Reducing the Damage: Metabolism Behaviour Aesthetic Medicine

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Age-Related Damage

According to the free radical theory of ageing, which is one of the most widely accepted theories, the physiological decline that occurs with age is, at least in part, due to accumulative oxidative damage to cells and molecules. This oxidative damage is induced by reactive oxygen (ROS) and nitrogen (RNS) species, which are highly reactive. ROS have been implicated as major initiators of tissue damage and can upregulate enzyme activity, signal transcription and gene expression of several compounds that can exert deleterious effects on proteins, lipids and DNA which in turn are responsible for all the age-related alterations in different tissues.

It is well established that inflammation and oxidative stress are key components of the ageing process [1–3], but how early in the pathological cascade these processes are involved or which specific molecular components are key is yet to be fully elucidated. However, oxidative stress is understood as a disparity between the rates of free radical production and neutralization, which occurs when the antioxidant mechanisms are overwhelmed. Increased free radicals may in turn lead to activation of a plethora of pro-inflammatory cytokines thereby activating the cascade that leads to further inflammation [4]. Thus, inflammation and oxidative stress could be considered as the twin evils, which may act synergistically.

Cellular enzymatic antioxidant defences which are present in young persons are able by scavenging ROS, to decrease the oxidative damage that could give rise to irreversible damages of structure and functions of cellular macromolecules. Loss of these defences with age enhances oxidative damage and has been suggested to contribute importantly to the ageing process and to the pathogenesis of many age-related diseases.

Free Radicals and Oxidative Stress

Free radicals are molecules containing one or more unpaired electrons in its external orbital *(radical)* and are able to maintain an independent existence (free) although this existence has a very low duration (generally10^{-6}–10^{-9} s). The presence of unpaired electrons renders these molecules extremely reactive, since it tries to interact with other molecules in its vicinity to pair these electrons and to stabilize [5], generating in the process changes that can alter its structure and function.

Free radicals might in play a fundamental role in metabolism, the generation of mutations, the ageing process and death, which together play a role in species evolution [1].

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In nature most free radicals derive from **oxygen** and are named oxygen reactive species (**ROS**), although other free radicals derive from nitrogen (reactive nitrogen species, **RNS**), from sulphur, etc.

The mitochondrial respiratory chain is skipping 2–5% from the oxygen that we are getting and this part contributes to the generation of free radicals.

Oxygen accepts one single electron transforming it to **superoxide anion** ($^{\bullet}O_2^-$) [5]. This radical $^{\bullet}O_2^-$ is transformed to hydrogen peroxide and oxygen through a dismutation reaction, which can be catalysed or not by the enzyme *superoxide dismutase (SOD)*:

Although its reactivity is limited, $^{\cdot}O_2^-$ is able to induce tissue damage through its pro-inflammatory actions. It generates also endothelial damage, increases capillary permeability, stimulates the production of chemotactic agents increasing the recruitment of neutrophils and stimulates the autocatalytic destruction of neurotransmitters and hormones [6].

The most reactive ROS is the hydroxyl radical ($^{\cdot}OH$), mainly generated through the **Fenton reaction**, **in which** H_2O_2 interacts with reduced transition metal ions, generally Fe^{+2} and Cu^{+}, present as part of the prosthetic groups of multiple enzymes and proteins [5, 7]. Its half-life is in the order of 10^{-9} s and reacts with practically any biomolecule that might be located in the proximity of its place of synthesis, giving rise to chain reactions like in the case of lipid peroxidation.

NO is a relatively stable radical that does not react quickly with the majority of biomolecules [5]. However, it reacts easily with transition metals and other radicals including oxygen, peroxyl radicals and $^{\cdot}OH$ radical. The interaction with the last two plays a very important role in the damaging capacities of NO.

NO is produced by the enzyme **nitric oxide synthase (NOS).** There are three different types of NOS: **nNOS** (type I, NOS-1) that is constitutively expressed in neural tissue; **iNOS** (type II, NOS-2) that is inducible and can be expressed in a great variety of cells and tissues, especially in the pro-inflammatory-agent-stimulated macrophages; and **eNOS** (type III, NOS-3) that is present in a constitutive way fundamentally in vascular endothelial cells that play a role in vasodilatation.

There is also a mitochondrial NOS (mtNOS) in which cytochrome C release is stimulated with an increase in lipid peroxidation that could induce Ca^{2+}-dependent apoptosis [8].

Hemoxygenase

Three HO isoforms have been described, localized in the smooth endoplasmic reticulum of cells.

The inducible isoform (HO-1) is undetectable under normal conditions but can be induced by several stimuli that produce oxidative stress or through NO by means of the activation of transfer factors like NF-κB and AP-1. It modulates the response of the liver tissue to those stressors [9]. Age is also associated with an increase in the expression of HO-1, possibly due to the increase of age-associated oxidative stress. The effect could also be linked to the activation of the age-associated increase in NF-κB [10].

Actions of ROS and RNS

Physiological

Free radicals seem to play a role in some physiological processes, such as genetic regulation, cellular replication, differentiation and apoptosis, probably acting as secondary messengers in the transduction signal pathways [3]. It is also known that the reactive species are one of the elements implicated in the response to pathogens (the oxidative "explosion" of neutrophils).

Harmful Effects

Living organisms are always exposed to a certain amount of reactive species due to its oxygen-dependent metabolism, and they use several mechanisms to fight against it. Oxidative stress takes place when the balance between

pro-oxidant and antioxidant species is altered in favour of the former.

DNA damage: Reactive species and especially the $^{\cdot}OH$ radical react with the double bonds of puric and pyrimidinic bases of DNA, generating derivatives of 8-OH-deoxyguanosine, which have a very high mutagenic activity and are used frequently as a marker of oxidative damage to DNA [11].

Oxidative damage to lipids: lipid peroxidation. It is a process by which lipids and especially polyunsaturated fatty acids (PUFA) are influenced by free radicals, originating an autopropagatable chain reaction that is able to oxidize all free fatty acids of the affected systems (cellular membrane, mitochondrial membrane, etc.).

During this process several characteristic molecules are generated such as lipoperoxides, hydroxynonenals, malondialdehyde, and other byproducts, many of which can be determined by several methods [12, 13] as markers of oxidative stress.

All these alterations are especially significant in the mitochondria, whose functionality depends on the existence of an intact membrane [14].

Protein Oxidation

Oxidative stress also affects proteins, altering its structure and function. Amino acid including the appearance of proteins undergo modifications induced by the reactive species, including carbonyl groups, hydroperoxides [15] and nitrosylated derivatives [8]. These processes induce alterations in the structure and function of the affected proteins and this could influence cellular physiology [16, 17].

Sources of Free Radicals and Reactive Species

The principal source of reactive species is the mitochondrial electronic transport chain that is composed of a *group of enzymes* whose coordinated activities are able to couple metabolic substrate oxidation with the generation of ATP in a process known as "oxidative phosphorylation". Nearly 2% of the total oxygen employed by the mitochondria is not completely reduced and escapes the system in the form of $^{\cdot}O_2^-$ and H_2O_2 [18].

The enzyme *xanthine oxidase* (XO) is another important source of reactive species. This is a cytosolic enzyme that produces $^{\cdot}O_2^-$ and H_2O_2 during the oxidation of hypoxanthine to xanthine during purine metabolism.

Enzymes with an oxidoreductase activity, such as NOS; those involved in prostaglandin and leukotriene synthesis, such as cyclooxygenase and lipoxygenase; and P450 cytochromes also generate reactive species. Neutrophil, phagocyte and microsome activities are other sources of reactive species.

Endogenous Antioxidants Against ROS and RNS

The organism has developed a series of defence mechanisms, generally considered as *antioxidants*, to protect itself from the action of free radicals and act either to prevent the formation of those radicals or neutralize it or facilitate the repair of the induced damage.

These antioxidants are as follows:

Superoxide Dismutase (SOD)

This enzyme comes from a broadly present family of metalloproteins in nature that are able to catalyse dismutation of 2 $^{\cdot}O_2^-$ to H_2O_2. They are highly important since they are in the first line of cellular defence against oxidative damage that can be caused by the superoxide ion and the reactive species that derive from its presence [19].

Glutathione Peroxidase (GPx)

GPx plays a fundamental role in detoxification of hydrogen peroxide and lipoperoxides that are generated in the cells [20]. GPx catalyses also the reduction of H_2O_2 and organic hydroperoxides H_2O and alcohol, respectively, using reduced glutathione (GSH) as donors of electrons.

The reduction of oxidized glutathione (GSSG) generated in these reactions is catalysed by the enzyme glutathione reductase (GR).

Catalase (CAT)

This enzyme catalyses the breaking down of H_2O_2 to H_2O. It is localized principally in the peroxisomes, and its tissular distribution is similar to SOD.

Glutathione

It is a tiolic tripeptide present in the majority of cells from plants or animals. It is one of the most abundant antioxidants of the cell, reacting directly with free radicals or through GPx. It seems also to act in the reduction of several cellular antioxidants, like vitamin E.

Vitamin E

It belongs to a family of highly lipophilic phenolic compounds that play a fundamental role in the protective action against lipid peroxidation of cellular membranes. The organism cannot synthesize this molecule and its presence in the body is only dependent of its intake with food.

Vitamin C

Vitamin C is a hydrosoluble molecule present in the cytosolic compartment of cells and in the extracellular fluid. Although it can interact directly with radicals $^{\cdot}O_2^-$ and $^{\cdot}OH$, it seems that its principal function is to participate in the recycling of vitamin E [21].

Other Antioxidants

Carotenoids are natural dyes present in many vegetal components (tomatoes, carrots, citrus, spinach, corn) capable of neutralizing $^1O_2$ and inhibiting lipid peroxidation. Flavonoids are a group of polyphenol antioxidants present in fruits, veggies and drinks (tea, wine, beer), capable of reacting with $^{\cdot}O_2^-$, $^{\cdot}OH$ and peroxyl radicals.

Oxidative Stress and Ageing

The amount of oxidative damage present in different macromolecules of an organism increases with age and the accumulation of damage throughout life could have crucial functional consequences [2]. There are studies that demonstrate that ageing is associated with an increase of oxidative damage to DNA [11], following an increase in the rate of mutations as well as in the amount of nitrosylated or oxidized proteins in several tissues [22]. The elimination rate through proteolysis of oxidized proteins is reduced, with the consequent accumulation of defective proteins [8]. The increase in oxidative damage to lipids is also increased [23]. All these elements might influence the alterations of age-related mitochondrial functions, inducing a reduction in ATP production and uncoupling of the respiratory chain, which is associated with a further increase in free radicals and closes the evil circle [16, 17, 24].

Apoptosis and Cellular Survival During Ageing

All living organisms need to have a regulated form of cellular death that could allow to control very closely several critical aspects for the maintenance of its homeostasis like the size of the tissue, the number of cells taking part in its composition or the protection against elements that could jeopardize its integrity. Apoptosis was described for the first time by Currie in 1972 [25], is also named programmed cellular death and is really a physiological way of programmed cellular suicide.

Apoptosis takes place under normal physiological conditions in development and embryogenesis to allow for organic remodelling,

metamorphosis or normal tissue exchange, as a defence mechanism, and also in a more later part of life during ageing.

Mechanisms regulating apoptosis are not completely elucidated but the involvement of two families of proteins has been demonstrated: members of the caspase family and the proteins of the Bcl-2 family.

Caspases are cysteine proteases very well maintained through evolution from nematodes until humans. Activation of caspases is one of the principal points in the regulation of apoptosis [26].

The **Bcl-2** family is composed of a series of genes that play a critical role in the control of mitochondrial integrity. Some members of the family, like Bax, Bak and Bad, are apoptosis inducers. On the contrary the expression of other members like Bcl-2 and Ced-9 prevents apoptosis. In any case it is well known that the principal role of Bcl-2 proteins is to control mitochondrial homeostasis: under certain conditions canals or pores are formed in the outer mitochondrial membrane, allowing the exit of mitochondrial contents, being among them cytochrome C, which, in addition to playing a fundamental role in the electron chain transport, is an essential component of the activation of caspase-9 in the cytosol.

Regulatory Elements for Oxidative Stress Damage: GH

Growth hormone (GH) is the most abundant anterior pituitary hormone that accounts for 4–10% of the wet weight of the anterior pituitary in the human adult, amounting about 5–10 mg per gland [27].

The circulating levels of this hormone decline during the first weeks after birth but reaches adult levels after 2 or 3 weeks of life. A substantial increase of GH during puberty has been observed. Spontaneous episodes of GH secretion occur every 3–4 h over 24 h, being these secretory peaks more frequent and smaller in females than in males. The highest secretion of GH occurs during the two first hours of nocturnal rest in the period of slow wave sleep.

Three hypothalamic hormones are involved in GH control: somatostatin (SS), GH-releasing hormone (GHRH) and ghrelin, which is also synthetized in the stomach [28]. SS has a direct inhibitory effect on GH release in response to all known stimuli [29].

Hypothalamic GHRH binds to specific receptors in the somatotropic cells stimulating GH secretion, cell proliferation and also GH-gene transcription [30]. Each episodic secretion of GH is determined by the release of GHRH to the portal circulation together with a decrease in somatostatin. This pulsatile pattern of GH secretion seems to be more important for the peripheral hormonal effects, than the total amount of GH secreted [31].

Ghrelin is another peptide with 28 amino acids, mainly synthetized in the stomach mucosa, but it is also produced in the hypothalamus, which has been found to stimulate GH release both in vivo and in vitro [32].

Actions of GH

GH acts on tissues on a receptor (GHR) that consists in a transmembrane protein codified by a gene located in chromosome 5 [33].

GH is an anabolic protein hormone that causes cells to grow and multiply by directly increasing the rate at which amino acids are used to synthesize proteins. Due to these effects, GH induces an increase in the growth rate of long bones and skeletal muscles during childhood and teenage years. GH also stimulates lipolysis, which is the breakdown of triglycerides into fatty acids and glycerol providing substrates for the neosynthesis of glucose and thus has a sparing effect on glucose utilization. GH also promotes fat catabolism [33].

GH deficiency in the adult has been recently recognized as a specific clinical syndrome characterized by a combination of metabolic and cardiovascular features that are more evident in women than in men [34]. The syndrome includes a high prevalence of dyslipidaemia, glucose intolerance, central obesity and hypertension. Early arteriosclerosis is found in this asymptom-

atic GH deficiency. All these are important contributory factors to the increased cardiovascular risk [35]. Adults with GH deficiency [36, 37] exhibit a diminution of lean body mass and an increase of adipose tissue, which means a reduction of the muscular force capacity. Increase of force and exercise capacity has been reported in elderly people when GH therapy is instituted [38, 39, 40].

Physiological Decrease of GH Secretion with Age

Ageing is associated with several changes and alterations in metabolism, body composition and organ function. Elderly people show bone mineral density loss, lean body mass and muscular strength reduction, adipose tissue increase, insulin resistance and glucose intolerance, etc. [37, 41, 42]. The similarities detected between all the consequences of GH deficiency (GHD) in adults and the changes shown by elderly people point to a possible relationship between age-related physical impairment and the GH/IGF-1 axis decline that physiologically occurs with age [37, 42, 43]. Old age could be a physiological state of GH deficiency. The hormones of the somatotropic axis, growth-hormone-releasing hormone (GHRH), growth hormone (GH) and insulin-like growth factor-1 (IGF-1), apart from their effects on somatic growth and metabolism [27], also exert some other actions on the cardiovascular system.

Indeed, experimental evidence demonstrates that GH treatment has beneficial effects on aged animals. It improves cerebral microvasculature [44], coronary blood flow and heart capillary density [45] in ageing rats. In humans, GH treatment is able to enhance lean body mass and muscular strength, reduces body fat [38, 46, 47], improves plasma lipid profile and increases bone mineral density [47]. However, the effect of GH on vascular function and structure in aged individuals is not well established.

The central nervous system is a target for growth hormone (GH) actions [48]. GH deficiency is associated with sleep disturbances, memory loss, feeling of diminished well-being and other cognitive impairments. Memory and cognitive performances of GH-deficient patients are ameliorated by GH replacement therapy [37, 44]. In animal models, GH has been shown to protect the brain and the spinal cord from different forms of neurodegenerative stimuli and promote neuronal survival after hypoxic-ischaemic injury [49, 50, 51, 52]. These neuroprotective effects of GH suggest that decreases in the hormonal levels with age [37] may affect the brain and may contribute to the ageing-associated deterioration of brain function [53, 54].

Metabolic Effects

It has been demonstrated that GH treatment to both GHD adults and elderly people is able to improve several parameters related to body composition [47, 55], for example, reducing abdominal obesity, which is a strong predictor of cardiovascular risk [56]. The increment in lean body mass in old GH-treated rats is associated with an increase in body weight gain as compared to the weight loss observed in old untreated animals confirming the preponderance of the anabolic properties of GH in the old animals over the lipolytic effects on fat tissue [57].

It has been previously reported [58] that there is a decrease in GH and IGF-1 production with age, but in the present study reduced plasma IGF-1 levels were only seen in males, whereas hepatic IGF-1 content was significantly reduced in both sexes [59]. GH administration was able to significantly increase the hepatic content and plasma IGF-1 levels.

Vascular Effects

GH-deficient patients show a greater risk of cardiovascular alterations [35] and endothelial dysfunction, including a reduced vascular endothelial-dependent relaxation [60].

Ageing is associated with both structural and functional changes that take place in the vascular wall [61, 62]. An increase in media-intima thick-

ness as well as changes in cellular and extracellular composition of the vessel wall [62] can be seen. Ageing is also associated with an impaired endothelium-dependent vasodilatation. Similar results have been obtained in experiments carried out in humans, measured as the response to brachial artery infusion of acetylcholine by plethysmography [63]. This endothelial dysfunction seems to be parallel to the general deterioration of the animals as shown by the correlation found between the maximal relaxation to acetylcholine and body composition parameters.

A decrease in endothelial NO availability due to reduced synthesis and/or major degradation by oxidative stress has been suggested to be an important mechanism underlying the altered response to endothelium-dependent agents during ageing [61, 62], which has been confirmed by our group [64, 65]. In addition, an increase in contracting factors which can counteract the effect of relaxing ones might also be involved in this altered endothelial function. In our previous study, the administration of GH to old rats produced an expected increase in plasma levels of IGF-1 that was accompanied by an improvement of endothelial function and vessel structure [57, 66].

The mechanisms underlying the beneficial effects exerted by GH involve an increase in endothelial NO availability that has been confirmed by our group [64, 65] This enhanced NO availability could positively influence vascular function and structure. These data confirm previous studies which show that GH can exert beneficial effects on cardiovascular system in aged animals [44, 45].

Effects on the CNS

The hippocampus, a brain region involved in spatial and episodic memory [67], may significantly contribute to ageing-associated decline in cognitive abilities [54, 68]. Although in most brain areas there is no massive neuronal loss with ageing [53, 69], a significant reduction in the number of neurons has been reported in the hilus of the dentate gyrus of the hippocampal formation in aged humans [54] and in 24-month-old Fischer 344 male rats [52].

It is well known that GH exerts important effects on CNS [48] increasing psychological capacity in adults [70], memory, concentration, alertness and capacity of work. Some neurotransmitters change also under GH treatments [71]. Receptors for GH exist in CNS at different levels: neurons, glia and endothelial cells in the vessels [72]. Under GH stimulation IGF-I is produced in the cerebral tissue [73] probably playing a local trophic role [74]. Emergence of new neurons in the brain is a well-documented phenomenon, especially in young animals, but the real significance of this fact is still unknown [75].

Estimation of the total number of hilar neurons by our group revealed that 24-month-old rats that were treated with GH had more neurons in the hilus than control animals treated with vehicle. GH is a neuroprotective factor for the brain and spinal cord of young animals [50, 51] and prevents hippocampal neuronal cell loss after unilateral hypoxic-ischaemic brain injury [51, 65, 76]. The neuroprotective effect of GH was observed in both sexes.

After GH treatment a clear inhibition of the apoptosis was observed, which was accompanied by a decrease in nucleosome levels and an increase in Bcl-2 levels.

The role of GH and IGF-I on the regulation of the production of free radicals and the antioxidant defences is controversial. It has been demonstrated that the administration of both GH and IGF-I exerts a protective effect on several experimental models of free-radical-induced tissue damage. Previous data showed in our laboratory confirm these effects in the liver, pancreas, heart and brain [64, 77, 78, 79, 80]. Effects can be due to direct actions of GH or mediated by IGF-1.

However, there are also data that indicate that the hormones of the somatotropic axis could also exert a negative influence on life expectancy and antioxidant defences. Transgenic mice that overexpress GH show a reduction in life expectancy as well as a phenotypic expression of premature ageing with an excess of oxidative stress damage [81]. On the contrary dwarf Ames mice that are deficient in GH, TSH and prolactin live longer

than the normal individuals of the same strain [81, 82]. Our explanation for the apparently controversial results is that what we expect when we proceed with replacement therapies with GH in old animals is to maintain the physiological levels of GH present in young adults. Both transgenic animals and acromegaly patients show very high levels of GH starting very early in life, thus exerting at least negative effects on the whole organism, whereas compensating for the lost levels of GH in old animals should replace the positive physiological actions of GH.

Since the paper of Rudman D and colleagues [47], which has been converted in a "classical reference", replacement therapy with GH in elderly people has been proposed, and many studies that have been carried out demonstrate beneficial effects of the treatment [83]. However, the studies have obtained positive results [37, 84]. The administration of GH to men older than 60 years of age restores normal levels of IGF-I reaching values present in young people [38], and this could be extremely beneficial.

Both in GH deficiency and in the elderly, replacement therapy with GH is able to increase lean body mass and to reduce body fat [38, 84, 85]. Replacement therapy in the elderly with GH also has beneficial effects on plasma lipid levels, since cholesterol values are reduced and also the relationship between LDL/HLD [86]. Our group has confirmed these results in old rats [64, 77, 79, 87].

There are also effects on the skin as has been described previously with an increase in cutaneous stiffness and thickness [47, 89, 90]. It has been also seen that when age-associated immunosenescence is treated with GH, a highly beneficial effect can be observed.

Melatonin

Melatonin, an indolic hormone secreted by the pineal gland, is a substance closely related to biological rhythms and has been used since decades for the induction of sleep and the treatment of jet lag. In addition to its role as a chronobiotic hormone, melatonin is a ubiquitous direct free radical scavenger and an important indirect antioxidant.

The pineal gland [91] is the link between light signals of the environment and the endocrine and nervous systems through the secretion of its hormone **melatonin**, which is regulated by the degree of environmental light.

Melatonin is synthesized from tryptophan in the pineal gland and then is secreted to the circulation, showing a circadian rhythm with maximal values present during the period of darkness. It seems that this more recently discovered action as antioxidant was actually its initial activity and appeared nearly simultaneously with the starting of life on earth as a way to reduce the oxidative damage in nearly all living beings [92].

Bright artificial light is able to reduce the amplitude of the nocturnal peak [91]. Besides these direct scavenging actions, melatonin stimulates also a host of endogenous antioxidant enzymes, including superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GRd), and inhibits the activity of nitric oxide synthase (NOS) thus making it possible to effectively fight not only against free radicals but also against inflammation, apoptosis and also several age-associated diseases.

In humans, melatonin levels start to descend from 25 to 35 years of age, and at 40–60 years the levels are about 35–50% of those present in young individuals [92, 93], and especially a reduction in the nocturnal peak has been detected [94].

In parallel with the reduction of melatonin levels, an increment in free radicals has been observed together with the reduction in antioxidant enzymes such as SOD, GRd and GPx, which are in part regulated by melatonin itself. Thus one of the reasons to have an age-related oxidative damage could be the reduction of melatonin [95].

Moreover, melatonin is a small, lipophilic and hydrophilic molecule, which allows it to easily cross biological barriers and membranes and diffuse throughout cell compartments, reaching the place where the free radicals and reactive species are generated in all tissues: the mitochondria.

Melatonin is critical for the regulation of circadian and seasonal changes in various aspects of physiology and neuroendocrine function [91,

92, 96]. In addition, melatonin is also produced at high levels in other organs (bone marrow, gastrointestinal tract) [97] and the immune system – especially lymphocytes [98]. Melatonin and its metabolites exert potent hydroxyl and peroxyl radical scavenging activity [99, 100, 101], and they increase the efficiency of the electron transport chain in the mitochondria and, as a consequence, reduce electron leakage and generation of free radicals. Melatonin also reduces the formation of 8-hydroxy-2′-deoxiguanosine, a marker of damaged DNA, more effectively than some classical antioxidants [101]. As age advances, the nocturnal production of melatonin decreases in animals of various species, including humans [93, 94]. Melatonin is an anti-apoptotic mediator. So melatonin supplementation suppresses NO-induced apoptosis by induction of Bcl-2 expression [102]. In previous publications, we found that melatonin treatment reduced NO levels and increases cytochrome C content in mitochondrial fraction of the liver of old and castrated female rats. Additionally melatonin treatment enhances hepatic antioxidant/detoxification systems, consequently reducing apoptotic rate [76, 88, 102].

Ageing is associated with a dysregulation of the immune system known as immunosenescence that is characterized by a decrease in the functional activity of NK cells, granulocytes and macrophages. Besides causing changes in innate immunity, ageing is associated with changes in cellular and humoral immunity. Reductions in CD3 and CD4 and rises of CD8 immune cells occur in elderly individuals. Changes in the expression and function of TLR (toll-like receptors) as a result of immunosenescence lead to increased secretion of pro-inflammatory cytokines and chemokines [98].

Immunosenescence

The ability of melatonin to revert age-associated thymic involution adds further support to the concept that it is a potential therapeutic agent for the correction of immunodeficiency states associated with ageing and possibly other immunocompromised situations like severe stress [98]. Immunopharmacological activity of melatonin has been demonstrated in various experimental models [79, 88]. Melatonin regulates the immune system by affecting cytokine production in immunocompetent cells [103]. Inasmuch as melatonin is able to stimulate the production of intracellular glutathione [104], its immuno-enhancing effect may be partly a result of its action on glutathione levels.

Melatonin and Diabetes Type 2

Diabetes type 2 appears in the fourth decade of life in persons with a genetic predisposition and that generally are overweight. The first indication of glucose metabolism impairment is the appearance of peripheral insulin resistance. The pancreas reacts initially with beta cell hypertrophy leading to hyperinsulinaemia, thus making it possible to maintain normoglycaemia, and later on, after some months or years, with beta cells getting exhausted or entering apoptosis with the corresponding reduction in plasma insulin levels and the appearance of hyperglycaemia. Drugs used until now allow the treatment of diabetes as a chronic disease but are not able to cure it. The elements responsible for insulin resistance in peripheral tissues are related with oxidative stress and inflammation and the same occurs in the endocrine pancreas itself. Melatonin when given both to experimental animals (1–10 mg/Kg) that show already insulin resistance and hyperinsulinemia and to humans (40–60 mg) with increased HOMA index is able to revert the situation, reducing plasma insulin levels and enhancing the islet production of insulin by reducing several inflammation- and oxidative-stress-associated factors present in both the peripheral tissues like muscle and fat tissue such as TNF-alpha, NF-kB and IL1/2. The administration of melatonin to experimental animals was able also to increase the pancreas genes of survival like SIRT1 and FoxO as well as genes of differentiation like PCNA, Pdx and Sei1 and to reduce apoptosis markers restoring its normal function [79, 80, 105].

These effects have been observed also in humans suffering from insulin resistance in which

melatonin besides of a reduction in plasma insulin levels a potentiation of the beta cell capacity to produce insulin, which reduces the risk of exhaustion of these cells and thus allowing the maintenance or restoration of normoglycaemia [105].

Oestrogens

Oestrogens have shown to exert fundamentally antioxidant effects but also under some circumstances pro-oxidant actions [106, 107], as well as anti-inflammatory actions in several experimental models [108]. They have also shown protective effects against peroxidative membrane damage [106, 109]. These hormones are able to protect hepatocytes undergoing oxidative stress [110], and they preserve hepatic integrity and function in several experimental models of liver injury in which oxidative damage is involved [111]. They have also shown to exert protective effects in other tissues, such as CNS [112, 113] and heart and skeletal muscle [114]. Moreover, it has been demonstrated that mitochondria isolated from brain and liver of female rats exhibit higher antioxidant gene expression and lower oxidative damage than males [115]. All these findings support the idea of oestrogens acting as protective agents against oxidative damage of different aetiologies, and our studies are in accordance with this, showing a protective effect of oestrogens against age-induced oxidative injuries in the liver but also in other tissues.

Both menopause and ovariectomy are known to induce deleterious effects on different organs and systems, such as cardiovascular system, plasma lipid profile and bone turnover [116, 117]. On the other hand, oestrogens have shown to exert positive effects on vascular function [117, 118], to have antioxidant properties in vitro [106, 109] and to play neuroprotective actions [112]. 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 oestrogens on the liver [119]. Moreover, hepatic tissue in both males and females contain oestrogen receptors and respond to these hormones [120].

The degree of damage found in these parameters is, in general, lower in intact females than in ovariectomized ones. Thus, a possible protective effect of oestrogens on oxidative and inflammatory age-induced liver injury can be suggested. Moreover, when ovariectomized female rats were treated with oestrogens, they showed a clear improvement in the values of all studied functions.

On the other hand, oestrogens have also shown to exert anti-inflammatory actions in different experimental models, such as experimental arthritis [121], uveitis [122], shock [123], amyloid-b-induced inflammatory reaction [124] and carrageenan-induced pleurisy [108]. All these data are in accordance with those found in the present experiment, in which treatment with oestrogens were able to reduce NO release in cells isolated from old rats.

Several mechanisms have been proposed to be involved in these protective actions of oestrogens. They have shown to maintain the level of endogenous antioxidants, such as GHS and other antioxidant enzymes [111, 115, 125]. Oestrogens are also able to inhibit the activation of the gene regulator NF-κB in different cell cultures [112, 126], which is involved in immune and inflammatory response. They may also act as direct free radical scavengers and iron chelators, since some of their protective effects are not mediated by the interaction with the classical ER [113, 114]. And oestrogens have shown to modulate the expression of cytokines and other molecules related to inflammation [108, 125].

A naturally occurring sexual dimorphism in the immune response has been shown by both clinical and experimental data [127, 128]. Females show, at least during the reproductive period, a more vigorous cellular and humoral immune response than males [129]. Furthermore, data suggest that physiological levels of oestrogen stimulate the humoral and cellular immune response, whereas male testosterone does the opposite [130]. Recent studies on many experimental models have demonstrated that sex hormones may regulate immune reactivity, and concretely T cell response, and the subsequent release of various cytokines [125]. Moreover,

oestrogen deprivation affects several aspects of the immune function. Thus, a decrease of NK activity has been found after oestrogen withdrawal in rhesus monkeys [131]. In fact, the results of the present work show that intact female rats demonstrate higher values of the immune parameters studied than ovariectomized in which these values decrease in some functions such as lymphoproliferation.

If a good immune system is a predictor of longevity [132], the better response found in females could explain in part the longer lifespan of female rats as compared to males, which was attributed to oestrogen [133]. Many studies have examined the cytoprotective role of oestrogens on the prevention of age-related diseases, but the mechanism(s) underlying its effects on the ageing process has not been elucidated. Oestrogens have been also found to exert a protective effect against oxidative stress and mitochondrial DNA damage. This protection against free-radical-mediated damage could explain the different lifespan of both genders [134]. Oestrogens have been reported to act as antioxidants in vitro [111, 135]. Ovariectomy caused an increase of oxidative stress in mitochondria and oestrogen replacement therapy completely prevented this effect [134]. Same effects can be seen in heart and skeletal muscle [114]. Although no data are available about its effect on immune cells, a similar mechanism could be suspected for them. But the physiological levels of these sex hormones are not high enough to show a direct antioxidant action. Oestrogens are also able to increase the antioxidant defences of the organism. Thus, oestrogens increase the expression of Mn-superoxide dismutase and glutathione peroxidase, two of the major antioxidant enzymes found in the mitochondria [134].

Phytoestrogens are plant-derived substances, with molecular structures similar to those of oestrogens, that share some of the effects of these hormones. Isoflavones, which is one group of phytoestrogens, are now being widely studied, since they seem to exert beneficial effects on health [136, 137]. Isoflavones are organic plant substances found in soy, legumes, fruits and vegetables, which have been suggested to exert beneficial effects on health. These compounds seem to reduce the incidence of various cancer types [136] and coronary heart disease [138].

Isoflavones have shown to behave both as agonist and antagonist of oestrogenic action, similarly, but not identically, to the action of a SERM. In the presence of a stronger oestrogen like estradiol, an antagonist action on both cell growth and oestrogen-induced protein synthesis has been observed [139, 140]. One of the possible explanations for the dual activity of isoflavones could be its interaction with oestrogenic receptor. A more intense affinity for receptor ERβ has been described. ERβ receptors have different transcriptional activities. It has been shown that isoflavone excretion, derived from vegetal food, is much higher in Japanese than in North American women and that this excretion is negatively correlated with the incidence of climacteric symptoms and breast cancer [141, 142]. In fact, in Western countries isoflavones are becoming an alternative treatment for climacteric alterations, such as hot flushes [143]. Since isoflavones show oestrogenic effects on several tissues, it has been tested for antioxidant activity in several tissues including the immune system.

Phytosoya®, a commercial soya extract, has been shown to protect against glucose-induced oxidation of human LDL [65]. It has also shown to protect against oxidatively induced DNA damage in different cell lines [144, 145]. Our group has shown that Phytosoya® is able to improve some parameters related to oxidative stress in several tissues that are altered by ageing and ovariectomy. And we also have shown that these effects are similar to that of oestrogen. The importance of these results rests in the fact that phytoestrogens are being increasingly proposed as a safer alternative to hormone replacement therapy, mainly in women who have some contraindication for being treated with oestrogens. Several mechanisms may be involved in this effect, such as a direct free radical scavenging [146], metal ion chelator activity [147], restoration of GSH levels [146] and modulation of the activity of antioxidant enzymes [137, 146, 148]. However, there are some discrepancies among the results of different studies carried out in order

to investigate these antioxidant properties of isoflavones, and some of them have been unable to demonstrate real antioxidant effects [149, 150]. It is necessary to point that these studies are difficult to compare, since they use different experimental designs and soya extracts, and on the other hand some women lack the enzymatic activity to hydrolyze the extracts in order to obtain the active aglycones. To elucidate the antioxidant properties of phytoestrogens and their intrinsic mechanisms of action, more work needs to be done.

Resveratrol

Resveratrol, a polyphenolic compound found in appreciable amounts in grapes and red wine, is currently a widely investigated molecule for its potentially beneficial effects on health and its capability to promote longevity [151]. In animal models, from lower metazoans to vertebrates, including small mammals, resveratrol administration has the remarkable property to prolong lifespan [151, 152, 153]. Several genes have been identified to play a role in the control of lifespan, including genes implicated in insulin-like signalling and genes coding for the Sir2/SIRT1 sirtuin family of deacetylases [154, 155]. In aged individuals, intense inflammatory activities characterized by the presence of cytokines, apoptotic cells, immune cell infiltration, amyloid deposits and fibrosis may cause a reduced function or failure in pancreas and other tissues [156]. Several factors are responsible for inflammation, including elevated nuclear-factor kappa B (NF-κB) activity, increased levels of cytokines such as tumour necrosis factor-alpha (TNF-α), interleukins (ILs), resistin, leptin and free fatty acids [157, 158, 159, 160]. NF-κB is a family of transcription factor that regulates expression of genes which are involved in immunity and inflammation. Sirtuins are NAD^+-dependent deacetylase enzymes related to histones and transcription factors like p53, FoxO family and PGC-1. Sirtuins and FoxO factors could remove NF-κB signalling and, thus, delay the ageing process.

Resveratrol supplementation has been shown to exert anti-inflammatory effects in various mammalian models of ageing [151, 161]. Previous studies have established that resveratrol can exert significant cardiovascular protective effects in various models of myocardial injury [162, 163, 164], hypertension [164, 165, 166] and type 2 diabetes [167, 168, 169].

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