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Review Article

Oxidative Stress, Nutritional Antioxidants, and Testosterone Secretion in Men

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Abstract

The biochemistry of testosterone synthesis within the Leydig cells of the human testes is well characterized. Reliance on the mitochondrial electron transfer system for the energy to drive testosterone synthesis exposes Leydig cell mitochondria to oxidative stress. Leydig cells experiencing oxidative stress exhibit reduced activities of antioxidant enzymes, increased lipid peroxidation, reductions in mitochondrial membrane potential required for testosterone synthesis, and reduced expression of the StAR steroidogenic acute regulatory (StAR) protein, culminating in inhibition of the synthesis and secretion of testosterone.

Evidence obtained from *in vitro*, laboratory, and animal experiments, and from human trials, provides strong support for the hypothesis that reducing oxidative stress releases Leydig cells from oxidative inhibition of testosterone synthesis and can improve testosterone status. Selected dietary antioxidants (e.g., the phytonutrients in pomegranates, phosphatidylserine, vitamin C, vitamin E, α-lipoic acid, zinc, and selenium) can contribute safely to oxidative stress reduction and enhanced androgenic status in otherwise healthy adult males. In this era of science-based medical decision-making, addressing oxidative stress and its potential role in undermining testosterone status deserves closer scrutiny.

Keywords: Low testosterone; Leydig cells; Oxidative stress; Antioxidants; Phosphatidylserine; Pomegranates

Abbreviations

SHBG: Steroid Hormone Binding Globulin; DHT: Dihydrotestosterone; the STAR protein: The Steroidogenic Acute Regulatory Protein; ERK1/2: Extracellular Signal-Regulated Kinase 1/2; LH: Luteinizing Hormone; AMP: Adenosine Monophosphate; 5'-Adenylic Acid; cAMP: Cyclic AMP; P450scc, CYP11A1: Cytochrome $P450_{\text{cholesterol side-chain cleavage enzyme}}$; NADPH: Reduced Nicotine Adenine Diphosphonucleotide; Cytochrome P450 17α-hydroxylase/17, 20-lyase: CYP17A1 hydroxylase and CYP17A1 lyase; 3β-HSD, HSD3B2: 3β-hydroxysteroid dehydrogenase/$\Delta^5 \rightarrow \Delta^4$ isomerase; 17β-HSD, HSD17B3: 17β-hydroxysteroid dehydrogenase; DHEA: Dehydroepiandrosterone; SRD5A1, SRD5A2, SRD5A3: Short-Chain Dehydrogenase/Reductases; GnRH: Gonadotropin-Releasing Hormone; MrOS: The Osteoporotic Fractures in Men Study; BACH: The Boston Area Community Health Survey; CRP: C-Reactive Protein; TNF-α: Tumor Necrosis Factor-α; MIP1α: Macrophage Inflammatory Protein 1α; MIP1β: Macrophage Inflammatory Protein 1β; ROS: Reactive Oxygen Species; SO: $\cdot O_2^-$, Superoxide; H_2O_2: Hydrogen Peroxide; $\cdot OH^-$: Hydroxy Radical; ·NO: Nitric Oxide; $\cdot ROO^-$: Peroxyl Radical; $\cdot ONOO^-$, Peroxynitrite; $\cdot {}^1O_2$: Singlet Oxygen; HOCl: Hypochlorous Acid; SOD: Superoxide Dismutase; GPx: Glutathione Peroxidase; MDA: Malondialdehyde; PON1: Paraoxonase 1; PON2: Paraoxonase 2; CCl_4: Carbon Tetrachloride; CYP1A2: Cytochrome P450 Isozyme CYP1A2; CYP3A: Cytochrome P450 Isozyme CYP3A; GR: Glutathione Reductase; MAM: Mitochondria-Associated Membrane; DHA: Docosahexaenoic Acid; Akt: Protein Kinase B; mTOR2: Mammalian Target of Rapamycin-2; HDL: High-Density Lipoprotein; LDL: Low-Density Lipoprotein; DHA: Dehydroascorbate; HO-1: Heme Oxygenase-1; Nrf2: Nuclear Factor-Erythroid 2-Related Factor 2

Testosterone Synthesis and Metabolism

Testosterone appears in the human circulation either free from binding to any carrier molecules, bound to albumin, or bound to steroid hormone binding globulin (SHBG). Bound and unbound forms can be measured and serum testosterone concentrations are expressed as either free testosterone, biologically active testosterone (unbound testosterone plus testosterone bound to albumin), or total testosterone (unbound testosterone plus testosterone bound to SHBG plus testosterone bound to albumin) [1,2]. Circulating testosterone concentrations, which determine the availability of testosterone to the tissues, reflect the net balance between testicular synthesis and secretion of testosterone and the conversion of circulating testosterone into 17β-estradiol, dihydrotestosterone (DHT), and excretory metabolites.

Testosterone Synthesis

The biochemistry of testosterone synthesis within the Leydig cells of the human testes is well characterized [3,4]. Testosterone anabolism in men is proportional to the plasma concentration of pituitary-derived luteinizing hormone (LH) [5] and is triggered by binding of LH to its plasma membrane receptors on Leydig cells of the testes [6] and subsequent activation of intracellular signaling

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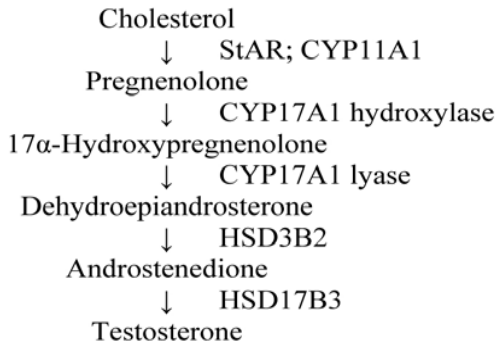

Figure 1: The Δ^5 testosterone synthesizing pathway in men (see text for full names of enzymes).

cascades that increase the conversion of adenosine monophosphate (AMP) to cyclic AMP (cAMP) and initiate the *de novo* synthesis of testosterone [7]. Testosterone biosynthesis begins with the activation of the steroidogenic acute regulatory protein (the StAR protein) by extracellular signal-regulated kinase 1/2 (ERK1/2) in response to the LH-induced increase in intracellular cAMP concentration (Figure 1) [8]. The StAR protein is a component of a transmembrane multi-protein complex (the transduceosome) that catalyzes the rate-limiting step in steroid biosynthesis [9], the translocation of cholesterol from the cytoplasm to cytochrome $P450_{\text{cholesterol side-chain cleavage enzyme}}$ (P450scc; CYP11A1) embedded within the inner mitochondrial membrane [9-13].

Three sequential reactions catalyzed by CYP11A1 convert cholesterol into pregnenolone, utilizing energy provided by mitochondrial reduced nicotine adenine diphosphonucleotide (NADPH) [3,4]. Pregnenolone diffuses from the mitochondria into adjacent smooth endoplasmic reticulum, where cytochrome P450 17α-hydroxylase/17,20-lyase (CYP17A1 hydroxylase and CYP17A1 lyase), 3β-hydroxysteroid dehydrogenase/$\Delta^5 \rightarrow \Delta^4$ isomerase (3β-HSD; the HSD3B2 isoform in human Leydig cells), and 17β-hydroxysteroid dehydrogenase (17β-HSD; the HSD17B3 isoform in human Leydig cells) drive the predominant "Δ^5" testosterone synthesizing pathway in men [3,4,14,15].

A minor "Δ^4" pathway converts a small amount of pregnenolone into testosterone following the conversion of pregnenolone into progesterone by the isomerase activity of HSD3B2 (Figure 2) [16].

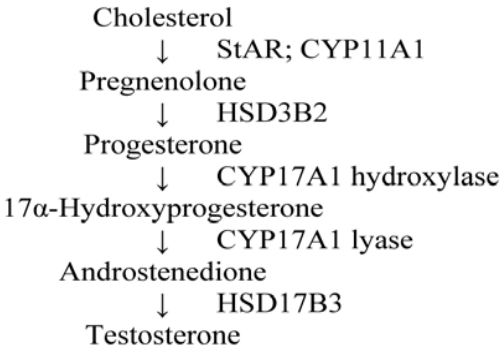

Figure 2: The Δ^4 testosterone synthesizing pathway in men (see text for full names of enzymes).

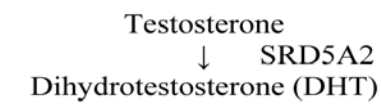

Figure 3: Testosterone conversion to DHT (see text for the full name of SRD5A2).

The CYP17A1 enzyme complex catalyzes the NADPH-dependent conversion of pregnenolone into 17α-hydroxypregnenolone and the subsequent conversion of 17α-hydroxypregnenolone into dehydroepiandrosterone (DHEA). HSD3B2 then catalyzes the 2-step conversion of DHEA into androstenedione; this reaction is rate-limiting for the entire pathway of *de novo* testosterone biosynthesis from pregnenolone [3,4] and the expression of HSD3B2 is upregulated by LH [12-17]. HSD17B3 then catalyzes the NADPH-dependent conversion of androstenedione into testosterone. Testosterone is metabolized to DHT in Leydig cells and peripheral tissues by several types of 5α-reductase enzymes (short-chain dehydrogenase/reductases; SRD5A1, SRD5A2 and SRD5A3; Figure 3); SRD5A2 has particularly high affinity for testosterone [3,4,14].

The Aromatase Complex, Testosterone, and 17β-Estradiol

Circulating testosterone is converted into 17β-estradiol by the aromatase enzyme complex embedded within the membranes of the endoplasmic reticulum of testicular Leydig and Sertoli cells, spermatocytes, prostate epithelial cells, bone, adipose tissue, and other organs in men (Figure 4) [17-21]. The aromatase complex also converts androstenedione to mildly estrogenic estrone, which can then be converted into 17β-estradiol by HSD17B3 (Figure 4) [3,4,18].

The rates of conversion of androstenedione and testosterone into 17β-estradiol are determined by both substrate availability and the level of aromatase expression and activity [22-25]. Testicular and prostatic aromatase activities account for only 15% to 20% of the 17β-estradiol in the circulation, while aromatase activity in nontesticular and nonprostatic tissues accounts for about 80% to 85% of the estrogens (predominantly 17β-estradiol) circulating in the adult male [26]. However, increased aromatase complex activity (as in obesity) increases the production of 17β-estradiol from circulating testosterone, increasing the serum total and free 17β-estradiol concentrations and reducing the serum total and free testosterone concentrations; conversely, decreased aromatase complex activity (such as that accompanying significant weight loss) decreases the production of 17β-estradiol from testosterone, decreasing the serum total and free 17β-estradiol concentrations and maintaining greater serum total and free testosterone concentrations [18,24-30].

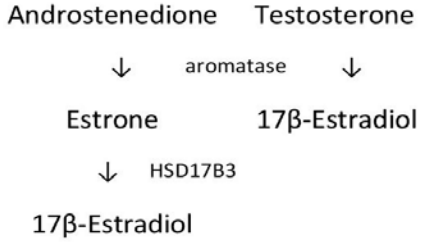

Figure 4: Testosterone and androstenedione conversion to 17β-estradiol (see text for full name of HSD17B3).

An elevated ratio of serum total 17β-estradiol concentration to serum total testosterone concentration stimulates the externalization of phosphatidylserine on the plasma membranes of human Leydig cells and initiates premature engulfment of healthy Leydig cells by testicular macrophages, reducing the amount of testosterone synthesizing tissue [31-33]. Elevated ratios of serum total 17β-estradiol concentration to serum total testosterone concentration are associated with "estrogenic erectile dysfunction," a disorder characterized by loss of libido and erectile impairment [34,35]. In addition, postprandial insulin sensitivity and the rate of postprandial glucose clearance decrease as the ratio of serum total 17β-estradiol concentration to serum total testosterone concentration increases [36].

Circulating Androgens, Age, and Male Sexual Function

Partly because their whole-body aromatase activity is greater and, therefore, older men convert testosterone to 17β-estradiol faster than younger men [23], aging is associated with increased serum total 17β-estradiol concentration [35,37] and decreased serum total testosterone concentration [22,35,37-52]. In many countries and among many ethnic groups, men more than 40 years old experience annual declines of between 0.5% and 2.5% in mid-morning serum concentrations of free testosterone, biologically active testosterone, and total testosterone [22,35,37-55]. This decline is caused by aging-associated attenuation of hypothalamic secretion of gonadotropin-releasing hormone (GnRH), resulting from hypothalamic hypersensitivity to the negative feedback inhibition of LH and testosterone on GnRH secretion, and a blunted response of testicular Leydig cells to testosterone-stimulating LH [56-62]. Together these changes downregulate testosterone production in the testes and decrease testosterone secretion into the circulation [57-62].

These declines carry clinical relevance. Male sexual performance and function are dependent upon testosterone adequacy [63-73], while relative testosterone inadequacy reduces male sexual desire, function, performance, and potency [63-67]. The strength, duration, and quality of erections, as well as the frequency of successful intercourse, are proportional to the serum free testosterone concentration [63-67,73,74] and successful erectile function may require a serum free testosterone concentration of at least 7 nmol/L (1 nmol/L = 28.8 ng/dL) [63,64,66]. In parallel with the aging-associated decline in circulating testosterone concentrations, the percentage of men who experience declining interest in sexual activity, impaired sexual performance, or moderate to severe erectile failure increases with increasing age after the 40th year [75].

"Testosterone Deficiency" and "Low Normal Testosterone"

Disordered testosterone physiology occurs along a continuum of severity. "Testosterone deficiency" reflects classical frank endocrine deficiency and is associated with clinical symptoms of hypogonadism, such as incomplete or delayed sexual development, sexual disorders, breast discomfort, gynecomastia, loss of body hair, reduced need for shaving, very small or shrinking testes, inability to father children, low or zero sperm count, loss of height, predisposition to fracture, low bone mineral density, hot flushes, and sweats [68]. Although definitions of "testosterone deficiency" vary, mid-morning serum total testosterone concentrations less than 7 nmol/L to 10.5 nmol/L are considered to reflect "testosterone deficiency" [68,76-78]. A less severe disorder of testosterone physiology, a condition of "low normal testosterone" (also called "Leydig cell impairment" [69]), is associated with physiological conditions that may include reductions in energy, motivation, initiative, self-confidence, concentration and memory, sleep quality, muscle bulk and strength; diminished physical or work performance; feeling sad or blue; depressed mood or dysthymia; mild anemia; and increased body fat and body mass index [68]. A diagnosis of "low normal testosterone" is considered to be consistent with a mid-morning serum total testosterone concentration between 7 nmol/L to 10.5 nmol/L and 12.2 nmol/L to 14 nmol/L [68,76-78].

Consequences of Low Normal Serum Testosterone Concentration

Low normal serum testosterone concentrations jeopardize men's health [79-88]. For example, in several prospective studies, remaining life expectancy was directly correlated with the initial serum total testosterone concentration [79], while the risk for premature death from any cause was inversely correlated with the initial serum total testosterone concentration [80-85]. In addition, during the 20-year prospective observational Rancho Bernardo Study of men aged 50 to 91 years living in southern California, men with initial serum total testosterone concentrations less than 8.4 nmol/L were at significantly greater risk for premature death from any cause, death from any cancer, death from any form of cardiovascular disease, or death from respiratory disease [86]. The results of a meta-analysis of the combined results of 11 previously-published epidemiologic observational studies indicated that the risk for suffering premature death from any cause was 35% greater among men with serum total testosterone concentrations less than 14 nmol/L [88].

Cardiovascular and cerebrovascular health are compromised by low normal serum testosterone concentrations [81,82,86,87,89-97]. For example, in the 5-year prospective observational Health in Men study of men aged 70 years or older in Australia, the combined risk for first stroke or first transient ischemic attack was doubled in men with initial serum total testosterone concentrations less than 11.7 nmol/L [89]. In a cross-sectional study of men with hypertension but free of evidence of clinical atherosclerosis in Athens, Greece, the risk for suffering either stroke, transient ischemic attack, peripheral artery disease, coronary artery disease, or premature death from cardiovascular disease was more than tripled in men with serum total testosterone concentrations less than 14 nmol/L [90]. In other studies, the rate of thickening of the carotid artery wall [91] and the risk for developing severe aortic atherosclerosis [92] were inversely correlated with the serum total testosterone concentration. The results of a meta-analysis of the combined results of 54 previously-published cross-sectional observational studies and 7 double-blind randomized placebo-controlled clinical trials indicated that the risk for developing any form of cardiovascular disease was inversely correlated with the serum total testosterone concentration [93].

Low normal serum testosterone concentrations also may be detrimental to skeletal integrity [49,98,99]. In a cross-sectional study of Swedish men aged 69 to 80 years, bone mineral densities of the hip, femoral trochanter, and spine were positively correlated with

the serum free testosterone concentration [49]. However, a more important threat to men's health is the relationship between low normal serum testosterone concentrations and functional physical disability [100-104]. For example, in the cross-sectional Toledo Study for Healthy Aging, the risk for frailty (the presence of at least 3 of the following: weakness, slowness, low activity, exhaustion, sarcopenia) was inversely correlated with the serum total testosterone concentration [103]. In a 4.5-year prospective cohort study of 5994 ambulatory men aged 65 years or older conducted within the Osteoporotic Fractures in Men (MrOS) study at six U.S. clinical centers, performance on a test of functional physical ability (ability to sit and stand rapidly without assistance) was inversely correlated with the serum total testosterone concentration [104].

Cognitive functioning also may be impaired in men with low normal serum testosterone concentrations [50,105]. For example, the 50- to 91-year old male participants in the Baltimore Longitudinal Study of Aging exhibited significant correlations between their initial serum total testosterone concentrations and measures of visual memory, verbal memory, and visuospatial functioning measured 10 years later [50].

Immune system functioning and inflammation also may be adversely affected by low normal serum testosterone concentrations [106-110]. In the cross-sectional Boston Area Community Health (BACH) survey of men aged 30 to 79 years residing in the Boston area, the serum total testosterone concentration was inversely correlated with the plasma concentration of C-reactive protein (CRP) [107], a plasma protein that is secreted during inflammation, stimulates the activation of immune system defense responses, and produces a persistently elevated degree of chronic systemic inflammation [108]. Consistent with that report, in a case-control study of hypogondal and eugonadal young men, the serum total testosterone concentration was inversely correlated with the plasma concentrations of the pro-inflammatory cytokines, tumor necrosis factor-α (TNF-α) and macrophage inflammatory proteins 1α and 1β (MIP1α and MIP1β) [109].

Oxidative Stress, Leydig Cells, and Testosterone Secretion

Healthy cellular metabolism requires the generation of metabolic energy within mitochondria without the production of collateral oxidative damage caused by the oxidizing byproducts of metabolism, including reactive oxygen species (ROS) such as superoxide (SO; $\cdot O_2$), hydrogen peroxide (H_2O_2), hydroxy radicals ($\cdot OH^-$), nitric oxide ($\cdot NO$), peroxyl radicals ($\cdot ROO^-$), peroxynitrite ($\cdot ONOO^-$), singlet oxygen ($\cdot {}^1O_2$), and hypochlorous acid (HOCl) [111-115]. About 2% to 3% of these ROS escape endogenous antioxidant mechanisms to oxidize cellular and circulating lipids, proteins, and nucleic acids [113-119]. ROS and other oxidizing molecules generated by environmental insults (e.g., ultraviolet irradiation, cigarette smoke, and air pollutants [120-123]), drugs [123], and ethanol [124] add to local and systemic oxidative stress.

Dependence on the mitochondrial electron transfer system for the energy to drive testosterone synthesis exposes Leydig cell mitochondria to oxidative stress [3,4], and the generation of ROS within Leydig cells increases when testosterone synthesis is stimulated [6,125]. Leydig cells exposed to oxidative stress exhibit reduced

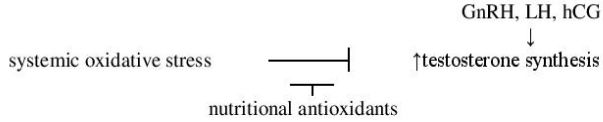

Figure 5: Hypothetical scheme illustrating oxidative inhibition of testosterone synthesis and its prevention by nutritional antioxidants.

activities of antioxidant enzymes (e.g., catalase, superoxide dismutase (SOD), and glutathione peroxidase (GPx)) [61,62,126,127], reduced intracellular glutathione content [61,62,128,129], increased secretion of the malondialdehyde (MDA) product of lipid peroxidation [61,62,127,128], increased oxidative modification of DNA [6], reductions in the mitochondrial membrane potential required for testosterone synthesis [130,131], and reduced expression of the StAR protein [130]. Oxidatively damaged Leydig cells are less sensitive to LH, with fewer LH receptors expressed per cell, and exhibit impaired activation of the StAR protein, reduced activities of several enzymes of the testosterone biosynthetic pathway (CYP11A1, 3β-HSD, CYP17A1 hydroxylase, CYP17A1 lyase, 17β-HSD), and inhibition of testosterone synthesis [61,62,127,129,132-134]. In contrast, a reduction in systemic oxidative stress induced by lifelong daily episodes of running reduces oxidative stress within Leydig cells and increases the rate of testosterone secretion in mice [135].

The aging-associated declines in testosterone production and circulating testosterone concentrations [56-62] are at least in part the consequences of cumulative oxidative stress within Leydig cells [136,137]. With advancing age, Leydig cells exhibit increasing degrees of intracellular lipid peroxidation [136,137] and decreasing expression of SOD, GPx, and glutathione [136-138].

This evidence provides strong support for the hypotheses that 1) systemic oxidative stress inhibits testosterone synthesis in Leydig

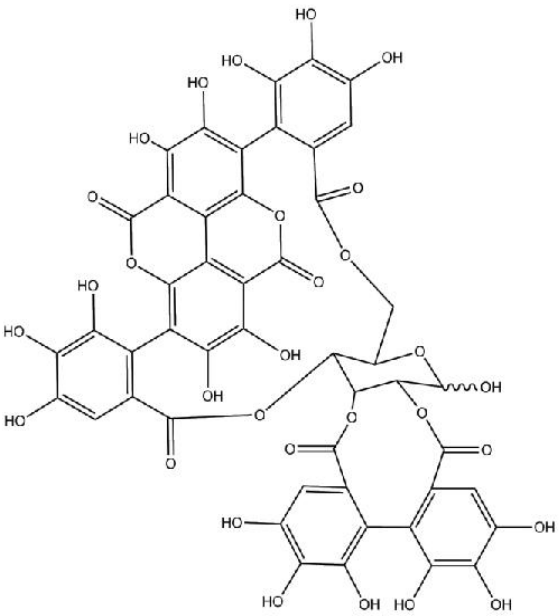

Figure 6: Punicalagin. By Markwdck (Own work) [CC0], via Wikimedia Commons. Downloaded from http://upload.wikimedia.org/wikipedia/commons/9/91/Punicalagin1.png on December 22, 2014.

cells and 2) reducing systemic oxidative stress releases testosterone synthesis from oxidative inhibition and improves testosterone status (Figure 5). Consistent with these hypotheses, several nutritional antioxidants (e.g., the phytonutrients in pomegranates, phosphatidylserine, vitamin C, vitamin E, α-lipoic acid, zinc, and selenium) have been observed to contribute to a reduction in systemic and local oxidative stress, stimulation or reversal of inhibition of testosterone synthesis, and enhancement of androgenic status.

Pomegranates, Punicalagins, Ellagitannins, Anthocyanidins, Anthocyanins, Proanthocyanidins, Procyanidins, and their Metabolites

Pomegranates and their extracts contain a large number of nutritional antioxidants, including punicalagins, hydrolyzable ellagitannins (ellagic esters of glucose), anthocyanins, ascorbic acid, and α-tocopherol [139-145]. Punicalagins and ellagitannins are large polyphenols (Figure 6) that are stable at stomach pH and pass into the small intestine unchanged [142,146], although a small fraction are hydrolyzed within the neutral pH environment of the human small intestine, forming combinations of ellagic and gallic acids [139,146-148]. Nonetheless, most large pomegranate polyphenols proceed to the large intestine, where microbial metabolism converts them into the smaller urolithins (Figure 7): urolithin A (3,8-dihydroxy-6H-dibenzo[*b,d*]pyran-6-one), urolithin B (3-hydroxy-6H-dibenzo[*b,d*] pyran-6-one), urolithin C (3,8,9-trihydroxy-6H-dibenzo[*b,d*]pyran-6-one) and urolithin D (3,8,9,10-tetrahydroxy-6H-dibenzo[*b,d*] pyran-6-one) [139,144,147,149-153]. Ellagic acid (Figure 8) and the urolithins are absorbed intact into the human bloodstream [144,147,151,153-155].

Pomegranate juice and methanolic and aqueous extracts of pomegranate fruits also contain mixtures of polyphenolic anthocyanidins (200 mg to 400 mg per L) [141,143,156]. Anthocyanidins are natural pigments responsible for the blue, purple, red and orange colors of many fruits and vegetables; the chromophore of 8 conjugated double bonds carrying a positive charge on the heterocyclic oxygen ring is responsible for the intense red-orange to blue-violet color produced by anthocyanidins under acidic conditions [157,158]. Of the more than 650 known anthocyanidins [157,158], pelargonidin, cyanidin, delphinidin, petunidin, peonidin, and malvidin (Figure 9) account for 90% of all dietary anthocyanidins [159], and 13 others (Table 1) account for most of the other 10% [160].

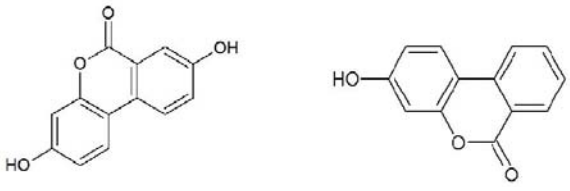

Figure 7: Left, urolithin A*; right, urolithin B#. *By NotWith (Own work) [CC BY-SA 3.0 (http://creativecommons.org/licenses/by-sa/3.0)], via Wikimedia Commons. Reprinted under the Creative Commons Attribution-Share Alike 3.0 Unported license from http://commons.wikimedia.org/wiki/File:Urolithin_A.png on December 22, 2014. #By NotWith (Own work) [CC BY-SA 3.0 (http://creativecommons.org/licenses/by-sa/3.0)], via Wikimedia Commons. Downloaded from http://upload.wikimedia.org/wikipedia/commons/f/fa/Urolithin_B.png on December 22, 2014.

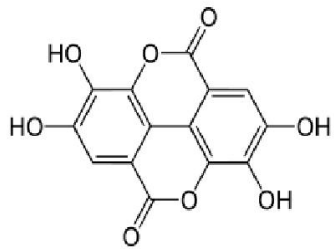

Figure 8: Ellagic acid. By Yikrazuul (Own work) [Public domain], via Wikimedia Commons. Downloaded from http://upload.wikimedia.org/wikipedia/commons/d/da/Ellagic_acid.svg on December 22, 2014.

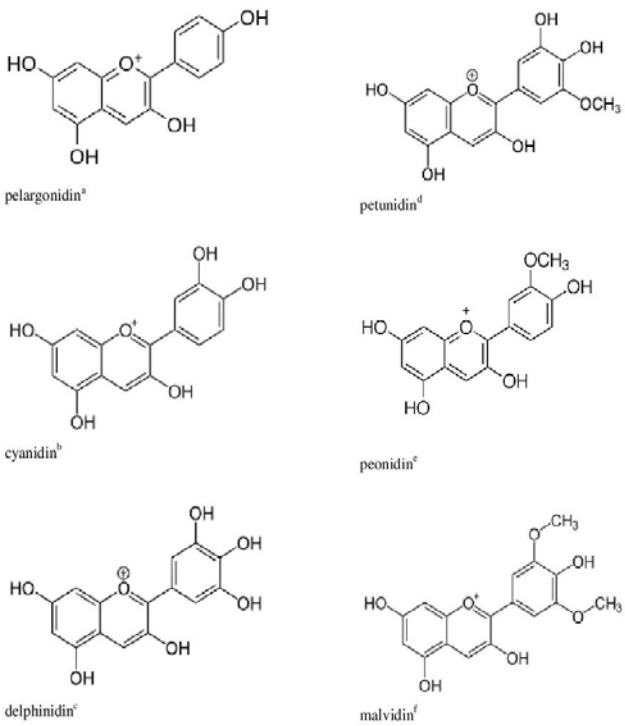

Figure 9: The 6 principal anthocyanidins. [a]By Pelargonidin.png: Edgar181 derivative work: Shakiestone (this file was derived from: Pelargonidin.png) [Public domain], via Wikimedia Commons). Downloaded from http://upload.wikimedia.org/wikipedia/commons/1/1a/Pelargonidin.svg on December 22, 2014. [b]By NEUROtiker (Own work) [Public domain], via Wikimedia Commons. Downloaded from http://upload.wikimedia.org/wikipedia/commons/8/8b/Cyanidin.svg on December 22, 2014. [c]By Yikrazuul (Own work) [Public domain], via Wikimedia Commons. Downloaded from http://upload.wikimedia.org/wikipedia/commons/5/5e/Delphinidin.svg on December 22, 2014. [d]By Yikrazuul (Own work) [Public domain], via Wikimedia Commons. Downloaded from http://upload.wikimedia.org/wikipedia/commons/1/1b/Petunidin.svg on December 22, 2014. [e]By Edgar181 (Own work) [Public domain], via Wikimedia Commons. Downloaded from http://upload.wikimedia.org/wikipedia/commons/e/eb/Peonidin.png on December 22, 2014. [f]By NEUROtiker (Own work) [Public domain], via Wikimedia Commons. Downloaded from http://upload.wikimedia.org/wikipedia/commons/d/d7/Malvidin.svg on December 22, 2014.

Anthocyanidins are unstable to light and are water-insoluble so that they usually do not occur in their free state but most often are linked to sugars, which provide stability and water solubility [158]. These glycosides are called anthocyanins [158]. The anthocyanins occurring in pomegranate juice in the largest amounts contain glucose that is covalently bound to the ring oxygen and are cyanidin-

Table 1: Structural variation among 13 minor anthocyanidins [160].

	Carbon Position						
Anthocyanidin	3	5	6	7	3'	4'	5'
Apigeninidin	H	OH	H	OH	H	OH	H
Aurantinidin	OH	OH	OH	OH	H	OH	H
Capensinidin	OH	OCH_3	H	OH	OCH_3	OH	OCH_3
Europinidin	OH	OCH_3	H	OH	OCH_3	OH	OH
Hirsutidin	OH	OH	H	OCH_3	OCH_3	OH	OCH_3
6-Hydroxycyanidin	OH	OH	OH	OH	OH	OH	H
6-Hydroxydelphinidin	OH	OH	OH	OH	OH	OH	OH
Luteolinidin	H	OH	H	OH	OH	OH	H
5-Methylcyanidin	OH	OCH_3	H	OH	OH	OH	H
Pulchellidin	OH	OCH_3	H	OH	OH	OH	OH
Riccionidin A	H	H	OH	OH	H	OH	H
Rosinidin	OH	OH	H	OCH_3	OCH_3	OH	H
Tricetinidin	H	OH	H	OH	OH	OH	OH

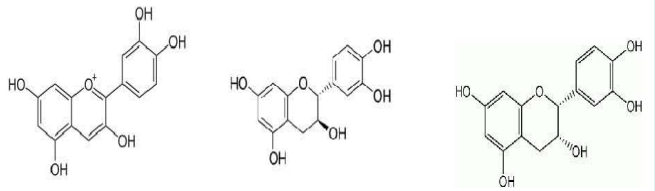

Figure 10: *Left*, cyanidin[a]; *Center*, catechin[b]; *Right*, epicatechin[c]. [a]By NEUROtiker (Own work) [Public domain], via Wikimedia Commons. Downloaded from http://upload.wikimedia.org/wikipedia/commons/8/8b/Cyanidin.svg on December 22, 2014. [b]By Edgar181 (Own work) [Public domain], via Wikimedia Commons. Downloaded from http://upload.wikimedia.org/wikipedia/commons/4/44/%28%2B%29-Catechin.png on December 22, 2014. [c]By selfmade by Shaddack, 23 October 2005 (Own work) [Public domain], via Wikimedia Commons. Downloaded from http://upload.wikimedia.org/wikipedia/commons/2/20/Epicatechin.png on December 22, 2014.

3-glucoside (chrysanthemin), cyanidin-3,5-diglucoside, delphinidin-3-glucoside (myrtillin), pelargonidin-3-glucoside (callistephin) and malvidin-3-glucoside (oenin) [145,159]. Anthocyanins are stable at stomach pH and, following their passage into the intestinal tract, most are absorbed into the bloodstream, although with low efficiency [159].

Pomegranates also contain small amounts of proanthocyanidins (polymers of flavan-3-ols such as cyanidin, catechin, and epicatechin) and procyanidins (polymers containing only epicatechin units) [161,162]. Cyanidin, catechin and epicatechin are extremely similar structurally (Figure 10) and procyanidins (Figure 11) are the most abundant type of proanthocyanidins in plants [161]. Humans absorb the epicatechin and catechin monomers from dietary proanthocyanidins and procyanidins with very low effiicency, producing plasma concentrations that are directly proportional to intake [163-166].

Antioxidant activity

Pomegranate polyphenols exhibit strong antioxidant potency [141,167-176]. The individual pomegranate polyphenols and their metabolites, including punicalagin, ellagic acid, gallic acid, and the urolithins, express antioxidant activities proportional to the number

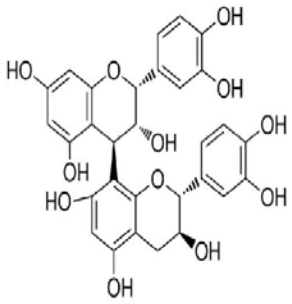

Figure 11: Procyanidin B2. By n/a [Public domain], via Wikimedia Commons. Downloaded from http://upload.wikimedia.org/wikipedia/commons/e/eb/Structure_of_Procyanidin_B2.png on December 22, 2014.

of hydrolyzable hydroxyl groups that are present [139-141,173,177-186]. Dietary supplementation with pomegranate polyphenols upregulates the expression and activities of paraoxonases 1 (PON1) [187] and 2 (PON2) [188], endogenous antioxidants that buffer the intracellular (PON2) and extracellular (PON1) environments from oxidizing conditions [187,188] and increase the systemic antioxidant capacity and reduce systemic oxidative stress in men [140,187,189-191].

Anthocyanidins and their glucosides contain multiple binding sites for unpaired free radical electrons [158,160,192] which confer potent antioxidant activity [159,192-198]. Following anthocyanin consumption, serum ORAC is directly proportional to the amount of anthocyanins consumed [159,199,200]. This increase in circulating antioxidant capacity is accompanied by increased circulating glutathione concentration [201]; decreased circulating concentrations of oxidized glutathione, oxidized proteins, and lipid peroxides [201]; and less oxidative damage to DNA in circulating white blood cells [201].

Antioxidant activity appears to be a general characteristic of proanthocyanidins [202]. In human studies, the consumption of 200 mg to 466 mg of total proanthocyanidins daily for several weeks has reduced the plasma concentration of oxidized LDL particles [203] and has increased the resistance of freshly-harvested LDL particles to *ex vivo* oxidation [166].

Activity against oxidative inhibition of testosterone synthesis

Pomegranate polyphenols downregulate the expression of hepatic CYP1A2 and CYP3A, slowing the rate of oxidative conversion of carbon tetrachloride (CCl_4) into the trichloromethyl radical that reacts with O_2 to produce the lipid peroxidizing trichloromethyl peroxyl radical and subsequent lipid peroxidation [204,205]. Although CCl_4 also reduces the testicular activities of the antioxidants, glutathione, GPx, glutathione reductase (GR), SOD, and catalase, while inhibiting LH-stimulated testosterone synthesis, these effects are prevented by the concurrent consumption of pomegranate polyphenols [206]. Consistent with these reports, the pomegranate polyphenol, ellagic acid, has prevented adriamycin-induced testicular lipid peroxidation and inhibition of testosterone synthesis [207]. These animal data indicate that pomegranate polyphenols reduce toxin-induced oxidative stress and deinhibit testosterone synthesis within the testes.

Antiaromatase activity

The addition of either urolithin B or 8-*O*-methylurolithin B (a product of hepatic processing of urolithin B [147,148]) to the culture medium of aromatase-overexpressing MCF-7aro estrogen receptor-positive breast cancer cells attenuated the stimulatory effect of androstenedione on aromatase complex activity [148]. 50% inhibition of androstenedione stimulation of aromatase complex activity was obtained with physiologically relevant concentrations of 8-*O*-methylurolithin B (2.5 μM) and urolithin B (5 μM). A classification system that compares the inhibitory potency of phytonutrients to the potency of aromatase complex-inhibiting drugs has been suggested [208]; according to these standards, urolithin B, at plasma concentrations that are achieved through the consumption of pomegranate polyphenols [147,151,153], is a "strong" inhibitor of the aromatase complex and contributes to the maintenance of serum testosterone concentrations [148,208].

Phosphatidylserine

Phosphatidylserine is the major acidic phospholipid in human membranes and constitutes 2% to 20% of the total lipid mass of human plasma and intracellular membranes [209-211]. Within the human testes, phosphatidylserine constitutes 1% to 2% of total tissue weight [211] and is a basic structural component of endoplasmic reticulum, nuclear envelopes, Golgi apparati, inner (cytosolic) leaflets of plasma membranes, and outer mitochondrial membranes [209-217].

Phosphatidylserine is synthesized within mitochondria-associated membrane (MAM) domains of the endoplasmic reticulum, almost entirely through the phosphatidylserine synthase 1-catalyzed substitution of serine for choline on docosahexaenoic acid (DHA)-enriched phosphatidylcholine [218-225]. In addition, a small amount of phosphatidylserine results from the replacement of ethanolamine with serine on phosphatidylethanolamine by phosphatidylserine synthase 2 [219-222,226], an enzyme whose expression is the greatest within testicular tissues [225,227].

The amount of oral phosphatidylserine that is taken up and incorporated into human cell membranes increases as phosphatidylserine intake increases [228-232]. Exogenous phosphatidylserine is transported from the plasma membrane's outer leaflet to its inner leaflet by a phosphatidylserine-specific ATP-dependent aminophospholipid translocase ("flippase") [219,222,229-236]. Maintenance of transmembrane phosphatidylserine asymmetry is critical to cell survival; in contrast, increased outer leaflet phosphatidylserine content is a required signal for the irreversible initiation of phagocytic engulfment of apoptotic cells in many cell types, including testicular cells [237-245]. In order to avoid inappropriately initiating apoptosis, healthy cells devote up to 8% of all ATP consumption to maintaining transmembrane phosphatidylserine asymmetry [230,246].

Testicular cells are enriched in phosphatidylserine [210] and require phosphatidylserine for the performance of normal testicular functions, including testosterone synthesis [247,248] and spermatogenesis [238]. In Leydig cells, testosterone synthesis is stimulated by phosphatidylserine through a sequence in which phosphatidylserine induces the translocation of cytosolic Akt (protein kinase B) to the plasma membrane and interacts directly with Akt to alter its conformation and allow it to be activated via phosphorylation by mTOR2 [249,250]. Phosphatidylserine-dependent activation of Akt is followed by Akt activation of protein kinase C [248-254], which participates in signaling pathways that culminate in testosterone synthesis through the primary "Δ^5" pathway (pregnenolone → 17α-hydroxypregnenolone → dehydroepiandrosterone → androstenedione → testosterone). Phosphatidylserine also stimulates the isomerase activity of HSD3B2 in the testes, increasing testosterone synthesis through the alternate "Δ^4" pathway (pregnenolone → progesterone → androstenedione → testosterone) [247,255,256]. By participating in the initiation of androgenic signaling cascades and through direct stimulation of the rate-limiting HSD3B2 enzyme, while not affecting the activities of any other "Δ^5" or "Δ^4" pathway enzymes (including 5α-reductase), phosphatidylserine can exert a substantial positive influence on testosterone status [3,4,247-257].

These androgenic responses to the presence of phosphatidylserine can be triggered in men. In a double-blind, randomized, placebo-controlled study, healthy men with initially "desirable" resting plasma free testosterone concentrations and participating in a prescribed exercise regimen added 600 mg of phosphatidylserine to their daily diets for 10 days [258]. Supplemental phosphatidylserine produced a 60% greater increase in resting plasma free testosterone concentration than was associated with the ingestion of placebo.

The antioxidant activity of phosphatidylserine contributes to its potentiating effect on Leydig cell testosterone production [131]. The antioxidant effects of phosphatidylserine are ubiquitous; human neurons cultured in the presence of phosphatidylserine (25 *μM*) exhibit significant reductions in electric shock-induced ROS production [260], rats fed phosphatidylserine exhibit upregulated antioxidant enzyme activities in the brain (SOD and catalase) and liver (SOD and GPx) [259], and the capacity of human high-density lipoprotein (HDL) particles to prevent the spontaneous oxidation of circulating low-density lipoprotein (LDL) particles [260,261] is proportional to the phosphatidylserine content of the HDL particles [261].

Vitamin C

Vitamin C is an essential nutrient that must be supplied through the diet because humans lack the enzyme, gulonolactone oxidase, and cannot synthesize vitamin C *de novo* [262]. Vitamin C is known to be an electron donor for a number of human enzymes that participate in hydroxylation reactions [263]. The best known of these reactions is the posttranslational hydroxylation of peptide-bound proline and lysine residues during formation and cross-linking of mature collagen [262,263]. In these reactions, vitamin C (as ascorbate) reactivates the enzymes by reducing the metal sites of prolyl (iron) and lysyl (copper) hydroxylases [262].

Ascorbate, the dominant form of vitamin C in humans [262], contains 2 enolic hydrogen atoms that provide electrons that are available for nonenzymatic transfer to biological oxidants [264] and provide the basis for the antioxidant properties of vitamin C [264]. Ascorbate readily scavenges (reduces) reactive oxygen and nitrogen species, including hydroxyl, peroxyl, nitroxyl, ferryl, thyl, α-tocopheryl, $\cdot O_2^-$, $\cdot ONOO^-$, and nitroxide radicals: $\cdot {}^1O_2$: hypochlorite:

and H_2O_2 generated from $\cdot O_2^-$ [264-270]. Oxidized ascorbate can be reduced back to ascorbate by transfer of its free radical electron to another receptor molecule (such as reduced glutathione, α-lipoic acid, or another molecule of oxidized ascorbate) or can be further oxidized to become dehydroascorbate (DHA) [263-271]. In turn, DHA can be recycled to ascorbate or can be converted into the excretory end product, 2,3-diketogulonate [264,267-271]

In humans, the total antioxidant capacity of the circulation is positively correlated with daily vitamin C intake [272,273] and is increased by dietary supplementation with vitamin C [274]. Supplemental vitamin C reduces systemic oxidative stress (reflected in deceased whole-body lipid peroxidation) [275] and large amounts of intracellular ascorbate provide protection against collateral oxidative damage secondary to generation of ROS during mitochondrial respiration [276]. The uptake of vitamin C by tissues, including the Leydig cells of the testes, is proportional to vitamin C intake [277-280]

Oral vitamin C increases LH secretion by isolated pituitary cells in the absence of hypothalamic LH releasing hormone [281] and stimulates testosterone synthesis [278,282] and increased serum total testosterone concentrations in otherwise unmanipulated healthy male rats. The concurrent consumption of vitamin C has prevented the inhibition of testicular antioxidant enzymes, the increase in the formation of lipid peroxidation products and oxidized protein carbonyls, and the suppression of testosterone synthesis commonly observed in laboratory animals exposed to cadmium [283], lead [284], cyclophosphamide [285-287], or arsenic [288,289]. In addition, these environmental toxins downregulate the activities of testicular HSD3B2 and HSD17B3 [283,285-291]; this effect is prevented by concurrent consumption of vitamin C [283,285,288,289], suggesting that vitamin C may directly upregulate testosterone synthesis in addition to protecting the synthetic pathway from oxidative inhibition.

Vitamin C Safety

The safety of vitamin C consumption is not in doubt. The consumption of 2500 mg daily for 7 days has been harmless [292] and a detailed systematic comparison of 29 published human clinical trials in which subjects had consumed supplemental vitamin C in amounts ranging from a single bolus of 4000 mg to daily consumption of 10000 mg for up to 210 days determined that the scientific evidence clearly substantiates that the daily intake of 2000 mg of supplemental vitamin C (in addition to that provided by the diet) indefinitely is safe and short-term intakes of up to 4000 mg daily are without adverse effects other than occasional bowel intolerance in some individuals [293]. In addition, the Cochrane Collaboration examination of the data obtained from 2490 subjects who consumed more than 1000 mg of supplemental vitamin C daily revealed no adverse effects that could be attributed to the vitamin [294]. Even intravenous injection of an amount of vitamin C sufficient to increase plasma vitamin C concentrations by approximately 2500% (to over 1200 μ*M*) was without adverse effect [295].

Two reports have generated concern that intakes of vitamin C greater than the RDA could promote urolithiasis in susceptible individuals [296,297]. In a short, 6-day study, the consumption of 2000 mg of supplemental vitamin C by individuals with histories of prior kidney stone formation produced a significant increase in the urinary excretion of oxalate, a putative biomarker of urolithogenic risk [296]. However, no signs of crystal formation were observed. In addition, the diagnostic reliability of increased urinary oxalate excretion as a direct biomarker of increased risk for kidney stone formation has been challenged in recent years [298-300].

Furthermore, when the risk of symptomatic kidney stones was studied for 14 years prospectively in a cohort of 85,557 women with no prior history of kidney stones (the Nurses' Health Study), vitamin C intake was not associated with increased risk for developing kidney stones [301]. Similarly, a 6-year prospective study of the relationship between the intake of vitamin C and the risk of symptomatic kidney stones performed in a cohort of 45,251 men aged 40 to 75 years old and with no prior history of kidney calculi (the Health Professionals Follow-Up Study) determined that vitamin C intake was not associated with increased risk for developing kidney stones [302].

A re-evaluation of the Health Professionals Follow-Up Study data after an additional 8 years of observation initially reported that compared to the effects of the consumption of less than 90 mg of vitamin C daily, the daily consumption of 1000 mg or more significantly increased the relative risk of forming a kidney stone by 41% [296]. Although seemingly impressive, this would increase an individual's likelihood of forming a kidney stone sometime during their lifetime from 4.8% to 6.7%. However, these investigators compared vitamin C intakes that can be achieved only through dietary supplementation to frankly deficient intakes; if correct, their conclusion implies that vitamin C deficiency is somehow protective against urolithiasis. Further analysis revealed that there was no increase in risk when the effects of the consumption of 1000 mg or more of supplemental vitamin C was compared to the effects of no supplementation with vitamin C [303]. In contrast, when the effects of the daily consumption of 218 mg or more of vitamin C from food were compared to the effects of the consumption of 105 mg or less of vitamin C from food, the relative risk for kidney stone formation increased significantly by 31%, suggesting that a confounding factor consumed with vitamin C-rich foods and beverages, and not vitamin C, is urolithogenic. Together, these updated findings confirm the safety of vitamin C consumption.

It should be noted that it has been reported that healthy human peripheral blood white blood cells exposed to supraphysiologic concentrations of vitamin C (in the order of 1000 μ*M*) exhibited impaired ability to synthesize DNA or phagocytose pathogens [304]. However, this concentration is 5- to 15-fold greater than the highest concentrations ever reported in human blood following oral vitamin C intake and has been equaled for only a few minutes following direct intravenous injection 1000 mg or more of ascorbate [292,295,305]. The findings in this report [304] cannot be considered representative of the safety of dietary supplementation with vitamin C.

Vitamin E

Vitamin E (α-tocopherol) is highly bioavailable in humans [306-313] and is the most powerful chain-breaking lipid-soluble dietary antioxidant [314]. In addition, the rate-limiting enzyme in the synthesis of glutathione, γ-glutamyl-cysteinyl synthetase, requires vitamin E as a cofactor [315]. The presence of vitamin E in the culture medium has attenuated iron-induced lipid peroxidation in cultured Leydig cells [316].

While vitamin E deficiency impairs the cAMP response to LH, thereby desensitizing the Leydig cell to LH and reducing the testosterone synthetic response to LH stimulation [317], Leydig cell responsiveness to LH is proportional to the amount of vitamin E to which the cells are exposed [316] and supplemental vitamin E administered to healthy male rats has increased the testosterone synthetic response to LH stimulation [318]. Consistent with these reports, dietary supplementation with vitamin E (483 mg daily for 8 weeks) has produced a 20% increase in testosterone synthesis in healthy men [319].

Chronic forced swimming exercise increases systemic oxidative stress in the Leydig cells of rats, as reflected in increased intracellular lipid peroxidation and downregulation of glutathione, SOD, GPx, and catalase [282,320,321]. In these cells, the increase in oxidative stress is accompanied by inhibition of testosterone synthesis [282,320,321]. The activities of testicular antioxidant enzymes also are inhibited, increasing intracellular oxidative stress, and testosterone synthesis is impaired, in rats administered cadmium [283,322,323] or chromium VI [324] and in mice administered sodium azide [325]. In addition, chromium VI inhibits the activities of HSD3B2 and HSD17B3, contributing to the impairment of testosterone synthesis [324]. In all of these model systems, concurrent dietary supplementation with vitamin E prevents both the increase in oxidative stress and the inhibition of testosterone synthesis [283,320,322-325].

Vitamin C and Vitamin E

The Leydig cells of rats fed the polychlorinated biphenyl, Arochlor-1254, exhibit decreased activities of antioxidant enzymes, increased generation of H_2O_2, lipid peroxidation products and other ROS, and inhibition of the StAR protein, $P450_{scc}$, HSD3B2, and testosterone synthesis [326-333]. The combined concurrent consumption of supplemental vitamin C and vitamin E attenuates or prevents these negative effects on testosterone status [330,331].

α-Lipoic Acid

α-Lipoic acid (5-(1,2-dithiolan-3-yl)-pentanoic acid; thioctic acid) is a disulfide compound that is synthesized within human mitochondria by lipoic acid synthase [334,335]. Exogenous (dietary) α-lipoic acid is absorbed into the human circulation and contributes to the human α-lipoic acid pool [336,337]. The most important dietary sources of α-lipoic acid are spinach and broccoli [338].

Mitochondrial enzymes of oxidative metabolism that require α-lipoic acid as a cofactor include pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, branched-chain α-ketoacid dehydrogenase, and the glycine cleavage system that converts glycine into pyruvate [339]. α-Lipoic acid must interact with these enzymes in order for aerobic metabolism to proceed [339]. In addition to its cofactor roles, α-lipoic acid penetrates both cell membranes and aqueous compartments throughout the body, allowing α-lipoic acid to act as a multi-purpose nonenzymatic antioxidant that protects mitochondria and surrounding cellular elements from oxidation by the free radicals produced by mitochondria during oxidative metabolism [335,340,341]. The sulfhydryl groups on the α-lipoic acid molecule provide strong antioxidant potency, directly exchanging free protons for free radical electrons in lipophillic environments (e.g., biological membranes) and exchanging free protons with hydroxyl ions and water during the deactivating reduction of free radical electrons in aqueous environments (e.g., biological fluids) [340]. During these reactions, α-lipoic acid becomes reduced to dihydrolipoic acid, an intermediate that retains some antioxidant potency before being either β-oxidized or *S*-methylated into excretory metabolites [337,338].

In addition to reducing oxidizing compounds, α-lipoic acid can recycle (reduce) other nonenzymatic antioxidants after they have become oxidized, prompting the descriptor, "antioxidant of antioxidants" [342-344]. α-Lipoic acid also stimulates increased activities of endogenous antioxidant enzymes, including SOD, catalase, GPx, and heme oxygenase-1 (HO-1) [342-353]. Conversely, elevated systemic oxidative stress downregulates the expression of lipoic acid synthase, increasing the need for dietary α-lipoic acid in order to forestall retardation of aerobic energy production [354].

An elevated level of systemic oxidative stress inhibits Leydig cell synthesis of testosterone [61,62,127,129,134]. Elevated oxidative stress caused by exposure to cadmium [291], cyclophosphamide [355], acrylamide [356], *bis-n*-butyl phthalate [357], or bisphenol-A (BPA) [358,359] inhibits the activities of testicular and Leydig cell glutathione, GPx, GR, SOD, catalase, HSD3B2, and HSF17B3, increases intracellular lipid peroxidation, and attenuates testosterone synthesis. Dietary supplementation of rats exposed to these agents with α-lipoic acid has prevented or reversed these detrimental effects on testosterone status [291,355,356,358,359]. Long-term intense forced swimming exercise also produces inhibition of antioxidant enzymes, elevated oxidative stress, inhibition of HSD3B3 and HSD17B3, and impaired testosterone synthesis within the testes of rats; concurrent consumption of α-lipoic acid attenuates or prevents these damaging consequences of exercise-induced oxidative stress [360].

Dietary α-lipoic acid is safe. In a multicenter, randomized, double-blind, placebo-controlled clinical trial, men and women with symptomatic diabetic sensorimotor polyneuropathy consumed either placebo or α-lipoic acid (600 mg daily, 1200 mg daily, or 1800 mg daily) for 5 weeks [361]. Among these subjects, the incidence of gastrointestinal upset was increased slightly by 1200 mg and 1800 mg of α-lipoic acid daily, although no other adverse reactions were observed. In contrast, supplementation with 1800 mg of α-lipoic acid daily for 7 months did not precipitate adverse reactions in men and women with advanced multi-site polyneuropathies accompanying diabetes [362]. In addition to the absence of adverse reactions during these trials [361,362], the safety of exogenous α-lipoic acid was demonstrated by the absence of adverse reactions during a study in which men and women with metabolically stable diabetes complicated by symptomatic diabetic sensorimotor polyneuropathy received intravenous injections of 600 mg of α-lipoic acid (5 injections per week for 3 weeks) [363].

Zinc

Chronic zinc deprivation generally results in a low intracellular zinc concentration [364,365], increased intracellular oxidative stress [366-369], and increased sensitivity to oxidative stress [365,366]. An abundance of zinc may protect protein sulfhydryls and DNA from oxidation, or reduce the rate of hydroxyl radical formation from H_2O_2, through the antagonism of redox-active transition metals, such

as iron and copper [226,368-372]. Protection of protein sulfhydryl groups from oxidative modification may involve reduction of sulfhydryl reactivity through direct binding of zinc to the sulfhydryl groups of cysteine residues, steric hindrance as a result of zinc binding to some other protein site in close proximity to the sulfhydryl group, or a conformational change in the protein resulting from zinc binding to some other site on the protein [226,368].

An increase in the intracellular concentration of free zinc ions following their oxidative stress-induced release from intracellular metallothionein releases the nuclear transcription factor, nuclear factor-erythroid 2-related factor 2 (Nrf2), from repression by Kelch-like ECH-associated protein 1 (Keap1) [373]; in turn, derepressed Nrf2 stimulates the expression of the *Gclc* gene that codes for glutamate cysteine ligase, the rate-limiting enzyme in the synthetic pathway for glutathione, resulting in increased *de novo* synthesis of glutathione [374-377].

Increased intracellular oxidative stress is associated with inhibition of testosterone synthesis by Leydig cells [61,62,127,129,132-134,366,378]. Conversely, zinc supplementation can prevent oxidative stress-induced inhibition of testicular antioxidant enzyme activities (with increased formation of lipid peroxidation products and oxidative modification of DNA) and of testosterone synthesis [378,379].

Selenium

Selenium expresses its biological activity after its incorporation into the twenty-first amino acid, selenocysteine, and the genetically-coded insertion of selenocysteine into the amino acid sequence of selenoproteins during translation of mRNA [380-386]. The abundance of individual selenocysteine-containing selenoproteins is directly responsive to dietary selenium availability [387-393].

At physiological pH, selenocysteine within selenoproteins is fully ionized and acts as a very efficient redox catalyst [380,385,394,395]. Several families of selenoproteins that act as redox enzymes and exploit the chemical properties of selenium have been identified, including the six selenium-dependent antioxidant GPx enzymes that catalyze the removal of hydroperoxides and peroxynitrites by glutathione [380,383-385,396,397]. The selenium-dependent thioredoxin reductases also catalyze the regeneration of ascorbic acid from oxidized dehydroascorbic acid (thereby contributing to intracellular antioxidation) [380,398]. Dietary supplementation with selenium increases intracellular thioredoxin reductase activity and increases the efficiency of regeneration of ascorbic acid [399].

By fostering increased systemic oxidative stress, dietary selenium deficiency impairs the ability of Leydig cells to synthesize testosterone in response to LH [400]. Conversely, supplemental selenium has been reported to attenuate or prevent the oxidative stress-induced inhibition of testosterone synthesis associated with exposure to cadmium [290,291], sodium azide [325], or di(2-ethylhexyl) phthalate [401,402].

Improving Testosterone Status does not Increase the Risk for Prostate Disease

Because the normal growth of prostate tissue, as well as the progression of certain intermediate and high-risk prostate cancers, are dependant on the presence of testosterone, it has been feared that improvement in testosterone status might increase the risk for the development of benign prostatic hyperplasia or prostate cancer [77]. Fortunately, the available scientific evidence confirms that testosterone has no impact on prostate health in adult men, at any age, in the absence of pre-existing prostate cancer [56,71,76,77,403,404]. Meta-analyses of the results of previously published studies have demonstrated that the risk for developing prostate cancer is independent of the serum concentrations of total or bioavailable testosterone [56,405] and that testosterone replacement therapy has no detrimental effect on prostate health [73]. In joint statements released in 2010 [76] and 2013 [77], the International Endocrine Aspects of Male Sexual Dysfunctions Committee and the Endocrine Subcommittee of the Standards Committee of the International Society for Sexual Medicine concluded that increasing circulating testosterone concentrations with exogenous testosterone has no effect on the risk for the development of prostate cancer, the progression of prostate cancer, the appearance of prostate-related symptoms, the development of benign prostate hyperplasia, or the serum prostate-specific antigen concentration.

Conclusion

Healthy serum testosterone concentrations in aging men promote health and longevity. This androgenic hormone is so important to maintenance of health that even "low normal testosterone" is associated with reductions in energy, motivation, initiative, self-confidence, concentration, memory, sleep quality, muscle bulk, and strength; diminished physical or work performance; feeling sad or depressed; mild anemia; and increased body fat and body mass index.

A growing body of evidence demonstrates that aging often is accompanied by excessive oxidative stress and enhanced oxidative damage within Leydig cells. In addition, oxidizing environmental insults add to local and systemic oxidative stress and contribute to alterations the redox environment of the aging Leydig cells. Oxidatively damaged Leydig cells exhibit decreased responsiveness to LH, with inhibition of testosterone synthesis. These observations support the hypothesis that ROS play an important role in age-related reductions in Leydig cell testosterone production.

Conversely, antioxidant defenses can be augmented by dietary supplementation with specific antioxidant and mitochondrial protective nutrients that reduce cell-wide oxidative damage, support redox balance within Leydig cells, release Leydig cells from oxidative inhibition of testosterone synthesis, and increase the rate of testosterone secretion. The available scientific evidence provides strong support for the hypothesis that reducing oxidative stress can safely improve testosterone status.

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