Cardioprotective effects of vitamin B complex and Ethinyl Estradiol in ovariectomized rats

Bassant M.M. Ibrahim[a], Hanan F. Al-Saeed[c], Aziza B. Shalby[b]

Departments of [a]Pharmacology, [b]Hormones, National Research Centre, Giza, [c]Department of Physiology, Faculty of Medicine for Girls, Al-Azhar University, Cairo, Egypt

Correspondence to Bassant M.M. Ibrahim, MD, Department of Pharmacology, National Research Centre, El-Bohouth Street (former El-Tahrir Street), Dokki, PO Box 12622, Giza, Egypt Tel: + 20 201 223 911 349; e-mail: bmmih1974@gmail.com

Received 27 May 2017
Accepted 24 July 2017

Journal of The Arab Society for Medical Research 2017, 12:39–45

Background/aim
Reduction of female sex hormones is associated with health disorders including cardiovascular diseases such as hypertension. Supplementation with vitamin B complex can lower the incidence of these disorders. The present study aimed to study the potential cardioprotective effects of vitamin B complex and ethinyl estradiol (EE) when administered alone or in combination to hormonal-deprived ovariectomized (OVX) rats.
Materials and methods
Fifty female Wister rats were classified into five equal groups: the first group included normal rats, the second group was OVX (positive control), and the third to fifth groups were OVX rats that were treated by EE (0.03 mg/kg), vitamin B complex (9.036 mg/kg), and a combination of both treatments, respectively. Treatment continued for eight successive weeks. Blood pressure (BP), cystathionine γ-lyase (CSE), reduced glutathione (R-GSH), superoxide dismutase (SOD), catalase (CAT), and interleukin-β6 (IL-β6) were measured.
Results
The OVX rats showed a significant increase in BP, reduction in CSE, R-GSH, SOD, and CAT, and increase in IL-β6 levels, whereas the treated OVX rats showed a significant decrease in BP, increase in CSE, R-GSH, SOD, and CAT, and reduction in IL-β6 levels.
Conclusion
Vitamin B complex and EE exert cardioprotective effects in OVX rats.

Keywords:
Blood pressure, cystathionine γ-lyase, ethinyl estradiol, ovariectomy, oxidative stress, vitamin B

J Arab Soc Med Res 12:39–45

1687-4293

Introduction

Cardiovascular disorders are the most common cause of morbidity and mortality worldwide. However, women tend to develop heart disease after menopause because of the cardioprotective effects of female sex hormones, which exert their influence through various mechanisms. One of them is enhancement of the cystathionine γ-lyase (CSE) action as it acts as a major H_2S-producing enzyme in the cardiovascular system [1]. H_2S is capable of inducing vasorelaxation and lowering blood pressure (BP) in rats because of almost a direct action on smooth muscle cells by activation of ATP-sensitive potassium channels and partially an endothelium-dependent mechanism [2]; therefore, the deficiency of CSE leads to decreased endogenous H_2S level, an age-dependent increase in BP, and impaired endothelium-dependent vasorelaxation [1]. It is well known that the CSE/H_2S system is involved in the development as well as the aging process. The messenger RNA and protein levels of CSE are lower in old rats and mice [3,4]. There is evidence showing that CSE protein expression is significantly reduced in mouse embryonic fibroblasts, which causes increased oxidative stress and accelerated genetic cellular deterioration with age [5]. H_2S incubation significantly reduces oxidative stress and protects the cells from genetic deterioration with age. It has also been reported that H_2S improves the function of senescent human umbilical vein endothelial cells [6].

It has been established that antioxidants are essential in any cardioprotective regimen as it was reported that the beneficial role of cardioprotective nutrients is because of their antioxidant as well as anti-inflammatory effects. They are also capable of immune regulation, endothelial cell protection, cell membrane stabilization, methylation, and epigenetic support. Sufficient levels of antioxidants from diet can help in the prevention of pathological changes associated with oxidative stress in the cardiac muscle. Vitamin B complexes are very important antioxidants [7]. B complex vitamins are important for many

biochemical processes in the body, especially the cardiovascular function and its protection. They play an important role in methylation chemistry, lipid regulation, energy production, free radical management, and inflammatory processes. Thiamine (vitamin B_1), pyridoxin (vitamin B_6), and cyanocobalamin (vitamin B_{12}) are naturally occurring B vitamins present in whole and unprocessed foods. B vitamins have been closely related to cardiovascular health status; cardiovascular risks are reduced in individuals with higher intakes of multivitamin supplements [8].

Studies carried out by Zhu *et al.* [9], as well as other studies have shown that estrogens (E2) protect cardiomyocytes as they increase CSE expression, and thus lead to endogenous H_2S generation in the myocardium; thus, their effects are associated with decreased oxidative stress and inflammatory status. The design of the present study is based on the hypothesis that ovariectomy (OVX) and reduction of estrogens in rats will cause cardiac disease and affect BP through reduction of CSE and oxidative stress as well as inflammatory process, which can be counteracted by the use of vitamin B complex and ethinyl estradiol (EE), either alone or in combination.

Materials and methods

Materials

Animals

Female Albino Wistar rats (150–175 g body weight) obtained from the animal house colony of the National Research Centre, Dokki, Giza, Egypt, were used in the experiment. All animals were housed in stainless-steel cages in a temperature-controlled (23±1°C) and artificially illuminated (12 h dark/light cycle) room free from any source of chemical contamination, and were provided with a standard laboratory diet and water *ad libitum*. Animal procedures were performed in accordance with the Ethics Committee of the National Research Centre and followed the recommendations of the National Institutes of Health Guide for Care and Use of Laboratory Animals [10].

Chemicals and drugs

(1) EE was purchased from Bayer Schering Pharma AG (Berlin, Germany).
(2) Vitamin B complex was purchased from Amriya Pharmaceuticals (Egypt). Each tablet contained thiamine HCl (vitamin B_1) 150 mg, pyridoxine HCl (vitamin B_6) 100 mg, and cyanocobalamin (vitamin B_{12}) 1 mg.
(3) Kits and chemicals for biochemical assay were of high analytical grade, products of Sigma (Massachusetts, USA), Merck Co. (Munich, Germany) and My Biosource (California, USA).

Apparatus

(1) A noninvasive BP monitor and thermostatically controlled heating cabinet (Ugo Basile, Varese, Italy).
(2) RT-qPCR for CSE.
(3) Spectrophotometer Co (JascoV-630; Jasco, Tokyo, Japan) and enzyme-linked immunosorbent assay apparatus (Thermo Fisher, Massachusetts, USA) were used for biochemical analysis.

Methods

Ovariectomy procedure

OVX was performed to ensure elimination of the protective effect of female sex hormones on the cardiovascular system. The animals were anesthetized with thiopental (50 mg/kg) according to Keefer *et al.* [11]. Bilateral OVX was performed using a double-dorsolateral approach according to Alkofahi and Atta [12]. The anesthetized rat was laid prone on the operating table and fixed using sticking plaster. The bulged area on the back was shaved bilaterally .The ovaries were found on both sides of the abdomen slightly below the kidney. Skin incision site was detected by placing a thumb at the uppermost proximal area of the thigh.The medial portion of the base of the distal phalanx was the incision site. A 1.5 cm skin incision was made to expose the dorsolateral abdominal muscles such as the external oblique muscle. Entry into the peritoneal cavity was gained by dissecting the muscle, which revealed the adipose tissue surrounding the ovary. The adipose tissue was pulled away until the ovary and uterine tubes were identified. The periovarian fat with the ovary was pulled away from the incision site gently to prevent detachment of a small piece of ovary. After identifying the ovary and uterine horn, ligation was performed at the distal uterine horn to remove the ovarian tissue completely in one action; the horn was returned to the abdominal cavity and the muscle and skin were sutured. All animals were left for 4 weeks before starting initiation of treatment with vitamin B complex or EE or their combination.

Study design

Animals were classified into five groups of 10 rats each. The first group included normal rats that received distilled water throughout the experiment. The second group was a positive control for which

OVX was performed and did not receive any treatment throughout the experimental period. The third to fifth groups were OVX and left for 4 weeks after surgery to ensure elimination of female sex hormones, and then the third group received EE at a dose of (0.03 mg/kg) according to and Czekaj and Nowaczyk-Dura [13]. The fourth group received vitamin B complex at a dose of (9.036 mg/kg); the human dose of vitamin B complex (one tablet administered twice daily) was converted into doses for rats according to Paget and Barnes [14]. The fifth group received a combination of both vitamin B complex and EE. Treatment continued for eight successive weeks.

Blood pressure measurement procedure

Training was conducted by the method described by Irvine *et al.* [15] as follows: plastic restraint tubes were placed in the animal cages for a few days before measurement to allow familiarity. The animals were then restrained in the tubes for 10–20 min/day for 5 days before recording BP. Animals were warmed for 30 min at 29°C in a thermostatically controlled heating cabinet (Ugo Basile) for better detection of the tail artery pulse; the tail was passed through a miniaturized cuff and a tail-cuff sensor.

The pulse was recorded by the sensor during automatic inflation and deflation of the cuff. Systolic and diastolic BP was defined as the cuff inflation and deflation. Baseline systolic BP of animals was measured indirectly before OVX at zero time using a noninvasive BP monitor (Ugo Basile) from the tail of conscious rats using the tail-cuff technique for which all animals were pretrained until BP was recorded steadily with minimal stress, and odds (means extreme ranges) in measurements of BP were excluded.

Then measurements were performed 4 weeks after OVX, then after 4 weeks of receiving vitamin B complex or EE or their combination, and finally at the end of the treatment period, which was eight successive weeks. The average of at least three measurements for each rat was determined on each occasion.

Sample preparation for the detection of cystathionine-γ-lyase expression, oxidative stress, and inflammatory biomarkers in the myocardium

Twenty-four hours after the last dose of treatment, the animals were killed under anesthesia, the thoracic cavities were opened, and then the hearts were dissected and homogenized in PBS (pH 7.4) using a glass-Teflon homogenizing tube (Janke & Kunkel; IKA-Werk, Staufen im Breisgau, Germany). The homogenate was centrifuged at 3000 rpm for 10 min and the supernatant was isolated for the detection of CSE, oxidative stress, and inflammatory biomarkers in the myocardium.

Cystathionine-γ-lyase expression in the myocardium: real-time quantitative reverse transcription PCR

Total RNA was extracted from each frozen sample using Qiagene Kit (Qiagene, West Virginia, USA) according to a standard protocol. The isolated total RNA was converted into complementary DNA using Moloney murine leukemia virus reverse transcriptase (Promega, Wisconsin, USA). Real-time PCR was performed using the Step One Plus Real-Time PCR System (Applied Biosystems, Foster City, California, USA) and an SYBR Green PCR Master Mix (Applied Biosystems) in a final volume of 10 µl under the following thermal cycling conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The sequences of PCR primer pairs used for each gene are shown in Table 1. Data were analyzed using the ABI Prism sequence detection system software and quantified using the v1·7 Sequence Detection Software from PE Biosystems (Foster City, California, USA). The relative expression of studied genes was calculated using the comparative threshold cycle method. All values were normalized to the β actin genes as an invariant endogenous control (reference gene). The relative quantification was then calculated by the expression $2^{-\Delta\Delta C_t}$ [16].

Biochemical parameters

Oxidative stress biomarkers

Measurement of reduced glutathione (R-GSH) content: 5% tissue homogenates were prepared in 20 mmol/l EDTA, pH 4.7, and 100 µl of the homogenate was added to 0.2 mol/l Tris-EDTA (1.0 ml, pH 8.2) buffer

Table 1 Sequence of the primers used for real-time PCR

Gene bank accession number	Primer sequence	
NM_001014035	Forward primer: 5′-CATGGATGAAGTGTATGGAGGC-3′	Cystathionine γ-lyase
	Reverse primer: 5′-CGGCAGCAGAGGTAACAATCG-3′	
NM_031144.3	Forward primer: 5′-TATCCTGGCCTCACTGTCCA-3′	β-Actin
	Reverse primer: 5′-AACGCAGCTCAGTAACAGTC-3′	

(Fluka; Switzerland) and 20 mmol/l EDTA, pH 4.7 (0.9 ml), followed by 20 µl of Ellman reagent (10 mmol/l DTNB in methanol). After 30 min of incubation at room temperature, absorbance was read at 412 nm. The R-GSH concentration was expressed as nano moles per milligram of tissue protein according to the method of Sedlak and Lindsay [17].

Measurement of superoxide dismutase (SOD) activity: The activity of SOD was evaluated according to the method of Alirezaei *et al.* [18] using the kits of Randox Co. (West Virginia, USA). This method uses xanthine and xanthine oxidase to generate superoxide radicals, which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyl tetrazolium chloride to form a red formazan dye. The SOD activity is then measured by the degree of inhibition of this reaction. One unit of SOD is that which causes 50% inhibition of the rate of reduction of 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyl tetrazolium chloride under the conditions of the assay. SOD levels were recorded at 505 nm and through a standard curve and expressed as unit per milligram of tissue protein.

Measurement of tissue catalase (CAT) activity: The reaction mixture (1 ml) consisted of 50 mmol/l potassium phosphate (pH 7.0), 19 mmol/l H_2O_2, and a 50 µl sample. The reaction was initiated by the addition of H_2O_2 and absorbance changes were measured at 240 nm (25°C) for 30 s. The CAT activity was expressed as the unit that is defined as micromoles of H_2O_2 consumed per minute per milligram of tissue protein according to the method of Kheradmand *et al.* [19] using the kits of Merck Co.

Inflammatory biomarkers

Measurement of interleukin-β6 (IL-β6): It was measured according to the manufacturer's instruction by the enzyme-linked immunosorbent assay kit supplied by My Biosource according to the method of Krakauer *et al.* [20].

Statistical analysis

The data are expressed as mean±SE for each group. Results of biochemical tests were analyzed using one-way analysis of variance, followed by the Tukey–Kramer test for multiple comparisons; *P* value of less than 0.05 was considered significant in all types of statistical tests. Results of BP records were analyzed using two-way analysis of variance, followed by Tukey–Kramer's test for multiple comparisons; *P* value of less than 0.001 was considered significant. Comparison between groups was performed during the same period of treatment for all groups. Graph Pad Software (GraphPad Software Inc., La Jolla, CA, USA) (version 6) was used to carry out the statistical tests.

Results

Systolic blood pressure records

The present study shows that systolic BP in normal rats did not show any significant change from baseline readings of the same group throughout the experiment. However, OVX, after 4 weeks, resulted in a significant increase in BP by 30% compared with the baseline measurements of the other four groups. The positive control group measurements after eight weeks of OVX and 4 weeks of treatment of the other groups increased by 30%, from readings of 4 weeks after OVX, whereas after 12 weeks of OVX and eight weeks of treatment of the other groups, it increased by 32% of the measurements obtained 4 weeks after OVX. The BP of all treated groups was significantly less than that of the positive control group as the EE group (0.03 mg/kg), the vitamin B complex group (9.036 mg/kg), and the combination group of both measurements showed a reduction in BP after eight weeks of OVX and 4 weeks of treatment by 11, 8, and 9% 4 weeks after OVX. After 12 weeks of OVX and eight weeks of treatment, all treated groups started showing a significant reduction by 13.2% for the EE group, 10.4% for the vitamin B complex group, and 13.5% for group treated with the combination 4 weeks after OVX. The results are shown in Table 2.

Cystathionine γ-lyase

The present study shows that OVX alone without treatment reduced CSE level significantly by 63% compared with normal rats, whereas treatment with EE, vitamin B complex, and their combination after OVX reduced CSE by 43, 25, and 3%, respectively, compared with normal rats. The potencies of vitamin B complex alone and its combination with EE therapy compared with EE therapy alone are 131.5 and 170.17%, respectively. The results are shown in Table 3.

Oxidative stress parameters, reduced glutathione, superoxide dismutase, and catalase

OVX alone without treatment reduced R-GSH, SOD, and CAT by 52.6, 93, and 61.38%, respectively, compared with normal rats, whereas treatment with EE alone, vitamin B complex alone, and their combination after OVX reduced R-GSH by 37.26, 35.87, and 23.42%, respectively, reduced SOD by 74.93, 53.67, and 17.97%, respectively, and CAT by 29.58, 14.72, and 5.32%, respectively. All results were compared with normal rats. In terms of the effects of vitamin B complex alone and its combination with EE

therapy compared with EE therapy alone on R-GSH, their potencies were 102.21 and 122%, respectively, whereas their potencies for their effect on SOD were 184.8 and 327.27%, respectively; for CAT, their potencies were 121.11 and 134.45%, respectively. The results are shown in Table 3.

Inflammatory biomarker: interleukin-β6

OVX alone without treatment increased the levels of IL-β6 significantly by 444% compared with normal rats, whereas treatment with EE, vitamin B complex, and their combination after OVX, increased the levels by 231.6, 194.1, and 106.3%, respectively, compared with normal rats. The potencies of vitamin B complex alone and its combination with EE therapy compared with EE therapy alone are 112.2 and 160.6%, respectively. The results are shown in Table 3.

Discussion

As estrogen maintains normal BP levels in women [21], its deficiency in menopausal women leads to elevated BP in addition to anxiety and memory impairment, which requires estrogen replacement therapy (ERT) [22]. Although ERT is somewhat palliative, yet, it has many risks such as breast cancer, heart disease as well as stroke [23].

OVX in rodents represents a model of depletion of estrogen, and thus it is used widely to study menopause-associated circumstances and available therapeutics [24]. This is why it has been used as a model to study the potential therapeutic effects of EE and vitamin B complex when administered each either alone or in combination on cardiovascular disorders after menopause has been induced in an animal model to mimic human menopause.

A study carried out by Zhu *et al.* [9] showed that ERT (30 μg E2β/kg/day) for 12 weeks in OVX rats led to increased H_2S production through CSE upregulation in the myocardium, which may explain the results of the cardiovascular-protective effects of estrogens in our study, evidenced by lowered BP as a result of increased CSE that was detected by gene expression of CSE in the myocardium of treated rats. Moreover, there is a higher risk of breast cancer because of the use of

Table 2 Effects of treatment with ethinyl estradiol (0.03 mg/kg) and vitamin B complex (9.036 mg/kg), either each alone or in combination, for eight successive weeks on systolic blood pressure of ovariectomized rats

Groups	Onset			
	Baseline	Four weeks after ovariectomized	Eight weeks after ovariectomized/4 weeks after treatment	Twelve weeks after ovariectomized/eight weeks after treatment
Normal		100.6±3.14	100±3.3	98.6±3.5
Positive control untreated ovariectomized			172±7[a]	175±7[a]
Ethinyl estradiol (0.03 mg/kg)			117.9±4.7[a,b]	115±4.1[a,b]
Vitamin B complex (9.036 mg/kg)	102±4.1	132.6±5.3[a]	121.9±4.8[a,b]	118.76±4.2[a,b]
Ethinyl estradiol+vitamin B complex			120.6±4.8[a,b]	114.4±3.6[a,b]

All results are expressed as mean±SE (N=10); Statistical analysis was carried out using two-way analysis of variance, followed by Tukey–Kramer's test for multiple comparisons; [a]$P<0.001$, significantly different from the negative control group; [b]$P<0.001$, significantly different from the positive control group.

Table 3 Effects of treatment with ethinyl estradiol (0.03 mg/kg), vitamin B complex (9.036 mg/kg), either given alone or in combination, for eight successive weeks on cystathionine γ-lyase, reduced glutathione, superoxide dismutase, catalase, and interleukin-β6

Groups	Parameters				
	Cystathionine γ-lyase	Reduced glutathione	Superoxide dismutase	Catalase	Interleukin β-6
Normal	1±0.01	82.73±1	3.95±0.2	206.5±3.8	30.88±1.7
Positive control untreated ovariectomized	0.37±0.01[a,c,d,e]	39.18±0.6[a,c,d,e]	0.27±0.01[a,c,d,e]	79.75±1.3[a,c,d,e]	168±3[a,c,d,e]
Ethinyl estradiol (0.03 mg/kg)	0.57±0.01[a,b,d,e]	51.9±0.8[a,b,e]	0.99±0.1[a,b,d,e]	145.4±3.9[a,b,d,e]	102.4±3.7[a,b,e]
Vitamin B complex (9.036 mg/kg)	0.75±0.01[a,b,c,e]	53.05±1[a,b,e]	1.83±0.1[a,b,c,e]	176.1±4.9[a,b,c,e]	90.83±4[a,b,e]
Ethinyl estradiol+vitamin B complex	0.97±0.02[b,c,d]	63.35±0.7[a,b,c,d]	3.24±0.2[a,b,c,d]	195.5±4[b,c,d]	63.73±0.7[a,b,c,d]

Results are expressed as mean±SE of blood pressure, N=10, $P<0.05$; Statistical analysis was carried out using one-way analysis of variance, followed by Tukey–Kramer's test for multiple comparisons. Treatment was started 4 weeks after ovariectomized and continued for eight successive weeks; [a]Significantly different from the negative control group; [b]Significantly different from the positive control group; [c]Significantly different from the group treated with ethinyl estradiol (0.03 mg/kg); [d]Significantly different from the group treated with group treated with vitamin B complex (9.036 mg/kg); [e]Significantly different from the group treated with a combination of ethinyl estradiol and vitamin B complex.

hormone replacement therapy, which is the same for the so-called 'bioidentical' or 'natural' hormones as it is for synthetic hormones.

Disturbed balance between production and elimination of reactive oxygen species through the antioxidant defense system stress leads to oxidative stress [25], which leads to many health problems including cardiovascular diseases. This was clear in our study, where increased oxidative stress biomarkers in OVX rats were concomitant with increased BP level and decreased CSE as well as increased inflammatory biomarker IL-β6.

As decreased estrogen levels after menopause are associated with augmented oxidative stress [26], it was concluded that the low incidence of oxidative stress in the cardiovascular system during premenopause was because estrogen levels exist in high concentrations, Chang *et al.* [27]; this was also evident in the present study, where treatment of OVX rats with EE for eight weeks improved the levels of oxidative stress biomarkers such as R-GSH, CAT, and SOD as well as the levels of CSE and inflammatory biomarker IL-β6, and consequently the BP was lowered.

The results of the impact of OVX in our study and the ameliorating effects of EE as well as vitamin B6 can be explained by their effects on oxidative stress as it was reported by Damy *et al.* [28] that cardiac and systemic GSH deficiency is accompanied by cardiac abnormalities of patients with cardiac diseases; this is because GSH counteracts oxidative damage and prevents heart diseases. Moreover, previous studies have indicated that GSH levels increased following treatment of amenorrhea or after menopause with oral contraceptives [29]. CAT is an antioxidant enzyme present in all animal tissues and it decomposes hydrogen peroxide and protects the tissue from highly reactive hydroxyl radicals [30]. It was found that the CAT level is associated inversely with coronary heart disease [31], which was proven in our study, where it was associated with low BP in all treated rats.

Therefore, supplementation of antioxidants in the diet is important because they exert a protective effect in menopausal and postmenopausal women impacts of oxidative stress adverse effects [32]. Supplementation with the B complex in diet maintains a normal balance between reduced glutathione and oxidized glutathione ratio. A low ratio means that there is a risk of oxidative stress, which is harmful for postmenopausal women [33].

The relation between level of oxidative stress biomarkers and CSE and consequently the cardioprotective effects of vitamin B complex are because of the fact that the synthesis of glutathione depends on the presence of cysteine synthesized from methionine through the effect of vitamin B_6-dependent enzymes cystathionine 13-syntase (EC 4.2.1.22) and cystathionine/lyase (EC 4.4.1.1) [34]. Moreover, vitamin B_1 was capable of protecting $NADP^+$-dependent dehydrogenase activities that supply NADPH and this helps to maintain GSH in the reduced form. Also, it prevents lipid peroxidation and ATP depletion, and thus protects the tissues and cells from tissue injury and cell death and prevents inflammation [35–38], which was obvious in this study because of reduced IL-β6.

Conclusion

Supplementation of the vitamin B complex as well as EE in the diet after menopause exerts a cardioprotective effect and when administered in combination, their effect is better than when each of these is administered alone; however, their effect in combination does not exert an additional synergistic effect. They don't increase the effect algebrically when used together.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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