Review

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Oxidative damage and the pathogenesis of menopause related disturbances and diseases

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Abstract: The postmenopausal phase of life is frequently associated in women with subjective symptoms (e.g. vasomotor) and real diseases (atherosclerosis with coronary ischemia, osteoporosis, Alzheimer-type neurodegeneration, urogenital dystrophy), which together determine the post-menopausal syndrome. Observations that oxidative damage by reactive oxygen/nitrogen species in experimental models can contribute to the pathogenesis of these disturbances stimulated research on the relationships between menopause, its endocrine deficiency, oxidative balance and the "wellness" in postmenopausal life. The connection among these events is probably due to the loss of protective actions exerted by estrogens during the fertile life. Most recent studies have revealed that estrogens exert an antioxidant action not by direct chemical neutralization of reactants as it was expected until recently but by modulating the expression of antioxidant enzymes that control levels of biological reducing agents. Also nutritional antioxidants apparently act by a similar mechanism. From this perspective it is conceivable that a cumulative control of body oxidant challenges and biological defenses could help in monitoring between "normal" and "pathological" menopause. However, as clinical studies failed to confirm this scenario in vivo, we have decided to review the existing literature to understand the causes of this discrepancy and whether this was due to methodologic reasons or to real failure of the basic hypothesis.

Keywords: antioxidant status; cardiovascular diseases; estradiol; menopause; osteoporosis; reactive oxygen species.

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Introduction

Epidemiologic evidences confirm the increasing incidence of chronic degenerative diseases, cardiovascular diseases (CVD), Alzheimer's disease (AD) and osteoporosis, in women after menopausal transition [1–4]. Menopause is defined as the phase of physiologic changes in aging women approaching reproductive senescence, triggered by cessation of ovarian endocrine function with drastic decline in estrogen secretion and definitive loss of endometrial cycling. The combination between declining of 17β-estradiol (E2) levels and advancing age is deleterious to women's organism, as witnessed by the epidemiology of CVD in females [1, 2]. Indeed premenopausal women have lower risk and incidence of CVD compared to age-matched men, with gradual disappearance of this sex-linked advantage after menopause [1]. An important issue which is more evident in the case of osteoporosis as compared to other menopause-related diseases is a highly variable individual sensitivity since only a fraction (about 40%) of the women undergoing menopause display a tendency to high bone turnover with rapid decline in bone mass density. This group represents the so-called "fast-looser" patients, who have high risk to develop overt osteoporosis. Factors that govern this population splitting are still unclear [5].

The direct link of estrogens, notably E2, in these processes is related to the loss of protective actions afforded by these hormones. Relevant to this consideration, cumulating epidemiological evidences suggest a tight association between severity/incidence of menopausal disturbances and endocrine deficiency as well as partial relief provided by hormone therapy [6, 7]. The detailed mechanisms whereby estrogens play protective effects on endothelium, bone and brain are not completely understood, but might relate to "systemic antioxidant actions" [8, 9]. Within this frame oxidative stress (OxS) is considered as the common pathogenic factor for all diseases and disturbances (e.g. vasomotor complains, CVD, osteoporosis, cognitive impairment and urogenital dystrophy, etc.) [8, 10–17] grouped together in the "menopausal syndrome".

Since the hypothesis that menopausal transition leads to an average increase of OxS, which in turn contributes to the higher risk of menopause-associated pathologies, is still disputed, in this report we review the current state of knowledge on this topic, to highlight the most relevant laboratory and epidemiological evidences supporting/refusing these assumptions and the progresses in understanding the underlying mechanisms.

Oxidative stress

Definition

OxS is widely defined as a derangement in the dynamic balance between oxidants and antioxidants, in favor of the former, leading to damages against biological structures and progress to disease [18]. The most abundant form of oxidants is the reactive oxygen species (ROS). Other species that display potent oxidizing ability are reactive nitrogen species (RNS), hypochlorous acid, chloramines and sulfur radicals [11]. Most of them are free radicals, i.e. unstable and highly reactive particles due to the lack of one or of a couple of electrons. They can achieve chemical stability by acquiring electrons from nucleic acids, lipids, proteins or nearly any neighbor molecular entity. These reactions induce changes in the final targets inevitably leading to important consequences in cell physiology, which in most instances are harmful to cells but can also be beneficial in some biological frameworks [11, 12]. For instance, among ROS hydrogen peroxide (H_2O_2) plays a dual role serving both as a toxic oxidant and as a fundamental signaling molecule that regulates cellular processes, such as proliferation, migration, anoikis, survival and autophagy [19]. Their steady state concentration appears as the determinant factor that determines whether ROS serve as pro-survival or deleterious players [11]. There is now a large consensus that high level of these reactive species, including H_2O_2, due to a poorly controlled generation causes damages that contribute substantially to aging and age-associated disease, as stated by the free radical theory of aging [20].

Origin of reactive species

Reactive species are also formed under basal conditions but pathologic conditions greatly stimulate their generation through activation of enzymes, such xanthine oxidase, dihydronicotinamide-adenine dinucleotide phosphate oxidase (NOX), lipoxygenase, cyclooxygenase, etc., localized in membranes and in subcellular organelles [11]. These pathways account for lower amount of ROS production than in mitochondria where 5% of oxygen flux gives rise to ROS, notably superoxide anion ($O_2^{\bullet-}$) even under physiologic conditions [21]. This keeps with the statement by Cadenas and Davies that the fundamental role of oxygen in aerobic organisms is "simply to act as a sink or dumping ground of electrons" [21]. Additional $O_2^{\bullet-}$ is produced during respiratory burst accompanying phagocytosis in the professional phagocytes of the bacterial defense system. This process requires large amount of energy (obtained by increasing the consumption of glucose and oxygen), enhanced NADPH production (via the pentose monophosphate shunt) and simultaneous activation of membrane NOX, giving rise to a supplemental generation of superoxide anion [11, 22].

These primary ROS (chiefly $O_2^{\bullet-}$ and H_2O_2) can generate other "secondary" ROS either directly or through metal- or enzyme-catalyzed reactions [11, 23, 24]. Superoxide anion is readily dismutated by mitochondrial superoxide dismutase (SOD) into poorly reactive H_2O_2 (which can also derive from peroxisome or the mitochondrial outer membrane monoamine oxidase) [21, 25]. This non-radical ROS in turn can be involved in different reaction pathways. By one side, it can be reduced to water by mitochondrial glutathione peroxidase or catalase or, upon crossing mitochondrial membranes, by cytosolic forms of these enzymes [11]. In case of ineffective detoxification, H_2O_2 can generate the highly reactive hydroxyl radical ($^{\bullet}OH$) or peroxyl radical ($^{\bullet}OOH$) in the presence of free reduced ions of iron (or other transition metals) [23]. Complex biological pathways that regulate iron metabolism dispose of intracellular free iron but excess of $O_2^{\bullet-}$ production close to active redox pools of Cu and Fe in mitochondria can allow occurrence of the Fenton reaction (Figure 1) [11, 26].

The hydroxyl radical can damage any biological molecules (proteins, lipids, DNA) with rate constants approaching diffusion limited rate [24]. Thus it is virtually impossible in vivo to find a compound with any more significant hydroxyl radical scavenging activity to protect the "victims" themselves from its attack [24]. Similar considerations apply to peroxynitrite (ONOO-) derived by the combination between nitrogen monoxide (NO) and superoxide ion, itself the main responsible of the cytotoxicity

$$Fe^{+++} + H_2O_2 \rightarrow Fe^{++} + {}^{\bullet}OOH + H^{+} \quad (1)$$

$$Fe^{++} + H_2O_2 \rightarrow Fe^{+++} + OH^{-} + {}^{\bullet}OH \quad (2)$$

Figure 1: In Fenton reaction iron and the hydrogen peroxide react together to generate hydroxyl radical (1) or hydroperoxyl radical (2).

ascribed to NO [27]. As outlined below in a very recent review Forman states that the only efficient protection mechanism cells can adopt against these potent radicals is to prevent their formation [24, 28].

The antioxidants

Several non-enzymatic and enzymatic machineries contribute to maintaining ROS/RNS concentration within a life-compatible range, with an intracellular "redox homeostasis" being mostly guaranteed by the thiol peptides glutathione (GSH) and thioredoxin (Trx). The redox state of these two thiol peptides is preserved by the cooperative work of a series of enzymes such as glutathione peroxidase (Gpx), Trx reductase, GSH-synthetase, etc. [11, 24] (Table 1). Notably, the posttranscriptional addition of GSH to protein cysteine, modulate the activity of intracellular signaling molecules, and inhibits the activity of many important enzyme [11].

The high rate constants of reaction involving reactive species implies that to be an effective scavenger of these molecules in vivo the putative antioxidant must be at high concentration (as in the case of intracellular GSH) or an enzyme. GSH (1–10 mM intracellular concentration)

Table 1: Origin, localization and main functions of the major antioxidants in human body.

Name	Origin (Diet/Endo)	Localization (IC/EC)	Main antioxidant functions
Glutathione system			
Glutathione synthetase (GSS) (+glutamate cysteine ligase)	Endo	IC	Synthesis of GSH
Glutathione (GSH)	Endo	IC	– Major substrate of peroxidases (GPx) – Scavenger of reactive species – Modulation of enzymes and signaling molecules activity via post-transcriptional modification of proteins (S-glutathionylation)
Glutathione peroxidases (GPxs)	Endo	IC[a]	Reduction of hydroperoxides
Gutathione *S*-transferases (GSTs)	Endo	IC	Detoxification of xenobiotics and other noxious compounds by conjugation with GSH
Glutathione reductases (GR)	Endo	IC	Reduction of glutathione disulfide (GSSG) to GSH
Thioredoxin system			
Thioredoxin (Trx)	Endo	IC	– Reduction of disulfides within oxidized cellular proteins – electron donors to Prxs
Thioredoxin reductases (TrxRs)	Endo	IC	Reduction of the oxidized form of Trx (regeneration of Trx)
Peroxiredoxins (Prxs)	Endo	IC	Reduction of hydroperoxides
Scavenging enzymes			
Catalases (CATs)	Endo	IC	Decomposition of H_2O_2 to water
Superoxide dismutases (SODs)	Endo	IC[a]	Dismutation of superoxide radical to either O_2 or H_2O_2
Low molecular weight antioxidants			
α-Tocopherol	Diet	EC/IC	Scavenger of hydroperoxyl radicals (chain breaking activity)
Ascorbic acid	Diet	EC/IC	– Reduction of tocopheryl radical to α-tocopherol – Potential scavenger of reactive species (but its concentration in vivo is too low)
β-Carotene	Diet	EC/IC	Potential scavenger of reactive species (but its concentration in vivo is too low)
Albumin	Endo	EC	– Sequestration of pro-oxidant metals – Trapping of reactive species
Uric acid	Endo	EC	– Chelation of pro-oxidant metals – Scavenger of peroxynitrite
Flavonoids (resveratrol, curcumin, etc.)	Diet	EC/IC	Promotion of antioxidant enzymes expression

[a]Some isoforms of these enzymes are also present in extracellular compartment. EC, extracellular; IC, intracellular; Endo, endogen.

and SOD (that act in combination with catalase and GSH peroxidase to decompose H_2O_2) seem to be the only two antioxidant capable to overcome these kinetic constraints [24, 29]. Low molecular weight antioxidants of endogenous (uric acid, albumin, etc.) or dietary origin (ascorbic acid, α-tocopherol, flavonoids, etc.) are able to retard and/or prevent oxidative damage, but generally unable to directly neutralize reactive species [24] (Table 1). Among them α-tocopherol is the only compound capable to compete with enzymatic machineries (with average reaction rate 100,000 times faster than other non-enzymatic components) providing effective chain breaking in lipid peroxidation thanks to its high concentration, membrane localization and relatively rapid kinetics of reaction, effectively protecting membranes from damage [24, 30]. This scavenging activity results in the formation of α-tocopheroxyl radical that can be efficiently reduced back to the innocuous form by ascorbic acid [31].

Considering the mechanism of action of dietary antioxidants, Forman et al. [24] and others [32, 33] proposed that they most likely act affecting signaling pathways to induce endogenous enzymatic antioxidant response, as in the case of the polyphenols curcumin (turmeric) and resveratrol (grapes) that work in vivo not through direct chemical reaction as in vitro but by eliciting expression of nuclear factor erythroid 2-related factor 2 (Nrf2) [24]. This transcription factor translocates to the nucleus to bind the antioxidant response element (ARE) and activate transcription of a battery of genes encoding phase II detoxification enzymes (including those involved in GSH and TRX systems) and other endogen enzymes (such as SOD and catalase) [34]. As we will discuss below, estrogens apparently work by a similar approach.

Laboratory markers of OxS

Reliable markers are required for quantitative assessment of OxS in vivo. In this instance the landscape is complicated by the high reactivity, short half-life and heterogeneity of reactive species making their quantification in biologic fluids highly challenging [28]. Several methods have been proposed as based on fluorescent probes, electron spin resonance, chemioluminescence which are capable to directly detect ROS/RNS, but unpractical in clinical studies [35].

The best non-invasive analytical approach to quantify these reactive species is to trace them by fingerprinting, analyzing in blood/urine/breath the concentration of stable by-products of oxidatively damaged biomolecules, which serve as proofs of the prior presence of reactive species at concentrations overwhelming antioxidant defense [35]. Drawbacks are posed by lack of consensus about the selection of OxS parameters to be determined, absence of reference values for specific markers and of careful methodological comparison among analytical approaches [35, 36]. A few acceptable markers are emerging with appreciable stability and tendency to accumulate during disease progression. We will now review their properties in relation to the molecules they are derived from. A more schematic overview of the most used OxS markers is shown in Table 2.

Table 2: Most frequently assessed peripheral markers of oxidative damage.

Marker	Oxidized biomolecules	Specimens[a]	Preferred methods	Other methods
F2-isoprostanes (F2-iso)	Lipids	Urine/serum/plasma/breath	GC-MS/MS LC-MS/MS	ELISA RIA
Malondialdehyde (MDA)	Lipids	Serum/plasma/urine	GC-MS/MS LC-MS/MS HPLC-FD	Spectrophotometric Fluorimetric
4-hydroxynonenal (4-HNE)	Lipids	Serum/plasma	GC/MS	ELISA
Lipid hydroperoxides (LOOH)	Lipids	Serum/plasma	GC/MS	Spectrophotometric Fluorimetric
Protein carbonyls	Proteins	Serum/plasma	HPLC	ELISA Spectrophotometric
Nitrotyrosine	Proteins	Serum/plasma/urine	GC-MS/MS HPLC-MS/MS HPLC-ED	ELISA
8-oxo-7,8-dihydro-2′-deoxyguanosine (8-OHdG)	DNA	Urine/serum/plasma	GC-MS/MS LC-MS/MS	ELISA

[a]Only the most commonly used samples are indicated. GC, gas chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; LC, liquid chromatography; HPLC, high pressure liquid chromatography; FD, fluorimetric detection; ED, electrochemical detection.

Lipid peroxidation

Polyunsaturated fatty acids (PUFAs) in plasma membranes are prone to oxidation by free radicals (mainly $OH^{\cdot}$) with perturbation of membrane function. Removal of a hydrogen atom from PUFA initiates a chain reaction mechanism that leads to the formation of several primary, i.e. lipid hydroperoxides (LOOH) and secondary (aldehyde) products which can be stable enough to serve as candidate markers. The stability of these by-products can be suitably enhanced by supplementing the samples with antioxidants, metal chelators or acid-organic solvents to inhibit enzyme reactions that might alter their concentrations. The chemistry associated with these reaction has been exhaustively reviewed [35, 37].

Reliable measurement of plasma/serum level of LOOH by GC/MS or oximetry is time-consuming and expensive [35]. Alternative spectrophotometric/fluorimetric methods are available: they are cheap, easy but have limited sensitivity. Although mainly semiquantitative, they allowed significant measurements on the response of the concentration of LOOH to administration of the lipid peroxidation initiator CCl_4 during investigations in in vivo animal models [36].

Alternatively the aldehydes 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA) can be measured. 4-HNE is a chief messenger of ROS-mediated cell toxicity modulating transcriptional factors and enzymes in signaling pathways during cell senescence, proliferation/differentiation, apoptosis, and necrosis [38]. 4-HNE is formed at low levels under physiological conditions but is markedly raised in pathological conditions [39]. 4-HNE-protein adducts can be accurately measured by time- and labor-consuming GC/MS [40] or by immunoblotting (qualitative) methods. An ELISA test was introduced recently but has not been validated for measurements in blood samples [41]. MDA is also considered an effective marker of lipid peroxidation and is easily measured in serum/plasma samples by a spectrophotometric assay based on complex formation with thiobarbituric acid (TBA) to form TBA reacting substances (or TBARS) [42]. This method gives a good estimate of lipid peroxidation in purified systems but is questionable in serum/plasma because additional compounds besides MDA form colored complexes with the reagent [36, 42, 43]. Improved sensitivity and specificity in the detection of MDA by the TBA assay are achieved by coupling with high pressure liquid chromatography (HPLC) plus fluorimetric detection, GC-MS/MS and LC-MS/MS [36, 42].

Attention is now focusing on F2-isoprostanes (F2-iso) which are derived from free-radical mediated peroxidation of arachidonic [35]. F2-iso and other isoprostane-like compounds (e.g. F4- and E4/D4-neuroprostanes) are the most reliable markers of lipid peroxidation and indicators of OxS in vivo in different diseases [44]. They exert specific functions in tissues (usually vasoconstriction), have widespread occurrence and are stable in biological fluids (plasma/breath condensate/urine). The preferred biological sample is urine because of its accessibility, low content of lipids (that could produce autoxidation artifacts) and low intra-individual variability compared to plasma, upon normalization for creatinine excretion [35, 36, 44]. Addition of antioxidants (e.g. butylated hydroxyl-toluene) and storage at −80 °C are recommended to avoid artifacts. GC-MS is the gold standard for quantitate F2-iso in biological samples, and along with GC-tandem MS (GC-MS/MS) and LC-MS are the only procedures reliable in distinguishing different isoforms and other prostanoids [35, 44]. Easy to perform ELISA and RIA immunoassays are also available, but although affording high-throughput analyses are less precise because polyclonal antibodies towards F2-iso exhibit cross-reactivity with many other molecules, thus overestimating its concentration [28, 44].

Protein oxidation

The detection of specific oxidative posttranslational modifications to proteins can be a help to decipher modulation patterns of complex signaling pathways [45]. OxS-driven damage leads to gross protein changes (fragmentation, crosslinkage) but also oxidation to protein carbonyls or modification of tyrosine residues [46]. As the circulating concentration of the most stable by-products reflects the intracellular degree of OxS, they can serve as a surrogate for this purpose [47]. As already discussed for lipoperoxidation markers, the reliability of protein oxidation products (including S-glutathionylation, carbonylation or tyrosine modification to 3-nitro-, 3-chloro- and 3-bromotyrosine) strictly depends on the procedure employed [35, 48]. In general, MS-based technologies are preferred while highly publicized spectrophotometric or ELISA assays still present severe limitations [35, 48].

Determination of carbonyl-groups in proteins represents the most commonly employed marker of protein oxidative damage. It is well-documented that it accumulates with aging and in several OxS-related diseaes [35, 49]. They are measured frequently in plasma or serum, where they are present in detectable amounts also in healthy individuals, while the tyrosine derivative 3-nitrotyrosine (formed by reaction with peroxynitrite or NO) which would be more specific for physiopathological assessment, is

difficult to measure in blood or urine because of its high instability in these fluids [50–52].

DNA/RNA oxidation

ROS can damage cellular DNA and generate a broad range of by-products including cross-linked nucleotides and fragmented filaments [49]. Typical changes involve guanine nucleotides which are oxidized at position 8, yielding the potential markers 8-oxo-7, 8-dihydro-2′-deoxyguanosine (8-OHdG) in DNA and 8-OHG in RNA. 8-OHdG, by far the most frequently assessed marker of nucleic acid damage, is promutagenic, since it mismatches with adenine if it is not repaired prior to DNA replication, contributing to pathogenesis of human diseases [53]. Urinary levels of 8-OHdG are widely employed in epidemiologic studies as potential diagnostic markers and disease risk factors, particularly for cancer [54]. Urinary determination is preferred because of the easy non-invasive collection and the stability at −80 °C [55]. The European Standards Committee on Urinary DNA Lesion Analysis made significant contributions to the analytical validation of this marker demonstrating that HPLC with electrochemical detection, GC-MS, and HPLC tandem SM are the best methods in terms of sensitivity and reproducibility [56]. Several investigations, including those on the CCl_4 rat model, employed commercial ELISAs to measure 8-OHdG [36] with results comparable with those obtained employing MS [57] upon introducing appropriate modifications to the assay procedure (e.g. temperature of incubation of primary antibody) despite concerns by many authors about the validity of the ELISA approach [56, 58].

Estrogen decline and increase in disease incidence. The role of OxS

By definition, menopause begins after 12-months of amenorrhea since the final menstruation. In established menopause circulatory concentration of the reproductive estrogen E2 drops to 10 pg/mL, compared with average values of 100–250 pg/mL in the fertile life. Clinical effects of the estrogenic deficiency in vivo are partly relieved by residual estrogens, like estranges which are produced by adipose tissue and by traces of androgens.

These endocrine changes are frequently associated with the severe pathologies of menopause, including disturbances in sleep/mood, vasomotor and genitourinary symptoms [1], ischemic coronary diseases [2, 59], Alzheimer related neurodegeneration [4, 60] and osteoporosis [61].

Estrogens, notably E2, display epidemiologically significant protection at cardiovascular and reproductive system, bone, liver, and brain, where the estrogen receptors ERα and ERβ are expressed at different levels [62]. These receptors are crucial for the actions of endogenous or exogenous E2 administered in hormone replacement therapy (HRT) in the attempt to restore premenopausal E2 levels minimizing the effects of ovarian deficiency. Although the real benefits of HRT on women's health are still debated [63, 64], available evidence suggest the efficacy in reducing the risk of CVD [65], incidence of osteoporosis [66], all-cause mortality [66, 67] and intensity of hot flashes and other menopausal disturbances [1, 67].

At the metabolic level, E2 does decrease the concentration of atherogenic plasma lipids, ameliorate endothelial function and inflammatory homeostasis, increase neuronal survival, etc…, emphasizing the multi-faceted modulatory actions of E2 [68].

During last decades, researchers hypothesized that menopause itself represents an important threat to women's health because it is accompanied by increased systemic OxS, triggered by the loss of E2 which has antioxidant activity in vitro on cell cultures, ex vivo and in animal models [14, 69–74]. The hypothesis of a protection by E2 against oxidative damage to cardiovascular, neuronal and bone tissue still waits a definitive scientific validation in human disease since epidemiologic studies failed yet to confirm the in vivo impact of these hormones in the systemic redox balance, as we discuss below.

Supporting evidences

E2 as a potent antioxidant in cell cultures and in ovariectomized animals

Disruption in systemic OxS at menopause could be ascribed to endocrine changes if E2 possesses potent antioxidant properties. The first evidence was provided by Sack et al. [74] who demonstrated increased resistance of LDL to oxidative modification and pro-atherogenic transformation in 18 postmenopausal women receiving intra-arterial infusion of E2, and in 12 women also receiving transdermal E2 for 3 weeks [74]. These effects were reversed after therapy discontinuation. Additional experiments confirmed that the oxidation of LDL by cells or by copper ions ex vivo [69, 70] is inhibited in a dose-dependent way by E2 administered to achieve super-physiological levels, but were not easily reproduced in vivo, with contrasting results [75–79].

Viña and others [80–82] shed light on these controversies demonstrating that the antioxidative activity of E2 is related to molecular signaling pathways affecting cellular redox homeostasis. The first hypothesis to explain the ability of E2 to counteract the ROS challenge, i.e. that it was due to the reducing propriety of the phenolic structure, has been rejected because E2 concentration in plasma is too low to afford any tangible effect by direct chemistry [80]. In contrast it is now appreciated that E2 binding to estrogen receptors stimulates the expression of mitochondrial and cytosolic SOD (respectively, MnSOD and Cu/Zn SOD) and Gpx [81, 82]. Consistently, estrogen deprivation in ovariectomized mice [82] or rats [81] has been extensively shown to correlate with down-regulation of these enzymes and increase of ROS-induced damage. Noteworthy, the administration of estrogens plus progestins after ovariectomy had beneficial effects on OxS levels in both animal models [81–83].

Along with these experimental evidences, clinical data in postmenopausal women also support the possibility that the protection by E2 against chronic degenerative diseases involves activation of antioxidant pathways as OxS is apparently involved in the onset of the major degenerative diseases of menopause by still poorly understood biochemical mechanisms.

CVD

Increased OxS is crucial in the pathogenesis of vascular diseases, including hypertension and atherosclerosis [11, 84]. Toxicity by the oxidative reactive species causes endothelial dysfunction, muscular proliferation and contraction, and destabilization of atherosclerotic plaques. The main trigger of damage to vascular walls is the loss in bioavailability of NO which mediates endothelium-dependent relaxation [11, 84].

E2 can guarantee suitable NO bioavailability by increasing the generation of NO (inducing NO synthase expression) [73] or decreasing the production of superoxide (whose reaction with NO produce peroxynitrite) [80, 81]. The antioxidant pathway takes place by means of E2-mediated stimulation of expression and activity of intra- as well as extra-cellular SOD [82]. Accordingly, a decrease in estrogens in postmenopausal women is related to a decrease in endothelium-dependent relaxation [85].

An additional antioxidant effect by which estrogens contribute to cardio-protection is related to the ability of the hormone to influence body fat distribution. During menopause the decrease in E2 circulating level promotes visceral/abdominal adiposity shifting fat distribution from gynoid (gluteal-femoral) to android (trunk-central) type [86, 87]. This redistribution is related to metabolic abnormalities such as glucose intolerance, hyperlipidemia and low grade inflammation, potentially capable of altering the systemic redox balance [88–90]. Indeed, solid epidemiological data confirm the relationships between E2, visceral/abdominal adiposity and OxS [90].

Osteoporosis

Menopause is clinically associated with increased incidence of osteoporosis and risk of fractures, because of increased bone turnover and loss of bone mass [61]. During female reproductive life, E2 protects the bone against erosion by preserving the crucial balance between bone resorption and formation, likely through interference with OxS [15, 91]. According to Almeida et al. [92], the ability of E2 to attenuate OxS relates to the mechanisms underlying the beneficial effects of these hormones on bone cells. Indeed, most probably by Erα-mediated activation of cytoplasmic kinases, E2 increases glutathione reductase activity in the bone marrow (a rich source of mesenchymal stem cells) of transgenic mice [92]. Besides, studies on ovariectomized rodents demonstrated that E2 deprivation causes a drastic decline in GSH, Trx and the enzymes involved in their recycling pathways [93]. Compellingly, the levels of these antioxidants were rapidly normalized by administration of a single dose of E2.

Therefore, the over-expression of endogenous antioxidant machineries is of paramount importance to prevent the deleterious action of ROS toward bone health. These reactive species (produced by either NOX or xanthine oxidase) stimulate osteoclast differentiation and bone resorption in murine calvarial and bone marrow cultures [94] and in osteoblasts/spleen cells cocultures [95], probably through stimulation of the osteoclastogenic nuclear factor-kappa β ligand (RANKL)-RANK signaling between osteoblasts and osteoclast precursors as in several in vitro studies [91, 94]. Binding of RANKL to RANK initiates osteoclast differentiation/activation, critical for their survival and promotion of bone resorption [96]. Derangements in this interaction are prominent in pathogenesis of postmenopausal osteoporosis [96].

Alzheimer's disease

AD, the most frequent neurodegenerative disorder associated with memory impairment/dementia in the elderlies, is more frequent in women than in men, with relative

risk between 1.2 and 1.5 [97]. Even if the time-course of the pathogenic progression is unclear, aggregation of amyloid β (Aβ) to form extracellular senile plaques, neurofibrillary tangles and mitochondrial dysfunction play primary roles in AD development and clinical progression [98]. Cerebrovascular lesions and defects in brain micro-circulation are frequent pathological traits of this disease [99] and are, to various extents, related to OxS [13, 15, 98, 100, 101].

Oxidative damage is present in vulnerable neurons long before the development of other neuropathological correlates [102] and is ascribed to toxic species released by dysfunctional mitochondria [103]. ROS are also responsible for the neurotoxicity by Aβ aggregates which generate H_2O_2 [100] through activation of microglial and neuronal NOX [104].

The coincident contributions of age (the foremost risk factor for AD), female gender and OxS call attention to menopause and ovarian failure in the pathogenesis of AD. Although longevity and survival difference favoring women might contribute to this gender difference, the possible contribution of declining ovarian function after menopause cannot be disregarded [4, 105].

In line with this hypothesis, hormone therapy in postmenopausal women may decrease AD risk and alleviate disease symptoms [106], in line with the neuroprotective activity of E2 in cultured neurons and in animal models [107, 108]. The putative beneficial effects of E2 include increase in cerebral blood flow, glucose transport and consumption and reduction in Aβ aggregation and deposition [109], with prominent modulation of metabolism and neurotoxicity of Aβ, and thereby the formation of senile plaques. According to recent evidences obtained from post-mortem human brains E2 decreases Aβ formation by enhancing the activity of cerebrocortical SOD, also preventing lipid peroxidation damages induced by Aβ [110]. Animal experiments also highlight the presence of mitochondrial estrogen receptors in the cerebral vessels [111], suggesting that E2 can act through both mitochondrial and nuclear genomes to enhance cerebral vascular mitochondrial function limiting the leak of ROS from the respiratory chain.

Contrasting evidences

Despite these experimental evidences, the validation of the hypothesis that menopause represents an important threat for women's health because its occurrence is accompanied by an increase of systemic OxS still requires solid and consistent epidemiological data on humans. Two points need investigation to verify the relationship between estrogens and OxS: the first whether the menopausal decline of E2 coincides with an increase of systemic OxS, the second whether administration of estrogens replacement therapy in postmenopausal women alleviates systemic OxS. So far results did not answer these queries in humans mostly because of limitations in the study design and shortcomings in the methodology used to detect OxS in vivo. We now briefly survey these points for future purposes.

The issue of study design

Longitudinal studies involving follow-up of women traversing menopause represent the most rigorous approach to analyze cause-effect relationships between menopause and OxS, but they are complicated to perform because of the high cost, drop-out of subjects during follow-up, and individual variability in duration and age of onset of menopause. Therefore, most of the available results arise from the less rigorous cross-sectional studies which compare parameters of OxS in pre- and post-menopausal women (Table 3). By this approach it is impossible to determine whether OxS is a consequence of menopause or vice-versa. Additionally data from cross-sectional studies require correction for differences in age and confounding features of the population – e.g. body mass index (BMI), life-style and ethnicity – by means of multivariate analysis, although this approach cannot definitely eliminate this potential bias. Age represents the main drawback in these studies because it is frequently different in subsets of women with different menopausal status. As aging is tightly linked to both OxS and menopausal event (as well as the correlated symptoms and diseases), cross-sectional investigations should be better designed with proper exclusion/inclusion criteria selecting women with similar age.

Additional factors to be taken into account include concomitant diseases, therapies (e.g. chemotherapy, anti-inflammatory, etc.), abuse of alcohol, physical activity, diet, smoking, BMI, all potentially affecting systemic OxS. Of peculiar relevance is fat body distribution which changes during the menopause transition, with increasing abdominal/visceral adiposity (even without gross changes in weight/BMI) [87]. Notoriously visceral fat is associated with OxS markers [79, 88–90]. Two other features strongly affecting reliability of cross-sectional studies are sample size and criteria for the definition of menopause. Sample size is frequently limited in population studies [9, 79, 112, 113] introducing the potential risk to report false-negative findings (type II error). Indeed, just a few studies deal with samples larger than 100 women overall [75, 78, 114–120]

and only two include more than 1000 subjects [75, 118] (Table 2). As for the definition of menopause, the Stages of Reproductive Aging Workshop (STRAW) criteria [121], recently revised and extended [122], classify the phases of women's reproductive life, based on changes in menstrual cycle length and frequency. Women are considered of reproductive age if the bleeding cycle is regular; in perimenopause if they report irregular menstrual cycles and/or 2–11 months of amenorrhea (absence of menstruation), and in post-menopause only after a 12-month period of amenorrhea. Among the studies we analyzed on menopause and its relationship to OxS, we found two reports that did not follow these recommendations [114, 116], classifying postmenopausal women as reporting more than 3 months of amenorrhea, including inevitably in this group also some perimenopausal women. This lack of uniformity with basic standards affects the reliability of data presented and limits the comparability with the other published studies.

Methodology shortcomings

As stated, the absence of a gold standard marker and of universally accepted reference intervals in the normal population are fundamental limitations to investigate OxS in vivo. To better clarify this issue we critically review the results of the nine largest studies comparing OxS in reproductive age and postmenopausal women. In three studies urinary F2-iso was measured by ELISA [75, 116, 119], eventually including a chromatographic pre-analytical step to improve the specificity of detection. Notably these studies failed to detect an increase in F2-isos in postmenopausal women compared to younger fertile counterparts. Moreover, in contrast with the expectations, in one case this marker correlated positively, with E2 values in postmenopausal women, even after adjustment for age, BMI and other confounders [75]. A potential interplay between endogenous sexual hormones (including E2, sex hormone-binding globulin, follicle stimulating hormones, etc.) and F2-iso, as reliably measured by GS-MS, was also prospectively investigated in BioCycle Study, a cohort investigation on 259 women aged 18–44 years followed for two menstrual cycles [123]. F2-iso displayed positive independent association with circulating E2 levels throughout the menstrual cycle. The reliability of the methodologies and markers employed in these studies cast doubts about the prevention of OxS by E2 at least in premenopausal women.

Investigations focusing on other lipoperoxidation markers did not aid in solving controversy about the

Table 3: Population-based studies dealing with the association between OxS markers and menopausal status (and/or E2 blood level).

First author (date of publication)	Sample size[a]	Marker (method)	Results	
			POST vs. PRE	[E2] vs. [OxS marker]
Abdul-Rasheed (2010) [112]	33	TBARS (spectrophotometry)	POST>PRE	
Cervellati (2011) [78]	123	LOOH (spectrophotometry)	No difference	No correlation
Lee (2015) [118]	2238	Homocysteine (not described)	No difference	
Paik (2012) [119]	176	F2-iso (ELISA)	No difference	
Pansini (2007) [79]	76	LOOH (spectrophotometry)	No difference	
Sánchez-Rodríguez (2012) [117]	187	TBARS (spectrophotometry)	POST>PRE	
Schisterman (2010) [123]	259[b] (only PRe)	1. F2-iso (GC-MS)		Positive correlation
		TBARS (spectrofluorimetry)		No correlation
Signorelli (2006) [114]	101	4-HNE (spectrophotometry)	POST>PRE	
		MDA (HPLC)	POST>PRE	
Sowers (2008) [116]	468	F2-iso (ELISA)	No difference	
Sowers (2008) [75]	1101[c]	F2-iso (ELISA)	No difference	No correlation
Tupikowska (2004) [113]	66	LOOH (spectrophotometry)	POST>PRE	
Victorino (2013) [9]	58	LOOH (spectrophotometry)	POST>PRE	
		MDA (HPLC) TBARS	No difference	
		(spectrophotometry)	No difference	
Yang (2009) [120]	226	TBARS (spectrophotometry)	No difference	
		8-OHdG (ELISA)	No difference	

[a]With some exceptions ([b] and [c]) the sample considered in the analysis include women in premenopause or in postmenopause not under hormonal therapy. [b]Only premenopausal women. [c]Premenopausal and perimenopausal included in the same group. TBARS, thiobarbituric acid reacting substances; LOOH, lipid hydroperoxides; F2-iso, F2-isoprostanes; GC-MS, gas chromatography mass spectrometry; MDA, malondialdehyde; 8-OHdG, 8-oxo-7,8-dihydro-2′-deoxyguanosine; 4-HNE, 4-hydroxynonenal; POST, postmenopausal women; PRE, premenopausal women.

antioxidant potential of E2 in vivo because of their marked inconsistency. Two works reported high level of the aldehydes [114, 117] (MDA assessed by HPLC and 4-HNE by ELISA, or of TBARS) in serum of premenopausal vs. postmenopausal women, while values of urinary TBARS and serum hydroperoxides did not vary between the two groups in other reports [9, 78]. Likewise no difference in homocysteine concentration was found in a large study involving 840 pre- and 1392 postmenopausal women [118] and in an investigation of urinary 8-OHdG (ELISA assay) in women at different menopausal status [120]. These results partially contrast with those recently obtained by comparing this DNA damage index level between women and men at various ages [124]. The authors observed gender difference only when subjects older than 50 years were considered, with a trend towards higher levels of 8-OHdG in women compared to men and interpreted the results as proof of the impact of estrogens decline on OxS.

HRT (and/or ERT) and OxS

Although efficacy and safety of hormone therapies were questioned by the results of some large randomized clinical trials [64, 125] prescription of both HRT (i.e. combination of estrogen and progestins) and ERT (i.e. only estrogens) is still popular for the prevention and treatment of typical menopausal complaints/diseases.

The literature is flooded with papers focusing on the impact of exogenous hormones on systemic redox balance. Two cross-sectional studies, by the same authors, investigated the influence of HRT on F2iso in postmenopausal women [75, 116], reporting a higher level of this marker in women under therapy compared to non-users. In particular, one of these studies showed significantly higher excretion of F2iso in 243 women using HRT than in 53 using ERT [116]. In the authors' view, the ability of progesterone to antagonize antioxidant action of E2 in vitro, as demonstrated by Ozacmak and Sayan [72], may account for these "surprising" results.

Contradictory data on the effects of hormonal therapy emerged from minor studies (total number of subjects lower than 100) employing different peripheral markers of OxS. Escalante Gómez and Quesada Mor found lower 8-OH-2dG levels in women receiving combined HRT compared to women who did not receive therapy [126], while TBARS were lower in women on ERT compared to non-users. Another recent study reported a decrease of TBARS, but unvaried levels of serum homocysteine and carbonyls in postmenopausal women under HRT compared to women receiving oral placebo [127]. At variance with this report, Unfer et al. [128] found no difference in serum TBARS between healthy postmenopausal women without/with HRT.

The picture that portrays the effects of exogenous sexual hormones on systemic OxS becomes even more confusing when the few longitudinal studies available are considered. In a first study F2-iso was assessed in 15 women on ERT and 15 on combined HRT after long-term hormone therapy (baseline), after 4 weeks pause and again 3 weeks after resumption of therapy [129]. Apparently both types of treatment did not significantly affect systemic oxidative stress in vivo. Telci and colleagues examined the influence of HRT on OxS [130] in 12 postmenopausal women who had received HRT for 6 months, and 13 postmenopausal women who did not receive HRT, as the control group. They found that after the period of therapy, protein carbonyls (but not nitrotyrosine) decreased.

Multiple reasons can account for the evident high level of discrepancy among these results. Indeed, besides the limitations due to low size of the sample and analytical short-comings, other sources of variation are represented by differences in administration routes (oral tablets or topical skin patches), types and doses of estrogens and progestins, time of start and duration of the administration, etc. Thus, also these studies cannot help in deciphering the effective impact of HRT/ERT on systemic OxS, and in shedding light on the in vivo antioxidant power of E2.

Conclusions

The menopausal syndrome involves a large array of subjective symptoms (hot flashes, vasomotor derangements, mood and sleep disturbances) and overt chronic degenerative diseases (osteoporosis, CV ischemic diseases, AD-type neurodegeneration and genitourinary dystrophy). The ability to select patients for preventive medical therapies would be important in this perspective. On the other side, there is ample evidence that the pathogenesis of the severe chronic degenerative diseases that complicate menopause is likely linked to accumulation of oxidative damages. Hence, a closer inspection in the relationships between menopause and OxS (and its chief endocrine estrogenic deficiency) have become an important task to accomplish. With this aim we have reviewed current literature.

What emerges clearly from our preceding discussions is that – despite promising data in vitro and in animal models – the role of OxS as a culprit for tissue damage in vivo in post-menopausal life is still far to be definitely proven. We have identified possible drawbacks

for the oncoming failures, which could be summarized as follows: i) individual sensitivity of the patients, so that real damage must be expected in only a fraction of the total population; ii) absence of appropriate study design on samples of appropriate size; iii) absence of gold standard marker and validated reference values for oxidative by-products in the normal population.

The first and the second points present intimate contact points and represent the main pitfall in this topic. In particular the lack of a longitudinal-type approach on this research field preclude the possibility to firmly establish that menopause might be a causative of systemic OxS. The reasons behind this absence in literature mostly lay in the difficulties in carrying out this type of investigation. For these purposes women should be recruited in the premenopausal phase and followed up until an established advanced menopause is attained, to allow time for chronic associated diseases to emerge. It is certainly not inappropriate to estimate a follow-up period of several years which makes this approach unpractical. The only possible alternative arises therefore through cross-sectional studies, which must be carefully planned with strict inclusion/exclusion parameters, well-defined criteria for menopause definition, maximal age-match as possible and adequate assessment of body fat mass and distribution, to respect the concept that fat itself behaves as an endocrine tissue and that can greatly influence OxS. Most likely, once these refined parameters will be accepted even the discriminating power of cross-sectional studies should increase enough to eventually allow the detection of bimodal distributions in the population, if it is true that issues of individual sensitivity are real problems.

This poor and intrinsically flawed epidemiological literature argued with the proposed association between menopause and systemic OxS, failing to support the commonly held hypothesis according to which estrogens act as potent antioxidants. In our view, however, this is only an apparent rejection, because the action of estrogens may be effective against reactive species proliferation and toxicity even when this is not reflected at systemic level. In these terms, hormones, as consistently seen in vitro, may exert a specific antioxidant protection towards those cells/tissues that are most vulnerable to the deleterious consequence of menopausal transition.

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