International Journal of Cardiology xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

International Journal of Cardiology

journal homepage: www.elsevier.com/locate/ijcard

Effect of hormone replacement therapy with the *anti*-mineralocorticoid progestin Drospirenone compared to tibolone on endothelial function and central haemodynamics in post-menopausal women

Cristiana Vitale [a,*], Caterina Mammi [a], Marco Gambacciani [b], Novella Russo [a], Ilaria Spoletini [a], Massimo Fini [a], Maurizio Volterrani [a], Giuseppe M.C. Rosano [a,c]

[a] *Department of Medical Sciences, IRCCS San Raffaele, Roma, Italy*
[b] *Department of Obstetrics and Gynecology, University of Pisa, Italy*
[c] *Cardiovascular & Cell Sciences Institute, St Georges Hospital Medical School, London, UK*

ARTICLE INFO

Article history:
Received 22 August 2016
Accepted 6 November 2016
Available online xxxx

Keywords:
Hormone replacement therapy
Prognosis
Endothelial function
Drospirenone
Pulse wave velocity
Augmentation index

ABSTRACT

Drospirenone (DRSP) is an antialdosterone agent with progestogenic and antiandrogenic effects. This compound, has been recently used in combination with 17β-estradiol (E2) as hormonal therapy in postmenopausal women and has been shown to exert a significant antihypertensive effect in hypertensive post-menopausal women. Aim of the present study was to compare the effect of DRSP/E2 with those of Tibolone (T) on endothelial function, arterial stiffness, and lipid profile of early postmenopausal women naïve on post-menopausal hormonal therapy. Twenty-four women met the inclusion criteria and entered the study. Women were randomized to receive either DRSP/E2 or T for 6 months. Blood pressure and heart rate were similar in both groups at baseline and at the end of the study. Compared to baseline, endothelial function assessed by Reactive Hyperemia (RH) significantly improved in women receiving E2/DRSP, whereas no significant differences between baseline and follow up were detected in women receiving Tibolone. Women receiving E2/DRSP showed a significant decrease in pulse wave velocity and Augmentation Index compared to baseline while no changes were observed in women receiving Tibolone. The capacity of sera to trigger endothelial cells apoptosis *in vitro* measured by cell death assay was significantly reduced by E/DRSP but not by T (HFA-E 70 ± 5,6% *vs* HFD-E 41 ± 4,5%, $p < 0{,}001$). In conclusion, the present study shows that the association of Estradiol and Drospirenone as hormonal replacement therapy significantly improves vascular parameters and the composition of sera relevant for vascular protection in early post-menopausal normotensive women. These effects are not shared by Tibolone.

1. Introduction

Post-menopausal estrogen deficiency is associated with an increased cardiovascular risk linked, among others, to the negative effect of ovarian hormones deprivation on cardiac functions and risk factors [1]. Despite early studies have consistently reported the effectiveness of hormone replacement therapy (HRT) in reducing cardiovascular events in early postmenopausal women, the cardiovascular benefits of HRT have been questioned by the results of one single randomized controlled study conducted mainly in late post-menopausal women [2–4]. Although the reasons for these conflicting results are not readily apparent, and are possibly related to patient selection and the progestin used, new alternative HRT strategies for postmenopausal women are clearly warranted [1–18].

The increased cardiovascular risk in post-menopause is mainly related to the change in blood pressure and lipid profiles and in vascular reactivity coupled with a progressive impairment of glucose tolerance [1–19]. An important step in the development of post-menopausal hypertension and glicaemic disorders is the activation of the renin-angiotensin-aldosterone system (RAAS) that, in addition to its effect on target organ damage and progression of atherosclerosis, plays a key role in the impairment of vascular stiffness and endothelial function [1–22]. Although studies on the RAAS focused mainly on angiotensin II and its inhibition, experimental and clinical studies have shown that also aldosterone has an independent and relevant role in the pathogenesis of cardiovascular and renal disease [23].

Drospirenone (DRSP) is a novel antialdosterone agent with progestogenic and antiandrogenic effects. This compound, has been recently used in combination with 17β-estradiol (E2) as hormonal therapy in postmenopausal women and has been shown to exert a significant antihypertensive effect in hypertensive post-menopausal women [24]. However, whether DRSP/E2 could exert protective cardiovascular

* Corresponding author at: Dipartimento di Scienze Internistiche Motorie e Sensoriali, Centro Ricerca Clinica e Sperimentale, IRCCS San Raffaele, Via della Pisana 235, Roma, Italy.
E-mail address: cristiana.vitale@sanraffaele.it (C. Vitale).

http://dx.doi.org/10.1016/j.ijcard.2016.11.149
0167-5273/

effects related to its *anti*-aldosterone properties independently on its antihypertensive action in normotensive early post-menopausal women has not been addressed.

It is known that the impairment of endothelial function is the initial step in both the progression of atherosclerosis and arterial hypertension and estrogens have been shown to improve endothelial function in early postmenopausal women, while the effect of progestins is controversial and mainly related to their chemical structure and scheme of administration [1,9,16,18]. After the publication of the WHI study, because of the translation of the overall study results into young postmenopausal women, alternative forms of postmenopausal hormone therapy have been suggested. Among these Tibolone has claimed a protective effect on cardiovascular functions without sharing the negative effect of classical estrogen/progestin therapy despite causing a 25% reduction of HDL cholesterol.

Aim of the present study was to compare the effect of DRSP/E2 with those of Tibolone on endothelial function, arterial stiffness, and lipid profile of early postmenopausal women naïve on post-menopausal hormonal therapy.

2. Material and methods

2.1. Study patients

Women were included in this study if they were early post-menopausal (>6 months <5 years since menopausal) aging between 45 and 60 years. Menopause was defined as absence of menstrual cycles from at least 6 months and plasma levels of FSH > 25 mIU/ml and E2 < 150 pmol/l). Only women with intact uterus were included. Women with hormonal treatment in the past, unstable angina and/or arrhythmia, recent acute MI (<3 months), hypertension (SBP/DBP > 140/90 mm Hg), renal or liver dysfunction as well as those with primary valvular, congenital heart disease, myocardial, pericardial or endocardial disease, congestive heart failure, history of intolerance or allergic response to medications similar to study drugs or women presenting with contraindications to study drugs were also excluded.

2.2. Study design

The study was double blinded, randomized DRSP/E2 *vs* Tibolone for parallel groups with a duration of 6 months. The study was approved by the local Ethics Committee. After having signed an informed consent form, patients underwent a blood sampling and a baseline evaluation of endothelial function, tonometry and pulse wave velocity before receiving the assigned treatment. Women were dispensed with study drugs after randomization and at 3 months. At 3 and 6 months patients were asked to return the unused drugs in order to assess compliance. All women underwent evaluations of vascular function and blood tests after 3 and 6 months of study treatment.

2.3. Blood sampling

Venous blood samples was taken after at least 10 h fasting, in a supine position after 20 min of rest with a Vacutainer system (Becton Dickinson, Meylan, France). Baseline blood samples were collected in tubes containing EDTA or trisodium citrate (1:9 vol/vol) and were immediately placed on ice and centrifuged within 1 h from collection. Plasma was divided into aliquots and stored at −80 °C until laboratory analysis. All serum were assessed in duplicate.

2.4. Cell culture study

Human umbilical endothelial cells (HUVECs) (Lonza, San Diego, CA.) were grown in EBM-2 medium supplemented with hEGF, h-FGF-B, VEGF, ascorbic acid, hydrocortison, *R*3-IGF-1, eparin, gentamicine and 2% FBS (EGM-2, Lonza) at 37 °C in an atmosphere of 95% air–5% CO_2. HUVECs at passage 6 were cultured for 18–24-48 h in presence of 20% sera. Lactate dehydrogenase (LDH) released from dying cells was detected using the LDH Cytotoxicity Detection kit (Roche Diagnostics GmbH, Penzberg, Germany) according to manufacturer's instructions. Maximum LDH release was assessed by adding a lysis solution to cells. The results were expressed as percentage of LDH released in each experimental condition over the maximum LDH release.

For quantification of apoptosis, fragmented DNA was measured by sandwich ELISA with antihistone-coated microtiter plates and peroxidase-conjugate *anti*-DNA antibodies using Cell Death detection kit (Roche, Japan) according to manufacturer's instructions. Briefly, after exposure to sera (48 h), cells washed in PBS and incubated in lysis buffer for 30 min at room temperature (RT) and centrifuged at 12,000 rpm for 10 min. The supernatants were transferred into a streptavidin-coated microplate and incubated with biotin-conjugated *anti*-histone and peroxidase-conjugated anti-DNA monoclonal antibodies for 2 h at RT. After washing, substrate solution was added to each well for 15 min. Absorbance was measured at 405 nm. The results were expressed as percentage of DNA fragmentation compared to a positive control (included in the assay kit).

After experimental treatment cells were collected and fixed in ice-cold 70% ethanol for 30 min at 4 °C, washed with phosphate-buffered saline (PBS) and incubated for 1 h at 37 °C in the dark with 1 ml of PBS containing propidium iodide (PI) (50 μg/ml) and RNAse (5 μg/ml). DNA staining by PI was measured acquiring 10^4 events/sample using a FACSCalibur Flow Cytometer (Becton Dickinson, Milan, Italy). Data were analysed using Cell Quest software. Cells undergoing apoptosis were expressed as the percentage of hypodiploid cells (sub-G1 population) over total cells. Apoptosis was assessed by flow cytometric analysis of cells after double staining with fluorescein isothiocyanate (FITC)-conjugated Annexin V and PI using the human Annexin V-FITC Kit (Bender MedSystems, Vienna, Austria) following manufacturer's instructions. Double negative healthy cells accumulate in the lower left quadrant. Early apoptotic cells exposing phosphatidyl serine residues on the extracellular portion of cell membrane but maintaining plasma membrane integrity result Annexin $V^+ PI^-$ and are displayed in the lower right quadrant while late apoptotic and necrotic cells, due to increased plasma membrane permeability result Annexin $V^+ PI^+$ double positive and are shown in the upper right quadrant.

Cells were incubated for 24 h with 20% of HFA-E and HFD-E sera. Total cell extracts were obtained by lysing the cells in RIPA lysis buffer (50 mM Tris–HCl pH 7.6, 100 mM NaCl, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1% Triton X-100, 10% glycerol, 100 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM sodium ortovanadate, 5 mM sodium pyrophosphate and protease inhibitors). Protein concentration was determined by BCA method, using known concentrations of BSA as a standard curve. 30 μg of proteins from each lysate were separated on a 10% SDS/PAGE gel and transferred to nitrocellulose membranes. Western blotting was performed by using *anti*-human Bax (Cell Signaling 1:1000), Caspase-3 (Upstate 1 μg/ml), Cleaved Caspase-3 (Asp175) (Cell Signaling 1:1000) and Tubulin (Santa Cruz Biotechnology 1:1000) as primary antibodies. The specific signal was detected with an enhanced chemiluminescence (ECL, Pierce) and quantified by densitometry. Results were normalized for Tubulin.

2.5. Endothelial function study

Endothelial function was assessed by reactive hyperemia using the Endo-path system. Evaluations were performed by experienced investigators unaware of the clinical data. The same investigator performed the 3 studies in each patient in order to avoid inter-observer variability. Studies were conducted in a quiet and temperature controlled room (22–23 °C). Patients were asked to avoid caffeine-containing drinks and to refrain from smoking for the 6 h preceding the study. Reactive hyperaemia was measured using the *endo*-path system that measures continuously and bilaterally hand blood flow using a validated pletismographic method. Patients were studied in the supine position after 15 min rest. Two probes for the measurement of hand blood flow were placed on both hands and blood flow was monitored for at least 5 min. A pneumatic torniquet was placed around the forearm distal to the target artery and was inflated to a pressure of 250 mm Hg for 5 min. Reactive hyperaemia was induced by sudden cuff deflation. Blood flow was measured continuously at baseline (5 min), during blood flow occlusion, during reactive hyperaemia, before and for 5 min after sublingual nitroglycerine (spray). The RH-PAT index was used to assess the presence of endothelial dysfunction. Treatment effect was assessed using the ratio between the maximum 30 s averaged value detected in the test arm during hyperaemic flow divided by the mean 60 s flow obtained before occusion.

2.6. Study of arterial stiffness and central aortic pressure

The elasticity of arterial vessels was assessed by means of pulse wave velocity and augmentation index this latter measurement allowed the calculation of the aortic central pressure. Although there exist devices able to measure both parameters simultaneneously we measured the two parameters separately using the two validated devices that are most reliable for each measurement.

Pulse wave velocity was measured with the Complior™ device. A tonometer probe was placed on the right carotid and right femoral arteries in order to detect the time delay between the peak of the blood pressure wave at the carotid and femoral level. The distance between the two measurement sites, blood pressure and heart rate were measured and recorded.

Central aortic pressure and Augmentation Index (Aix) were measured by applanation tonometry using the Sphygmocor™ device. A tonometer probe was placed on the right radial artery in order to detect and record the pressure waveform. Augmentation index and pulse wave velocity were detected semi-automatically and the results were stored on a magnetic support. The central aortic pressure was derived from the measurement of augmentation index and recorded.

2.7. Statistical analysis

Analysis of covariance for repeated measurements using baseline values as constant covariate was used to test statistical difference between measurements. A p value <0.05 was accepted as the level of statistical significance. Correlation between variables was calculated with Pearson's or Spearman's correlation coefficient. For these analysis a p value <0.005 was considered for statistical significance. The power of the study was calculated on the basis of preliminary studies from our group that have shown a 40% improvement of endothelial function with estrogen and hormone replacement therapy and no effect with Tibolone.

Please cite this article as: C. Vitale, et al., Effect of hormone replacement therapy with the *anti*-mineralocorticoid progestin Drospirenone compared to tibolone on endothelial function and central..., Int J Cardiol (2016), http://dx.doi.org/10.1016/j.ijcard.2016.11.149