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Effect of catecholestrogen administration during adriamycin-induced cardiomyopathy in ovariectomized rat

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Abstract

The therapeutical beneficial effect of estrogen-derived metabolites or catecholestrogens is controversial. These molecules are produced during estrogen therapy based on 17-β-estradiol treatment. The metabolization of 17-β-estradiol is carried out in brain, kidney or liver, and triggers different products such as 2- and 4- hydroxyestradiol (2OH and 4OH). These products have shown antioxidant properties against oxidative stress (OS) in several experimental models. Different noxious side effects related to those metabolites have also been observed upon estrogen therapy. In this sense, catecholestrogens seem to be implicated in tumoral and mutagenic process after long treatment with estrogens substitutive therapy.

In our study, we have verified that 2OH and 4OH have antioxidant and cardioprotective effects against adriamycin (AD)-induced cardiomyopathy in ovariectomized (OVX) rats. Catecholestrogens diminished the lipid peroxides and carbonyl protein (CO) content, and different enzymes related to cell injury (creatinine kinase, lactate dehydrogenase, aspartate aminotransferase, alanine aminotransferase) in cardiac tissue from OVX-, AD-, and OVX + AD-treated rats. All these changes were correlated to a recovery on reduced glutathione (GSH), glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT) in heart tissue.

The present study showed that 2OH and 4OH reduced all the parameters related to OS, antioxidant depletion and cardiac injury in OVX rats treated or not with AD.

Keywords: *Catecholestrogens, oxidative stress, ovariectomy, adriamycin, cardiomyopathy*

Introduction

During the last decade, different studies have been carried out in order to assess the antioxidant properties of 17-β-estradiol and its related metabolites. Different studies have shown that those molecules may exert a cytoprotective or noxious side effect due to long treatment upon estrogen therapy.

In several tissues, such as brain, kidney or liver, 17-β-estradiol is metabolized to catecholestrogens, 2- and 4-hydroxyestradiol (2OH and 4OH), by the NADPH-dependent cytochrome P450-linked monooxygenase system [1]. These metabolites are rapidly O-methylated by the ubiquitously present catechol-O-methyl transferase to 2-methoxyestradiol [2]. This last metabolite and 2OH are the most potent biologically metabolites of 17-β-estradiol [3]. Catecholestrogens show antioxidant properties against the lipid peroxidation induced by chelatable iron or copper [4–6], $FeSO_4$-treated phospholipids [7] and Fe^{3+}-ADP-adriamycin [8], hydrogen peroxide [9] or 2, 2′-azobis [10]. It has also been shown that catecholestrogens induce different intracellular signalling pathways related to cellular proliferation and differentiation [11]. Other authors propose

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that catecholestrogens inhibit angiogenesis [12]. In parallel with the cytoprotective properties of those products, different studies have shown that long catecholestrogen treatment generates oxidative stress (OS) that progress to severe cancer [13–15].

In the present study, we followed an experimental model of cardiomyopathy induced by AD. AD is an antineoplasic anthracycline drug that induces OS in several tissues including heart [16–18].

The aim of the present study was to evaluate the role of 2OH and 4OH against OS and cardiopathy induced by adriamycin (AD) in ovariectomized (OVX) rats.

Material and methods

Animals

Fifty-four female Wistar rats (Charles River, Barcelona, Spain) (250–275 g corresponding to two months and fifteen days old) received laboratory food and water *ad libitum*. The animal room was controlled at constant temperature (22 ± 2°C), humidity and light cycle (8–20 h). The animals ($n = 54$) were divided in the following groups: (1) Control (C), (2) Sham Operated (SO), (3) OVX, (4) AD, (5) OVX + AD, (6) OVX + 2OH, (7) OVX + AD + 2 OH, (8) OVX + 4OH and (9) OVX + AD + 4OH.

Treatments

The OVX was done by bilateral procedure as described by Pomeau-Delille [19]. The treatments were administered to animals when the absence of ovary cycle was verified. This period was usually lasting twenty days. The animals from control and SO maintained a normal body weight during all the study.

The AD (Pharmacia & Upjohn) was administered intraperitoneally twice (at 0 and 9 days) at a doses of 10 mg/kg body weight. The 2OH and 4OH were administered subcutaneously at 0.9 mg/kg body weight in dimethyl sulfoxide every two days until sacrifice. This concentration represents 2.5×10^3 times more than the physiologic concentration, that ranges from 100 pM to 5 nM. The solvent had no effect in the studied parameters. The animals were sacrificed under anaesthesia 18 days after the administration of treatments according to previous study [20]. Blood samples were obtained from the vascular trunk of the neck. The plasma fraction was used for the determination of different parameters related to cardiac damage. The heart was extracted and froze immediately at −40°C in phosphate buffer solution.

Hemodynamic parameters

The arterial mean pressure (PAM) and the cardiac frequency (FC) were measured using a cylindrical device for the measure of pressure (LE 5100 Pressure Cylinder) coupled to a digital device (LE 5000 Digitalis Pressure Meter, LETICA).

Biochemical parameters

Lipid peroxidation products measurement. The levels of lipid peroxidation products were determined using a commercial assay (LPO-586, Byoxitech). The heart was homogenated in 20 mM Tris HCl pH 7.4 and centrifuged at 10000 × *g* during 15 min at 4°C. The assay is based on the reaction of a chromogenic reagent, N-methyl-2phenylindole, with MDA at 45°C. The results were expressed in relation to the protein content.

Protein carbonylation. The measurement of protein oxidation (CO) was done following a modification of the procedure described by Oliver et al. [21] The heart was homogenated in 20 mM Tris-HCl pH 7.4 and centrifuged at 10000 × *g* during 10 min and 4°C. Samples were deproteinized (v/v) with 500 µl 20% trichloroacetic acid during 10 min at 4°C. The resulting supernatant was incubated with 500 µl 10 mM dinitrophenylhidrazine (DNPH) in 2N HCl during one hour at room temperature. Samples were precipitated with 500 µl 20% trichloroacetic acid during 10 min at 4°C and centrifuged at 10000 × *g* during 5 min. The supernatant was extracted twice with 1 ml of ethanol/ethylacetate (1:1) (v/v). It was added 1 ml of 6 M guanidine and incubated during 15 min at 37°C. The samples were measured at 360 nm using a coefficient of extinction $\varepsilon = 21\ M^{-1} \times cm^{-1}$.

Reduced glutathione. The measurement of reduced glutathione (GSH) was determined using a commercial assay GSH- 400 (Byoxitech S.A). Briefly, all mercaptans present in the sample react with 4- chloro-1-methyl-7-trifluromethyl-quinolinium methylsulfate in a first chemical reaction. The second step consisted of the β-elimination reaction under alkaline conditions of the product from the first reaction into a chromogenic thione-derived product.

Glutathione peroxidase, catalase and superoxide dismutase measurement. Glutathione peroxidase (GPx) was measured according to the method described by Flohé and Gunzler [22]. Catalase (CAT) was determined following the method described by Aebi [23]. Superoxide dismutase (SOD) was assessed according to the method published by Sun et al. [24]

Table I. Effect of 2-or 4-hydroxyestradiol (2OH or 4OH) on aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine kinase (CK) and lactate dehydrogenase (LDH) activities in plasma from ovariectomized (OVX) rats treated or not with adriamycin (AD).

	AST u/l ($x10^{-2}$)	ALT u/l	CK u/l ($x10^{-3}$)	LDH u/l ($x10^{-2}$)
Control	1.24 ± 0.15	24.9 ± 2.88	3.29 ± 0.27	8.27 ± 1.99
Sham Operated	1.24 ± 0.18	23.4 ± 2.38	3.96 ± 0.56	8.54 ± 1.61
OVX	1.77 ± 0.13$^{\blacklozenge\blacklozenge\blacklozenge}$	30.6 ± 2.91$^{\blacklozenge\blacklozenge}$	5.65 ± 0.85$^{\blacklozenge\blacklozenge}$	9.82 ± 0.11$^{\blacklozenge\blacklozenge}$
AD	3.35 ± 0.34$^{\blacklozenge\blacklozenge\blacklozenge}$	40.2 ± 1.46$^{\blacklozenge\blacklozenge\blacklozenge}$	6.87 ± 0.74$^{\blacklozenge\blacklozenge\blacklozenge}$	12.3 ± 1.45$^{\blacklozenge\blacklozenge\blacklozenge}$
OVX + AD	3.70 ± 0.34$^{\blacklozenge\blacklozenge\blacklozenge}$	45.8 ± 2.72$^{\blacklozenge\blacklozenge\blacklozenge}$	7.31 ± 3.20$^{\blacklozenge\blacklozenge\blacklozenge}$	15.1 ± 4.42$^{\blacklozenge\blacklozenge\blacklozenge}$
OVX + 2OH	1.44 ± 0.37^{++}	25.7 ± 1.96^{+++}	4.07 ± 0.66^{+++}	8.28 ± 1.51^{+}
OVX + 4OH	1.46 ± 0.18^{+}	27.0 ± 1.19^{+++}	4.19 ± 0.45^{+++}	8.24 ± 3.38^{+}
OVX + AD + 2OH	2.00 ± 0.59***	35.7 ± 4.55***	4.18 ± 0.87***	12.7 ± 2.19**
OVX + AD + 4OH	2.17 ± 0.38***	37.2 ± 1.85***	4.58 ± 0.46***	13.3 ± 2.04*

Values are expressed as mean ± S.E.M. ($^{\blacklozenge\blacklozenge\blacklozenge}p \leq 0.001$; $^{\blacklozenge\blacklozenge}p \leq 0.01$ vs. SO), ($^{+++}p \leq 0.001$; $^{++}p \leq 0.01$; $^{+}p \leq 0.05$ vs. OVX) and ($^{***}p \leq 0.001$; $^{**}p \leq 0.01$ and $^{*}p \leq 0.05$ vs OVX + AD).

Nitric oxide production in heart. Nitric oxide (NO) production was evaluated using a commercial non-enzymatic assay from Byoxitech. This assay is based on the chemical reduction of nitrate to nitrite by granulated cadmium that reacts with the chromogenic Greiss reagent.

Parameters related to cardiac injury. Different parameters related to cardiac injury such as the activity of creatinine kinase (CK), lactate dehydrogenase (LDH), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were automatically analyzed in plasma (Axon, Bayer).

Statistical analysis

All results were expressed as mean ± S.E.M. Differences between groups were assessed by the one-way analysis of variance (ANOVA). If the variances between groups were homogenous (Levene's test), groups were subjected to the multiple comparison Bonferroni's test. If the equal variances were not assumed the significance was evaluated by the Dunnett's T3 test. The statistical significance was set at $p \leq 0.05$ and indicated using superscript symbols in the figures.

Results

Evaluation of cardiac injury

OVX or AD significantly enhanced cardiac damage ($0.001 \leq p \leq 0.01$) showing an additive effect on ALT, CK and LDH activity in plasma from OVX + AD-treated rats ($p \leq 0.01$) (Table I). The 2OH and 4OH administration had a significant cytoprotective effect in OVX and OVX + AD-treated rats ($0.001 \leq p \leq 0.05$).

Hemodynamic parameters

OVX, AD and OVX + AD-treated rats significantly increase PAM and FC parameters ($p < 0.001$). The 2OH or 4OH administration significantly reduced PAM and FC in OVX and OVX + AD-treated rats. Ovariectomy reduced the production of NO in heart ($p < 0.001$). By contrast, AD significantly enhanced NO production in heart from AD and OVX + AD-treated rats ($p < 0.001$ and $p < 0.01$ respectively). The 2OH or 4OH administration significantly enhanced NO in OVX rats ($p \leq 0.05$ and $p \leq 0.001$ respectively). The body weight increased and diminished significantly in OVX and AD-treated rats, respectively ($p \leq 0.01$). The 2OH or 4OH administration diminished body weight in OVX rats ($p \leq 0.05$) (Table II).

Table II. Effect of 2-or 4-hydroxyestradiol (2OH or 4OH) on arterial mean pressure (PAM), cardiac frequency (FC), nitric oxide (NO) production and body weight in ovariectomized (OVX) rats treated or not with adriamycin (AD).

	PAM mmHg	FC s.p.m	Cardiac NO μMNO/mg protein	Body Weight Kg
Control	129.95 ± 16.25	386.62 ± 33.91	0.0433 ± 0.0012	0.209 ± 0.012
Sham Operated	128,26 ± 12.54	386.42 ± 32.18	0.0434 ± 0.0009	0.207 ± 0.010
OVX	135,633 ± 5.063$^{\blacklozenge}$	365.86 ± 35.57$^{\blacklozenge}$	0.0401 ± 0.0005$^{\blacklozenge\blacklozenge\blacklozenge}$	0.233 ± 0.015$^{\blacklozenge\blacklozenge}$
AD	143,667 ± 12.44$^{\blacklozenge\blacklozenge}$	445.67 ± 11.0$^{\blacklozenge\blacklozenge\blacklozenge}$	0.0499 ± 0.0010$^{\blacklozenge\blacklozenge\blacklozenge}$	0.166 ± 0.017$^{\blacklozenge\blacklozenge}$
OVX + AD	148,667 ± 2.44$^{\blacklozenge\blacklozenge\blacklozenge}$	462.53 ± 27.8$^{\blacklozenge\blacklozenge\blacklozenge}$	0.0457 ± 0.0017$^{\blacklozenge\blacklozenge}$	0.206 ± 0.018
OVX + 2OH	127.333 ± 6.892^{+++}	386.46 ± 14.62^{+}	0.044 ± 0.00230^{+}	0.214 ± 0.012^{+}
OVX + 4OH	131.033 ± 3.206^{+++}	396.6 ± 11.997^{+}	0.0447 ± 0.0009^{+++}	0.206 ± 0.015^{+}
OVX + AD + 2OH	140.967 ± 3.695***	361.87 ± 13.61***	0.0462 ± 0.0015	0.202 ± 0.016
OVX + AD + 4OH	139.567 ± 8.441***	373.73 ± 15.96***	0.0469 ± 0.0012	0.208 ± 0.017

Values are expressed as mean ± S.E.M. ($^{\blacklozenge\blacklozenge\blacklozenge}p \leq 0.001$; $^{\blacklozenge\blacklozenge}p \leq 0.01$; $^{\blacklozenge}p \leq 0.05$ vs. SO), ($^{+++}p < 0.001$; $^{++}p < 0.01$; $^{+}p \leq 0.05$ vs. OVX) and ($^{***}p < 0.001$ vs OVX + AD).

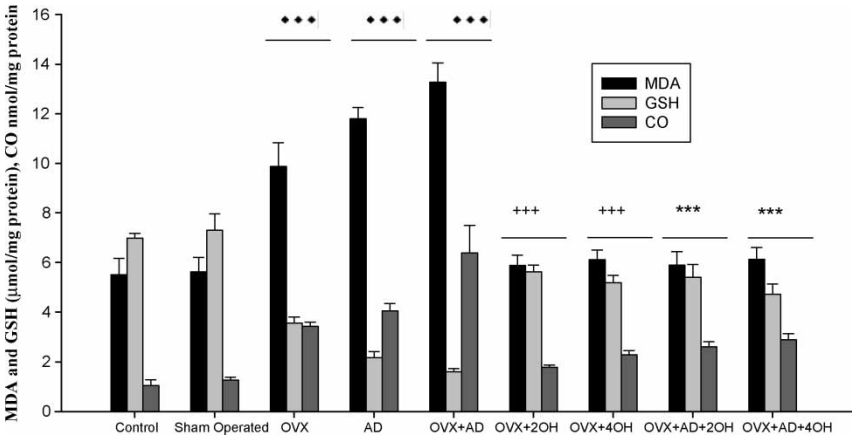

Figure 1. Effect of 2- or 4- hydroxyestradiol (2OH or 4OH) on lipid peroxidation products (MDA), reduced glutathione (GSH) and protein carbonylation (CO) content in heart tissue of ovariectomized (OVX) rats treated or not with adriamycin (AD). OVX and AD enhanced OS in heart ($p \leq 0.001$). The administration of 2OH and 4OH had a significant cytoprotective effect in OVX and OVX + AD-treated rats ($p \leq 0.001$). Values are expressed as mean ± S.E.M. ($^{\blacklozenge\blacklozenge\blacklozenge}p \leq 0.001$ vs SO); ($^{+++}p \leq 0.001$ vs OVX) and ($^{***}p \leq 0.001$ vs OVX + AD).

Evaluation of oxidative stress

OVX and AD enhanced MDA and CO contents in cardiac tissue ($p \leq 0.001$). The administration of 2OH and 4OH had a significant cytoprotective effect in OVX and OVX + AD-treated rats ($p \leq 0.001$), (Figure 1).

Evaluation of antioxidants

GSH, GPx, CAT and SOD content in heart tissue is presented in Figures 1 and 2. All of them followed a similar profile with OVX and/or AD experimental interventions. OVX, AD and OVX + AD decreased the GSH content (Figure 1) and CAT, SOD and GPx activities (Figure 2) ($p \leq 0.001$). The administration of 2OH and 4OH recovered the GSH content and the activities of all antioxidant enzymes found in sham operated animals ($p \leq 0.001$).

Discussion

The administration of AD or OVX induced cardiomyopathy associated with OS. The 2OH or 4OH administration reverted all the parameters related to OS and cardiac injury.

It has been previously shown that the administration of AD induces cardiomyopathy associated with an exacerbation of OS [16–18]. In this study, we have found that the absence of estrogens by OVX enhanced different parameters of OS, cardiac injury and haemodynamic disturbances (Tables I and II).

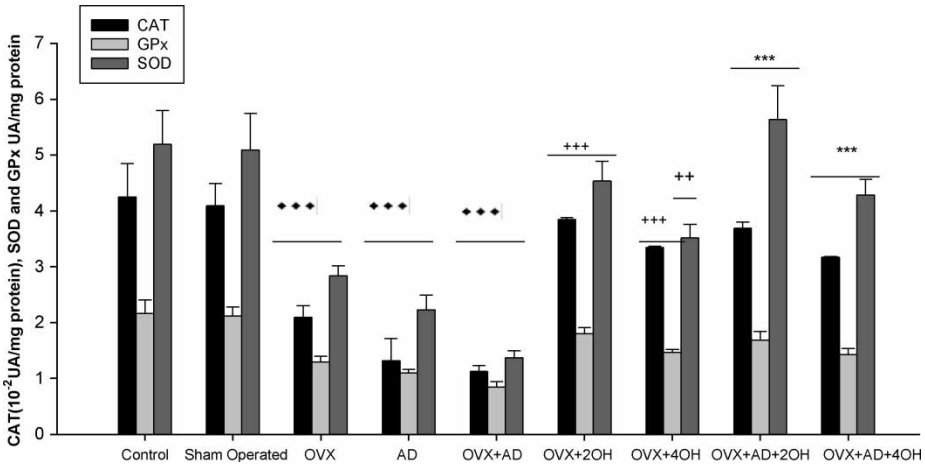

Figure 2. Effect of 2- or 4- hydroxyestradiol (2OH or 4OH) on glutathione peroxidase (GPx), catalase (CAT), and superoxide dismutase (SOD) activities in heart tissue of ovariectomized (OVX) rats treated or not with adriamycin (AD). OVX, AD and OVX + AD decreased the activity of CAT, SOD and GPx ($p \leq 0.001$). The administration of 2OH and 4OH recovered the activities of all antioxidant enzymes found in sham operated animals ($p \leq 0.001$). Values are expressed as mean ± S.E.M. ($^{\blacklozenge\blacklozenge\blacklozenge}p \leq 0.001$ vs. S.O), ($^{+++}p \leq 0.001$; ^{++}p0.01 vs. OVX) and ($^{***}p \leq 0.001$ vs OVX + AD).

These results suggest that estrogens are related to the maintenance of the correct cardiac function.

OVX increased the body weight (Table II) and different parameters related to OS in cardiac tissue (Figures 1 and 2). In this sense, OVX reduced antioxidant status (GSH, CAT, SOD and GPx activities) and enhanced MDA and CO content in heart. The induction of OS in OVX rats seem to be related to the lack of estrogens and its antioxidant capacity. The administration of 2OH or 4OH showed a beneficial effect in the reduction of body weight, OS and cardiac injury. In this sense, we must emphasize that the reduction of estrogens due to OVX was sufficient to induce OS and cardiac injury. AD treatment was used to exacerbate cell injury induced by OVX. Nevertheless, the study showed that both experimental models of cardiac injury have different effect on hemodynamic disturbances. In this sense, OVX and AD exerted an opposite effect on FC and NO production in heart. This observation may be related to a different underlying mechanism of cell damage by OVX or AD. In addition, although catecholestrogens exerted an opposite effect on hemodynamic disturbances caused either by OVX or AD, 2OH and 4OH were able to reduce cardiomyopathy in OVX-and OVX + AD-treated animals. Catecholestrogens with an ortho-diphenol structure have been reported to be more potent antioxidants than their monophenolic precursors in different experimental models [4,5,8,25–27]. Muraoka and Miura [10] demonstrated that catecholestrogens had more antioxidant capacity than α-tocopherol against ADP-Fe^{3+}-induced lipid peroxidation. 2OH administration prevented ATP and GSH depletion induced by ter-butylhydroperoxide [28]. In this study, the authors suggested that catecholestrogens decrease the lipid peroxidation by metal chelation [28]. It has been previously shown that estrogens interfer with iron-inducing lipid peroxidation coupled to Fenton's reaction In addition, 2OH interacts with cytochrome P450 which prevents superoxide radicals formation [28]. The addition of 2OH prevented *in vitro* Cu^{2+}-dependent oxidation of human LDL suggesting potential cytoprotective properties of estrogens against atherosclerosis [29]. We have not found any report showing the effect of catecholestrogens on the activity of antioxidant enzymes. In our study, 2OH and 4OH recovered GSH content (Figure 1) and CAT, SOD and GPx activities in OVX and OVX + AD treated animals (Figure 2). Different studies have related the prolonged estrogens treatment with the presence of carcinogenic and mutagenic effects [13–15]. These authors describe that the transformation of estradiol to 2-hydroxyestrone, 2OH, 4-hydroxyestrone and 4OH give raise quinonic forms that interact with DNA and lead to carcinogenic adducts [13,30]. Markides et al [31]. propose that pro- or antioxidant activities of estrogens and their metabolities are determined by a balance between their reduction potential and concentration, and their free radical scavenging capacity that blocks lipid peroxidation. Low catecholestrogens concentrations (100pm–100nM) are associated with oxidant effects while high concentrations show antioxidant properties Other authors propose that the beneficial effects of catecholestrogens depend on the nature of free radical and with the physicochemical environment in which it is generated. Ruiz-Larrea et al.[4] have observed that phenolic estrogens has more antioxidant capacity than catecholestrogens whether radicals are generated in aqueous phase while catecholestrogens are more effective if radicals are produced in lipophilic phase. In conclusion, our study showed that 2OH and 4OH exerted a beneficial effect against OS and cardiomyopathy induced by OVX and AD.

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