardiac level of this cytokine. However, both single and combination therapy with E2 and P4 reduced the amount of this cytokine, but these changes were not significant.

Similar to TNF-α, the cardiac level of IL-6 was also increased by the induction of diabetes (Dia vs. Sham-Con, $p < 0.001$, Fig. 4), and ovariectomy did not have any effect on this cytokine. Although P4, similar to E2, was able to reduce the level of IL-6, unlike E2 ($p < 0.05$), this decrease was not significant in the P4 group compared to the Veh group. Also, E2 in combination with P4 was not able to significantly reduce the level of IL-6 in the heart compared to the E2+Veh group (Fig. 4).

The cardiac level of IL-10 is shown in Fig. 5. T2D lowered the level of IL-10 in the heart (Dia vs. Sham-Con, $p < 0.001$) and this decrease was intensified by the ovariectomy (OVX + Dia vs. Dia, $p < 0.05$). Conversely, E2 therapy resulted in a significant increase in this cytokine compared to the Veh group ($p < 0.05$). While P4 either individually or in combination with E2, could not correct the changes in this cytokine.

Single therapy with E2 and P4 was able to change the inflammatory balance

As shown in Fig. 6, the TNF-α/IL-10 ratio increased with the induction of diabetes (Dia vs. Sham-Con, $p < 0.001$), and this

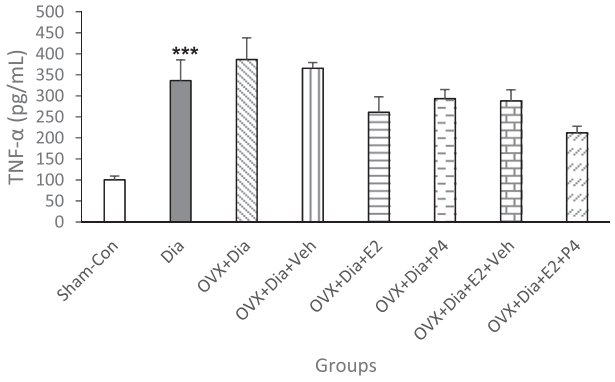

Fig. 3 The effect of diabetes, ovariectomy, E2 and P4 on the level of TNF-α in the heart. Data are expressed as mean ± S.E.M., n = 6 rats/group. ***$p < 0.001$ vs. Sham-Con. Abbreviations: Dia: diabetes; E2: 17β-estradiol; OVX: ovariectomized rats; P4: progesterone; Sham-Con: Sham-control; Veh: vehicle.

increase intensified when the ovaries were removed (OVX + Dia vs. Dia, $p < 0.001$). In contrast, hormone therapy with E2 and P4 resulted in a reduction in this ratio compared to the Veh group ($p < 0.001$ and $p < 0.05$, respectively). However, combination therapy did not change significantly in TNF-α/IL-10 compared to the E2+Veh group. The observed changes in the IL-6/IL-10 ratio were similar to those of the TNF-α/IL-10 ratio, so this ratio was also increased by induction of diabetes compared to the sham-control group ($p < 0.05$, Fig. 7). This ratio was also higher in the OVX diabetic group than in the diabetic group ($p < 0.05$, Fig. 7). The effects of treatment with E2 and P4 on this ratio were similar to that of TNF-α/IL-10 ratio, so that individual use of E2 and P4 reduced the ratio (OVX + Dia + E2 and OVX + Dia + P4 vs. OVX + Dia + Veh, $p < 0.001$ and $p < 0.05$, respectively, Fig. 7), and when combined therapy was performed, there was no change in this ratio compared to the E2+Veh group.

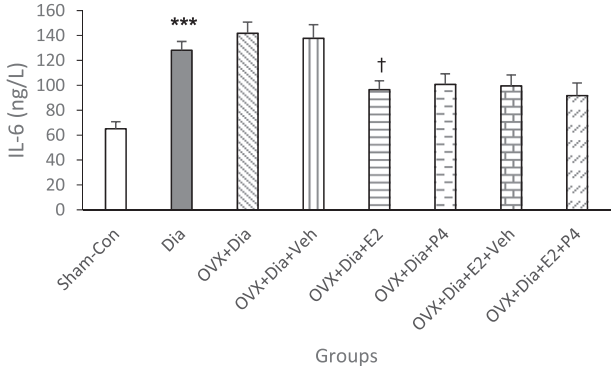

Fig. 4 The effect of diabetes, ovariectomy, E2 and P4 on the level of IL-6 in the heart. Data are expressed as mean ± S.E.M., n = 6 rats/group. ***$p < 0.001$ vs. Sham-Con. †$p < 0.05$ vs. OVX + Dia + Veh. Abbreviations: Dia: diabetes; E2: 17β-estradiol; OVX: ovariectomized rats; P4: progesterone; Sham-Con: Sham-control; Veh: vehicle.

Discussion

Since both E2 and P4 are produced in the ovaries and released into the bloodstream, and so far, their combined therapeutic effects on cardiovascular dysfunction caused by diabetes have not been determined, in the present study, the cardiovascular protective effects of these female sex hormones in OVX diabetic animals (menopause model) were examined with an emphasis on the role of cytokines and metabolic parameters. The main findings of this study include: (1) Cardiovascular dysfunction in diabetes was caused by changes in lipid profiles, insulin resistance, blood pressure, and cytokines. (2) Diabetes along with menopause caused exacerbations of metabolic and cardiovascular disorders, which may be due to changes in cytokines. (3) Hormone therapy with E2 improved cardiovascular dysfunction caused by diabetes and menopausal status by making changes in metabolic parameters as well as changes in cardiac cytokines, in which E2 plays a more important role in these effects. (4) The combined use of E2 and P4 eliminated the cardiovascular protective effects of E2 in T2D after menopause.

Studies have shown that reduced body weight and increased water and food intake happens in such condition, and E2 therapy reduces the water and food intake [18]. P4 therapy has also been reported to prevent weight loss in diabetic animals [19]. Sex hormones are known to regulate food intake by increasing the anorexigenic effect of leptin and cholecystokinin [20].

The induction of T2D and ovariectomy led to defects in lipid profile and, unlike P4, E2 therapy decreased LDL and TC. Additionally, combined therapy improved lipid profile so that, if the target is TG reduction, combined therapy would be more effective. Studies have shown that OVX diabetic rats have a defect in their lipid profile and confirmed the successful induction of T2D by lipid profile defects [21]. Injection of E2 in

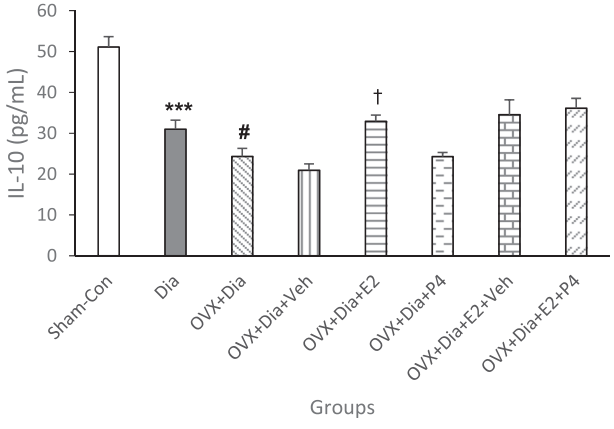

Fig. 5 The effect of diabetes, ovariectomy, E2 and P4 on the level of IL-10 in the heart. Data are expressed as mean ± S.E.M., n = 6 rats/group. ***$p < 0.001$ vs. Sham-Con. #$p < 0.05$ vs. Dia. †$p < 0.05$ vs. OVX + Dia + Veh. Abbreviations: Dia: diabetes; E2: 17β-estradiol; OVX: ovariectomized rats; P4: progesterone; Sham-Con: Sham-control; Veh: vehicle.

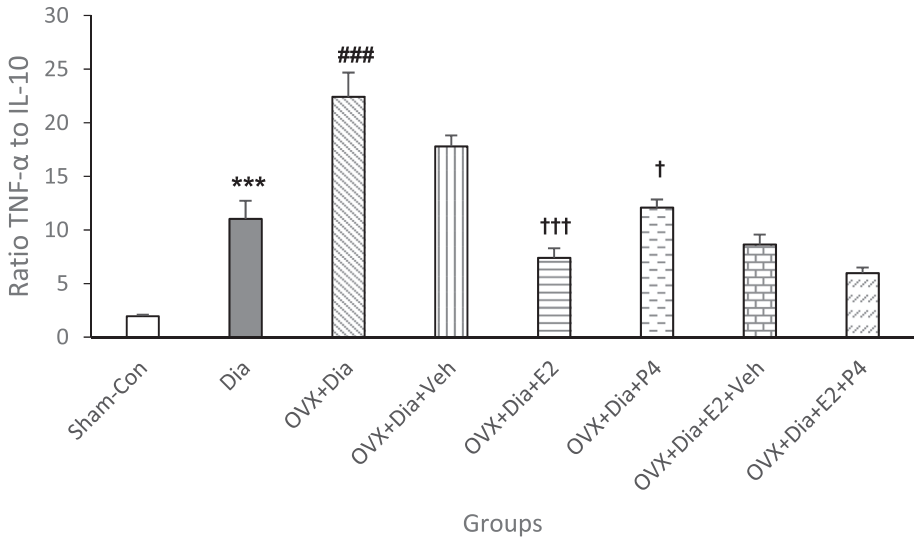

Fig. 6 The effect of diabetes, ovariectomy, E2 and P4 on the ratio of TNF-α to IL-10 in the heart. Data are expressed as mean ± S.E.M., n = 6 rats/group. ***$p < 0.001$ vs. Sham-Con. ###$p < 0.001$ vs. Dia.†$p < 0.05$ and †††$p < 0.001$ vs. OVX + Dia + Veh. Abbreviations: Dia: diabetes; E2: 17β-estradiol; OVX: ovariectomized rats; P4: progesterone; Sham-Con: Sham-control; Veh: vehicle.

PMW and OVX rats can improve dyslipidemia [22]. Nevertheless, it has been shown in a study that P4 -treated animals were more likely to have higher TC and lower HDL. Defects in cellular metabolism of cholesterol [23] and insulin secretion disorder [24] that are present in diabetes have been shown to increase the metabolism of lipids and transfer them from fat tissue to the plasma. Sex hormones affect the levels of lipid markers by making changes in the genes involved in the metabolism of lipoproteins, such as the lipoprotein lipase enzyme and liver receptors of lipoproteins [25]. For example, E2 therapy reduces the activity of lipoprotein lipase, and the use of progestins reduces HDL in premenopausal women, which is associated with an increase in liver lipase activity [26].

T2D and ovariectomy reduced the uterine weight, and E2 therapy prevented this decrement. Also, when E2 and P4 were used in combination, P4 resisted the effects of E2. Diabetes decreases E2 level and uterine weight [18]. Uterophic effects are a clear physiological response to E2 injection, and E2 stimulates the cells in both endometrium and myometrium,

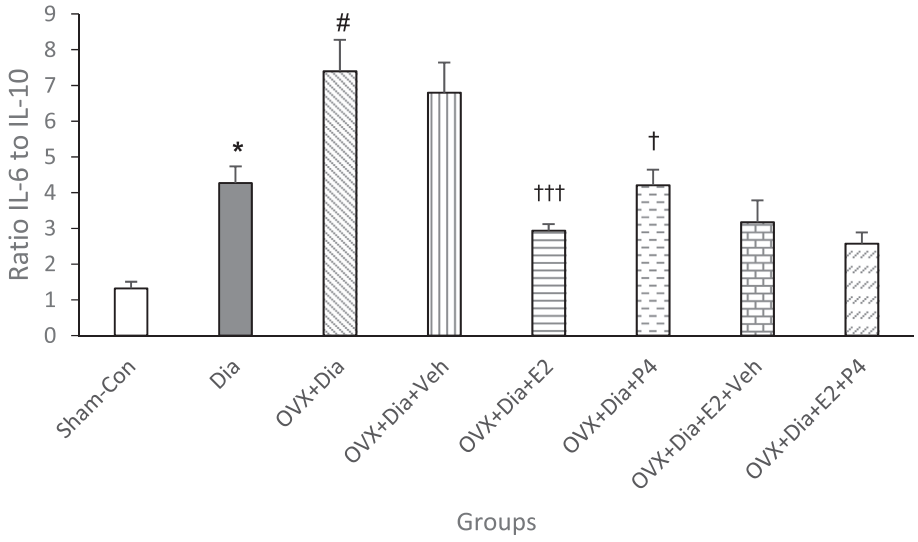

Fig. 7 The effect of diabetes, ovariectomy, E2 and P4 on the ratio of IL-6 to IL-10 in the heart. Data are expressed as mean ± S.E.M., n = 6 rats/group. *$p < 0.05$ vs. Sham-Con. #$p < 0.05$ vs. Dia. †$p < 0.05$ and †††$p < 0.001$ vs. OVX + Dia + Veh. Abbreviations: Dia: diabetes; E2: 17β-estradiol; OVX: ovariectomized rats; Sham-Con: Sham-control; P4: progesterone; Veh: vehicle.

leading to increased uterine growth [27]. On the other hand, P4 can suppress E2 effects on cell growth and uterine metabolism [28]. Probably one of the mechanisms by which the P4 opposes the effects of E2 is through the reduction of estrogen receptors (ERs) in the uterine tissue.

Ovariectomy exacerbated the destructive effects of diabetes on the pancreas. Treatment with E2, unlike P4, has been shown to improve FBG and insulin resistance. Given that there was no significant difference between the combined and the E2+Veh groups, it can also be concluded that P4 in combination with E2 eliminates the beneficial effects of E2 on insulin resistance. Evidence suggests that reduction of E2 along with T2D causes an increase in dysfunction of beta-pancreatic cells [29], and in humans similar to rodents, the loss of E2 results in metabolic imbalances [30]. P4 has been shown to cause insulin resistance [31] and reduce insulin sensitivity in gestational diabetes by reducing the expression of glucose transporter 4 (GLUT4) in muscle and fat tissues [32]. However, inconsistent with our results, in another study, the use of P4 led to a reduction in blood glucose in diabetic animals [19], which could be due to differences in the sex and age of the animals and the type of injury.

Diabetes mellitus in animals with and without ovaries resulted in elevated MBP and cardiac indices, and E2 therapy reduced these disorders. P4 only reduced atherogenic and cardiac risk indices, and it had no effect on other indices. Laboratory studies in adult female rats show that ovariectomy induces hypertrophy [29] and increases blood pressure in both healthy and diabetic rats [33]. The antiatherosclerotic effects of E2 have been identified in a variety of animal models [34]. Additionally, the simultaneous use of E2 and P4 inhibits the progression of atherosclerosis in OVX animals [33]. However, in a study, P4 in combination with E2 was not able to inhibit the formation of atherosclerotic plaques [34], which could be due to the difference in type and duration of diabetes and dosage. Both E2 and P4 receptors have been detected in the heart tissue, and they regulate the metabolism of cardiac proteins [35]. E2 affects cardiac hypertrophy by preventing phosphorylation of P38. Additionally, one of the reasons why P4 is not able to reduce hypertrophy is its artificial nature and the fact that it probably has androgenic effects [36].

T2D and the creation of an animal menopause model led to increased Ang II, TNF-α, IL-6 and decreased IL-10. Since there is no significant difference between the combined groups and E2+Veh in IL-6 and IL-10 indices, it can be concluded that the effects observed in the combined group are likely to be due to the opposite effect of P4 on E2 effect. The role of the renin-angiotensin system (RAS) in diabetic cardiomyopathy has been proven in both laboratory and clinical studies [37] and it has been observed that the cardiac level of Ang II increases in diabetes [38]. Studies have shown that both E2 and P4 reduce the expression and alter the activity of the AT1 angiotensin receptor [39], and probably, in this way, reduce the effects of Ang II. Furthermore, E2 and P4 therapy resulted in a decrease in TNF-α/IL-10 and IL-6/IL-10 ratios. Therefore, P4 can be used instead of E2 when the inflammatory balance is considered. The concentration of inflammatory cytokines is higher in obese and insulin-resistant individuals [40] and E2 reduction that occurs during menopause is associated with increased expression and secretion of inflammatory cytokines of TNF-α, and IL-6 [41]. E2 has also been shown to reduce inflammatory markers [42]. Unlike our study, P4 has been shown to normalize the changes of inflammatory cytokine in diabetes, inhibit macrophages [43] and reduce IL-6 production [44]. Some available information suggests that sex hormones can inhibit inflammation by modulating nuclear factor κB (NFκB) trafficking to the nucleus [45] and reducing the levels of P65 protein and transmitting it to the nucleus [44].

Conclusion

In summary, the present study showed that the induction of T2D in ovariectomized (menopause model) animals leads to biochemical and physiological abnormalities, leading to both increased blood sugar and dyslipidemia, possibly due to changing the pancreatic function and increasing insulin resistance. In addition, these conditions led to cardiovascular dysfunction, including cardiac hypertrophy and atherosclerosis. The possible mechanism for these changes was the increase in angiotensin II and the loss of inflammatory balance in favor of inflammatory cytokines in postmenopausal T2D. Although E2 alone was able to improve blood indices, pancreatic function, responses of peripheral tissues to insulin, and cardiac function, the use of P4 alone and its combination with E2 was ineffective in many cases and sometimes eliminated the cardiovascular protective effect of E2. Therefore, it is suggested that in postmenopausal diabetes, if the goal is to improve cardiovascular disorders, E2 should be used alone (not in combination with P4). Determining the mechanism of inhibitory effects of P4 on the cardiovascular protective effects of E2 is suggested in future studies.

Ethical issues

The study was performed according to the Institutional Animal Care Committee of Kerman University (No.95/105) and was in accordance with the guidelines of the National Institutes of Health on the care and use of animals.

Conflicts of interest

There is no conflicts of interest.

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