Review

Oxidative Stress and Antioxidation in Testicular Leydig: Implications for Male Fertility

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Review

Oxidative Stress and Antioxidation in Testicular Leydig: Implications for Male Fertility

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Abstract: Leydig cells, pivotal for male fertility, undergo significant oxidative stress due to their proximity to macrophages and reactive oxygen species (ROS) generated by extracellular structures. This stress disrupts Leydig cell function, impairing steroidogenesis and spermatogenesis, ultimately leading to male infertility. Oxidative stress triggers apoptosis and inhibits testosterone production, crucial for spermatogenesis regulation. Understanding the mechanisms of oxidative stress and antioxidation on Leydig cells is vital. Some studies highlight the role of antioxidants in mitigating oxidative damage, emphasizing critical pathway of Sirt1 in oxidative stress and Nrf2 in antioxidation. Future research should focus on screening antioxidant substances and optimizing antioxidant dosages for Leydig cell protection and testosterone restoration, thereby enhancing reproductive performance. This review provides insights into combating male infertility by addressing Leydig cell oxidative stress and antioxidation.

Keywords: Leydig cells; spermatogenesis; testosterone; oxidative stress; antioxidants

1. Introduction

Male infertility caused by oxidative stress in Leydig cells has become a prominent problem. In the interstitial tissues adjacent to the testicular seminiferous tubules, Leydig cells serve as important endocrine cells. Their pivotal role in steroidogenesis is crucial for male fertility, as is their contribution to spermatogenesis [1,2]. Testosterone synthesized by Leydig cell exerts diverse effects on male development across the whole reproductive stages. Specifically, during the fetal stage, testosterone secretion by fetal Leydig cell is imperative for the morphogenesis of the genital tract and external genitalia, as well as the migration of the testes into the scrotum. In puberty, the testosterone plays a critical role in facilitating spermatogenesis, maintaining the structure integrity of the blood-testicular barrier, and ensuring fertility maintenance [2]. Due to the proximity of Leydig cell to macrophages in the testis, Leydig cell highly susceptible to reactive oxygen species (ROS) produced by extracellular structures [2,3]. Activated macrophages release ROS, which can oxidize adjacent tissues and cells, including sensitive Leydig cells to oxidative stress [3]. Furthermore, Leydig cell also be important components of innating immune responses by releasing of cytokines, chemokines, and other substances that defenses against pathogens and potential self-impairments [4]. when the homeostatic balance between antioxidant and oxidative stress induced by ROS is disrupted, Leydig cells become vulnerable to oxidative stress, leading to severe dysfunction in steroidogenesis and spermatogenesis, ultimately causing in male infertility [2]. The excessive production of oxygen free radicals induces oxidative stress, which overwhelms the antioxidant capabilities of cells, leading to homeostatic imbalance of ROS and antioxidation. Studies have demonstrated that oxidative stress damages the testicular cells by promoting lipid peroxide, triggering apoptosis, disrupting mitochondrial function,

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and reducing testosterone secretion [5]. Decreased testosterone production by Leydig cells can result in impaired steroidogenesis, compromised spermatogenesis, and ultimately male infertility [6,7].

The review summarizes the detrimental effects of oxidative stress on leydig cells, and explores the antioxidative mechanism of common antioxidants. By providing an overview of oxidative and antioxidative stress in Leydig cells, this review offers valuable insights for developing strategies to prevent male infertility resulting from oxidative stress.

1. Pathways of ROS Production in the Organism and Potential Harm to Male Fertility

Elevated ROS levels in the male reproductive tract, stemming from both endogenous and exogenous factors, disrupt the balance between oxidant production and the scavenging activity of antioxidant enzymes, thus triggering oxidative stress, as illustrated in Figure 1. Overall, ROS disturbed the regulation of metabolic hormone (Leptin, Insulin and T3), hormone (Cortisol) and immune (Proinflammatory cytokines) stress, and then indirectly impact on the regulation of hypothalamus-pituitary gland-Testes axis (H-P-T axis). Meanwhile, the ROS directly disturb the regulation of H-P-T axis. Finally, ROS is harm to male fertility. Some synthetic and natural antioxidants have been widely used to alleviate oxidative stress of Leydig cell.

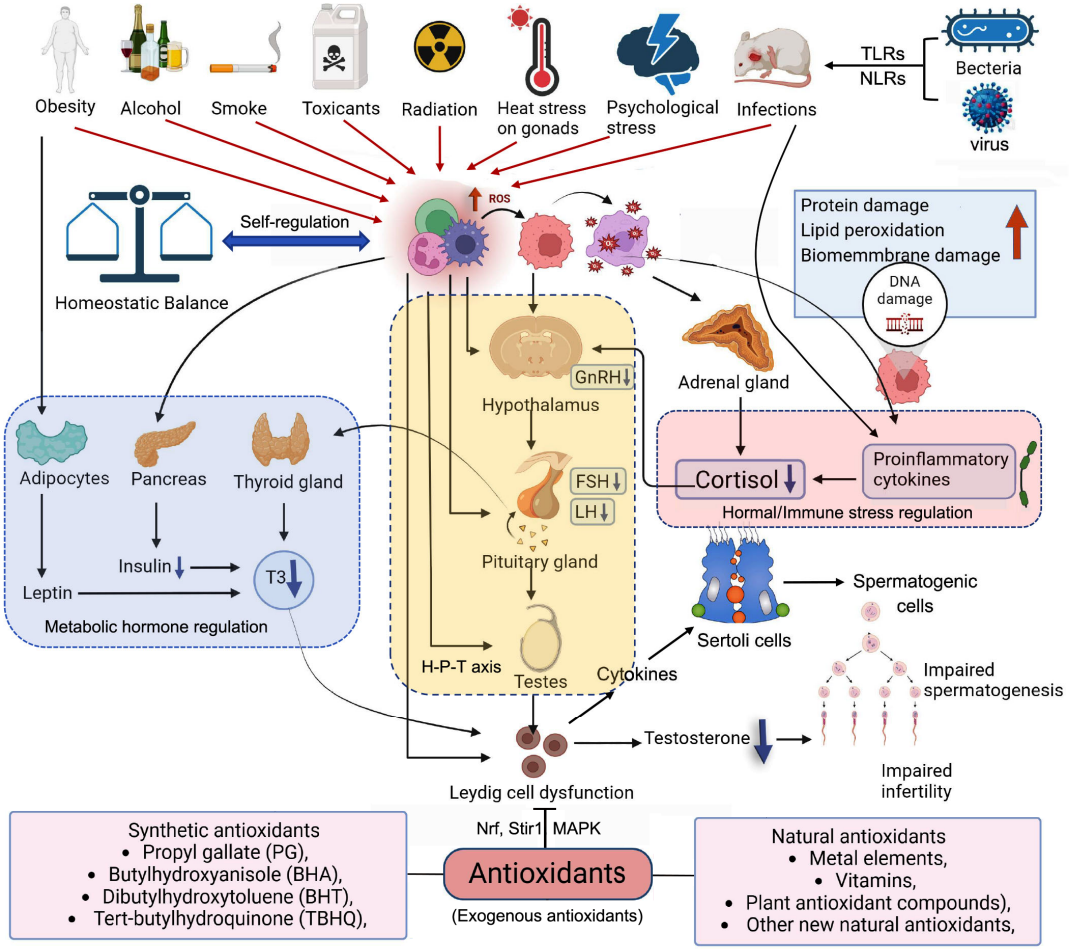

Figure 1. Both exogenous and endogenous ROS disturbed the regulation of metabolic hormone, hormonal or immune stress, and H-P-T axis, and then impact on Leydig cell function. TLRs: Toll-like receptors; NLRs: Nod-like receptors; ROS: reactive oxygen species; GnRH: Gonadotropin releasing hormone; FSH: Follicle stimulating hormone; LH: Luteinizing hormone; T3: Tri-iodothyronine; H-P-T axis: Hypothalamus-pituitary gland-Testes axis; Nrf: Nucleus factor-E2 related factor; Stir1: Silent information regulator of transcription 1; MAPK: Mitogen activated protein kinase.

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Various sources of ROS originate from testicular cells, with disruptions observed in the mitochondrial electron transport chain (ETC) and mitochondrial/microsomal cytochrome P450 enzymatic reactions, representing consequences of the oxidative stress [74]. The suppression of StAR protein transcription and subsequent the reduce of cholesterol transport to the inner mitochondrial membrane for conversion into 17-hydroxypregnenolone is a consequence of oxidative stress, resulting in impaired Leydig cell steroidogenesis [75]. By suppressing the transcription of essential enzymes such as P450scc, CYP17, and 3β-HSD, ROS, particularly H_2O_2 originating from medullary cells and testicular macrophages, induces mitochondrial dysfunction, thereby compromising steroidogenesis [76]. Therefore, ROS disturb the process of testosterone synthesis by inhibiting the transcription of enzymes in ETC, and steroidogenic acute regulatory protein (StAR), that is a translocator protein (TSP) co-factor and participate in the formation of transduceosome to transport cholesterol into mitochondria (Figure 2). The presence of oxidative stress (OS) in spermatogonia is also correlated with diminished levels of cellular antioxidant enzymes, such as glutathione peroxidase (GPX), superoxide dismutase (SOD), malondialdehyde (MDA), and catalase (CAT), along with the progression of apoptotic processes [65,77].

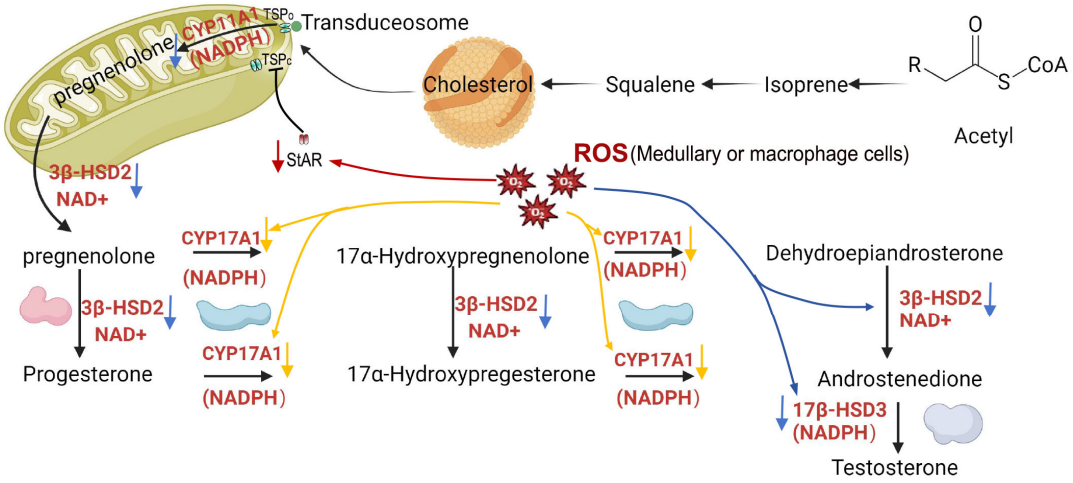

Figure 2. ROS disturb the process of testosterone synthesis. CYP: cytochrome P; 3β-HSD2:3-beta-hydroxy steroid dehydrogenases 3; 17β-HSD3:17-beta-hydroxy steroid dehydrogenases; StAR: steroidogenic acute regulatory protein; TSPc: Close status of translocator protein; TSPo: Open status of translocator protein. ↓:ROS inhibit the transcript of StAR;↓:ROS inhibit the transcript of CYP11/17A1(NADPH);↓:ROS inhibit the transcript of 3β-HSD2(NAD+).

3. Function of Leydig Cells

3.1. Involvement in the Regulation of Spermatogenesis

Leydig cells are in the connective tissues surrounding the seminiferous tubules of the testes, and mature Leydig cells are typical steroid hormone-secreting cells characterized by abundant slippery endoplasmic reticulum, large and numerous mitochondria, and the presence of lipid droplets in the cytoplasm, containing essential substances for steroid hormones synthesis. The hallmark enzyme of Leydig cell is 3β-hydroxysteroid dehydrogenase. Leydig cells play pivotal roles in supporting germ cells, regulating spermatogenesis, and secreting testosterone to promote sperm maturation. Beyond their roles in supporting germ cells and regulating spermatogenesis, Leydig cells serve crucial functions in reproductive physiology. They maintain the integrity of immune barrier for germ cells by promoting the tight junction of blood-testis barrier. Additionally, Leydig cells secrete testosterone that is essential for promoting the development, differentiation of reproductive organs, as well as maintaining secondary sexual characteristics and sexual function.

Spermatogenesis is a major physiological function of testes, and serves as the basis of male reproduction. Abnormalities in spermatogenesis leads to male infertility. A series of intricate and

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interacting processes involve in regulation in spermatogenesis [8]. Leydig cells, alongside peritubular myeloid cells and Sertoli cells, form a distinct somatic cell population responsible for orchestrating spermatogenesis, with Leydig cells playing a vital regulatory role in this process (Figure 3).

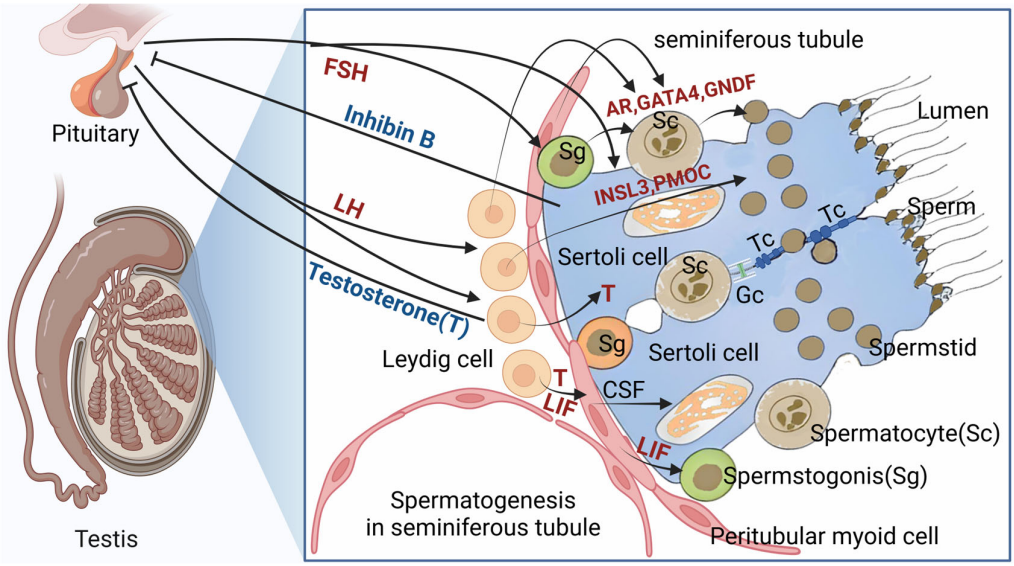

Figure 3. Endocrine and paracrine secretion of Leydig cell paly a important role in the biological process of spermatogenesis. AR: Androgen receptor; GATA4: GATA binding protein 4; GNDF: glial cell derived neurotrophic factor; INSL3: Insulin-like factor 3; PMOC: Pro-opiomelanocortin; CSF: Colony stimulated factor; FSH: Follicle simulated factor; LH: luteinizing hormone; LIF: Leukemia inhibitory factor; Tc: The tight conjunction between Sertoli and Sertoli cell; Gc: Gap conjunction between Sertoli and Sertoli cel.

3.2. Production of Testosterone and Indirect Regulation in Spermatogenesis

Several studies have highlighted that hormones and autocrine signals play a crucial role in continuous and asynchronous sperm production [8]. Androgens secreted by Leydig cells in the testes play a fundamental role in male sexual differentiation and behavioral patterns [9,10], serving as the cornerstone for sustaining, initiating, and modulating spermatogenesis [11–14]. Testicular cells treated with androgen-specific knockdowns induced androgen receptor binding in Leydig cells to control spermatogenesis. suggesting an irreplaceable role for Leydig cell in maintaining normal male reproduction [15–18]. These studies have confirmed that normal spermatogenesis is dependent on testosterone concentration to be maintained by Leydig cells.

Testicular Leydig cells play a pivotal role in the indirect regulation of spermatogenesis, primarily mediated by steroidogenic proteins such as StAR, members of the cytochrome P450 family (P450scc, P450c17), the peripheral-type benzodiazepine receptor, hydroxysteroid dehydrogenases (17β-HSD, 3β-HSD), and the luteinizing hormone receptor (LHR), which collectively facilitate testosterone biosynthesis [36]. The synthesis of testosterone in Leydig cells of the testis commences with the mobilization of cholesterol from lipid droplets and/or plasma membranes. This cholesterol is then shuttled into mitochondria, where it is enzymatically converted into pregnenolone. Subsequently, pregnenolone is processed by enzymes present in the smooth endoplasmic reticulum (ER) to yield steroidal products [19].

The production of testosterone can be outlined in the following sequential steps: In response to the hypothalamic gonadotropin-releasing hormone (GnRH), the pituitary gland secretes luteinizing hormone (LH). LH binds to LH receptor (LHR) on the Leydig cell, activating G proteins and stimulating the production of cyclic adenosine 3',5'-monophosphate (cAMP), ultimately initiating steroidogenesis [20]. In the subsequent stages, cAMP prompts the translocation of cholesterol to the mitochondria, marking a pivotal step in steroid synthesis. Following this, pregnenolone is

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synthesized from the cholesterol in the mitochondria [21], which is then enzymatically transformed in the smooth endoplasmic reticulum to produce testosterone [22,23].

Testosterone diffuses from leydig cells, via androgen-binding proteins translocated to Sertoli cells where it binds to androgen receptors to regulate spermatogenesis. At least four crucial processes in spermatogenesis depend on testosterone: maintenance of BTB, meiosis, Sertoli-sperm adhesion, and sperm release [24–29]. This suggests that leydig cells play an indirect regulatory role in spermatogenesis [30].

3.3. Other Factors Regulating Spermatogenesis via Leydig Cells

There has been a large body of research that shows that Leydig cells regulate spermatogenesis through the secretion of growth factors and steroidogenesis [31,32]. Furthermore, testicular Leydig cells produce cytokines and hormones that also contribute to regulation of spermatogenesis [33], In order for hormones and cytokines to be able to regulate spermatogenesis, they must bind to specific receptors on Leydig cells (Table 1) including estrogens, interleukin 1α (IL-1α), inhibin, insulin-like peptide 3 (INSL3), insulin-like growth factor 1 (IGF1), transforming growth factor β (TGFβ), and thyroid hormones [9,10,34–37]. Studies suggest that leydig cell dysfunction adversely affects not only spermatogenesis and sex differentiation, but also gonadal development, and androgen production

Table 1. The receptors on Leydig cell associated with spermatogenesis.

Receptors/ Cytokines	*Species*	Characteristic	cited document
AR	Humans, Rats, Dogs	Synergizes with androgens to regulate spermatogenesis	[36,38–41]
ER	Human, Rat	Maintains sperm count and function	[42–44]
LGRS	Rats	Facilitates the stimulatory effect of INSL3 on cAMP production during spermatogenesis.	[45,46]
OTR	Human, monkey	Modulates oxytocin levels within the male reproductive tract.	[38]
LHR	Human, rat, flounder	Synergizes with LH to promote testosterone production	[47]
FSHR	Human, Rat, Stallion	Mediates the effects of follicle stimulating hormone on spermatogenesis	[47–49]
LIFR	Rats	Maintenance of germ cell numbers, sperm motility, seminiferous tubule structure and steroid formation	[50]
TNFR	Rats	Harmonizes spermatogenesis	[51]
IGFR	Rats, Horses, Dogs	Regulates testicular function and germ cell development	[52–54]
TGF-βR	Human, Rat, Boar	Controls meiosis and early spermatogenesis	[55–57]
PGR	Humans, cats, sea bream	Regulates the role of progesterone in spermatogenesis	[58–60]
KIT	Rats	Promotes proliferation, migration and postnatal spermatogenesis of primordial germ cells	[61–63]
Cubn	Rats	Facilitates endocytosis and nutrient uptake in both germ cells and somatic cells, essential for the sustenance of spermatogenesis.	[64]

4. The Impairment of Oxidative Stress on Leydig Cell Functions

4.1. Stimulation of Leydig Cell Apoptosis

Apoptosis, a crucial aspect of normal cellular functioning, involves the programmed elimination of cells, leading to a reduction in their overall number. Oxidative stress may damage the cell's DNA, leading to the activation of death receptors in the cell. Nevertheless, in response to oxidative stress, genes in apoptotic signaling may be weakened, thereby impeding the signaling required for normal

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apoptosis. Thus, intense oxidative stress can then lead to necrosis or cancerous transformation of cells during the course of the disease.

Exposing to H_2O_2 can provoke DNA damage and deteriorate the functional performance as well as hormone secretion, particularly leydig cells producing testosterone. Several studies have shown that in vitro, even at physiological concentrations, H_2O_2 interferes with leydig cells' normal function and increases the abnormal levels of intracellular oxidative stress and then lead to cell apoptosis. The accumulation of excessively produced H_2O_2 in leydig cells in vivo can lead to oxidative stress, often resulting from hormonal disturbances or other conditions that compromise cellular function [65].

4.2. Inhibition of Testosterone Production

When the concentrations of oxygen and its derived free radicals surpass the intrinsic antioxidant defenses of a cell, it results in a state of oxidative stress. ROS comprise oxygen radicals like hydroxyl radicals and superoxide anions [3], as well as substances that, although not radicals in their nature, are capable of generating free radicals in the context of cellular and tissue environments [66].

The crucial role of luteinizing hormone in stimulating testosterone production in Leydig cells is imperative for spermatogenesis. The testes rely on adequate levels of testosterone to ensure proper spermatogenesis and the survival of germ cells. Disruptions in testosterone production, whether partial or complete, can have significant adverse effects on the spermatogenic process, potentially leading to its malfunction or complete cessation.

Multiple investigations have revealed a direct correlation between oxidative stress-mediated cellular damage and overall testosterone production. Alleviating the inhibition of Leydig cells and testosterone synthesis by oxidative stress may improve the state of testosterone production.

4.3. Other Harmful Effects

First, oxidative stress has an impact on cellular metabolism, and once oxidative stress occurs, it initiates a stress-adaptive response in the cellular life mechanism [67]. This response regulates cellular metabolism in response to the appropriate stress stimulus [68]. However, when the oxidative stress level is higher, this adaptive response is not effective in rescuing cellular damage. For example, high levels of H_20_2 in the cell give rise to oxidative damage to proteins and DNA [69]. Similarly, the more a cell adapts to oxidative stress, the more it gradually reduces its metabolic capacity and function [70].

Second, oxidative stress affects mitochondria and thus the process of cellular esterification. Mitochondria play a major role in adenylate conversion, and red blood cells contain a large number of adenylate molecules [71]. If the cell is subjected to some stress, it may lead to mitochondrial damage, which can perturb cellular metabolism. And this perturbation will precisely trigger oxidative stress [72].

ROS impedes the phosphorylation of mTOR/AKT/ERK1/2 and LH/FSH signaling, consequently suppressing Leydig cell differentiation. This, in turn, results in reduced testosterone (T) levels and spermatogenesis due to lower FSH concentrations [73].

5. Current Status of Research on Antioxidant Stress Substances for Leydig Cells

5.1. Classification of Antioxidant Substances

The principle of antioxidant is to slow down or inhibit cellular oxidative damage by scavenging free radicals, there are a large number of varieties of antioxidants, which have different effects on the cell or the organism, the following table (Table 2) classifies them according to their sources and summarizes their functional roles [78,79].

Table 2. Classification of antioxidant substances.

Source	Species	Characteristic	Reference
Synthetic antioxidants	(1) Propyl gallate (PG), (2) Butylhydroxyanisole (BHA),	Although it has a strong antioxidant capacity, it is capable of producing hydrogen peroxide at	[80]

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	(3) Dibutylhydroxytoluene (BHT), (4) tert-butylhydroquinone (TBHQ),	physiological pH and temperature. Hydrogen peroxide itself is highly oxidizing and quite toxic to cells.	
Natural antioxidants	(1) Metal elements: copper, iron, zinc and manganese	They are cofactors of a series of important antioxidant enzymes, binding with enzymes to make enzymes with strong catalytic activity, by catalyzing superoxide radicals, thus eliminating ROS and preventing their damage to proteins, DNA and lipids.	[81,82]
	(2) Vitamins: Mainly vitamins A, B, C, E and K.	It not only has strong antioxidant ability, but also has a good protective effect on the structure of biological membranes.	[83,84]
	(3) plant antioxidant compounds: mainly polysaccharides, alkaloids, polyphenols, flavonoids and saponins five kinds of compounds.		[85–91] [92,93]
	(4) Other new natural antioxidants: Astaxanthin and antioxidant peptides	Antioxidant substances capable of traversing the blood-brain barrier can neutralize free radicals within the body, thereby rejuvenating cellular function and promoting overall organism vitality.	[94–96]

5.2. Current Research on Antioxidant Substances against Oxidative Stress

Despite the abundance of antioxidant compounds, the research investigating the application of antioxidants to Leydig cells for combating oxidative stress and mitigating the adverse effects of ROS on male spermatogenesis and reproductive function remains conspicuously limited [97–100], and the following table (Table 3) summarizes the roles and pathways of antioxidant stress substances that have been used in leydig cells in recent years. In conclusion, Melatonin and Vitamin E is effective and affordable, and Nrf2 signal pathway is in antioxidation.

Table 3. Antioxidative stress substances for Leydig cells.

	Antioxidant	Oxidative inducer	Animals	Effect	Pathway	Reference
In Vivo Experiments	Adrenomedullin, N-Acetylcysteine	LPS	Rats	Promoted cellular autophagy and attenuated focal death of LPS-exposed leydig cells	ROS-AMPK -mTOR	[88,101]
	Adrenomedullin, N-Acetylcysteine	LPS	Rats	Reduction of LPS-induced ROS overproduction	MAPK / NF-κB	[78,79,88,101]
	Melatonin	Cis-platinum	Mouse	Through the MT1/MT2 melatonin receptors, melatonin modulates the Sirt1/Nrf2 signaling axis, serving as a pivotal protective agent against cisplatin-induced apoptosis in mouse mesangial cells.	Sirt1 / Nrf2	[92,102–106]
	Lepidium sativum L	Aluminum oxide	Rats	Significantly reduced levels of ROS production and increased sperm motility and viability in mice	—	[107]
	Resveratrol	H_2O_2	Mice	Resveratrol safeguards against H2O2-triggered oxidative stress, thereby mitigating the decline in Leydig cell viability and functional proficiency. Lower concentrations of resveratrol had cytoprotective effects on oxidatively stressed leydig cells.	Nrf2-ARE	[108–121]
	Lycopene	Diethylphosphite	Mice	Lycopene is capable of enhancing antioxidant potential by modulating the Nrf2 signaling pathway, subsequently alleviating DEHP-induced damage to Leydig cells.	Nrf2 pathway	[122]
Cellular experiments	Vitamin E	Environment	Rats	Vitamin E exhibits a notable protective effect against oxidant-mediated lipid peroxidation in cultured Leydig cells, as well as preserving the cells' capacity to produce testosterone in vitro.	—	[123]

5.3. Typical Pathways against Oxidative Stress in Leydig Cell

Silent information regulator of transcription 1 (SIRT1), a critical regulator in the oxidative stress pathway, it belongs to a conserved subclass of histone deacetylases that are dependent on nicotinamide adenine dinucleotide (NAD^+) [85–87]. Recent research has furthermore validated the

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pivotal role of SIRT1 in conferring resilience against oxidative stress-induced injuries [92,93], and its mechanism may be related to its deacetylation effect on different target genes and target proteins [88–91].

Oxidative stress induces apoptosis in Leydig cells, an important mechanism as contributing to male reproductive impairment. Activation of autophagy serves to protects Leydig cells [124,125], the SIRT1 pathway antagonizes oxidative stress and activates cellular autophagy. The reduction of ROS mediated by Sirt1 involves a complex signaling network, including the Sirt1/FOXOs, Sirt1/NF-κB, Sirt1/NOX, Sirt1/SOD, and Sirt1/SOD. Sirt1/SOD and Sirt1/eNO. Among these biological factors, eNOs and SOD occupy a downstream position relative to FOXOs, which activate Sirt1 and, subsequently initiate the signaling pathway via a positive feedback loop [94–96]. Therefore, the augmentation of various antioxidant molecules helps to mitigate the adverse effects of oxidative stress [126,127].

Several investigations have demonstrated the capacity of Sirt1 to modulate several crucial regulatory factors (Figure 4), including the nuclear factor-κB (NF-κB), the forkhead box O1 transcription factor (FOXO1), the nuclear factor erythroid 2-related factor 2 (Nrf2), the tumor suppressor P53, as well as the peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α). Additionally, it has been found to regulate the activity of the hypoxia-inducible factor-1α (HIF-1α) and the adenosine 5'-monophosphate (AMP)-activated protein kinase (AMPK), and endothelialnitric synthase (eNOS), Ku70, P66Shc, and many other target genes and target proteins, which in turn play important roles in oxidative stress injury [97–100,124,125].

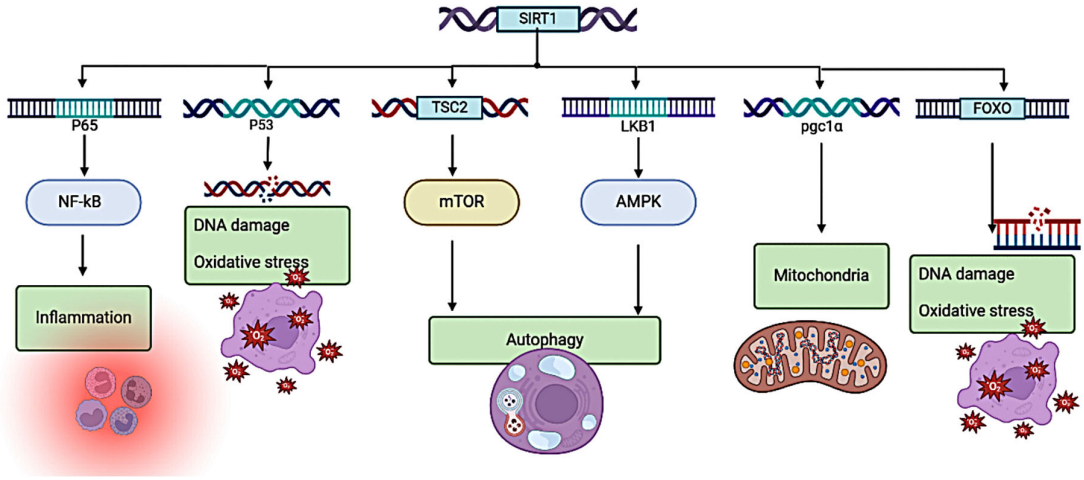

Figure 4. SIRT1 is a hub in these antioxidative stress pathways. P65/53: lymphocyte protein tyrosine kinase56/53; NF-kB: Nuclear factor-k-gene binding; TSC2: Tuberous sclerosis complex 2; LKB1: Liver kinase B1; mTOR: Mechanistic target of rapamycin; AMPK: (AMP)-activated protein kinase; PGC1α: peroxisome proliferator-activated receptor γ coactivator 1α; FOXO: forkhead box O.

6. Conclusions

Maintaining an optimal balance between oxygen-derived free radicals and antioxidants is necessary for Leydig cells to protect themselves against oxidative insults. Under normal conditions, Leydig cells rely on numerous antioxidants to uphold their antioxidant defenses, providing protection or restoration when confronted with oxidative stress. When managing oxidative stress, it is imperative to administer antioxidants at the appropriate dosage or concentration. Additionally, assessing whether the antioxidants can restore the testosterone-producing capacity of Leydig cells impaired by oxidative stress is of great importance for their potential application in livestock and poultry production. Further research on antioxidants should delve into greater depth, aiming to effectively mitigate oxidative damage to Leydig cells and enhance reproductive outcomes, such as spermatogenesis, in male animals at an affordable cost and with easy accessibility.

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