

## Review

# Antioxidant systems and oxidative stress in the testes

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## Introduction

Spermatogenesis is an extremely active replicative process capable of generating approximately 1,000 sperm a second. The high rates of cell division inherent in this process imply correspondingly high rates of mitochondrial oxygen consumption by the germinal epithelium. However, the poor vascularization of the testes means that oxygen tensions in this tissue are low<sup>1</sup> and that competition for this vital element within the testes is extremely intense. Since both spermatogenesis<sup>2</sup> and Leydig cell steroidogenesis<sup>3,4</sup> are vulnerable to oxidative stress, the low oxygen tension that characterizes this tissue may be an important component of the mechanisms by which the testes protects itself from free radical-mediated damage. In addition, the testes contain an elaborate array of antioxidant enzymes and free radical scavengers to ensure that the twin spermatogenic and steroidogenic functions of this organ are not impacted by oxidative stress. These antioxidant defence systems are of major importance because peroxidative damage is currently regarded as the single most important cause of impaired testicular function underpinning the pathological consequences of a wide range of conditions from testicular torsion to diabetes and xenobiotic exposure. This chapter sets out the specific nature of these antioxidant defence systems and also reviews the factors that have been found to impair their activity, precipitating a state of oxidative stress in the testes and impairing the latter's ability to produce viable spermatozoa capable of initiating and supporting embryonic development.

## Antioxidant Enzymes

Despite the low oxygen tensions that characterize the testicular micro-environment, this tissue remains vulnerable to oxidative stress due to the abundance of highly unsaturated fatty acids (particularly 20:4 and 22:6) and the presence of potential reactive oxygen species (ROS)-generating systems. ROS generation can be from the mitochondria and a variety of enzymes including the xanthine- and NADPH- oxidases,<sup>5,6</sup> and the cytochrome P450s.<sup>7</sup> These enzymes specialize in the professional generation of ROS or produce these

toxic metabolites as an inadvertent consequence of their biochemical activity. In order to address this risk, the testes have developed a sophisticated array of antioxidant systems comprising both enzymatic and non-enzymatic constituents. Concerning the enzymatic constituents of this defence system, the induction of oxidative stress in the testes precipitates a response characterized by the NFκB mediated induction of mRNA species for superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione-S-transferase (GST) activities.<sup>8</sup> The fundamental biochemistry of these antioxidant enzymes is summarized in Figure 1 and involves the rapid conversion of superoxide anion ( $O_2^{\cdot-}$ ) to hydrogen peroxide ( $H_2O_2$ ) in the presence of SOD in order to prevent the former from participating in the formation of highly pernicious hydroxyl radicals. The  $H_2O_2$  generated in this manner is a powerful membrane permeant oxidant in its own right that has to be rapidly eliminated from the cell in order to prevent the induction of oxidative damage to lipids, proteins and DNA. The elimination of  $H_2O_2$  is either effected by catalase or glutathione peroxidase, with the latter predominating in the case of the testes.<sup>9,10</sup> GST on the other hand involves a large and complex family of proteins that catalyse the conjugation of reduced glutathione via the sulfhydryl group to electrophilic centres on a wide variety of substrates in preparation for excretion from the cell. This activity is critical in the detoxification of peroxidised lipids as well as the metabolism of xenobiotics.

Given the importance of SOD in this defence strategy, it is not surprising that the testes contain not only the conventional cytosolic (Cu/Zn) and mitochondrial (Fe/Mn) forms of SOD but also feature an unusual form of extracellular SOD, (SOD-Ex) which is produced by both Sertoli and germ cells, particularly the former. There is also some evidence that the germ cells may stimulate the secretion of SOD-Ex by Sertoli cells through the actions of cytokines such as interleukin-1α.<sup>11</sup> The importance of the cytosolic form of SOD (SOD1) was recently emphasised in studies of SOD1-knockout mice subjected to testicular heat stress. This treatment induced significantly enhanced levels of DNA strand breakage and cytochrome C leakage from the mitochondria of germ cells in these animals compared with the wild-type controls.<sup>12</sup> Similarly, the importance of the mitochondrial form of SOD (SOD2) in controlling  $O_2^{\cdot-}$  leakage from testicular mitochondria has been emphasised by the finding that the mRNA for this enzyme is markedly higher in the testes than the liver, unlike GPx and catalase.<sup>13</sup> Moreover, SOD-2 mRNA levels are developmentally and translationally regulated with maximal levels of expression in early post-meiotic germ cells.<sup>13</sup>

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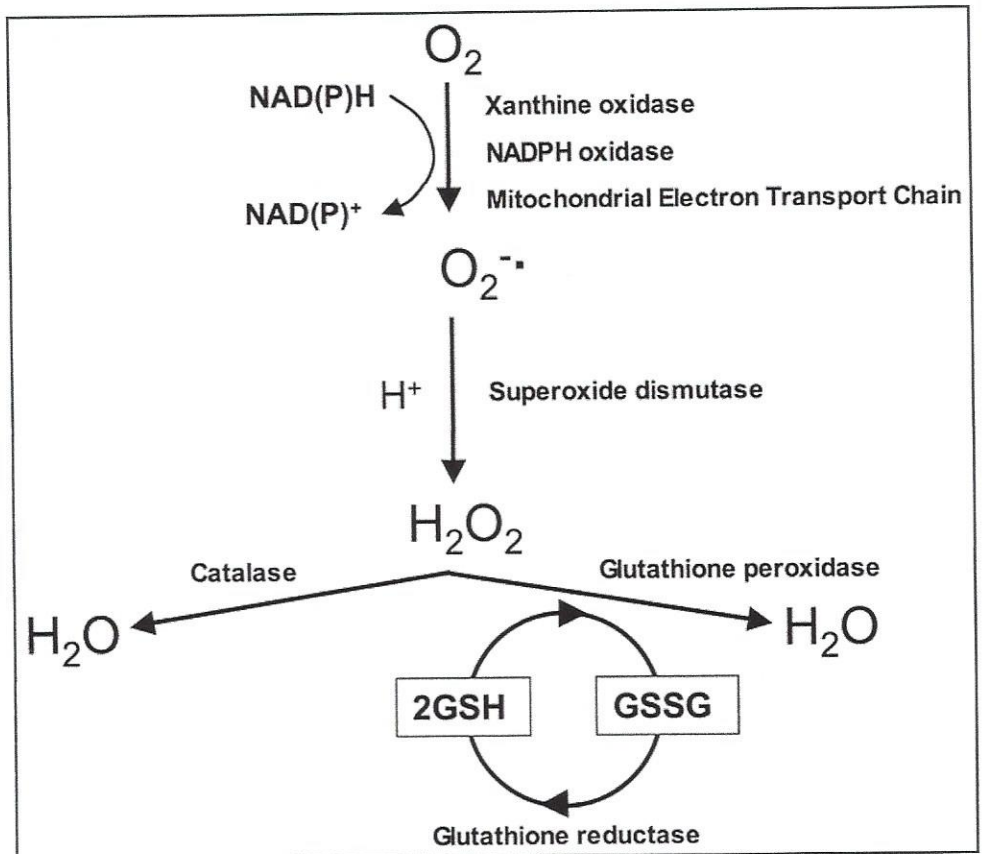
Figure 1. Major pathways of reactive oxygen species generation and metabolism. Superoxide can be generated by specialized enzymes, such as the xanthine or NADPH oxidases, or as a by-product of cellular metabolism, particularly the mitochondrial electron transport chain. Superoxide dismutase then converts the superoxide to hydrogen peroxide which has to be rapidly removed from the system. This is generally achieved by catalase or peroxidases, such as the glutathione peroxidases which use reduced glutathione (GSH) as the electron donor.

Although catalase is of limited importance in the testes, there are several isoforms of GPx in this tissue that use glutathione (GSH) as a source of electrons to reduce  $H_2O_2$  to water. They are concentrated in the mitochondria, nucleus and acrosomal domain of differentiating spermatozoa.<sup>14</sup> The phospholipid hydroperoxide GPx (PHGPx) is one of the most important GPx isoforms in a testicular context and is highly expressed in both spermatogenic and Leydig cells.<sup>15</sup> Since most forms of GPx are selenium dependent it is possible to gauge the importance of these enzymes in the support of testicular function by examining the impact of selenium deficiency on male reproduction. Animals fed on a selenium deficient diet exhibit a significant reduction of testicular GPx activity and an accompanying loss of germ cells from the germinal epithelium of the testes.<sup>16</sup> Moreover, selenium administration prior to the creation of oxidative stress in the testes using the torsion/detorsion model (see later) to create ischemia-reperfusion injury, has been found to suppress lipid peroxidation and improve the histopathological profile.<sup>17</sup>

### Small Molecular Mass Antioxidants

In addition to the major ROS processing enzymes, the testes rely heavily on small molecular weight antioxidant factors for protection against oxidative damage. These factors include ions and a wide variety of free radical scavengers, the nature of which are reviewed below.

**Zinc.** Zinc is an acknowledged antioxidant factor that as well as being a core constituent of free radical scavenging enzymes such as SOD and a recognized protector of sulfhydryl groups, is also thought to impair lipid peroxidation by displacing transition metals such as iron and copper from catalytic sites.<sup>18</sup> In keeping with such a central antioxidant role, this element has a profound effect on the level of oxidative stress experienced by the testes. Thus rats fed a zinc deficient diet experience a decrease in testicular antioxidant potential and a concomitant increase of lipid peroxidation in this tissue.<sup>19</sup> Conversely, zinc administration will counteract the oxidative stress created in the testes by exposure to lead,<sup>20-21</sup> as well as the peroxidative damage induced by ischemia-reperfusion as a consequence testicular torsion-detorsion.<sup>22</sup> Zinc administration has also been shown to attenuate the testicular oxidative DNA damage induced by



cadmium as well as the decline in sperm production and testosterone secretion induced by this heavy metal.<sup>23</sup>

**Vitamins C and E.** It has been recognized since the 1940s that vitamin E ( $\alpha$ -tocopherol) is a powerful lipophilic antioxidant that is absolutely vital for the maintenance of mammalian spermatogenesis.<sup>24</sup> It is present in particularly high amounts in Sertoli cells and pachytene spermatocytes and to a lesser extent round spermatids.<sup>25</sup> Vitamin C (ascorbic acid) also contributes to the support of spermatogenesis at least in part through its capacity to reduce  $\alpha$ -tocopherol and maintain this antioxidant in an active state. Vitamin C is itself maintained in a reduced state by a GSH-dependent dehydroascorbate reductase, which is abundant in the testes.<sup>26</sup> Deficiencies of vitamins C or E leads to a state of oxidative stress in the testes that disrupts both spermatogenesis and the production of testosterone.<sup>24</sup> Conversely, ascorbate administration to normal animals stimulates both sperm production and testosterone secretion.<sup>27</sup> This vitamin also counteracts the testicular oxidative stress induced by exposure to pro-oxidants such as arsenic, PCBs (Arochlor 1254), cadmium, endosulfan and alcohol.<sup>28-32</sup> Furthermore, endogenous ascorbate levels decrease dramatically when oxidative stress is induced in the testes by, for example, chronic exposure to lead, chromium, cadmium or aflatoxin.<sup>33-25</sup> Vitamin E has also been shown to suppress lipid peroxidation in testicular microsomes and mitochondria<sup>36,37</sup> and to reverse the detrimental effects of oxidative stress on testicular function mediated by exposure to such factors as ozone, iron overload, intensive exercise or exposure to aflatoxin, PCB, cyclophosphamide and formaldehyde.<sup>28,32,33,38-42</sup> Furthermore testicular vitamin E levels have also been shown to fall significantly when oxidative stress is induced by exposure to pro-oxidant stimuli such as chromium.<sup>34</sup>



**Melatonin and cytochrome C.** The pineal hormone melatonin (N-acetyl, 5-methoxytryptamine) also plays a major role in protecting the testes from oxidative stress, given the significant stimulatory effect of pinealectomy on the oxidative damage recorded in the testes as a consequence of induced hyperthyroidism.<sup>43</sup> Melatonin has two major attributes that set it apart from most other antioxidants. Firstly, it undergoes a two electron oxidation when acting as antioxidant, rather than the one electron oxidation favoured by many free radical scavengers. As a result, this compound cannot redox cycle and inadvertently generate free radicals. Secondly, melatonin is readily soluble in both lipid and aqueous environments and can readily cross the blood-testes barrier to protect the germinal epithelium. Melatonin levels in seminal plasma are depressed in infertile patients exhibiting poor motility, leukocytospermia, varicocele and non-obstructive azoospermia, all of which are conditions associated with oxidative stress in the male tract.<sup>44</sup> Moreover, the intraperitoneal injection of melatonin has been shown to alleviate oxidative stress in the testes following the experimental induction of a left sided varicocele.<sup>45</sup>

Another small molecular mass free radical scavenger that has recently been shown to play a major role in reducing  $H_2O_2$  is a testes-specific form of cytochrome C. This cytochrome C isoform is also a powerful activator of apoptosis, providing additional protection to the testes by virtue of its ability to facilitate the depletion of damaged germ cells.<sup>46</sup>

### Disruption of the Antioxidant Status of the Testes

Notwithstanding the antioxidant protection afforded to the testes in order to support its dual functions of steroidogenesis and sperm production, a wide variety of endogenous and exogenous factors are known to perturb these defences and generate a state of oxidative stress. In the following section, some of these factors are reviewed.

**Cryptorchidism.** The elevated temperatures associated with experimental cryptorchidism are associated with oxidative stress in the testes and a reduction in SOD and catalase activities.<sup>47</sup> Consistent with these findings, direct exposure of spermatogenic cells to elevated temperatures was found to induce high rates of apoptosis via mechanisms that were associated with elevated levels of  $H_2O_2$  generation and could be ameliorated by the addition of catalase.<sup>48</sup> Moreover the consequence of heat stress on spermatogenic cells was exacerbated in SOD1-knock out mice via mechanisms that could be reversed by the addition of Tiron, a superoxide anion radical scavenger.<sup>49</sup> The clinical significance of this finding can be seen in the high levels of DNA damage and ROS generation seen in the spermatozoa of patients with a history of cryptorchidism.<sup>50</sup>

**Testicular torsion.** Testicular torsion is a relatively common, painful condition that must be treated rapidly if the testes are not to suffer permanent damage. Prolonged torsion leads to testicular ischaemia and high levels of oxidative stress in the ipsilateral testes associated with NO and  $H_2O_2$  production, increased lipid peroxide formation, isoprostane accumulation, antioxidant enzyme depletion and increased rates of mitochondria-mediated apoptosis in the germ line.<sup>51-53</sup> Even short periods of ischaemia, for 3 hours or less, can lead to a high levels of oxidative stress in the testes, depletion of testicular glutathione levels and the consequent disruption of spermatogenesis. Significantly, the level of peroxidative damage observed in testicular tissue increases following detorsion, indicating the induction of reperfusion injury.<sup>54</sup> The biochemical basis for reperfusion injury

is thought to involve a key metabolic enzyme, xanthine dehydrogenase, which becomes converted to a xanthine oxidase during ischaemia, due to oxidation of essential -SH groups and/or a limited proteolytic clip. As soon as the tissue is reperfused with blood, the xanthine oxidase is suddenly presented with oxidizable substrate in the form of xanthine/hypoxanthine and starts to generate copious amounts of ROS. The latter then induce high levels of peroxidative damage via mechanisms that are enhanced by the local release of transition metals. Although this scheme of events was developed to explain the tissue injury associated with conditions such as myocardial infarction, it also applies to the testicular injury associated with torsion-detorsion. The general notion that the testicular damage precipitated by temporary ischaemia is associated with oxidative stress is supported by the sudden induction of lipid peroxidation and the concomitant suppression of endogenous antioxidant activities including SOD, catalase and glutathione peroxidase.<sup>17,55-57</sup> In addition, the tissue injury induced by testicular torsion/detorsion can be dramatically alleviated by pretreatment with exogenous antioxidants such as selenium, resveratrol, L-carnitine, caffeic acid phenethyl ester and garlic extract.<sup>17,57-60</sup> Finally the enzyme purportedly associated with reperfusion injury, xanthine oxidase, can be inhibited by allopurinol and the latter is known to reduce the testicular damage associated with testicular torsion.<sup>56</sup> Notwithstanding the importance of xanthine oxidase-mediated oxidative stress it should also be noted that neutrophil infiltration into the testes following torsion may represent yet another source of uncontrolled free radical generation responsible for mediating the pathophysiological consequences of temporary testicular ischaemia.<sup>61</sup>

One of the major issues associated with the clinical management of unilateral testicular torsion is whether the ipsilateral testis should be removed in order to preserve the contralateral testis. In animal models prolonged testicular torsion results in excessive ROS generation, depletion of antioxidant enzymes and the appearance of oxidative damage in the contralateral testes.<sup>62-64</sup> In light of these data, surgical removal of the ipsilateral testes would seem warranted if the period of ischaemia has been extensive.

**Varicocele.** The impaired venous drainage to the testes seen with varicocele is also associated with the disruption of spermatogenesis via mechanisms involving the induction of oxidative stress. In clinical studies, the presence of a varicocele has been shown to correlate with excess ROS generation by the spermatozoa, high rates of DNA damage in these cells and depleted antioxidant levels in the seminal plasma.<sup>65-67</sup> In a recent study, surgical correction of left sided varicocele was shown to significantly improve sperm concentration, total count, morphology and motility in concert with significant improvements in the antioxidant status of the spermatozoa and seminal plasma.<sup>68</sup> This reduction in oxidative stress secondary to varicocele excision was accompanied by a reduction in both protein carbonyl expression and DNA damage in the spermatozoa.<sup>68</sup> Independent studies have also shown that the testicular expression of 4-hydroxy-2-nonenal modified proteins (another marker of oxidative stress) is significantly higher in patients that responded positively to varicolectomy, suggesting that surgical treatments are capable of reducing oxidative stress in the testes.<sup>69</sup> Immunocytochemical analyses of 8-hydroxy-2'-deoxyguanosine expression in the testes of varicocele patients also revealed particularly high levels of oxidative DNA damage in the spermatogonia and spermatocytes that



correlated well with the severity of the varicosity.<sup>70</sup> The general concept that testicular pathologies associated with varicocele are linked with the induction of oxidative stress has been confirmed in animal models. Thus, creation of experimental bilateral varicocele in rats is associated with increases in lipid peroxidation and NO generation and a corresponding decrease in testicular antioxidant status.<sup>71</sup> Moreover, the pathological consequences of experimental varicocele induction can be significantly reversed by the concomitant administration of an antioxidant, melatonin.<sup>45</sup>

The site of free radical generation in varicocele patients is still open to conjecture. On the one hand, enhanced free radical generation by the spermatozoa and/or precursor germ cells has been repeatedly suggested,<sup>65,72,73</sup> on the other, there is evidence to suggest that excess free radical generation may involve the spermatic vein itself.<sup>74</sup> The excess generation of free radicals by the spermatozoa may be an indirect consequence of impaired spermatogenesis/epididymal function resulting in the retention of excess residual cytoplasm. The presence of excess cytoplasm has been positively correlated with the generation of ROS by human spermatozoa, via mechanisms involving the facilitated supply NADPH to oxidases in the sperm plasma membrane.<sup>75</sup> These enzymes, including NOX5 and DUOX, both of which have been identified in human spermatozoa,<sup>5,76</sup> are normally deprived of sufficient NADPH to drive free radical generation; what hexose monophosphate shunt activity there is, being largely devoted to the maintenance of glutathione reductase activity.<sup>77</sup> However, when excess residual cytoplasm is present the limited substrate availability is no longer an issue and free radical generation can be initiated. The relevance of this model to the oxidative stress detected in cases of varicocele is clearly suggested by the effects of varicocelectomy. Thus, not only do the spermatozoa produced by such patients exhibit high levels of ROS in association with cytoplasmic retention but also surgical correction of this condition both prevents cytoplasmic retention and suppresses ROS generation.<sup>73,78,79</sup> A causative association between these events therefore seems likely.

**Hyperthyroidism.** The induction of hyperthyroidism in rats is associated with oxidative stress in the testes as reflected by increased lipid peroxidation, elevated GSH levels and induction of antioxidant enzymes. The oxidative stress appears to be associated with a thyroxine dependent increase in mitochondrial activity and concomitant leakage of electrons from the mitochondrial electron transport chain.<sup>80,81</sup> The oxidative stress precipitated by hyperthyroidism can be exacerbated by pinealectomy removing melatonin, an important testicular antioxidant, from the redox equation.<sup>43</sup> These data resonate with clinical studies indicating that hyperthyroidism is associated with poor semen quality, particularly impaired motility, that normalize when the patients' thyroid dysfunction is corrected and euthyroidism established.<sup>82</sup> It should also be noted that hypothyroidism can induce oxidative stress in the testes as reflected by enhanced levels of H<sub>2</sub>O<sub>2</sub> production and increased carbonyl generation.<sup>83</sup> Clearly, normal testicular function is highly dependent on a functional thyroid system.

**Diabetes.** Experimental induction of diabetes in animal models has been shown to impair testicular function and decrease male fertility. Thus, diabetogens such as streptozotocin, enhance ROS generation and induce both lipid peroxidation and protein carbonyl expression in the testes. Moreover the oxidative stress associated with the diabetic condition is associated with DNA damage in the

male germ line and high rates of embryonic loss in mated females (dominant lethal effect). These effects could be attenuated by the administration of antioxidants such as ascorbic acid, melatonin, taurine or an herbal mixture containing extracts from *Musa paradisiaca*, *Tamarindus indica*, *Eugenia jambolana* and *Coccinia indica*.<sup>84-86</sup> In light of recent data showing an increased level of DNA damage in the spermatozoa of diabetic patients compared with non-diabetic controls,<sup>87</sup> causative links between diabetes, oxidative stress in the male germ line and DNA damage appears both likely and clinically, extremely important.

**Infection.** Another factor that may cause oxidative stress in the testes is infection. Experimental models of infection, involving the intraperitoneal injection of bacterial lipopolysaccharide (LPS), induced lipid peroxidation in the testes and rapidly depleted this tissue of antioxidant enzyme activity in the form of SOD, catalase and the glutathione peroxidase-reductase couple. This oxidative stress was associated with the transient generation of pro-inflammatory mediators such as interleukin 1 $\beta$ , inducible nitric oxide synthase and cyclo-oxygenase-2.<sup>88</sup> The same experimental infection model has also been used to demonstrate the particular sensitivity of Leydig cell steroidogenesis to oxidative stress induced by bacterial LPS. In these studies, the oxidative stress induced by LPS stimulated lipid peroxidation in Leydig cell membranes as well as significant reductions in steroidogenic acute regulatory protein (StAR) and 3 $\beta$ -hydroxysteroid dehydrogenase isomerase (3 $\beta$ -HSD) activity. Moreover, these effects were associated with the disruption of Leydig cell mitochondrial function and, specifically, the inhibition of StAR-mediated cholesterol transfer activity.<sup>89</sup>

**Physical exertion.** Physical exercise has been shown to up-regulate antioxidant activities in the testes of aging rats and may represent a practical way in which the detrimental effects of age on testicular function can be ameliorated.<sup>90</sup> A similar case could be argued for the ability of moderate exercise to ameliorate the degree of oxidative damage inflicted on the testes by chronic ethanol ingestion.<sup>91</sup> However, excess exercise can have the opposite effect, causing oxidative stress in the testes and generating high levels of lipid peroxidation in association with significant declines in the activities of key antioxidant enzymes including SOD, catalase, GST and GPx.<sup>92</sup> Such stress has a significant inhibitory effect on the both steroidogenesis and germ cell differentiation within the testes. The fact that these effects can be reversed by the administration of an antioxidant,  $\alpha$ -tocopherol succinate, confirms the importance of oxidative stress in the aetiology of such exercise-dependent testicular dysfunction.<sup>40</sup>

**Reproductive hormone imbalance.** The immediate endocrine environment of the testes has a major impact on the antioxidant status of this organ. Treatments including exposure to cyclophosphamide or dimethane sulfonate that diminish the intratesticular concentration of testosterone, inhibit the testicular expression of antioxidant enzymes such as GPx, SOD and catalase.<sup>93,94</sup> Furthermore, these suppressive effects on antioxidant expression, as well as the disruption of spermatogenesis, can be reversed by the administration of exogenous gonadotrophin to artificially elevate intratesticular testosterone levels.<sup>94,95</sup> Suppression of intratesticular testosterone with exogenous steroids, including both androgens and estrogens, similarly results in the suppression of antioxidant enzyme expression, a concomitant increase in peroxidative damage, the disruption of spermatogenesis and an increase in germ cell apoptosis.<sup>95-97</sup>



Intriguingly, the suppression of antioxidant activity in response to exogenous steroid treatment largely affects the Leydig cells that contain most of the catalase and GPx activities. Testicular SOD activities that are largely confined to the seminiferous tubules did not change dramatically under these circumstances.<sup>95</sup> It is therefore possible that the site of free radical generation in response to gonadotrophin withdrawal involves electron leakage from the inhibited steroidogenic pathway of the Leydig cells. These free radicals then attack the germ cells within the seminiferous tubules leading to extensive apoptosis and the disruption of spermatogenesis. The fact that aminogluthethimide, an inhibitor of the P450 cholesterol side-chain cleavage, induces extensive lipid peroxidation in the testis supports this contention.<sup>95</sup> Interestingly, over-stimulation of the Leydig cells by chronic exposure to hCG (100 IU/day for 30 days in rats) also stimulates high levels of ROS production from these cells, that in turn stimulate lipid peroxidation, reduction in antioxidant enzyme activities, germ cell apoptosis and the consequential disruption of spermatogenesis.<sup>98</sup> Thus, as we saw with the involvement of the thyroid gland in the control of testicular function, a stable redox environment depends on an appropriately balanced gonadotrophic support, either hyper- or hypo- gonadotrophism will induce a state of oxidative stress in the testis.

**Retinoids.** While fluctuations in Leydig cell steroidogenesis may be one source of free radical generation in the testes, another is the Sertoli cell population. The latter has been shown to generate ROS following stimulation with all trans-retinoic acid (RA), a vital cofactor for spermatogenesis. Exposure of rat Sertoli cells to RA led to activation of ROS generation, lipid peroxidation and, ultimately, a loss of cell viability.<sup>99</sup> There is also some evidence to suggest that retinol might stimulate ROS generation in rat Sertoli cells<sup>99,100</sup> and that this effect is accompanied by an upregulation of testicular antioxidant enzymes including SOD, GPx and catalase.<sup>101</sup> There may be nothing particularly specific about this effect since retinoids have been shown to stimulate ROS generation in a variety of other cellular systems.<sup>102</sup> Nevertheless, the free radical generation triggered by retinoids in the testes may explain the testicular degeneration induced by hypervitaminosis A in the rat<sup>103</sup> and the association between excess beta carotene intake and infertility in human males.<sup>104</sup>

**Impact of xenobiotics.** A wide variety of different xenobiotics have also been shown to induce oxidative stress in the testes in concert with the suppression of antioxidant mechanisms. A summary of these testicular toxicants is provided in Table 1. Heavy paternal smoking, for example, is known to generate oxidative DNA damage in the male germ line in association with a 32% reduction in the  $\alpha$ -tocopherol content of the seminal plasma.<sup>105</sup> The role of oxidative stress in the genesis of this DNA damage is supported by the observation that in individuals subjected to an ascorbate depleted diet, the seminal plasma ascorbate levels decreased by a half, while DNA damage levels in the spermatozoa increased by 91%. Repletion of the ascorbate levels in the diet had the reverse effect and decreased DNA damage by 36%.<sup>106</sup> Experimental exposure of rats to cigarette smoke also induces lipid peroxidation in the testes in association with disturbances in testicular antioxidant enzyme activity.<sup>2</sup> The testicular damage induced by cigarette smoke exposure in rats is certainly oxidative in nature because it can be reversed by concomitant exposure to an antioxidant (caffeic acid phenethyl ester).<sup>107</sup>

In addition to smoking, excessive alcohol consumption also has a negative effect of testicular function through the induction of oxidative stress and the concomitant disruption of testicular antioxidant status.<sup>108,109</sup> Furthermore, the ability of antioxidants such as vitamin C or lecithin to ameliorate this pathology, confirms the importance of oxidative stress in this context.<sup>110-111</sup> In addition to inducing low sperm counts and poor sperm motility, it also appears that the oxidative stress created in the Leydig cells as a consequence of chronic alcohol exposure diminishes the steroidogenic capacity of the testes, lowering circulating testosterone levels.<sup>112</sup>

Table 1 also highlights a number of metals that are known to induce oxidative stress in the testes and compromise male infertility. Chromium, for example, is a testicular toxicant that stimulates lipid peroxidation and suppresses antioxidant enzyme activities as well as ascorbate levels in the testes.<sup>113</sup> Additional studies in monkeys have also shown that chromium administration decreases not only inhibit the classical array of antioxidant enzymes in the testes but also diminishes the testicular content of GSH as well as vitamins A,E and C, while H<sub>2</sub>O<sub>2</sub> production and hydroxyl radical formation are increased.<sup>34</sup> Additional transition metals such as iron also induce lipid peroxidation, protein carbonyl expression and lipid soluble antioxidant depletion in testicular tissue with the consequent disruption of spermatogenesis.<sup>114,115</sup> Significantly, iron intoxication of male mice also induces a dominant lethal effect characterized by high levels of embryonic loss in females mated to iron-exposed males. In this situation, the oxidative stress induced in the testes by acute iron overload must have so damaged the DNA in the spermatozoa that the resulting embryos were non-viable.

Heavy metals such as lead, cadmium and uranium have a similar effect on the testes disrupting spermatogenesis via mechanisms that involved the induction of lipid peroxidation, depletion of ROS scavengers and disruption of testicular antioxidant enzyme activity.<sup>35,116-118</sup> Arsenic has also been shown to induce peroxidative damage in the testes elevating protein carbonyl expression and decreasing tissue GSH content and inhibiting  $3\beta$ - and  $17\beta$ -hydroxysteroid dehydrogenase activities. The importance of oxidative stress in the testicular toxicity associated with arsenic was emphasised by the ability of ascorbate to reverse these changes.<sup>119</sup> Similarly, vanadate is a testicular toxicant that induces lipid peroxidation in the testes along with significant suppression of testicular SOD and catalase and the disruption of  $3\beta$ - and  $17\beta$ -hydroxysteroid dehydrogenase activities.<sup>120</sup>

In addition to the above, Table 1 lists a wide variety of different industrial and environmental toxicants that are all capable of compromising male fertility by inducing a state of oxidative stress in the testes. These compounds include phthalate esters,<sup>121,122</sup> sulfur dioxide,<sup>123</sup> sodium fluoride,<sup>124</sup> a range of environmental estrogens (e.g., PCBs,<sup>2,39,125-127</sup> methoxychlor,<sup>128,129</sup> bisphenol A<sup>130,131</sup> nonylphenol<sup>132</sup>), chemotherapeutic agents (e.g., adriamycin,<sup>135</sup> cisplatin<sup>136,137</sup> cyclophosphamide<sup>138-140</sup>) hexachlorocyclohexane,<sup>141-142</sup> 2,4,6-trinitrotoluene,<sup>143</sup> aflatoxin,<sup>144</sup> lindane,<sup>145</sup> quinalphos<sup>146</sup> endosulfan,<sup>147</sup> diethyl maleate,<sup>148</sup> monensin,<sup>149</sup> formaldehyde,<sup>150</sup> alloxan,<sup>151,152</sup> streptozotocin,<sup>86</sup> acrylamide<sup>153</sup> and ozone.<sup>154</sup> In addition to this list of xenobiotic chemicals that can induce oxidative stress in the testes, physical factors such as static magnetic fields<sup>155</sup> and electromagnetic radiation in its various forms from heat<sup>12,156</sup> to X-ray<sup>157</sup> irradiation, can also trigger a state of oxidative stress in



Table 1 List of compounds capable of inducing oxidative stress in the testes

Compound	Ref.	Compound	Ref.
Smoking	2, 105–107	Nonylphenol	132–134
Alcohol	108–111	Adriamycin	135
Chromic acid	34, 113	Cisplatin	136–137
Iron	114, 115	Cyclophosphamide	138–140
Lead	35	Hexachlorocyclohexane	141–142
Cadmium	116	Trinitrotoluene	143
Uranium	118	Aflatoxin	144
Arsenic	119	Lindane	145
Vanadate	120	Quinalphos	146
Phthalate esters	121, 122	Endosulfan	147
Sulfur dioxide	123	Diethyl maleate	148
Sodium fluoride	124	Monensin	149
PCB/PCN	2, 39, 125–127	Formaldehyde	150
Methoxychlor	128, 129	Alloxan	151, 152
Bisphenol A	1, 130, 131	Streptozotocin	86
		Acrylamide	153
		Ozone	154

MDA, malondialdehyde; XO, xanthine oxidase; GPx, glutathione peroxidase; MPO, myeloperoxidase; iNOS, inducible nitric oxide synthase.

testicular tissue. Given the variety and prevalence of chemical and physical factors that can generate oxidative stress in the male gonad, there is an urgent need to identify antioxidants that can supplement the tissue's own antioxidant strategies to rescue the testes from the consequences of ROS attack.

### Antioxidant Therapy

In order to determine the relative potential of different antioxidants to address oxidative stress in the testes, the testicular torsion-detorsion model has been repeatedly used. Typically this model involves the application of antioxidant therapy prior to the creation of a brief period of oxidative stress and subsequent comparison of various testicular attributes (lipid peroxidation, histopathology, DNA damage or antioxidant enzyme status) with sham operated controls (Table 2). Such analyses have recorded significant protection against oxidative stress for factors as garlic extract,<sup>57</sup> caffeic acid phenethyl ester (CAPE),<sup>60</sup> N-acetyl cysteine,<sup>158</sup> pentoxifylline,<sup>159</sup> erdostein,<sup>160</sup> resveratrol,<sup>58</sup> dexpathenol,<sup>161</sup> L-carnitine<sup>59</sup> and propofol anaesthetic.<sup>162</sup>

A variety of antioxidants have also been assessed for their ability to counteract oxidative stress in the testes created by alternative mechanisms. For example: (1) CAPE has been shown to protect the testes from the oxidative stress created by exposure to dizocilpine (MK-801), a drug that is commonly used to induce schizophrenia,<sup>163</sup> and cigarette smoke<sup>107</sup> (2) lycopene, the red plant antioxidant that is a major constituent of tomatoes, is capable of reversing the oxidative damage induced in rat testes following exposure to cyclosporin A<sup>164</sup> or cisplatin<sup>137</sup> (3) extracts from the herb *Lycium barbarum*, effectively protect the testes from the oxidative damage induced by heat stress and significantly suppress the oxidative DNA damage induced in mouse testicular cells by H<sub>2</sub>O<sub>2</sub><sup>165</sup> (4) MTEC (an

aqueous-methanol extract of *Musa paradisiaca*, *Tamarindus indica*, *Eugenia jambolana* and *Coccinia indica*) protects against the oxidative testicular damage resulting from induced diabetes<sup>84</sup> (5) lecithin administration protects the testes from the oxidative stress induced by chronic ethanol exposure<sup>111</sup> (6) lipoic acid has been shown to inhibit the oxidative damage resulting from exposure to cyclophosphamide,<sup>140</sup> adriamycin<sup>135</sup> and X-irradiation<sup>157</sup> (7) complex feruloyl oligosaccharides released from wheat bran have also been shown to protect the testes from the oxidative stress associated with alloxan-induced diabetes in rats<sup>152</sup> and (8)  $\beta$ -carotene ameliorates cadmium induced oxidative stress in the testes, suppressing lipid peroxidation and restoring SOD, GST and GSH to normal physiological levels.<sup>166</sup>

One of the most effective antioxidants for the protection of testicular function is melatonin. This evolutionarily conserved compound has been shown to reduce oxidative stress in the testes induced by ethanol,<sup>167</sup> indomethacin,<sup>168</sup> X-irradiation,<sup>169</sup> streptozotocin-induced diabetes,<sup>86</sup> and cisplatin.<sup>136</sup> In vitro studies have also shown that melatonin and its immediate precursor N-acetyl-serotonin could inhibit ascorbate-Fe (II) induced lipid peroxidation in rat testicular microsomes and mitochondria.<sup>170,171</sup>

The administration of antioxidants such as resveratrol, ascorbate or cocoa rich in flavanols to normal animals, not suffering from induced oxidative stress, also appears to improve testicular function, suggesting that oxidative stress is a consistent feature of testicular physiology.<sup>172,173</sup> In light of such results, antioxidants have frequently been administered to infertile men in the hope of improving the quality of the semen profile. Very few properly controlled double blind crossover trials have been conducted in this context. However where these conditions have been met, the results have been extremely promising.<sup>174,175</sup>

### Conclusions

Oxidative stress is a major factor in the aetiology of male infertility. At the level of the isolated spermatozoon, ROS attack can induce lipid peroxidation and DNA fragmentation disrupting both the motility of these cells and their ability to support normal embryonic development.<sup>176–182</sup> At the level of the testes, oxidative stress is capable of disrupting the steroidogenic capacity of Leydig cells<sup>183</sup> as well as the capacity of the germinal epithelium to differentiate normal spermatozoa.<sup>184</sup> A large number of independent clinical studies have demonstrated a correlative relationship between male infertility and evidence of oxidative stress in the ejaculate.<sup>180,185</sup> Moreover the literature reviewed in this chapter reveals an abundance of experimental data in animal models demonstrating a causal relationship between the induction of oxidative stress in the testes and the impairment of male reproductive function. However these two lines of evidence have not yet come together. Although oxidative stress is clearly a dominant feature in the aetiology of male infertility, the underlying causative mechanisms remain unresolved. The plethora of physical, chemical, and pathological factors that can apparently contribute to the induction of oxidative stress in the testes is impressive and



Table 2 Impact of antioxidants on testicular damage caused by testicular torsion/detorsion

Antioxidant	Outcome	Ref.	Antioxidant	Outcome	Ref.
Garlic extract	↓ MDA ↓ XO ↑ histopathology	57	Pentoxifylline	↓ MDA ↓ Germ cell apoptosis	159
Caffeic acid phenethyl ester	↓ MDA ↑ histopathology ↑ GPx	186	Erdostein	↓ MDA ↑ GPx ↑ Catalase ↑ histopathology	160
Caffeic acid phenethyl ester	↓ MDA ↓ MPO ↓ iNOS ↑ histopathology	60	Resveratrol	↓ Germ cell apoptosis	58
N-acetylcysteine	↓ MDA ↑ GPx	158	Dexpathenol	↑ histopathology	161
Morphine	↓ MDA ↑ GPx ↑ SOD ↑ Catalase	187	L-carnitine	↓ MDA ↑ histopathology	59
			Propofol	↑ histopathology	162

MDA, malondialdehyde; XO, xanthine oxidase; GPx, glutathione peroxidase; MPO, myeloperoxidase; iNOS, inducible nitric oxide synthase.

suggests that the clinical picture will be extremely complex, with each individual being subject to a unique range of causative factors as a result of differences in occupational and environmental exposures, the presence of other pathological factors such as infection or diabetes, and genetic factors that could influence everything from the way in which specific xenobiotics are metabolised to the endocrine environment in which the testes have to function.

That there are so many factors capable of inducing oxidative stress in the testes strongly suggests that this is a vulnerable tissue that is both highly dependent on oxygen to drive spermatogenesis and yet highly susceptible to the toxic effects of reactive oxygen metabolites; in this context, the testis is very like the brain. While the testes clearly do possess highly specialized antioxidant defence enzymes such as extracellular SOD, PHGPx etc, there are clear benefits to be gained by treating susceptible individuals with exogenous antioxidants. Despite the evident clinical market for an antioxidant preparation specifically designed to support male reproductive health, it is remarkable how little effort has gone into the development of such a preparation and how poor most of the clinical trials in this area have been. In animal models an impressive range of antioxidant preparations has been examined and compounds identified that are clearly capable of crossing the blood testes barrier and protecting the germinal epithelium and Leydig cells from oxidative stress. The future imperatives for this area are to go beyond the superficial phenomenology that characterizes most of the clinical research in this area in an attempt to (i) gain insights into the underlying causes of oxidative stress in the male reproductive tract and (ii) develop optimized antioxidant preparations to treat pathologies arising from an imbalance in the

redox status of these tissues. The journey will be long and difficult but ultimately more rewarding than the empirical approach that characterizes the current approach to treating the infertile male.

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