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RESEARCH ARTICLE

Effect of chronic treatments with GH, melatonin, estrogens, and phytoestrogens on oxidative stress parameters in liver from aged female rats

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Abstract The aging theory postulates that this process may be due to the accumulation of oxidative damage in cells and molecules. The present study has investigated the effect of castration in old female rats on various parameters related to the antioxidant properties of several cellular fractions obtained from the liver, and the influence of several chronic treatments on it, both in intact and castrated animals. Sixty-one 22-month-old Wistar female rats, were used. About 21 intact animals were divided into three groups and treated for 10 weeks with GH, melatonin or saline, and 40 ovariectomized (at 12 months of age) animals were divided into five groups and treated for the same time with GH, melatonin, estrogens (Eos), phytoestrogens (Phyt) or saline. All animals were sacrificed at 24 months of age by decapitation. The activity of glutathione peroxidase (GPx) in cytosolic fraction, glutathione-S-transferase (GST) in cytosol and microsomal fractions, and the levels of Nitric oxide (NO) and cytochrome C in mitochondrial and cytosol fractions of liver were determined. A decrease in GST activity was detected in cytosol and in the microsomal fraction in ovariectomized animals as compared to intact rats. The activity of GPx was also decreased in ovariectomized as compared with the intact group. NO level was increased and cytochrome C decreased in the mitochondrial fraction of the liver in ovariectomized females as compared with the intact group, respectively. No significant changes after melatonin or GH treatments were found in GPx, GST activity and NO level in mitochondrial fraction in the intact group. Administration of GH, melatonin, Eos and Phyt in the ovariectomized groups significantly increased the GPx, and GST activity in the cytosol and microsomal fraction and decreased the level of NO in the mitochondrial fraction as compared with the untreated rats. A significant increase in the level of cytochrome C in the mitochondrial fraction and a decrease in the cytosol fraction were also found with all treatments. The administration of GH, melatonin, Eos and Phyt to castrated females seem to reduce oxidative changes in the liver from old ovariectomized rats.

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Introduction

Aging is associated by several changes in structure and function of different organs and tissues, and this process also affects the liver (Sohal 1993; Gasbarrini et al. 1998; Anantharaju et al. 2002). Furthermore, the sensitivity of liver to different damages, such as anoxia/reoxygenation injury (Gasbarrini et al. 1998) or drugs toxicity (Palomero et al. 2001), is increased with aging.

According to the free radical theory of aging, which is one of the most widely accepted, the physiological decline that occurs with age is, at least in part, due to accumulative oxidative damage to cells and molecules (Harman 1992; Sohal et al. 2002; Troen 2003). This oxidative damage is induced by reactive oxygen (ROS) and nitrogen (RNS) species, which are highly reactive molecules. These molecules, which are mainly generated by mitochondrial oxygen metabolism, can exert deleterious effects on proteins, lipids, and DNA (Reiter 1998; Reiter et al. 1999). In fact, an increase in the amount of oxidative damage to various macromolecules with age has been reported (Sohal et al. 2002). Hepatocytes are very rich in mitochondria and have a high respiratory rate, which means that these cells may be exposed to large amounts of ROS.

Reactive oxygen species have been implicated as major initiators of tissue damage and can up-regulate enzyme activity, signal transcription, and gene expression (Sen and Packer 1996; Wang and Martindale 1998). Many of these processes depend on the balance between the intracellular concentrations of pro-oxidant species and antioxidant compounds. By scavenging ROS, cellular enzymatic antioxidant defense decrease the risk of oxidative damage that could give rise to irreversible alterations of structure and functions of cellular macromolecules. Oxidative damage of the macromolecules increases with age and has been suggested to contribute importantly to the aging process and the pathogenesis of many age-related diseases (Wang et al. 2003). Antioxidant enzymes, such as superoxide dismutase (SOD-1), or catalase are closely linked with the cellular responses to various oxidative stresses. Glutathione plays a fundamental role in cellular defense against ROS by its participation in a variety of essential cellular reactions including enzymatic elimination of H_2O_2 and organic peroxides by glutathione peroxidase (GPx), maintaining the sulphydryl groups of proteins in the reduced state, transport of amino acids by the γ-glutamyl cycle (Slivka and Mytilineou 1987; Wu et al. 2004). Glutathione S-transferase (GST) is one of several detoxification enzymes. GSTs are divided into two evolutionarily distinct families: the cytosolic, soluble enzymes and the membrane-associated proteins in eicosanoid and glutathione metabolism. Representatives from most classes of soluble GSTs have been well studied and shown to have GST, peroxidase, and isomerase activities (Maeda and Crabb 2005). The liver plays a major role in the glutathione transferase–catalyzed formation of glutathione S-conjugates as the initial step of the mercapturic acid pathway, involved in detoxication and of xenobiotics (Sies et al. 1998).

Epidemiological data suggest that the rate of progression of chronic hepatic disease is higher in men than in women, suggesting a possible protective effect of estrogens (Eos) on the liver (Pinzani et al. 2001). Moreover, hepatic tissue in both males and females contain Eos receptors and respond to these hormones (Grandien et al. 1997). Eos have been shown to have in vitro antioxidant effects on membrane phospholipid peroxidation (Sugioka 1987). A positive correlation between GSH-Px activity and in vitro E_2 production by human ovaries incubated with FSH, and an inverse correlation between E_2 and lipid peroxide production with aging have been reported (Massafra et al. 2000). Eos, like vitamin E, possess ROS-scavenging chain-breaking antioxidant activity as hydrogen donors from their phenol-hydroxyl ring (Sugioka 1987). Other steroids tested, such as progesterone or testosterone had no significant antioxidant properties (Mooradian 1993).

Phytoestrogens (Phyt) are plant-derived compounds with molecular structures similar to those of Eos that share some of the effects of these hormones. Isoflavones, which is one group of Phyt, are now being widely studied, since they seem to exert beneficial effects on health (Adlercreutz et al. 1995; Kris-Etherton et al. 2002). Flavonoids have been shown to inhibit lipid peroxidation processes,

inherent in the auto-oxidation of lonoleic acid, oxidation of low-density lipoproteins, and peroxidation of microsomal, mitochondrial membranes. It is likely that several functions of flavonoids account for these effects on lipid peroxidation: flavonoid acting as metal chelators, chain breaking antioxidants, and/or free radical scavengers (Laughton and Halliwell 1989; Ursini et al. 1994). However, little is known about the role of Eos on the aging liver and the possible action of Phyt on it.

Melatonin, which is secreted by the pineal gland, is a powerful scavenger of oxygen free radicals, hydroxyl radicals, and peroxyl radicals (Tan et al. 2000; Cuzzocrea and Reiter 2001; Allegra et al. 2003; Jaworek et al. 2005). During the last decade, the antioxidant melatonin has been shown to possess genomic actions, regulating the expression of several genes. Melatonin also influences both antioxidant enzyme activity and cellular mRNA levels for these enzymes (Rodriguez et al. 2004). Melatonin is able increases the efficiency of the electron transport chain thereby limiting electron leakage and free radical generation, and promotes ATP synthesis. Via these actions, melatonin preserves the integrity of the mitochondria and helps to maintain cell functions and survival (Leon et al. 2005). It seems that at least some of melatonin's antioxidative actions are a result not of the melatonin direct action, but of the melatonin metabolites (Tan et al. 2007). Melatonin levels decline gradually over the life-span and may be related to lowered sleep efficacy, very often associated with advancing age, as well as to deterioration of many circadian rhythms. Melatonin exhibits immunomodulatory properties (Carrillo-Vico et al. 2005) and, a remodeling of immune system function is an integral part of aging. Finally, because melatonin is a potent free radical scavenger, its deficiency may result in reduced antioxidant protection in the elderly which may have significance not only for aging per se but also may contribute to the incidence or severity of some age-related diseases (Karasek 2004).

Human ageing is associated to a declining activity of the GH/IGF-I axis and to several changes in body composition, function, and metabolism, which show strict similarities with those of younger adults with pathological GH deficiency. The age-related changes of the GH/IGF-I axis activity are mainly dependent on age-related variations in the hypothalamic control of somatotroph function, which is also affected by changes in peripheral hormones and metabolic input. The term 'somatopause' indicates the potential link between the age-related decline in GH and IGF-I levels and changes in body composition, structural functions and metabolism which characterize ageing (Lanfranco et al. 2003).

The aim of the present study was to investigate the effect of aging and ovariectomy on various parameters related to oxidative stress in different fractions of the liver obtained from old female rats and the influence of chronic exogenous administration of the above mentioned hormones on these.

Materials and methods

Animals

Sixty-one female Wistar rats of 22 months of age were used in the present study. Twenty-one of them remained intact and the other forty were ovariectomized at 12 months of age, according to the following procedure: rats were anaesthetized with Equithesin and two small incisions (8 mm) were made through the skin and the muscle back walls in parallel with the animal body line. The ovaries were then located and a silk thread (Silkam®, Braun-Aesculap, Germany) was tightly tied around the oviduct, including the ovarian blood vessels. The oviduct was sectioned and the ovary removed, taking good care in leaving the knot intact. The muscle wall was then sutured with a synthetic absorbable thread (Safil®, Braun-Aesculap, Tuttlingen, Germany) and the skin with metallic clips. The animals were given a standard laboratory rat diet (A.04; Panlab, Barcelona, Spain) and water ad libitum, in a light and temperature controlled room. Intact animals were divided into three experimental groups (n = 7 each group). The ovariectomized rats were divided into five experimental groups: non-treated, treated with GH (2 mg s.c./Kg/day Saizen®, Serono, Madrid, Spain), melatonin (1 mg/kg/day N-Acetyl-5-methoxytryptamine, Sigma-Aldrich, St. Louis, MO, USA, EEUU). Melatonin was diluted in the drinking water. To increase melatonin solubility in water it was previously diluted in ethanol, 20 mg in 10 ml and kept in the darkness to avoid the damage of the hormone. Final alcohol concentration of the bottle 1%. Estradiol valerianate given sc (Sigma; 125 μg/week, diluted in sunflowerseed oil); and treated with

a soy extract containing 10% of isoflavones (Phytosoya®, Arkochim, Spain; 312 mg/kg/day in the drinking water, pre-diluted in ethanol; final ethanol concentration in the bottle: 1%). All non-treated animals received the correspondent vehicles doses. Six of the intact rats were submitted to sham operation following the same procedure, without removing ovaries. All the animals received humane care according to the Guidelines for Ethical Care of Experimental Animals of the European Union. After 10 weeks of treatment, rats were sacrificed by decapitation, and liver was collected and processed as described later.

Preparation of liver homogenates

The sample of liver was homogenized in 4–8 vol. (per weight tissue) of cold buffer e.g.: 50 mM Tris–HCl buffer (pH = 7.5), containing 5 mM EDTA and 1 mM 2-mercaptoethanol. The homogenate was centrifuged at 8,000 *g* at 2°C for 15 min. The supernatant was used for the assay of GPx activity.

Isolation of liver mitochondria

The liver samples were put in an ice-cold homogenization buffer (0.32 M sucrose, 1 mM K^+EDTA, and 10 mM Tris–Cl, pH = 7.4), containing 2.5 mg/ml of fatty acid-free bovine serum albumin (BSA) and 0.3 mM phenylmethylsulfonyl fluoride (PMSF, a protease inhibitor). The sections were minced and homogenized with 2 ml of homogenization buffer per gram of tissue, using a Teflon-glass homogenizer. The homogenate was centrifuged at 500 *g* at 4°C for 5 min. The pellet was washed with the homogenization buffer and centrifuged at 500 *g* for 5 min (4°C). The supernatant was centrifuged at 10,000 *g* at 4°C for 15 min. The mitochondrial pellet was washed once and then re-suspended in homogenization buffer. After centrifugation the supernatant was used for cytochrome C determination. The mitochondrial pellet was incubated with lysis buffer and used for NO release and cytochrome C determination.

Isolation of microsomal and cytosol fractions from liver

The liver samples were rinsed with 0.9% NaCl solution, weighed, and homogenized in 500 µl PBS buffer. The homogenate was centrifuged at 10,000 *g* at 4°C for 30 min. The supernatant was further centrifuged at 105,000 *g* at 4°C for 60 min. The pellet was washed once with 50 mmol/l Tris–HCl buffer (pH = 7.4) by centrifugation at 105,000 *g* at 4°C for 60 min. The supernatant as the cytosol fraction and the microsomal fraction was used for the assay of GST the activity.

Biochemical determinations

Glutathione-S-transferase and GPx activity were measured by commercially available kits (Oxis Research, Foster city, CA, USA). GST activity was measured spectrophotometrically by measuring formation of the conjugate of reduced glutathione (GSH) and 1-chloro-2.4-dinitrobenzene (CDNB) at 340 nm. Briefly, 10 µl of cytosol or microsomal fraction was transferred into a 190 µl incubation mixture consisting of 10 µl GSH, 180 µl CDNB in potassium phosphate buffer (pH = 6.5). The product formation was continuously measured for 5 min. GPx activity was measured spectrophotometrically, in which GPx activity was coupled to the oxidation of NADPH by glutathione reductase. Each 770 µl reaction mixture consisted of 350 µl buffer (pH = 7.6), 350 µl NADH reagent containing *β*-nicotinamide-adenine dinucleotide phosphate, GSH, glutathione reductase, and 70 µl homogenate. The reaction was initiated by an addition of 350 µl of tert-Butyl Hydroperoxide. The decrease in optical density at 340 nm due to the oxidation of NADPH was monitored for 3 min in this coupled assay at +25°C. The units of enzymatic activity were calculated using an extinction coefficient of 6,220 M/cm for NADPH.

Nitric oxide (NO) release from the mitochondria was measured by the Griess reaction as NO_2^- concentration after NO_3 reduction to NO_2^- as currently performed in our laboratory (Castillo et al. 2005). Briefly, samples were deproteinized by the addition of sulfosalicylic acid, were then incubated for 30 min at 4°C, and subsequently centrifuged for 20 min at 12,000 *g*. After incubation of the supernatants with Escherichia coli NO_3 reductase (37°C, 30 min), 1 ml of Griess reagent (0.5% naphthylenediamine dihydrochloride, 5% sulfonilamide, 25% H_3PO_4) was added. The reaction was performed at 22°C for 20 min, and the absorbance at 546 nm was measured, using $NaNO_2$ solution as standard. The

measured signal is linear from 1 to 150 μM ($r = 0.994$, $P < 0.001$, $n = 5$), and the detection threshold is ~2 μM.

Cytochrome *C* content of cells was determined with a commercially available Elisa kit (Oncogene Research Products, San Diego, CA, USA). This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for cytochrome *C* has been pre-coated onto a micro-titer plate. Standards and sample were pipetted into the wells and any cytochrome C present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for cytochrome *C* was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and color developed in proportion to the amount of cytochrome *C* bound in the initial step. The color development was stopped and the intensity of the color was measured at 450 nm during 30 min.

Protein determination was performed by Bradford method. The basis of this method is the addition of Coomassie brilliant blue dye to proteins. This union induces a shift in maximum dye absorbance from 465 to 595 nm. Absorbance is measured at 595 nm and compared with a known standard curve.

Statistical analyses

The results are presented as the means ± SE, obtained by combining the results from each cell preparation. Mean comparison is to be done by the Friedman's analysis of variance of ranks, followed by a two-tailed Wilcoxon's rank sum test for paired data to identify the source of the found differences; a confidence level $\geq$ 95% ($P < 0.05$) should be considered significant.

Results

As shown in Table 1, ovariectomy induced an evident and significant decrease in activity of GST in the cytosol and microsomal fraction of liver compared to intact old females. The activity of GPx was decreased also in old ovariectomized females when compared to age matched intact rats (Fig. 1a). Ovariectomy led also to an increase in the NO content in liver mitochondrial fraction as compared to the intact group (Fig. 2) In addition to the alterations in oxidative stress markers, ovariectomy also caused a decrease of cytochrome C content in mitochondria and an increase in the cytosol fraction, which implies mitochondrial dysfunction.

When ovariectomized old females were treated with melatonin, growth hormone, Eos or Phyt, the activity of GST and GPx were significantly increased as compared with non-treated rats (Table 1, Fig. 1b). In contrast, no significant changes after melatonin or GH treatments were found in GPx, or GST activity in the intact group of the same age (Table 1, Fig. 1a).

Administration of GH, melatonin, Eos, and Phyt to the ovariectomized groups significantly decreased the level of NO in the mitochondrial fraction as compared with the untreated rats. In the intact old female group the NO content in the mitochondrial fraction was slightly decreased after melatonin or GH treatment, but without statistical signification.

Figure 3 shows, that cytochrome C content was significantly increased in mitochondrial fraction and decreased in cytosol fraction in the ovariectomized groups after melatonin, GH, Es or Phyt administration.

Discussion

The liver plays the central role metabolizes ingested nutrients and xenobiotics. This organ has many closely related physiological functions including cellular metabolism and the biotransformation of ingested chemicals (Waziers et al. 1988; Vincenzini et al. 1991). Aging of the liver is associated with a variety of functional alterations (Popper 1986) such as reduced liver mass, decline in hepatic blood flow, accumulation of lipofuscin (the aging pigment), and a decline in the hepatic clearance capacity of metabolized hydrophobic compounds. Many studies have shown a decrease in hepatic protein turnover, including impaired protein synthesis and protein degradation (Schmucker 1990). Aging has been shown to alter the expression and activity for hepatic gluconeogenic, glycolytic, and nitrogen-metabolizing enzymes (Dhahbi et al. 1999).

Several epidemiological studies have demonstrated a lower incidence of cirrhosis and its complications in the liver of women than in men. But as menopause arrives, the morbility of the disease

Table 1 Glutathione S-transferase enzyme activity in liver tissue from intact and ovariectomized old female rat

Enzymes	Intact animals			Ovariectomized animals				
	Without treatment	+GH	+Mel	Without treatment	+GH	+Mel	+Eos	+Phyt
				Microsomal fraction				
Glutathione S-transferase, mU/mg protein	20.44 ± 0.48	20.39 ± 1.73	20.77 ± 2.91	15.27 ± 1.68*	19.04 ± 0.27#	22.54 ± 1.15♦	21.58 ± 1.12#	23.24 ± 1.37#
				Cytosol fraction				
Glutathione S-transferase, mU/mg protein	48.21 ± 3.85	46.99 ± 7.76	48.18 ± 2.31	36.54 ± 1.28*	50.23 ± 4.59Δ	60.45 ± 4.39♦	59.92 ± 7.12♦	51.55 ± 5.68♦

* $P < 0.05$ Intact compared with ovariectomized (OVX)

# $P < 0.05$ OVX treated compared with OVX control

♦ $P < 0.01$ OVX treated compared with OVX control

Δ $P < 0.03$ OVX treated compared with OVX control

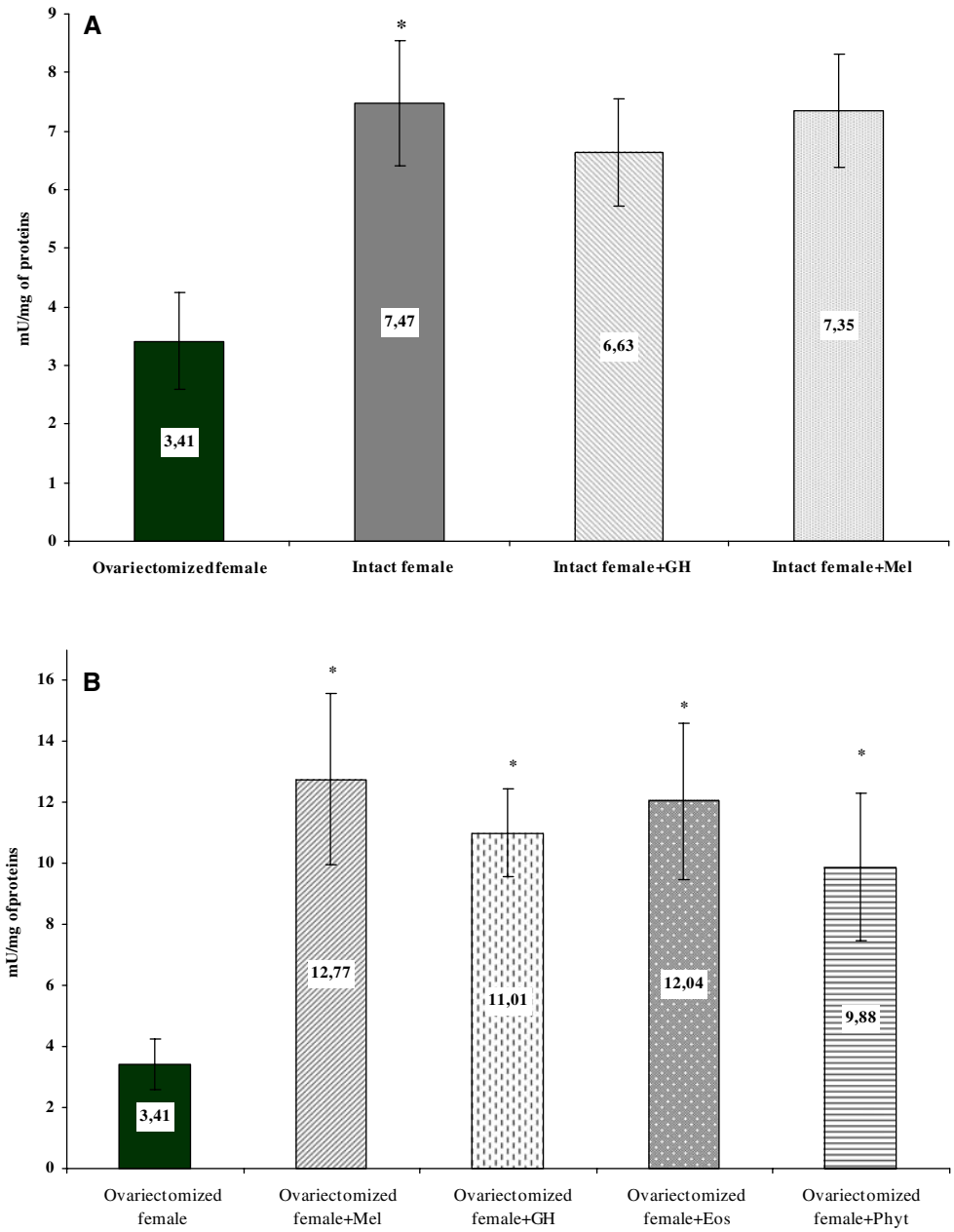

Fig. 1 Activity of cellular glutathione peroxidase (mU/mg of proteins) in liver homogenates. Values are expressed as mean ± SEM

increases gradually in women (Poynard et al. 1997; Poynard et al. 2001; el-Serag 2001). Women treated with tamoxifen, that can be considered a potent antiestrogen, are at increased risk of developing a fatty liver and non-alcoholic steatohepatitis (Saibara et al.1999).

Oxidative stress has been implicated in the pathogenesis of several alterations due to ageing and menopause, and can arise through the increased production of lipid peroxides and/or a deficiency of antioxidant defense. There are several mechanisms by which mammalian cells defend themselves against oxidative stress, such as GSH and antioxidant scavenging enzymes such as cellular Cu, Zn SOD-1, catalase (CAT), glutathione peroxidase (cGSH-Px) and GST (Kasapoglu and Ozben 2001). Measurement

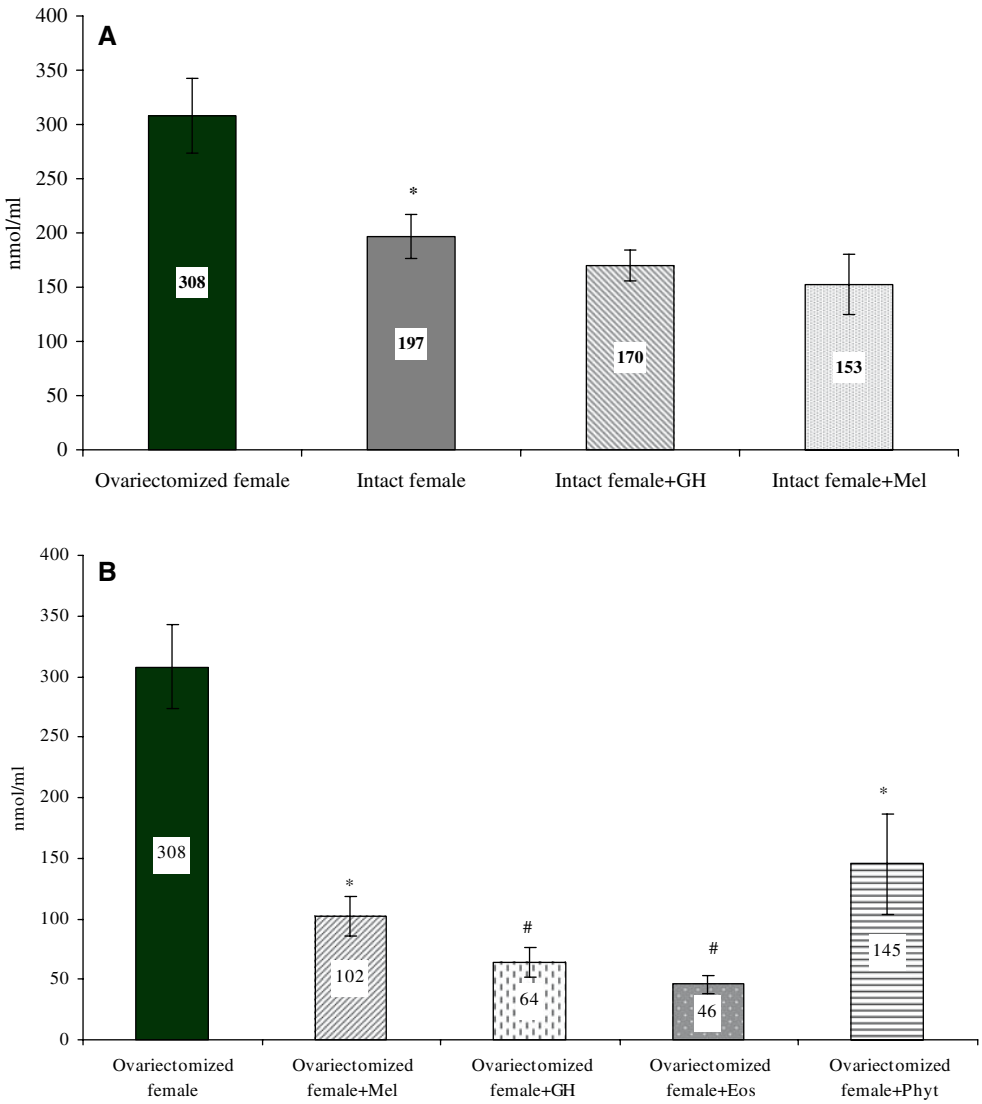

Fig. 2 Nitric oxide release (nmol/ml) in mitochondrial fraction of liver. Values are expressed as mean ± SEM

of the activity of both glutathione-dependent enzymes may serve as an estimation of the degree of functioning of the endogenous antioxidant system. GSH is a cosubstrate of cGSH-Px and GST, and when reacting with peroxides, it is converted to glutathione disulfide. GSH is thus responsible for protecting cellular thiol residues against oxidation. The levels of GSH fluctuate under various physiological conditions, including age, and are usually accompanied by increased lipid peroxidation with elevated production of malonyldialdehide (Wu et al. 2004). In the present study, activity of GPx and GST have been found to significantly decrease in ovariectomized female rats, as compared to intact ones of the same age. Our findings are in accordance with previous studies, in which it has been shown that menopause is associated with an increase in oxidative stress and a decrease of some antioxidants, such as ascorbic acid, alpha-tocopherol, total thiols (Vural et al. 2005) and antioxidant enzymes such as SOD, CAT and GPx (Ha 2004). Furthermore, the activities of GSH reductase and GSH peroxidase also diminish with age (Hazelton and Lang 1985). Our group has previously found that hepatocytes isolated from old intact female rats show alterations in several parameters related to oxidative stress such as LPO, CO,

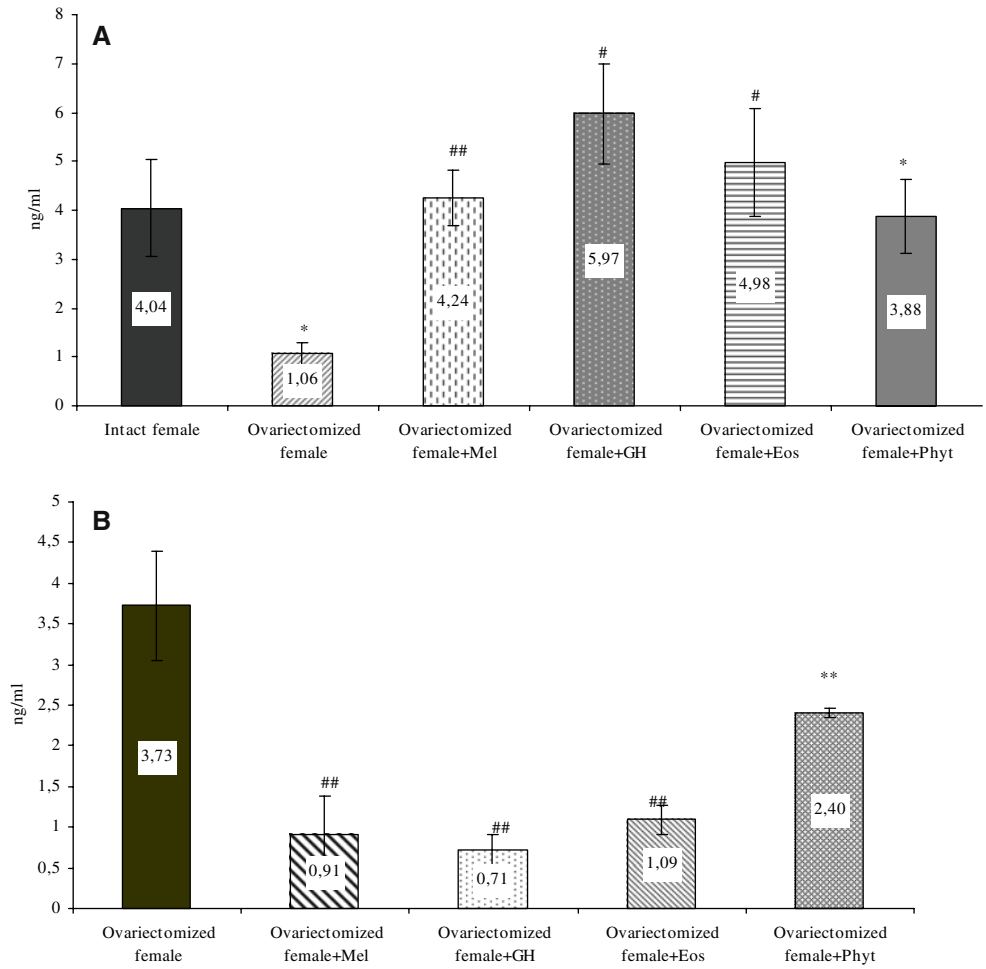

Fig. 3 The level of cytochrome C (ng/ml) in mitochondrial (a) and cytosol (b) fraction of liver. Values are expressed as mean ± SEM

cGMP, and synthesis of PLC, and that these changes were significantly more pronounced in age-matched old ovariectomized females (Castillo et al. 2005).

In animal cells, mitochondria are major sites of AOS formation and major targets of AOS-induced damage (Kokoszka et al. 2001; Toescu et al. 2000). Significant fractions (~2%) of oxygen are converted to the superoxide radicals and its reactive metabolites (ROS) in and around mitochondria (Inoue et al. 2003).

Mitochondrially associated NO synthases (i.e., eNOS, nNOS, or iNOS bound to mitochondria) or mtNOS (a possibly distinct NOS) may produce NO locally at mitochondria and, thus, has the potential to regulate respiration (Giulivi et al. 1998; Brown and Borutaite 2002; Acuna Castroviejo et al. 2005). Both free radicals, O2 and NO, are metabolized into the mitochondrial matrix through the formation of relatively stable and non-radical species, H_2O_2 and ONOO (Poderoso et al. 1996). Peroxynitrite and possibly NO itself inhibit cytosolic and mitochondrial aconitase, the latter being a component of the Kreb's cycle and, thus, required for cellular respiration. Apart from inhibiting respiration, NO has two other effects on mitochondria relevant to the induction of cell death: (i) induction of ROS and RNS production from mitochondria, and (ii) induction of mitochondrial permeability transition (MPT) by RNS (Brown and Borutaite 2002).

An increased mitochondrial formation of ROS triggers the intrinsic pathway by increasing the permeability of the outer mitochondrial membrane through the opening of transition pores. The opening of the permeability transition pore is favored by

oxidative stress through oxidation of intracellular glutathione and other critical sulfhydryl groups (Chernyak 1997). As a result of this process, cytochrome *C* moves from the intermembrane space into the cell's cytoplasm (Liu et al. 1996) where it joins another factor (Apaf-1). In the presence of dATP this complex polymerizes into an oligomer known as 'apoptosome'. The apoptosome activates a protease (caspase-9), which in turn activates caspase-3. The cascade of proteolytic reactions also activates DNAses and in the end the process results in cell death (Li et al. 1997). Others have indicated that a sustained production of NO• triggered by increased levels of Ca2+ may release cytochrome C from mitochondria to the cytosol, an event that has been implicated in cell apoptosis (Ghafourifar et al. 1999).

In the present study, NO release in the mitochondrial fraction was found to be increased in liver isolated from ovariectomized females rats, in accordance with previous studies of our group performed in primary cultures of hepatocytes obtained from old female rats in which the increase was also more marked in ovariectomized animals (Castillo et al. 2005) and with other studies showing also that iNOS activity and NO release were increased with ovariectomy (Lee et al. 2005; Koyuncu et al. 2006). In addition to stimulating NO levels, our data show that ovariectomy can also decrease cytochrome C content in mitochondrial fraction and increase it in cytosol. Many studies have showed that ovariectomy is able to induce changes in the mitochondrial functions. Functional assays of mitochondrial citrate synthase, complex I and IV, key rate-limiting steps in energy production, showed decreased enzyme activity in ovariectomized female rats (Stirone et al. 2005; Feng and Zhang 2005).

Estrogens have been shown to be powerful antioxidants, effectively preventing lipid peroxidation. The chemical structure of Eos allows for donation of a H^+ atom to a peroxyl radical. This property of Eos allows free radical scavenging and may exert its effect by interfering early or during the propagation phase of lipid peroxidation (Subbiah et al. 1993; Ayres et al. 1996). Ovariectomy caused an increase of oxidative stress in mitochondria and Eos replacement therapy completely prevented this effect (Borras et al. 2003). Same preventive effects of Eos from damage have been described in heart and skeletal muscle (Persky et al. 2000), uterus (Diaz-Flores et al. 1999), and liver (Huh et al. 1994). In an in vitro model of liver damage, E2 suppressed NF-kB in cultured hepatocytes undergoing oxidative stress (Green and Simpkins 2000; Omoya et al. 2001). In addition to its protective role in preventing lipid peroxidation, E2 has been shown to protect against DNA damage.

Phytoestrogens are naturally occurring plant chemicals that can produce an Eos-like effect in the body, and have been used as a natural alternative to hormone replacement therapy to reduce menopausal symptoms (Eden 1998). Flavonoids suppress the free radical-induced tissue damage caused by oxyradicals in Chinese hamster V79 cells, human cardiovascular cells, and neurons. Also, there is a good correlation between antioxidant properties of and radioprotection by flavonoids (Bors et al. 1996). Some flavonoids stimulate superoxide dismutase, as shown in patients with liver cirrhosis and probably are responsible for the hepatoprotective effects of these compounds (Huang et al. 1992). Phyt may maintain NO production in endothelial cell by enhancing the activity and the expression of eNO synthase by interacting with Eos receptors. This, however, does not rule out other possible mechanisms through which Phyt may also regulate NO production (Zhai et al. 2001).

Female rats maintain a certain degree of Eos secretion until late in their lives (Vom Saal et al. 1994). Other authors have also suggested that females, owing to the maintained estrogenic secretion are partially protected against some age related changes (Viña et al. 2003). This might explain why the intact animals are less affected than the castrated, and why the alterations induced in castrated were more marked than in intact. Since the alterations were more pronounced in castrated than in intact animals the beneficial effects of the various treatments were also more evident in the former as has been previously demonstrated by our group for other age related parameters associated with oxidative stress (Castillo et al. 2004). In our actual data when old ovariectomized female rats were treated with the isoflavone-containing extract or with estradiol, they showed a clear and significant improvement in the values of all studied functions.

In the present study, we show that effect of the commercial isoflavone mixture Phytosoya® is similar to that of Eos, at the dosages used. The importance's of these results lies in the fact that Phyt are being

increasingly proposed as a safer alternative to hormone replacement therapy, mainly in women who have some contraindication for being treated with Eos. And the present data confirm the beneficial effects of these substances.

It has been previously reported that there is a decrease in GH and IGF-1 production with age (Corpas et al. 1993; Raynaud-Simon 2003), and this fact has been proposed to be related to some of the changes that accompany the process of aging. Ovariectomy did not seem to exert any additional effect on the somatotrophic axis, since no differences in plasma levels and hepatic content of IGF-1 between intact and ovariectomized old female rats were found (Castillo et al. 2005). GH regulates the synthesis of many proteins that are specifically or preferentially expressed in the liver, such as plasma proteins, secretory proteins, serine protease inhibitors, P450 enzymes, and GST (Mode et al. 1992; Srivastava and Waxman 1993). The effects of GH on hepatic drug metabolism, for example, are complicated by the fact that the mode of GH administration exerts different effects on different genes (Tollet-Egnell et al. 2001). Both in vitro and in vivo studies suggest that GH status modulates antioxidative mechanisms (Brown-Borg et al. 2002; Brown-Borg and Rakoczy 2003). Matsubara et al. 1972 reported that the respiratory cytochrome content of isolated mitochondria was decreased in GH-deficient animals (hypophysectomized) in parallel with a decrease in respiratory activities, and that subsequent administration of GH increased the cytochrome contents (Matsubara et al. 1972).

The decrease of melatonin during aging can be associated with a decrease in the levels of GH and IGF-I, which may lead to evident metabolic changes in different organs. Many studies have examined the cytoprotective role of melatonin on the prevention of age-related diseases. Melatonin exerts potent hydroxyl and peroxyl radical scavenging activity. It strongly promotes the activity of exogenous and endogenous antioxidants (Reiter 1995). Chronic treatment of aged mice with a low dose (0.1 mg/kg body weight daily for 3 months) of melatonin reversed the age-related reduction in brain levels of GSH and GPx (Manda and Bhatia 2003). Melatonin regulates mitochondrial respiration and bioenergetics and protects mitochondria from excess NO by controlling the activity of mtNOS (Acuna-Castroviejo et al. 2005).

In this study, the administration of GH and melatonin was accompanied by an improvement of liver function in ovariectomized female rats. As a result, GH or melatonin treatments were able to enhance activity of some enzymes of the glutathione system in different cellular fraction. Treatment with melatonin and GH also induced a decrease in NO release and mitochondrial membrane damage (decreased cytochrome *C* level in the cytosolic fraction) in old ovariectomized female rat.

In conclusion, these results clearly indicate the influence of the hormonal status on liver susceptibility to lipid peroxidation. In addition, we have demonstrated that Eos, phytosoya, melatonin, and growth hormone can modulate the antioxidant status exerting a protective effect on the age- and ovariectomy induced liver injuries.

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