Oxidative Medicine and Cellular Longevity

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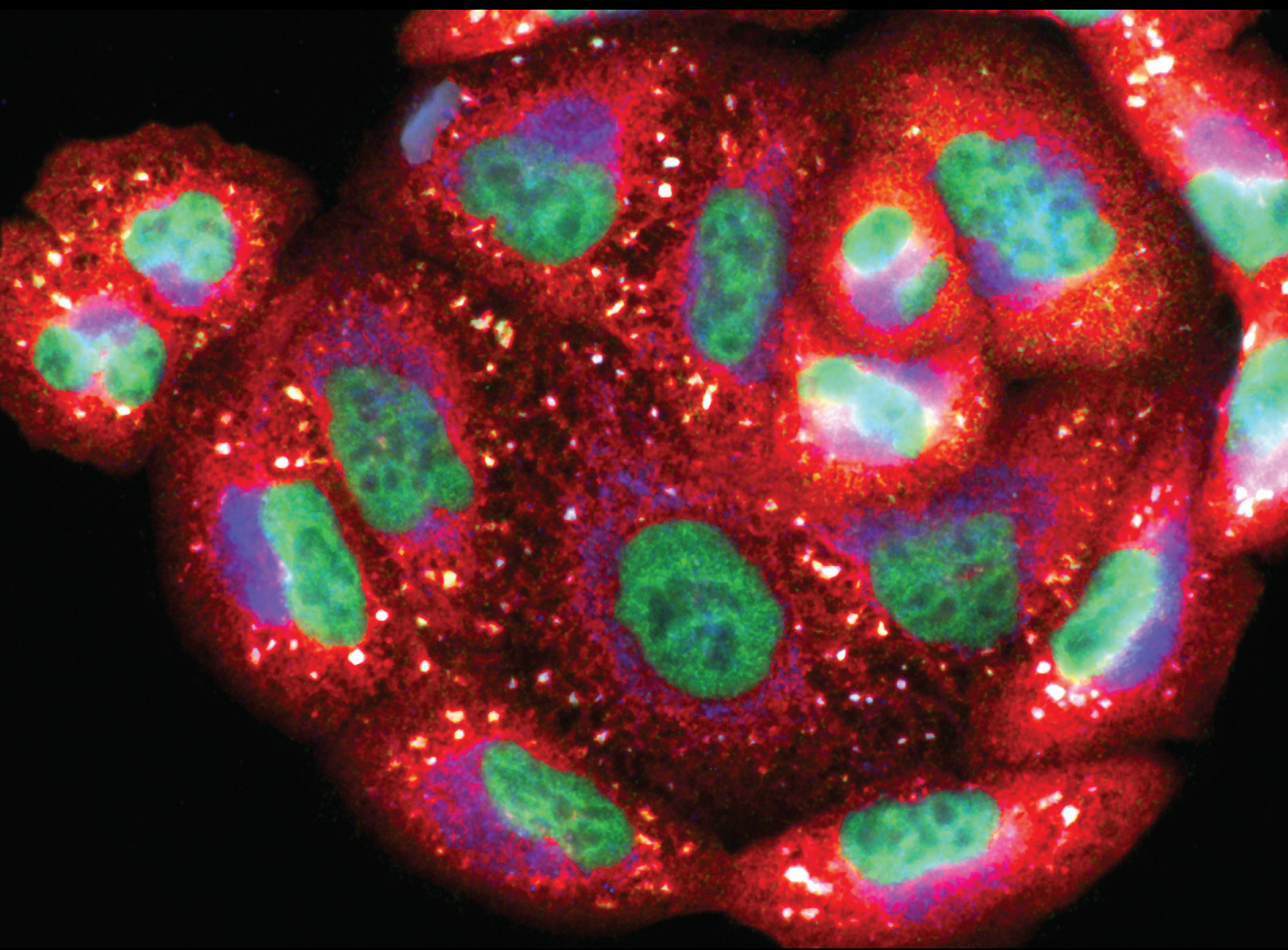

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Review Article

Relevance of Fatty Acids to Sperm Maturation and Quality

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Almost 50% of infertility cases are associated with human male infertility. The sperm membrane is a key structure influencing sperm morphology and function in normal and pathological conditions. The fatty acid profile determines the performance not only of sperm motility but also of acrosomal reaction and sperm-oocyte fusion. This review presents available knowledge on the role of fatty acid composition in human sperm and spermatogenesis and discusses the influence of dietary fatty acids on the sperm fatty acid profile. Recent studies in biological sciences and clinical researches in this field are also reported. The topic object of this review has potential application in medicine by identifying potential causes of infertility.

1. Introduction

Fatty acids (FAs) esterified to phospholipids are part of the cell membrane and contribute to the structural components. In addition to being part of cell membrane phospholipids, FAs are an energy source and as precursors of bioactive lipid mediators have a strong influence on cellular responses and functions [1]. Balancing of lipid rafts and the release of secondary messengers [2] are involved in FA control of intracellular and extracellular signaling pathways in numerous types of cells and tissues [1]. Thus, FAs could contribute to and take part in disease incidence, severity, and outcome [1, 3, 4]. In particular, FAs have crucial roles in biophysical, biochemical, and signaling processes that act as sensing mechanisms and stimulus transduction, thus participating in epigenetic control pathways [5–7].

FAs are classified, according to the presence of double bonds in their chain structure, as saturated FAs (SFAs) in the absence of double bonds, as monounsaturated fatty acids (MUFAs) when a single double bond is present, and as polyunsaturated FAs (PUFAs) when having two or more double bonds. The position of double bonds in unsaturated FA is often written using the n-x notation where x indicates the position of the first carbon with a double bond counting from the methyl end of the chain (n-) [8, 9]. Membrane fluidity, flexibility, fusion, fission, and curvature largely depend on the FA composition of phospholipids [10]. Phospholipids with a high amount of PUFA will increase membrane flexibility due to the multiple double bonds, whereas saturated or *trans*-FAs would make it rigid [11].

It is well known that the Western diet is relatively poor in n-3 PUFAs and rich in n-6 PUFAs [12]. Because arachidonic acid (ARA, C20:4n-6) is a precursor to proinflammatory mediators, the role of an increased dietary intake of ARA, or of its metabolic precursor (linoleic acid (LA), C18:2n-6), in elevating the inflammatory process is debated [1, 13].

In this review, the role of FA composition in sperm quality and spermatogenesis efficiency, together with the effects of dietary FAs on the sperm FA profile, is reviewed. The relevance of FA composition to the quality of sperm parameters is discussed in light of the potential application in medicine by identifying potential causes of infertility.

2. Fatty Acids: Sources, Synthesis, and Function—Distribution in Reproductive Tissues

Alpha-linolenic acid (ALA, C18:3n-3) and LA, respectively, as n-3 an n-6 PUFAs, are essential fatty acids (EFAs) that cannot be synthesized by animals and share the same

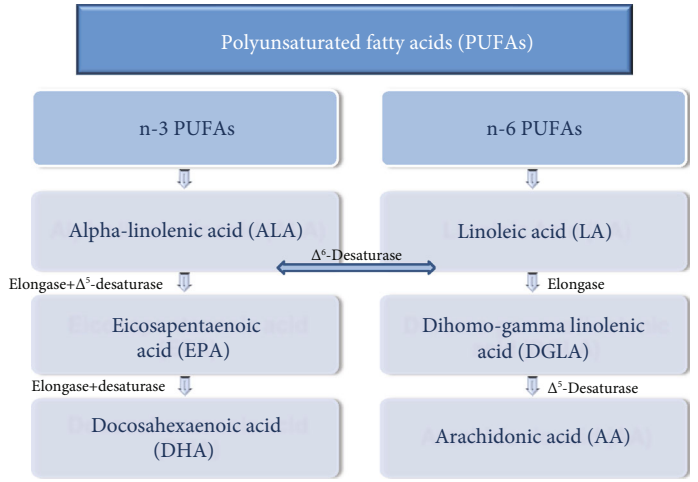

Figure 1: Summary of elongation and desaturation occurrence of polyunsaturated fatty acid (PUFA) metabolism.

elongation and desaturation process (Figure 1) in the metabolic pathway of PUFAs [14]. This process is catalyzed by elongating and desaturating enzymes, specifically elongases 2 (Elovl2) and 5 (Elovl5) and Δ^6-desaturase (FADS1) and Δ^5-desaturase (FADS2). FADS1 introduces a double bond to ALA and LA to extend into long-chain (LC) PUFA (LC-PUFA) [15, 16]. It is the rate-limiting enzyme in the synthesis of LA to ARA (Figure 1) and ALA to eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) [17]. Furthermore, because n-6 and n-3 FAs compete for the same enzyme pathways, their metabolism is largely affected by the availability of the substrates and by the affinity of ALA and LA for the different enzymes. Moreover, the elongation/desaturation rate of EFAs (Figure 1) differs between species and within the same species, and it is affected by sex, hormonal status, intestinal biome, genetic strain, and feed [18].

Numerous studies have demonstrated that EPA and DHA are poorly synthesized in most animal species and in particular humans [19]. Notably, the LC-PUFA production in men is 15% lower than that in women [20]. The biosynthesis of LC-PUFAs also varies among human populations, and the levels of LC-PUFAs in African American men were found to be higher compared to European Americans [21]. These differences are linked to polymorphisms in the FADS gene leading to higher capacity to biosynthesis of LC-PUFAs from LA and ALA.

PUFAs and their metabolites act as secondary messengers in the cell membrane. In fact, after binding to growth factors and hormones and to membrane receptors thereafter, phospholipase A_2 is activated and releases ARA, EPA, and DHA from the sn-2 position of the phospholipids. These molecules become substrates for eicosanoid biosynthesis: the ARA, via cyclooxygenases (COX), leads to the synthesis of 2-series prostanoids (prostaglandin E_2, prostacyclin I_2, and thromboxane A_2), while the activity of lipoxygenases (LOX) on ARA generates 5-hydroperoxyeicosatetraenoic acid, which in turn produces the 4-series leukotrienes [12]. Moreover, EPA and DHA are converted by the same enzymes, e.g., COX and LOX, to 3-series prostanoids (prostaglandin E3, prostacyclin I3, and thromboxane A3) and 5-series leukotrienes, respectively [22]. These two classes of EFA metabolites are not interconvertible and are metabolically and functionally distinct, where they have opposite physiological functions; n-6 FA derivatives have prothrombotic, proaggregatory, and proinflammatory properties while the n-3 metabolites have anti-inflammatory, antiproliferative, and antiatherosclerotic activity [12]. As a consequence, the balance of EFAs is important for good equilibrium and function of several tissues and biological machinery.

Also, other types of metabolites originating from PUFAs were recently discovered, such as nonenzymatic free radical/ROS-mediated PUFA metabolites (i.e., isoprostanoids) which are known as the secondary products of lipid peroxidation. Lately, it has become accepted that these molecules not only serve as markers of oxidative damage but also exhibit a wide range of bioactivities [23].

Other mediators termed as specialized proresolving mediators (SPM) are metabolites derived from enzymatic oxidation of EFAs including DHA and EPA. In particular, resolvin D (RvD), protectins and maresins, and resolvin E are DHA and EPA derivatives, respectively [24]. Nevertheless, the interaction of n-3 and n-6 FAs and their lipid mediators in the context of inflammation is complex and still not properly understood [1].

The testes and sperm have a characteristic lipid composition that is highly enriched in PUFAs, predominantly docosapentaenoic acid (DPA, 22:5n-6) in rodents and DHA in humans [25] and other mammals [26–28]. LA and ALA together with their metabolites, EPA and DHA, are deposited in reproductive tissues and potentially influence the reproductive function and fertility. As reported above, in the sperm plasma membrane, lipid composition and the degree of PUFA unsaturation are relevant to the membrane fluidity, flexibility, and receptor function. Such features are largely involved in the membrane fusion events occurring in fertilization. Really, it would be taken into consideration that the lipid component of the spermatozoon membrane, as a part of the membrane microdomains (plasma membrane microdomains are involved in sperm motility, ability

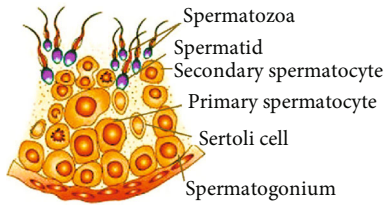

Figure 2: Spermatogenesis process: germ and Sertoli cells. The mitotic phase is represented by spermatogonia and the meiotic phase by primary and secondary spermatocytes and spermatids.

to penetrate the *zona pellucida*, and other capacitation-dependent changes), influences the membrane characteristics that are required for reaching and fusing with the oocyte. Additionally, it has been shown that the n-3 and n-6 PUFAs are essential for the reproductive activity, representing about 30% to 50% of the total FA amount in the membrane of mammal spermatozoa, and contribute to acrosome responsiveness [28].

In assessing the influence of PUFAs on male reproduction capability, the activity of metabolites generated from PUFAs (PUFA metabolism is reported above) should also be taken into consideration. On this point, prostaglandins and SPM are involved in the regulation of inflammation and infection, with these last ones being processes involved in affecting male fertility [25, 26]. Moreover, the skipped diene structure of the PUFA makes them susceptible to peroxidation and possibly alters the membrane characteristics. In this regard, reduction in human semen quality, as a consequence of smoking, infection, irradiation, varicocele, oligozoospermia, and drug exposure, has been linked to oxidative stress and lipoperoxidation [25].

3. Relevance of Fatty Acid Metabolism in Spermatogenesis

The process of spermatogenesis consists of a sequence of proliferative phases and differentiation and subsequent division to mitotic, meiotic, and spermatogenic stages (Figure 2). Each stage involves different cell types, including spermatogonia, spermatocytes, and spermatids, where lipid droplets increase throughout spermatogenesis [29]. Such phenomena demonstrate an intimate association between lipid metabolism alterations and fertility during spermatogenesis. FAs accumulate in testicular cells through two distinct processes: passive diffusion through the lipid bilayer and/or protein-facilitated transport mediated by CD36 glycoprotein, which is widely expressed in Sertoli cells [30].

The Sertoli cell, which is the supporting cell of spermatogenesis (Figure 2), has an important role in the endocrine and paracrine control of spermatogenesis. Functionally, it provides the cells of the seminiferous epithelium with nutrition, conveys mature spermatids to the lumen of seminiferous tubules, secretes androgen-binding protein, and interacts with endocrine Leydig cells. Throughout spermatogenesis, a dynamic and constant alteration in the membrane lipid composition of Sertoli cells occurs [31].

Liver and testicular cells convert dietary essential FAs (LA and ALA) to derivatives ARA, EPA, DPA, and DHA by alternating steps of elongation and desaturation [25]. As mentioned, these modifications include both Δ^5- and Δ^6-desaturases and elongases (Figure 1) specifically Elovl2 and Elovl5. Particularly, germ cells are known to be rich in PUFAs, more than the Sertoli cells, while the Sertoli cells are more active in converting the EFAs to DPA and DHA than germ cells [25]. This correlates well with the high expression of Δ^5-desaturase and Δ^6-desaturase in rat Sertoli cells and low expression in germ cells [32]. Human Sertoli cells can actively convert the 18-and 20-carbon PUFAs into their 22- and 24-carbon metabolites, and somehow, the conversion of n-3 FAs into 22- and 24-carbon metabolites is preferred by these cells over n-6 FAs in the metabolism, explaining to an extent the reason for high concentration of DHA in sperm [33].

In order to keep the energy of the seminiferous tubule at homeostasis, Sertoli cells react in response to several metabolic *stimuli*, through signaling cascades. For instance, the AMP-activated kinase is responsive to the global energetic status, the hypoxia-inducible factors are sensitive to oxygen concentration, and the peroxisome proliferator-activated receptors (PPARs) are influenced by FA availability in Sertoli cells. The development of metabolic diseases, including obesity and type II diabetes mellitus, induces these changes both directly and indirectly and as a consequence affects the Sertoli cell function and eventually male reproductive health [34].

During epididymal maturation, the lipid composition of the sperm membrane is remodeled, where the level of FA saturation is increased from the caput to the cauda epididymis, while the proportion of PUFAs remains similar along the epididymis [35]. The relative content of DHA is higher in epididymal versus testicular sperm in mice [36]. In addition, DHA is concentrated on the head or tail of the sperm and the levels vary among different species, where in human, the sperm head contains higher concentration of DHA [37]. If deficient, acrosome biogenesis is halted after the release of proacrosomal granules. It is further suggested that DHA is essential for the delivery of membrane protein misplaced syntaxin 2 for proper proacrosomal vesicle fusion [38].

The incorporation of PUFAs to semen extender is very crucial as it has different effects on semen quality for different animal species [28, 39]. Furthermore, seasonal differences in sperm FAs might in part explain the dismissal of equine spermatozoa for cryopreservation and cooled storage [40] in certain time of the year. Martínez-Soto et al. [41] suggested the spermatozoa and seminal FA profile as predictors of cryopreservation success in humans. n-3 PUFA, especially DHA, content in membrane FA was shown to have a direct association with sperm motility and viability after freezing/thawing, whereas MUFA abundance was inversely correlated with these sperm parameters. The subtemperature stress on the sperm demonstrates that the procedures may be designed to modify the lipid composition and/or antioxidant capacity of the ejaculate to make it more viable when cryopreserved.

The age of mammalians also modifies the sperm PUFA content. The proportion of PUFAs, namely, DHA, in the intact sperm, seminal fluid, and sperm head was lower in

semen collected from mature bulls than that from young bulls. The finding indicates that age differentiates the rate of absorption and/or metabolism of PUFA that could influence spermatogenesis. Reduced proportions of major FAs in mature bulls might reduce membrane fluidity, which subsequently may affect the quality for cryopreservation and/or oocyte-sperm fusion through fertilization [42].

The role of FAs in spermatogenesis was confirmed also by studies on the enzymes involved in the FA metabolism. HELO 1 is an enzyme expressed in the testis and involved in the elongation of LC-PUFAs (ARA into adrenic acid (AdA) [43]).

Stearoyl-CoA desaturase 2 (SCD2) is the predominant Δ^9-desaturase in the testis, and the Sertoli cells are the main site of its expression. Furthermore, both SCD1 and SCD2, as well as Δ^5- and Δ^6-desaturases, are highly expressed in the epididymis from sexually mature rats where the desaturase expression in Sertoli cells is hormonally regulated These desaturase enzymes can be induced by insulin, dexamethasone, and follicle-stimulating hormone [32].

In recent studies, lack of dietary n-3 PUFAs affected the spermatids. FADS2-KO mice fed with a PUFA-deficient diet except LA and ALA failed to produce mature spermatids and as a result created a defect on the acrosome formation [38]. Iizuka-Hishikawa et al. [11] reported that the loss of lysophosphatidic acid acyltransferase 3 caused a drastic reduction of DHA-containing phospholipids in mouse spermatids and led to excess cytoplasm around its head, which is normally removed by surrounding Sertoli cells via endocytosis in the final stage of spermatogenesis.

The sphingolipids of rodent spermatogenic cells (spermatocytes, spermatids) and spermatozoa consist of nonhydroxylated very long-chain and 2-hydroxylated very long-chain versions of VLC (C26 to C32) PUFAs that are not present in Sertoli cells. Recently, Santiago Valtierra et al. [44] investigated the role of elongase 4 (Elovl4) and fatty acid 2-hydroxylase (Fa2h), in rat testes with postnatal development and germ cell differentiation. Spermatocytes displayed the highest Elovl4 protein levels and activity. Fa2h mRNA was shown to be produced exclusively in germ cells, in particular round spermatids. Additionally, late spermatids, which result from elongation and head shape modifications, were shown to be enriched in Fa2h protein. For this reason, the distinctive expression of Elovl4 and Fa2h is associated with the abundance of n-V and h-V in the sphingolipid of rat spermatocytes and spermatids, respectively. Previously, Zadravec et al. [45] reported that the lack of Elovl2 was associated with a complete arrest of spermatogenesis, with seminiferous tubules displaying only spermatogonia and primary spermatocytes without further developing into germinal cells in mice.

Several hormones such as luteinizing hormone (LH) and adrenocorticotropin hormone (ACTH) may potentially change unsaturated FA composition in the testis by altering the activities of the enzymes [46]. In response to LH stimulation, together with increased testosterone secretion, the stored lipid is quickly depleted. Administering ACTH was prone to modification of Δ^5-desaturase activity in testicular cells of normal mature rats. The total FA composition of the Sertoli cells isolated from ACTH-treated rats showed a significant increase in the relative percentage of LA and a decrease in 20- and 22-carbon PUFA biosynthesis suggesting an ACTH inhibitory effect on Δ^5- and Δ^6-desaturases [47].

Oxidative stress, as a serious damaging factor for male reproductive function, is particularly reputed to be a causative factor for male infertility due to its deleterious effects on the developing germ cells and sperm function [48]. In particular, free radicals and/or reactive oxygen species (ROS) are able to attack PUFAs in cell membranes altering their structure, function, and permeability. The injury induced by ROS in the germinal and testis cell membrane may lead to cell death, abnormality, and motility loss [49].

Certain oxidized PUFA metabolites, malondialdehyde [50, 51] and recently 4-hydroxynonenal [52], are suggested to be valuable biomarkers to monitor lipid peroxidation in sperm. Although useful, the application is problematic as it lacks specificity and sensitivity especially when utilized for *in vivo* measurements. Many of these limitations were resolved with the discovery of PUFA nonenzymatic oxygenated metabolites mainly isoprostanoids which are known as the intermediate products of lipid peroxidation.

Reports suggest that the type of diet potentially contributes to male fertility. Among the nutrients, supplemented carbohydrates and proteins do not have a remarkable effect on male fertility [53]. On the other hand, human and animal studies demonstrated that high intake of unsaturated, saturated, and *trans*-FAs inversely affected semen quality [54, 55].

Overall, during spermatogenesis, membrane remodeling takes place and cell membrane permeability and fluidity change. Lipids are important components of the germ cell membrane, where the volume and ratio fluctuate in different phases of spermatogenesis. Abnormal lipid metabolism can cause spermatogenic dysfunction and consequently male infertility. Furthermore, membrane lipids of germ cells are mainly composed of cholesterol, phospholipids, and glycolipids, which play critical roles in cell adhesion and signal transduction during spermatogenesis. In addition, retaining the membrane flexibility of the sperm tail is crucial for the sperm movement. High level of PUFAs in the sperm membrane assures higher fluidity of sperm cells, hence increasing the kinetic traits of the sperm [56–58].

An insight into the correlation of membrane lipid composition with spermatogenesis helps us better understand the mechanisms of spermatogenesis and provide new approaches to the diagnosis and treatment of male infertility. The sperm FA profile and the beneficial and detrimental effects of dietary FAs are the current focus of research in the field of nutrition and male reproduction. For all these purposes, the FA profile has been proposed as a marker of semen quality for patients with different semen parameters; this could be useful to obtain reference values that can be translated into the clinical practice [59].

4. Fatty Acids in Human Spermatozoa

Among the several studies on FA level in spermatozoa (Table 1), Zalata et al. [60] showed a comprehensive profiling of 26 different FAs, including SFAs, MUFAs, and n-6- and n-

Table 1: Sperm fatty acid levels in controls compared to abnormal seminal conditions.

Fatty acid category	Fatty acid	Controls	Infertile men	References
SFAs	Palmitic acid (C16:0)	19 (healthy)	26* OAT (mol% of total quantity)	Gulaya et al. [63]
		0.87 (controls)	1.92* AT, 4.44* OAT (nmol/10^6 sperm)	Khosrowbeygi and Zarghami [69]
		26.4 (normozoospermic)	37* Asthen (nmol/10^8 sperm)	Tavilani et al. [66]
SFAs	Stearic acid (C18:0)	17 (healthy)	8* Asthen (mol% of total quantity)	Gulaya et al. [63]
		13 (normal)	17* Asthen, 15.3*Oligo (gr%)	Aksoy et al. [65]
		0.49 (controls)	1.05* AT, 2.71* OAT (nmol/10^6 sperm)	Khosrowbeygi and Zarghami [69]
		4 (normal)	14* Asthen (nmol/10^8 sperm)	Tavilani et al. [66]
MUFAs	Oleic acid (C18:1)	9 (normal)	11* Oligo (mol%)	Zalata et al. [60]
		11 (normozoospermic)	13* Asthen (wt% of total)	Conquer et al. [62]
		10 (normal)	11* Oligo, 11* OA (gr%)	Aksoy et al. [65]
		0.28 (controls)	0.41* Asthen, 0.65* AT, 1.67* OAT (nmol/10^6 sperm)	Khosrowbeygi and Zarghami [69]
n-3 PUFAs	α-Linolenic acid (C18:3)	0.31 (fertile)	0.14* OAT (% of total fatty acids)	Safarinejad et al. [70]
n-3 PUFAs	Eicosapentaenoic acid (C20:5)	0.62 (fertile)	0.31* OAT (% of total fatty acids)	Safarinejad et al. [70]
n-3 PUFAs	Docosahexaenoic acid (C22:6)	9.5 (fertile)	6.55* OAT (% of total fatty acids)	Safarinejad et al. [70]
		21.5 (normal)	16* Oligo (mol%)	Zalata et al. [60]
		14 (normozoospermic)	8* Asthen (wt% of total)	Conquer et al. [62]
		16 (healthy)	5.3* Asthen (mol% of total quantity)	Gulaya et al. [63]
		25 (normal)	18* Asthen, 20* Oligo (gr%)	Aksoy et al. [65]
		0.84 (controls)	1.65* AT (nmol/10^6 sperm)	Khosrowbeygi and Zarghami [69]
		32 (normal)	17* Asthen (nmol/10^8 sperm)	Tavilani et al. [66]
		22.4 (normozoospermic)	17.5* Asthen, 15.3* Oligo, 13.6* OA	Martínez-Soto et al. [41]
n-6 PUFAs	Linoleic acid (C18:2)	3.1 (fertile)	5.22* OAT (% of total fatty acids)	Safarinejad et al. [70]
		0.2 (controls)	0.48* AT (nmol/10^6 sperm)	Khosrowbeygi and Zarghami [69]
		8.4 (normal)	4.4* Asthen (nmol/10^8 sperm)	Tavilani et al. [66]
n-6 PUFAs	Arachidonic acid (C20:4)	1.76 (fertile)	3.18* OAT (% of total fatty acids)	Safarinejad et al. [70]
		0.2 (controls)	0.40* AT, 0.65* OAT (nmol/10^6 sperm)	Khosrowbeygi and Zarghami [69]

SFAs: saturated fatty acids; MUFAs: monounsaturated fatty acids; PUFAs: polyunsaturated fatty acids; Asthen: asthenozoospermia; AT: asthenoteratozoospermia; OAT: oligoasthenoteratozoospermia; OA: oligoasthenozoospermia. *Significant difference between the controls and infertile group. Control definition and units of measure are reported as indicated in the related reference sources.

3-PUFAs. Most of the investigations included a limited number of patients and/or analyzed FAs in sperm [61–67], or FA content was compared between spermatozoa and seminal plasma [37, 63, 68]. In such comparisons, Zerbinati et al. [59] showed that DHA was 6.2 times higher in the corresponding isolated spermatozoa than in seminal plasma from normozoospermic samples, while myristic, palmitic, palmitoleic, vaccenic, linoleic, eicosadienoic, dihomo-γ-linolenic, and docosapentaenoic acids were about 2.0 times higher in spermatozoa compared to seminal plasma. In contrast, behenic, lignoceric, oleic, and mead acids were lower in spermatozoa compared to seminal plasma.

In human semen, about thirty FA molecular species were identified [59] ranging between SFAs, MUFAs, and PUFAs (n-6 and n-3 PUFAs), which have been shown to be specifically associated with sperm parameters. In particular, distinct FA compositions were related to specific seminal conditions (Table 2).

Several studies investigated sperm FA proportion comparing fertile and infertile subjects or normozoospermic and nonnormozoospermic individuals. Khosrowbeygi and Zarghami [69] measured elevated levels of palmitic, stearic, oleic, linoleic, arachidonic, and DHA in spermatozoa from patients with modified sperm parameters compared to

Table 2: Seminal fatty acid levels in normozoospermia compared to abnormal seminal conditions.

Fatty acid category	Fatty acid (common name and number of carbons and double bonds)	Fatty acid contents[§]			
		Normozoospermia	OAT	AT	Varicocele
SFAs	Myristic acid (C14:0)	0.42	0.23**	0.26**	0.27**
	Palmitic acid (C16:0)	23.97	18.98**	19.58**	20.67**
	Stearic acid (C18:0)	22.05	25.53**	24.30**	23.92**
	Arachidic acid (C20:0)	1.16	1.58**	1.53**	1.26
	Behenic acid (C22:0)	0.71	0.29	0.05	0.05**
	Lignoceric acid (C24:0)	0.08	0.1	0.09	0.07
	Cerotic acids (C26:0)	0.09	0.10	0.09	0.12
MUFAs	Myristoleic acid (C14:1n-5)	0.12	0.23	0.11	0.18
	Palmitoleic acid (C16:1n-7)	0.34	0.24**	0.25**	0.28
	Vaccenic acid (C18:1n-7)	3.39	3.40	3.20	3.61
	Oleic acid (C18:1n-9)	16.61	22.44	19.21	17.53
	Gondoic acid (C20:1n-9)	1.40	2.39**	2.04**	1.84*
	Erucic acid (C22:1n-9)	0.05	0.41**	0.43**	0.45**
	Nervonic acid (C24:1n-9)	0.28	0.23	0.06	0.05
n-3 PUFAs	α-Linolenic acid (C18:3)	0.32	0.48	0.40	0.41
	Eicosatrienoic acid (C20:3)	0.11	0.15	0.09	0.07
	Eicosapentaenoic acid (C20:5)	0.40	0.21	0.11	0.11
	Docosapentaenoic acid (C22:5)	0.72	0.43	0.65	0.61
	Docosahexaenoic acid (C22:6)	12.82	3.42**	7.67**	8.33**
n-6 PUFAs	Linoleic acid (C18:2)	3.59	3.22	3.67	3.67
	γ-Linolenic acid (C18:3)	0.05	0.13	0.11	0.12**
	Eicosadienoic acid (C20:2)	0.65	0.63	0.81	0.79
	Dihomo-γ-linolenic acid (C20:3)	4.11	3.54	4.07	4.11
	Arachidonic acid (C20:4)	4.77	5.59	5.51	5.23
	Docosadienoic acid (C22:2)	0.10	0.20*	0.16*	0.18*
	Adrenic acid (C22:4)	0.85	0.93	0.91	0.93
	Osbond acid (C22:5)	0.84	1	1.01	1.05

SFAs: saturated fatty acids; MUFAs: monounsaturated fatty acids; PUFAs: polyunsaturated fatty acids; AT: asthenozoospermia; OAT: oligoasthenoteratozoospermia; controls compared to AT, OAT, and varicocele (*$P < 0.01$, **$P < 0.001$). [§]Fatty acid contents are reported as percentage of total FAs by weight for samples of whole seminal fluid and are referred to Zerbinati et al. [59].

normozoospermic subjects. Safarinejad et al. [70] found higher levels of n-6 PUFAs (LA and ARA) but lower levels of n-3 PUFA (ALA, EPA, and DHA) in spermatozoa and in blood plasma of infertile compared to fertile men. Other authors reported a lower seminal n-6/n-3 ratio in fertile men compared to the infertile ones, probably due to a significantly high amount of total n-3 PUFAs [41]. Recent investigation supports this observation where total n-3 PUFAs of normozoospermic individual's semen were significantly higher than those from men with oligozoospermia, asthenozoospermia, and oligoasthenozoospermia [71]. In addition, in normozoospermic subjects, it was shown that about 50%, 30%, and 20% of the total FAs were composed of SFAs, PUFAs, and MUFAs, respectively. Notably, four specific FAs (palmitic, stearic, and oleic acids and DHA) accounted for 74% of the total FA mass (palmitic acid, 24%; stearic acid, 22%; oleic acid, 16%; and DHA, 12%), and the single amount of DHA corresponded to 43% of the total PUFA content [59]. In this regard, in human sperm cells, DHA and palmitic acid were shown to be the predominant PUFA and SFA, respectively [37].

The amount of PUFAs, particularly DHA, in the sperm membrane augments as the sperm matures; it represents 20% of FA content in mature sperm compared with only 4% in immature germ cells [72]. Additionally, Haidl and Opper [73] reported a higher percentage of PUFAs in human sperm recovered from the cauda than in that recovered from the caput epididymis. Moreover, sperm DHA content has been positively correlated with sperm motility [63]. Many studies reported a high concentration of DHA in spermatozoa of normozoospermic subjects, but the concentration widely ranges from 4% to 30% [59, 65, 66, 74]. Calamera et al. [64] showed no differences in sperm DHA levels between normozoospermic and asthenozoospermic subjects.

Measurement of SFAs in seminal plasma showed that sperm concentration was positively correlated with palmitic

acid but negatively correlated with stearic acid and elaidic acid [37, 68]. Seminal stearic acid was also negatively correlated with sperm motility [59]. Of the SFAs, palmitic acid was found to be the major type in human spermatozoa [60, 75]. Moreover, stearic acid but not palmitic acid was higher in oligozoospermic and asthenozoospermic subjects, compared to normozoospermics [59].

Discrepancies in the reported levels of FAs may be explained by differences in the methods of sperm preparation and/or method of measurement. Of note, it has been suggested that the dietary habits (both FAs and antioxidants) could deeply affect the FA profile of sperm [33].

Nonetheless, lifestyle and health status were suggested to affect FA sperm level; however, no association was found between the seminal FA profile and smoking habit [59], but negative correlations between the body mass index and levels of spermatozoon DHA and palmitic acid were reported [76].

To understand the role of the FA profile in male infertility, FA quality and quantity should be investigated in different pathological conditions such as anatomical or genetic abnormalities, systemic or neurological diseases, and infections. Varicocele remains the most common diagnosis seen in infertile men [77]. In this regard, a consistent reduction of DHA levels was found by Tang et al. [74] in infertile men with varicocele compared to fertile men, and Zerbinati et al. [59] observed that a group of patients with varicocele had a reduced number of sperm and motility with a modified seminal FA profile compared with the normozoospermic group. The varicocele group also showed significantly higher levels of elaidic acid, compared to normozoospermic individuals.

In summary, the data mentioned above make clear the relevant role of FAs in sperm function and suggest them as markers of sperm alterations. Our research group has pointed out the possible relation of FAs and sperm pathologies [78]. We investigated three groups of men: fertile, idiopathic infertility, and infertile with varicocele. Infertile men had higher levels of semen ROS than fertile men. High levels of semen ROS can cause sperm dysfunction, DNA damage, and reduced male reproductive potential [78]. Spermatozoa are susceptible to nonenzymatic oxidative damage because the plasma membranes are rich in PUFAs thus generating the prostaglandin-like end product known as isoprostanes (IsoPs). The infertile varicocele group, despite having a similar low sperm quality as idiopathic infertile patients compared to fertile men, had increased seminal levels of F_2-isoprostanes (F_2-IsoPs), a specific class of IsoPs, and high percentage of sperm immaturity; this suggests that an appropriate FA composition is needed for sperm maturation [79]. The association of sperm immaturity and high levels of seminal F_2-IsoPs has been detected also in a patient carrier of round-headed sperm, a systematic sperm defect characterized by round nuclei with immature chromatin [80].

Compared to PUFAs, *trans*-FAs are associated with sperm quality in a different manner. Chavarro et al. [81] reported that semen levels of *trans*-FAs are inversely related to sperm concentration, and Zerbinati et al. [59] showed that oligoasthenozoospermic men had higher levels of seminal elaidic acid compared to normozoospermic subjects. As previously reported, they also found upregulated levels of elaidic acid in varicocele that could have deleterious consequences in these patients.

In conclusion, different FA contents in spermatozoa and seminal plasma have been described in individuals with pathological conditions compared to fertile men. It can be surmised that the FA profile could represent a good marker in male infertility and proper dietary integration of FAs may be a potential therapy for infertility.

5. Spermatozoa, Fatty Acids, and Diet

A recent metaregression analysis reported a significant decline in total sperm counts between 1973 and 2011 globally [82]. These data strongly suggest a notable decline in male reproductive health, with crucial implications for human reproduction and perpetuation of the species. Investigating modifiable lifestyle factors that influence human fertility is of major clinical and public health importance for understanding the problem [83]. Indeed, several observational studies that explored the associations between dietary patterns, food and nutrient consumption, and sperm quality suggest that adhering to a healthy diet (e.g., the Mediterranean diet) may improve male sperm quality parameters [84].

As noted, testis maturation, germ cell development, and function of sperm are related to lipid composition. PUFAs cannot be endogenously synthesized by humans and therefore must be obtained from food such as nuts, seeds, vegetable oils (source of LA and ALA), seafood (source of EPA and DHA), and meat and dairy (source of ARA). Dietary FAs influence the sperm FA profiles, and it appears to be the most sensitive to dietary n-3 PUFAs (ALA, EPA, and DHA) [33]. Consuming these foods modified the semen quality and FA sperm composition [33], whereas increased intake of SFAs or *trans*-FAs is reported to lower male reproductive ability in humans and animals [85]. Jensen et al. [86] observed that 701 young Danish men from the general population have a dose-response association between increased intake of saturated fat and decreased total sperm count and sperm concentration. In addition, a diet supplemented with fish oil increased DHA in the testis of rodents [87] and accumulated in the sperm membrane of humans [67]. In fertile individuals, administration for 4 weeks of high level of menhaden oil (50 ml) rich in DHA+EPA on a daily basis [88] had no effect on sperm motility, but semen phospholipid EPA increased.

Conquer et al. [89] studied serum and spermatozoon concentration of FAs and spermatozoon motility in asthenozoospermic men. In this double-blind, randomized, placebo-controlled study, both 400 and 800 mg/day DHA regimens increased serum DHA concentration but unaffected the spermatozoon DHA concentration and motility. In general, infertile men had lower concentrations of n-3 FAs in spermatozoa than fertile men [70] while it is suggested that oligoasthenoteratospermic men with low levels of EPA and DHA may benefit from n-3 FA supplementation [67]. After 32-week supplementation of 1.84 g of EPA (0.72 g) plus DHA (1.12 g) per day, oligoasthenoteratozoospermic men with low levels of EPA and DHA showed increased spermatozoon number, motility, and morphology,

but the treatment had no effect on semen volume or serum sex hormone concentrations [67]. Supplementation with DHA+EPA (990 mg/d and 135 mg/d, respectively) for 10 weeks to healthy subjects demonstrated no effect on sperm parameters but prevented DNA fragmentation [90]. Esmaeili et al. [37] found improvement of male sperm parameters after 4 weeks of n-3 PUFA diet, and the response was time-dependent and dose-dependent. Recently, González-Ravina et al. [91] demonstrated the importance of DHA supplementation as a means of improving sperm quality in asthenozoospermic men.

Recent gene knockout strategies as well as analyses of human genetic disorders have unveiled several important molecules involved in the uptake and trafficking of DHA; however, the mechanism of how the lipid profile affects the male reproductive system is not well understood [11].

It should be underlined that the sperm membrane enriched of long-chain PUFAs is more prone to oxidation [92, 93] due to the numerous skipped diene formations in the structure. Accordingly, for a better response in the intervention studies, it is probably more effective to supplement the subjects with a strong antioxidant with high-dose PUFA to avoid unnecessary oxidation.

In general, an increased content of n-3 PUFAs is expected to influence the regulation of PPARγ, apoptosis, eicosanoid formation, and hormone activity [37]. It has been reported that the inclusion of nuts in a Western diet significantly improved the total sperm count, vitality, motility, and morphology, and it was explained by the reduction in sperm DNA fragmentation [84]. *In vitro* and animal studies have shown that n-3 PUFAs are important substrates in early reproductive events, including improved fecundity, oocyte maturation, and embryo implantation [94, 95], and aid in restoring fertility and spermatogenesis in male rodents [96].

Several dietary studies related to PUFA supplementation have also demonstrated their capability to sustain sperm motility, viability, and fertility during chilling and freezing as well as improving testis development and spermatogenesis in a variety of livestock species [28]. In rats fed with a high-fat diet, olive oil (a source of MUFAs) and krill oil (a source of n-3 PUFAs) partially counteracted the negative effects of a high-fat diet and improved sperm quality, by increasing gamete motility, reducing oxidative stress, and slightly improving mitochondrial respiration efficiency [97]. Dietary supplementation with pomegranate seed, containing the PUFA punicic acid (18:3, n-5), in cloned goats improved sperm motility and viability following freezing-thawing and maintains developmental competency [98]. A diet enriched with vitamin E, zinc, selenium, folic acid, and n-3 PUFAs for at least two months improved sperm quantity and quality, especially sperm count and motility, and modified physical and functional properties of the sperm cell membrane in healthy dogs [57]. Studies on a rabbit model showed that the dietary supplementation of n-3 PUFAs and antioxidants [99, 100] largely altered the sperm membrane and improved the motility rate and the sperm speed. Moreover, rabbits treated with a diet supplemented with 10% of extruded flaxseed or 3.5% of fish oil showed a higher distribution of DHA and EPA in the testes and sperm membranes compared to controls [101].

In addition, in mice, either excessively high or insufficient n-3 PUFA consumption prior to conception until adulthood may cause adverse long-lasting effects on reproductive maturation and function of the progeny [102]. Finally, in the Seba's short tailed bat (*Carollia perspicillata*) concomitant to an increase in sperm velocity, the level of FA saturation increased from the caput to the cauda epididymis, while the proportion of PUFAs remained similar along the epididymis. Food treatments did not affect the sperm FA composition suggesting the presence of a specific endogenous mechanism [35].

Despite the relevance of n-3 PUFAs in male fertility, as reported above, it has been shown that PUFAs with 24–30 carbon atoms of the n-6 family in the testis are indispensable for normal sperm formation and fertility in male mice and that the investigated changes in n-6 fatty acid composition cannot be compensated by increased C22:6n-3 content [45].

Dietary fats may influence testicular function. However, most of the published literature on this field used semen quality parameters as the only proxy for testicular function. Minguez-Alarcón et al. [103] reported in healthy young Spanish men that MUFA intake was inversely associated with serum blood levels of testosterone and inhibin B whereas a positive association was observed between the intake of n-6 PUFAs and LH concentrations. In addition, the intake of *trans*-FAs was associated with lower testosterone. The intake of n-3 PUFAs was positively related to testicular volume while the intake of n-6 PUFAs and *trans*-FAs was inversely related to testicular volume.

Rats fed with an EFA-deficient diet developed testicular atrophy, and inclusion of LA did not prevent this incident and in fact they became infertile [104]. Separation of Sertoli cells and germ cells from rats fed with a fat-free diet for 9–14 days showed a shift in the lipid profile of both cell types towards a typical EFA deficiency pattern [105].

EFA deficiency has been associated with induced FA desaturase expression and activity in several tissues, but in the testis of sexually mature rats, none of the desaturases (SCD1, SCD2, Δ^{5}-desaturase, or Δ^{6}-desaturase) were induced in response to lowered contents of PUFAs [106]. This also applied to the caput epididymis, while EFA deficiency sensitivity was regained in the cauda epididymis; the desaturases were upregulated. A significant increase in the number of abnormal spermatozoa was observed in the cauda epididymis. It is suggested the alterations may be caused by the distortion of FA distribution in the spermatozoa, as well as in the epididymal tissue. On the other hand, a low-fat diet has been shown to decrease serum levels of androgens in human [107]. Thus, it cannot be excluded that the increased sperm abnormalities observed is an androgen-dependent effect induced by the diet [106].

Opposite to PUFAs, *trans*-FAs and SFAs appeared to have an effect on spermatogenesis. The association between *trans*-FAs, infertility, and fetal life has been reviewed by Çekici and Akdevelioğlu [108]. *trans*-FAs are found in commercially baked and fried foods, which accumulate in the testis, and high consumption is related to poor semen quality [33]. Previous studies reported [54, 55] that dietary *trans*-FA intake may be related to lower semen quality that

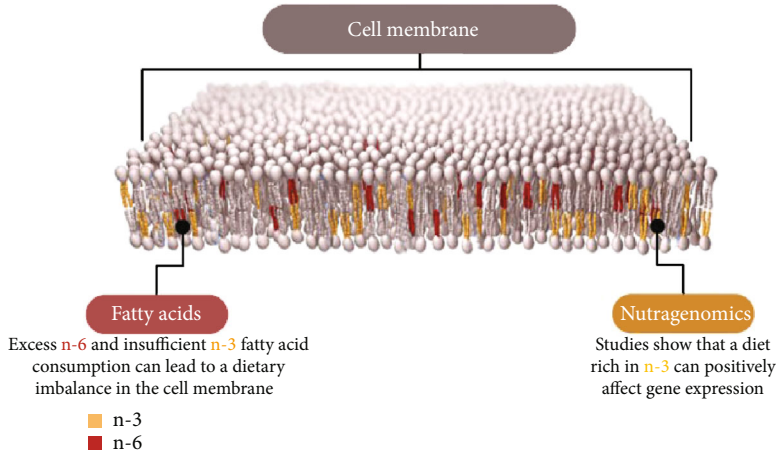

Figure 3: Incorporation of dietary n-3 and n-6 fatty acids in the cell membrane.

eventually becomes linked to the ability of *trans*-FAs to inhibit the activity of desaturases and, as a consequence, limit the incorporation of LC-PUFAs into sperm membranes [109]. Eslamian et al. [53] conducted a case-control study to investigate the association of FA intakes and asthenozoospermia. They found that high intake of SFAs and *trans*-FAs was positively related to the odds of having asthenozoospermia. Dietary intake of n-3 PUFAs, but not of MUFAs and n-6 PUFAs, was inversely associated with asthenozoospermia.

Furthermore, dietary *trans*-FAs in the male are reported to decrease the chance of fertilization [110]. A study conducted on 141 couples undergoing assisted reproduction techniques reported that sperm from men with the highest *trans*-FA intake (1.20% of total energy intake) gave the lowest rate of fertilization. *trans*-FA intake correlated positively with low total testosterone and calculated free testosterone concentration but had a negative correlation with testicular volume [110] suggesting an effect on testicular function. Men in the top 25% of *trans*-FAs intake have been reported to have 37% lower total sperm count, 15% lower testosterone levels, and 4% less testicular volume than men with the lowest *trans*-FA consumption [103]. Similarly, *trans*-FA exposure in male mice caused *trans*-FA accumulation in the testes leading to lower serum testosterone concentrations and sperm count. Inhibition of spermatogenesis and testicular degeneration are severe reproductive disorders associated with *trans*-FAs in rodents [111, 112].

trans-FAs may affect semen quality that can also involve and influence PPARs. These have some similarity with steroid and thyroid hormone receptors, which are ligand-activated nuclear transcription factors. Both PPARα- and PPARγ-responsive genes are involved in lipid homeostasis, especially glucose and lipid homeostasis. *trans*-FAs inhibit the primary function of PPARγ on sperm metabolism by downregulating PPARγ mRNA expression. Such adverse effects of *trans*-FAs have been claimed to be responsible for infertility [37].

Given that lipids are composed of majority of the sperm plasma membrane, this information may open new possibilities for the development of male diagnostic tools [113].

6. Conclusions and Perspectives

As single molecules or as components of molecules, FAs play multiple biological roles ranging from participation in cell membrane composition to energy suppliers and signaling molecules [114].

FAs, available for cellular function and membrane composition, can derive from exogenous sources or *de novo* synthesis. In particular, dietary sources of ALA, DHA, and EPA are crucial to maintain an adequate supply in n-3 PUFA metabolism [114, 115].

FAs, as a component of membrane lipid, are implicated in the modulation of biomembranes, and PUFAs heavily influence membrane permeability and elasticity (Figure 3). Thus, membrane PUFA composition plays a relevant role in different processes such as vesiculation, lipid flip-flops, and last but not least lipid-protein interactions. In particular, an increase in EPA and DHA is suggested to modify membrane stability and the composition in membrane-associated proteins by decreasing the MUFA/PUFA ratio. Such effects could be linked to the relevance of hydrocarbon chain length or *trans*-/*cis*-double bond and maintenance of the lipid array. Moreover, FAs work as energy suppliers and storage of lipophilic compounds. Both of these lipid features are connectable to cellular and spermatic biology [116].

Spermatogenesis is a complex process that involves the development of spermatozoa in the seminal tubules. The differentiation of spermatogonia into spermatozoa requires the participation of several cell types and the correct FA profile that contributes to a normal spermatogenetic process [117]. The importance of lipid composition, especially phospholipids, in the plasma membrane and semen plasma for spermatozoon function has since long been recognized [72]. PUFA level influences sperm maturation, motility, and acrosome reaction [118], and men with different seminal characteristics due to reproductive pathologies such as varicocele, infections, or others had shown different FA profiles [59]. Particularly, PUFAs may modulate oxidative stress, ROS production, and the inflammatory processes in spermatogenesis.

Sperm FA profiles and the beneficial and detrimental effects of dietary fatty acids are the current focus of research in the field of nutrition and male reproduction. In humans, diet is difficult to standardize, and research is mainly focused on the effect of dietary changes on male reproduction traits using an *in vitro* approach that does not take into account the dietary effect on spermatogenesis or on animal models.

Deep knowledge of how dietary lipid affects sperm lipid membrane composition, which in turn is relevant for sperm functionality, could improve the comprehension of sperm plasma membrane turnover and the susceptibility to oxidative damage. Such information will help to develop personalized nutraceutical treatments to improve male reproductive efficiency.

Abbreviations

FAs:	Fatty acids
SFAs:	Saturated FAs
MUFAs:	Monounsaturated FAs
PUFAs:	Polyunsaturated FAs
DPA:	Docosapentaenoic acid
DHA:	Docosahexaenoic acid
LA:	Linoleic acid
ALA:	Alpha-linolenic acid
EFAs:	Essential fatty acids
EPA:	Eicosapentaenoic acid
ARA:	Arachidonic acid
Elovl:	Elongation of very long-chain fatty acid protein
Δ^5-Desaturase:	Delta-5 desaturase
Δ^6-Desaturase:	Delta-6 desaturase
Δ^9-Desaturase:	Delta-9 desaturase
FADS1:	Fatty acid desaturase 1
FADS2:	Fatty acid desaturase 2
COX:	Cyclooxygenases
LOX:	Lipoxygenases
SCD:	Stearoyl-CoA desaturase
LH:	Luteinizing hormone
ACTH:	Adrenocorticotropin hormone
ROS:	Reactive oxygen species
trans-FAs:	*trans*-Fatty acids
PPAR:	Peroxisome proliferator-activated receptors.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Research Article

Effect of Different Exercise Loads on Testicular Oxidative Stress and Reproductive Function in Obese Male Mice

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This study is aimed at investigating the effect of different exercise loads on the reproductive function of obese male mice and the underlying mechanisms. Male mice with high-fat diet-induced obesity were divided into obesity control (OC), obesity moderate-load exercise (OME), and obesity high-load exercise (OHE) groups. The OME and OHE groups were subjected to swimming exercise 5 days per week over a duration of 8 weeks, with the exercise load progressively increased to 2 h per day in the OME group and 2 h twice per day in the OHE group. In the OC group mice without exercise regimen, we observed a decrease in mRNA expression of antioxidant enzymes, increase in free radical products, upregulation of mRNA and protein expression of nuclear factor-κB and proinflammatory cytokines, inhibition of mRNA and protein expression of testosterone synthases, decrease in the serum testosterone level and sperm quality, and increase in sperm apoptosis. Although both moderate-load exercise and high-load exercise reduced body fat, only moderate-load exercise effectively alleviated obesity-induced oxidative stress, downregulated the expression of nuclear factor-κB and proinflammatory cytokines, and reversed the decrease in mRNA and protein expression of testosterone synthases, serum testosterone level, and sperm quality. These changes were not observed in the OHE group mice. Obesity-induced testicular oxidative stress and inflammatory response decreased testosterone synthesis and sperm quality. Moderate-load exercise alleviated the negative effect of obesity on male reproductive function by decreasing testicular oxidative stress and inflammatory responses. Although high-load exercise effectively reduced body fat, its effects on alleviating oxidative stress and improving male reproductive function were limited.

1. Introduction

Over the last four decades, the number of people with obesity worldwide has increased rapidly from 105 million in 1975 to 641 million in 2014 [1]. In addition, infertility rates have increased parallelly with obesity rates [2, 3]. In some countries with high obesity incidence, monitoring of the total sperm count and sperm motility in males indicated an annual decrease of 1.5% [4, 5]. Increasing evidence suggests that obesity damages reproductive health in males and causes late-onset male hypogonadism [6, 7], which is characterized by low serum testosterone levels and relevant symptoms (poor libido, erectile dysfunction, diminished sperm quality parameters, and reproductive dysfunction) [8–10]. The mechanisms through which obesity affects male reproductive function are complex. Previous reports indicate that oxidative stress [11] and inflammatory responses [12–14] are associated with impaired function of Leydig cells. Furthermore, according to human [15] and animal studies [16], when oxidative stress and inflammatory responses in the semen of obese males are increased, the sperm motility is reduced, morphological defects are increased, and DNA damage and apoptotic rate of germ cells are increased [17]. However, it remains unclear whether there is a correlation between obesity, oxidative stress, and inflammatory response.

The effects of exercise on weight loss and body fat reduction are well-known, and exercise load is known to be positively correlated with body fat reduction; however, reports

about the effects of exercise-mediated body fat reduction on improvement in male reproductive function are inconsistent [18, 19]. We have previously reported that 8 weeks of moderate- or high-load exercise effectively reduced body fat, but the negative effects of obesity on male reproductive function were alleviated only by moderate-load exercise and not by high-load exercise [20]. Studies have shown that exercise load is closely related to oxidative stress [21]. Low-load exercise does not cause oxidative stress injury. Moderate-load exercise increases free radicals associated with an increased oxygen intake; as a positive adaptive response, it can also stimulate the expression and activity of antioxidant enzymes and enhance the body's antioxidant capacity [22]. However, due to an increased oxygen consumption during the heavy-load exercise, a large number of free radicals is produced through various mechanisms. Since the free radicals accumulate in excess, it exceeds the body's ability to resist oxidative stress, attacks biological macromolecules and membrane structures, and causes oxidative damage to the body that could be linked to exercise-related hypoandrogenemia and diminished sperm quality [23]. Therefore, we hypothesized that the inconsistencies in the effect of different exercise loads are related to oxidative stress and the inflammatory response. This study provides an experimental basis to further determine the mechanisms by which exercise and obesity affect male reproductive function, and it also provides a theoretical basis to develop effective prevention methods.

2. Materials and Methods

2.1. Animals. Fifty male C57BL/6L mice (age, 4 weeks; weight, 16-19 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) under permit number SCXK (Beijing) 2016-0006. All mice were housed under controlled experimental conditions ($22 \pm 5^{\circ}C$, $50 \pm 10\%$ relative humidity, and 12 h light/12 h dark cycle) and provided with food and water ad libitum. Each cage contained no more than five mice. All animal experiments in this study were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Animal Ethics Committee of Shenyang Sport University.

2.2. Obese Mouse Model. Ten mice were randomly selected to form a normal control (NC) group that was provided a normal diet (ND), while the remaining 40 mice were fed with a high-fat diet (HFD). The ND and HFD were formulated according to previously reported nutrient formulations [16], and the feed formulations were provided by Jianmin Company Ltd. (Shenyang, China). After 10 weeks of feeding, six obesity-resistant mice were eliminated from the HFD group, while the remaining mice reached body weights of more than 120% of the mean body weight of the NC group mice, thereby satisfying the criteria for an obese animal model [16]. The mice that satisfied the criteria were stratified according to their body weight and randomly assigned to the following three groups: obesity control (OC), 10 mice; obesity moderate-load exercise (OME), 12 mice; and obesity high-load exercise (OHE), 12 mice. Differences in the body weights of mice within the three groups were not significant ($P > 0.05$).

2.3. Exercise Intervention. The mice in the OME and OHE groups were subjected to 8 weeks of exercise intervention, which involved free swimming without interference in a plastic pool of diameter of 45 cm, water depth 60 cm, and water temperature of $32 \pm 1^{\circ}C$. A previously described exercise program [16] was adopted, which consisted of 2 days of acclimatization training followed by 8 weeks of proper swimming training. The exercise load was progressively increased during the training period, with an initial duration of 20 min once per day in the OME group and 20 min twice per day (6 h interval between the two sessions) in the OHE group. During weeks 1 and 2, the training time was increased in increments of 10 min until reaching 120 min per day and 120 min twice per day at the end of week 2 in the OME and OHE groups, respectively. These exercise loads were maintained for the subsequent 6 weeks of training.

2.4. Sample Collection. To observe the adaptive responses of mice to long-term exercise, sample collection was performed 36–40 h after the last exercise session in the OME group and the OHE group. The mice in both groups were fasted for 12 h before sample collection to eliminate the effect of exercise-induced stress responses and diet on the various indicators. Each mouse was weighed and subsequently anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg body weight; Sinopharm Chemical Reagent Co., Ltd., Shanghai, China). Blood samples were collected from the orbital venous plexus and centrifuged for 20 min ($4^{\circ}C$, 900 g) to separate the serum, which was stored at $-80^{\circ}C$ before serum testosterone testing. Simultaneously, rapid separation of the testis and epididymis was performed, and the sperm count, motility, and apoptosis rate in the epididymis were measured and assessed [16]. The separated testis was immersed in liquid nitrogen for rapid freezing and stored at $-80^{\circ}C$ until further use. The peritesticular, perirenal, and mesenteric fat was separated and weighed on an electronic balance to determine the abdominal fat content of each mouse.

2.5. Cauda Epididymal Sperm Count and Motility Measurements. The epididymis was removed from one side of the mice and placed in 1.0 mL of HEPES buffer. The epididymis was then cut at the junction between the corpus and the cauda epididymis, and the cauda was placed in a well containing 1.0 mL of HEPES buffer. The epididymis was cut into several segments with a pair of scissors and then gently pressed to release the semen from the vas deferens to mixing with the buffer. The number of sperm per microliter was recorded using a hemocytometer (15 μL per side). Sperm count and motility were assessed in accordance with the World Health Organization guidelines (≥200 sperms were counted per sample). The sperm count was determined using a hemocytometer. Sperm motility was assessed blindly under a light microscope by classifying 200 sperms per animal as either progressive motile, nonprogressive motile, or immotile. Motility was expressed as percentage of total

Table 1: Primer sequences for real-time PCR.

Gene	Forward primer	Reverse primer
Cat	5′-ATTGCCGTCCGATTCTCC-3′	5′-CCAGTTACCATCTTCAGTGTAG-3′
Gpx1	5′-CAGTTCGGACATCAGGAGAA-3′	5′-AGAGCGGGTGAGCCTTCT-3′
Sod1	5′-ACTTCGAGCAGAAGGCAAGC-3′	5′-GTCTCCAACATGCCTCTCTTCAT-3′
Nf-kb	5′-ACCTGAGTCTTCTGGACCGCTG-3′	5′-CCAGCCTTCTCCCAAGAGTCGT-3′
Tnfα	5′-ACGGCATGGATCTCAAAGAC-3′	5′-GTGGGTGAGGAGCACGTAGT-3′
IL1β	5′-GCTGCTTCCAAACCTTTGAC-3′	5′-AGCTTCTCCACAGCCACAAT-3′
Il10	5′-CTGTCATCGATTTCTCCCCTGTG-3′	5′-TGGTCTTGGAGCTTATTAAAATCAC-3′
Nr5a1	5′-TTGGGTCAGAGGTCATCCTT-3′	5′-CAACAGTGGACTTCCTGCTTC-3′
Star	5′-TGCCGAAGACAATCATCAAC-3′	5′-CAGGTCAATGTGGTGGACAG-3′
Cyp11a1	5′-CCTTTATGAGATGGCACACAA-3′	5′-GATGCTGGCTTTGAGGAGTG-3′
Gapdh	5-GACAACTTTGG-CATTGTGGA-3′	5′-ATGCAGGGATGATGTTCTGG-3′

motile sperm (progressive motile and nonprogressive motile sperms) [16].

2.6. Hormone Measurement. Samples for concentration measurement of serum total testosterone were processed using a commercial enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's protocol, and their absorbance was measured using a Multiskan GO 1510 (Thermo Fisher Scientific, Waltham, MA, USA). The ELISA kit was supplied by R&D Systems (Minneapolis, MN, USA). The detection limit of the testosterone kit was 0.75–24 ng/mL. The intra-assay coefficient of variation (CV) was less than 10%, and the interassay CV was less than 15% for the ELISA kit. All measurements were conducted in the Key Laboratory of Exercise Science of Shenyang Sport University.

2.7. Measurement of Sperm Apoptosis. After membrane removal, the other epididymis of each mouse was cut into pieces and incubated in saline at 37°C for 10 min to disassociate the sperm. The sample was centrifuged at 400 *g* for 5 min after filtering, and then the supernatant was discarded to collect the cells. Phosphate-buffered saline was added to form a sperm suspension, and 5 μL of Annexin V-fluorescein isothiocyanate and 5 μL of propidium iodide were added. The suspension was then mixed gently and incubated at 20°C away from light for 10 min. Measurements were performed using a flow cytometer (CytoFLEX; Beckman Coulter, Brea, CA, USA) within 1 h, with a minimum of 10000 spermatozoa examined during each measurement. Forward scatter/side scatter gating was adopted to eliminate disturbances from cell debris and cell aggregation. After sorting the spermatozoa and cell debris using scatter signals, the live, dead, and cells were distinguished on a bivariate fluorescent signal scatter plot. The excitation wavelength used was 488 nm; green fluorescence (480–530 nm) was detected using the FL1 channel, while red fluorescence (580–630 nm) was detected using the FL2 channel. The positive cell rate and mean fluorescence intensity were analyzed using CellQuest software (BD Biosciences, Franklin Lakes, NJ, USA). The percentage of early apoptotic sperm relative to the total sperm count was calculated for each group.

2.8. Determination of Oxidative Stress in Testicular Tissue. Testicular homogenate of 5% or 10% was prepared and centrifuged. Subsequently, the following indicators with reference to the product specifications of Nanjing Jiancheng Bioengineering Institute were determined using the supernatant: nitric oxide (NO; nitrate reductase method), nitric oxide synthase (NOS; colorimetric method), reduced glutathione (GSH), glutathione peroxidase (GSH-PX; colorimetric method), malondialdehyde (MDA; TBA method), catalase (CAT; visible light), hydrogen peroxide (H_2O_2), total superoxide dismutase (T-SOD; hydroxylamine method), and total antioxidant capacity (ABTS method).

2.9. Isolation of RNA and Real-Time PCR Analysis. An RNA extraction agent (Vazyme Biotech Co., Ltd., Nanjing, China) was used to extract total RNA from the testis of each mouse according to the manufacturer's instructions. Subsequently, a reverse transcription kit (Promega, Madison, WI, USA) was used for the reverse transcription of 1 μg of total RNA to cDNA in a 96-well thermal cycler (Applied Biosystems, Foster City, CA, USA). The target mRNA content was measured in a real-time PCR amplification system (Applied Biosystems) using a real-time PCR amplification kit (Promega) in accordance with the manufacturer's instructions. All primers were designed and synthesized by Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China). The primer sequences for the target genes are shown in Table 1. Each sample was amplified in triplicate; GAPDH was used as the housekeeping gene, and the $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression levels.

2.10. Western Blotting. After weighing the separated testis from each mouse, RIPA lysis buffer and phenylmethylsulfonyl fluoride, a protease inhibitor, were added, and the testis tissue was cut into pieces and homogenized in an ice bath. The homogenate was centrifuged, and the supernatant was removed for protein quantification in a microplate reader

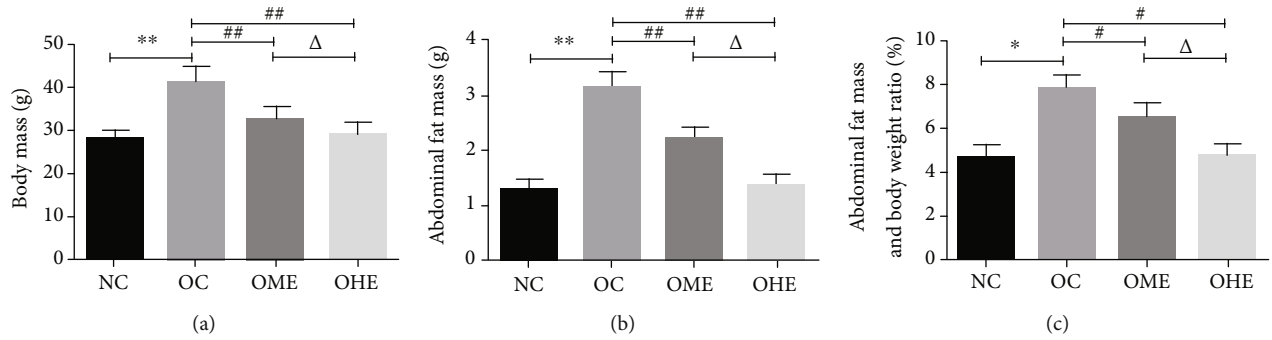

Figure 1: Effect of high-fat diet and exercise on body weight and abdominal fat content. Data are mean ± SE; NC: normal control; OC: obesity control; OME: obesity moderate exercise; OHE: obesity high exercise, vs. NC: $^{*}P < 0.05$, $^{**}P < 0.01$; vs. OC: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$; vs. OME: $^{\triangle}P < 0.05$, $^{\triangle\triangle}P < 0.01$.

(Thermo Fisher Scientific) using the BCA protein assay kit (Beijing Dingguo Changsheng Biotechnology Co., Ltd., Beijing, China). The target protein was separated by sodium dodecyl sulfate gel electrophoresis; 30–50 μL of protein lysis buffer was added to each well. The separated target protein and the internal control β-actin were transferred onto a nitrocellulose membrane and blocked for 1 h using 5% nonfat dry milk blocking buffer. After adding the primary antibody (rabbit anti-mouse; ABclonal, Wuhan, China), the nitrocellulose membrane containing the target protein was incubated overnight (12 h) at 4°C. The target proteins included NF-κB (ABclonal, A2547), tumor necrosis factor- (TNF-) α (11948; Cell Signaling Technology, Danvers, MA, USA), IL-1β (12426; Cell Signaling Technology), IL-10 (5261; Cell Signaling Technology), SF-1 (10976; Santa Cruz Biotechnology, Dallas, TX, USA), StAR (58013; Abcam, Cambridge, UK), P450scc (175408; Abcam), and β-actin (sc-1496; Santa Cruz Biotechnology). Subsequently, the membrane was incubated at 20°C for 1 h with the fluorescent dye-labeled secondary antibody (IRDye@ 800CW-labeled goat anti-rabbit; LI-COR, Lincoln, NE, USA) at 1 : 15000 dilution. Finally, the nitrocellulose membrane was inserted into the Odyssey infrared imaging system (LI-COR) to quantitatively analyze the protein bands using Image Studio imaging software provided with the system. The final result was reported as the target protein content/β-actin content ratio [16].

2.11. Statistical Analysis. The data are expressed as mean ± SE. Multiple group comparisons were performed by one-way analysis of variance (one-way ANOVA) followed by a Student-Newman-Keuls post hoc test to conduct multiple comparisons. The results were considered significant for P values of <0.05. These analyses were performed using SPSS 18.0 software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Effect of HFD and Exercise on Body Weight and Abdominal Fat Content. After 18 weeks of high-fat diet feeding, the body weight (Figure 1(a)), abdominal fat content (Figure 1(b)), and liposome ratio of the OC group (Figures 1(b) and 1(c)) were significantly higher than those of the NC group. After 8 weeks of exercise intervention, the body weight (Figure 1(a)), abdominal fat content (Figure 1(b)), and lipid ratio of the OME and OHE groups were significantly lower than those of the OC group; the decrease in the OHE group was higher than that in the OME group (Figures 1(a)–1(c)).

3.2. Effects of Obesity and Exercise on Testosterone Level and Sperm Quality. Compared with those in the NC group, the OC group had a significantly decreased serum testosterone level (Figure 2(a)), sperm count (Figure 2(b)), and sperm activity (Figure 2(c)), along with a significantly increased sperm apoptosis rate (Figures 2(d) and 2(f)). After 8 weeks of exercise intervention, the serum testosterone level (Figure 2(a)), sperm count (Figure 2(b)), and sperm motility of mice (Figure 2(c)) in the OME group were significantly increased, while the sperm apoptosis rate was decreased (Figures 2(d) and 2(g)). The serum testosterone level (Figure 2(a)), sperm count (Figure 2(b)), sperm motility (Figure 2(c)), and sperm apoptosis rate (Figures 2(d) and 2(h)) of the OHE group did not change significantly but were significantly higher than those of the OME group (Figures 2(a)–2(d) and 2(h)).

3.3. Effect of High-Fat Diet and Exercise on Testicular Oxidative Stress. Compared with those of the NC group, the antioxidant indexes T-AOC, CAT, GSH-Px, and GSH of the testis tissue of the OC group decreased significantly (Figures 3(a) and 3(c)–3(e)) and the oxidative stress products MDA, H_2O_2, and NO increased significantly (Figures 4(a)–4(d)). After 8 weeks of exercise intervention, the antioxidant indexes T-AOC, SOD, CAT, GSH-Px, and GSH of the OME group showed a significant recovery (Figures 3(a)–3(e)) and oxidative stress products MDA, H_2O_2, NOS, and NO showed a significant regression (Figures 4(a)–4(d)), but these changes did not occur in the OHE group (Figures 3(a)–3(e) and 4(a)–4(d)), and the values of most those indicators were significantly different from those of the OME group (Figures 3(a)–3(e) and 5(a)–5(d)).

3.4. Effect of Obesity and Exercise on the mRNA Expression of Antioxidant Enzymes. The mRNA expression of SOD and GSH-Px in the testicular tissues of the OC group was

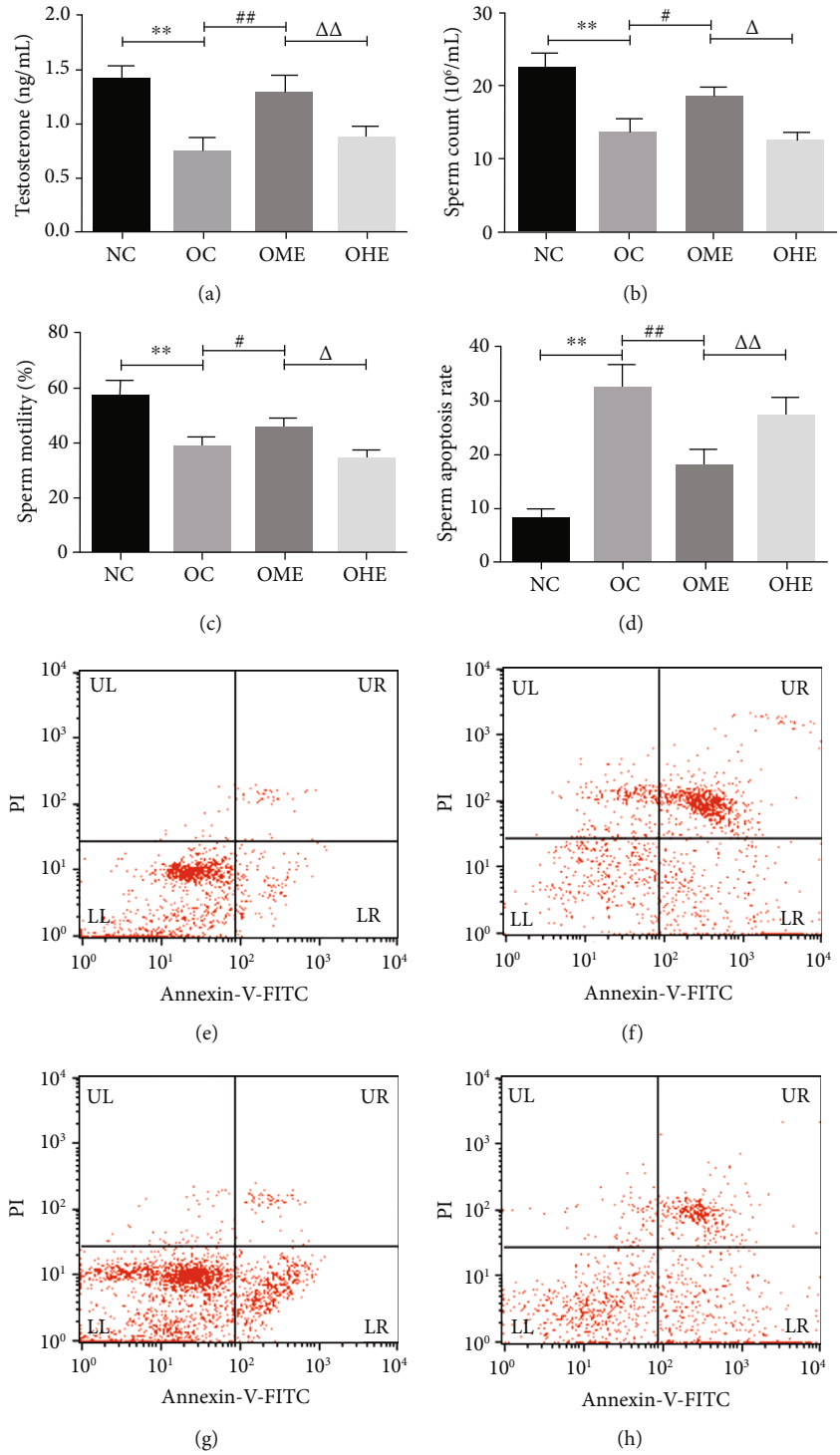

Figure 2: Effects of obesity and exercise on testosterone level and sperm quality. Data are mean ± SE; NC: normal control; OC: obesity control; OME: obesity moderate exercise; OHE: obesity high exercise. (e) Normal control (NC) group; (f) high-fat diet obesity control (OC) group; (g) obesity moderate-load exercise (OME) group; (h) obesity high-load exercise (OHE) group. Upper left (UL) region: necrotic cells; upper right (UR) region: late apoptotic cells; lower left (LL) region: live cells; lower right (LR) region: early apoptotic cells. vs. NC: $^{*}P < 0.05$, $^{**}P < 0.01$; vs. OC: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$; vs. OME: $^{\triangle}P < 0.05$, $^{\triangle\triangle}P < 0.01$.

significantly lower than of the NC group (Figures 5(a)–5(c)). After 8 weeks of moderate-load exercise, the mRNA expression of SOD and GSH-Px in the OME group showed a significant recovery (Figures 5(a)–5(c)). In the OHE group, there were no significant changes in the three antioxidant enzymes (Figures 5(a)–5(c)); there was a significant difference in the mRNA expression of SOD and GSH-Px between the OHE and OME groups (Figures 5(a)–5(c)).

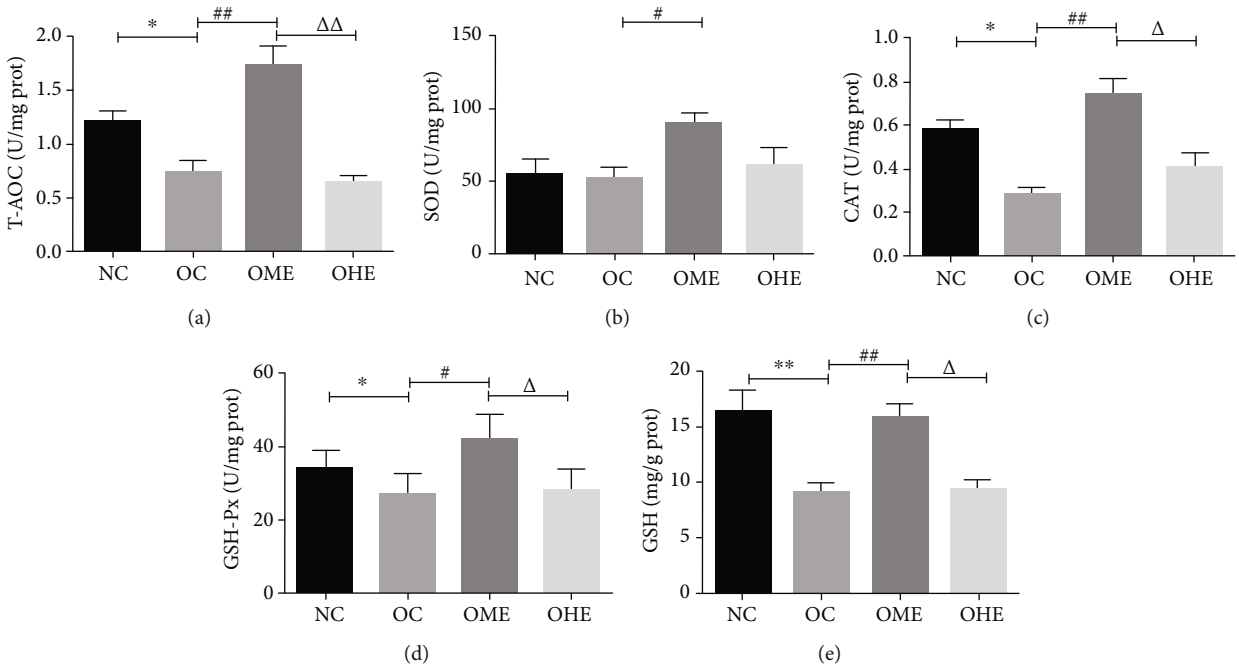

Figure 3: Influence of high-fat diet and exercise on the testicular antioxidant system. T-OAC: total antioxidant capacity; SOD: superoxide dismutase; CAT: catalase; GSH-Px: glutathione peroxidase; GSH: glutathione. Data are mean ± SE; NC: normal control; OC: obesity control; OME: obesity moderate exercise; OHE: obesity high exercise, vs. NC: $^{*}P < 0.05$, $^{**}P < 0.01$; vs. OC: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$; vs. OME: $^{\triangle}P < 0.05$, $^{\triangle\triangle}P < 0.01$.

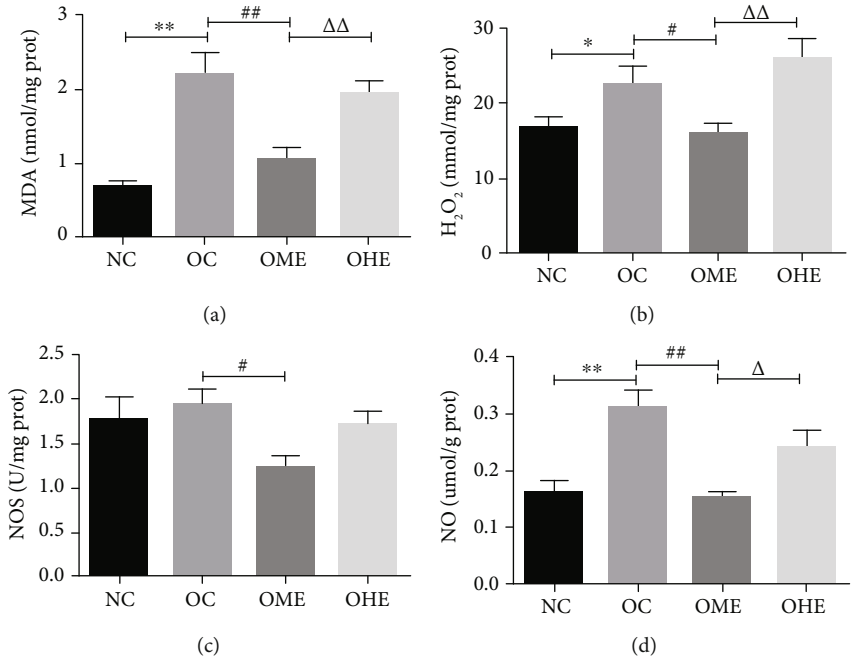

Figure 4: Effect of high-fat diet and exercise on the testicular oxidative stress product. MDA: malondialdehyde; H_2O_2: hydrogen peroxide; NOS: nitric oxide synthase; NO: nitric oxide. Data are mean ± SE; NC: normal control; OC: obesity control; OME: obesity moderate exercise; OHE: obesity high exercise, vs. NC: $^{*}P < 0.05$, $^{**}P < 0.01$; vs. OC: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$; vs. OME: $^{\triangle}P < 0.05$, $^{\triangle\triangle}P < 0.01$.

3.5. Effect of Obesity and Exercise on mRNA and Protein Expression of NF-κB, TNF-α, IL-1, and IL-10 in the Testicular Tissue. In the testicular tissue of the OC group, the mRNA and protein expression of NF-κB, TNF-α, and IL-1 increased significantly (Figures 6(a)–6(f)), while the mRNA and protein expression of the anti-inflammatory cytokine IL-10 decreased significantly (Figures 6(g) and 6(h)). Compared with that of the OC group, the mRNA and protein expression of NF-κB, TNF-α, and IL-1 in the OME group decreased significantly (Figures 6(a)–6(f)), while

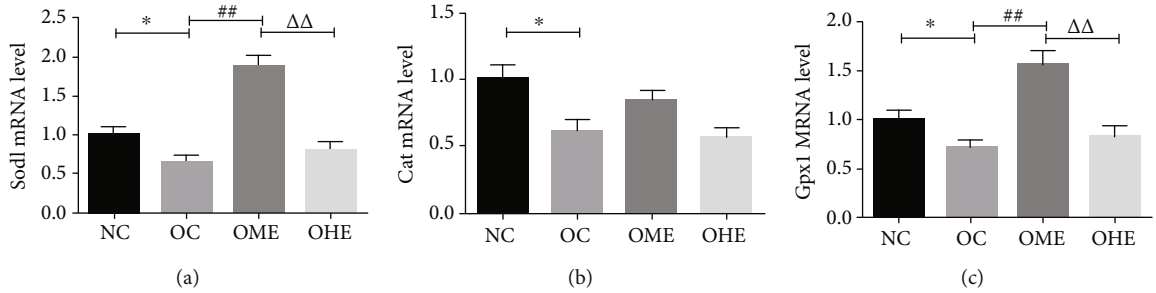

Figure 5: Effect of obesity and exercise on the mRNA expression of antioxidant enzymes. T-OAC: total antioxidant capacity; SOD: superoxide dismutase; CAT: catalase; GSH-Px: glutathione peroxidase. Data are mean ± SE; NC: normal control; OC: obesity control; OME: obesity moderate exercise; OHE: obesity high exercise, vs. NC: $^{*}P < 0.05$, $^{**}P < 0.01$; vs. OC: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$; vs. OME: $^{\triangle}P < 0.05$, $^{\triangle\triangle}P < 0.01$.

the mRNA and protein expression of IL-10 increased significantly (Figures 6(g)–6(h)). In the OHE group, there were no significant changes in the mRNA and protein expression of NF-κB, TNF-α, IL-1, and IL-10 (Figures 6(a)–6(h)); however, there were significant differences between the expression levels in the OME and OHE groups (Figures 6(a)–6(h)).

3.6. Effect of Obesity and Exercise on mRNA and Protein Levels of SF-1, StAR, and P450scc in the Testicular Tissue. Figure 7 shows that the mRNA and protein levels of SF-1, StAR, and P450 in the OC group were significantly lower than those in the NC group (Figures 7(a)–7(f)). The mRNA and protein levels of SF-1, StAR, and P450scc increased significantly in the OME group (Figures 7(a)–7(f)), while those in the OHE group were not significantly changed (Figures 7(a)–7(f)). There were significant differences in the mRNA expression of SF-1, StAR, and P450 and protein levels of SF-1 and P450 between the OME and OHE groups (Figures 7(a)–7(f)).

4. Discussion

To understand the mechanisms by which exercise affects reproductive function in men with obesity, we conducted a serial of experiments. Our previous studies indicated that both long-term moderate- or high-load exercise could effectively reduce body fat and alleviate leptin resistance. Interestingly, only the moderate-load exercise could alleviate the negative effects of obesity on the male reproductive function [20]. We hypothesized that this phenomenon might be related to the oxidative stress and the inflammatory response, as supported by our results, which are as follows: male obesity disrupted the balance between oxidation and antioxidation in the testicular tissue, induced oxidative stress, upregulated NF-κB, and triggered the inflammatory response, which reduced testosterone biosynthesis and sperm quality, thereby negatively affecting male reproductive function. Moderate-load exercise effectively alleviated the high oxidative stress induced by obesity, downregulated the expression of NF-κB and proinflammatory cytokines, and improved testosterone biosynthesis and sperm quality. However, high-load exercise did not alleviate the levels of obesity-induced oxidative stress and inflammatory response in the testicular tissue and did not significantly improve the reduced male reproductive function. Therefore, the oxidative stress-inflammatory response triggered by high-load exercise may have offset the inhibitory effects of body fat reduction on oxidative stress. Therefore, it is speculated that variations in exercise regimens have different effects on the male reproductive function caused by obesity via the inhibition/stimulation of oxidative-stress inflammatory response.

In addition to being one of the main factors affecting male infertility [24, 25], oxidative stress is closely related to obesity and exercise [26, 27]. Studies have shown that oxidative stress markers are positively correlated with the body mass index and body fat percentage [28]. Increased oxidative stress induced by excessive fat accumulation is an early promoter and key pathogenic mechanism of obesity-related metabolic syndrome [29]. Obesity can trigger systemic oxidative stress [30], which includes testicular and sperm oxidative stress, thereby resulting in the reduction of testosterone synthesis, spermatogenesis, and sperm quality [15, 26]. This study had similar results, that is, in obese male mice, the serum testosterone levels were reduced; sperm quality parameters were decreased; sperm apoptosis was increased; NO, NOS, H_2O_2, and MDA levels in the testicular tissue were significantly increased; T-AOC concentration was decreased; catalase and GSH activities and mRNA expression were decreased; and SOD mRNA expression was significantly decreased, although the SOD activity did not change significantly. The mechanisms through which obesity induces oxidative stress in testicular tissue remain unclear. A previous study showed that elevated levels of glucose and free fatty acids led to an increase in mitochondrial ROS. Obesity induces excessive accumulation of lipids in adipocytes, which causes an increase in the substrate load in the mitochondria, promotes the expression of NADPH oxidase subunits, and leads to increased ROS, reduced SOD and GSH-PX activities, and increased oxidative stress in the mitochondria [31]. In obese mice, immunohistochemical results revealed increases in the number of Leydig cells, the number and volume of lipid droplets in the cells [10], and the level of MDA, an oxidative stress marker and lipid peroxidation product. Among the membrane structures of Leydig cells, the mitochondria and endoplasmic reticulum are rich in polyunsaturated fatty acids, which are highly prone to ROS attacks, resulting in the production of large amounts of MDA [32–34]. Obesity

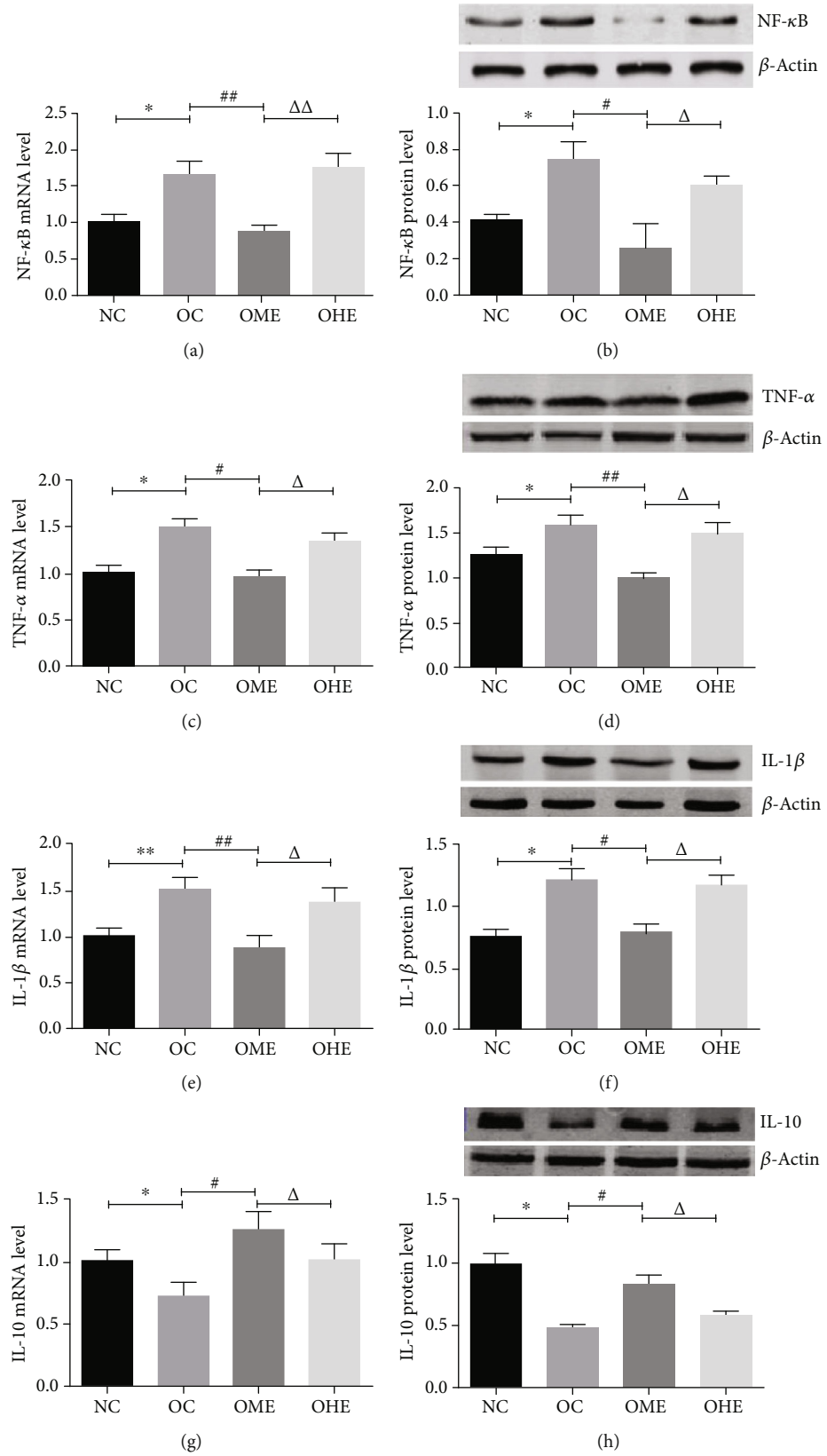

Figure 6: Effect of obesity and exercise on the mRNA level and protein expression of NF-κB, TNF-α, IL-1β, and IL-10 in the testicular tissue. NF-κB: nuclear factor κB; TNF-α: tumor necrosis factor-α; IL-1β: interleukin-1β; IL-10: interleukin-10. Data are mean ± SE; NC: normal control; OC: obesity control; OME: obesity moderate exercise; OHE: obesity high exercise; vs. NC: $^{*}P < 0.05$, $^{**}P < 0.01$; vs. OC: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$; vs. OME: $^{\triangle}P < 0.05$, $^{\triangle\triangle}P < 0.01$.

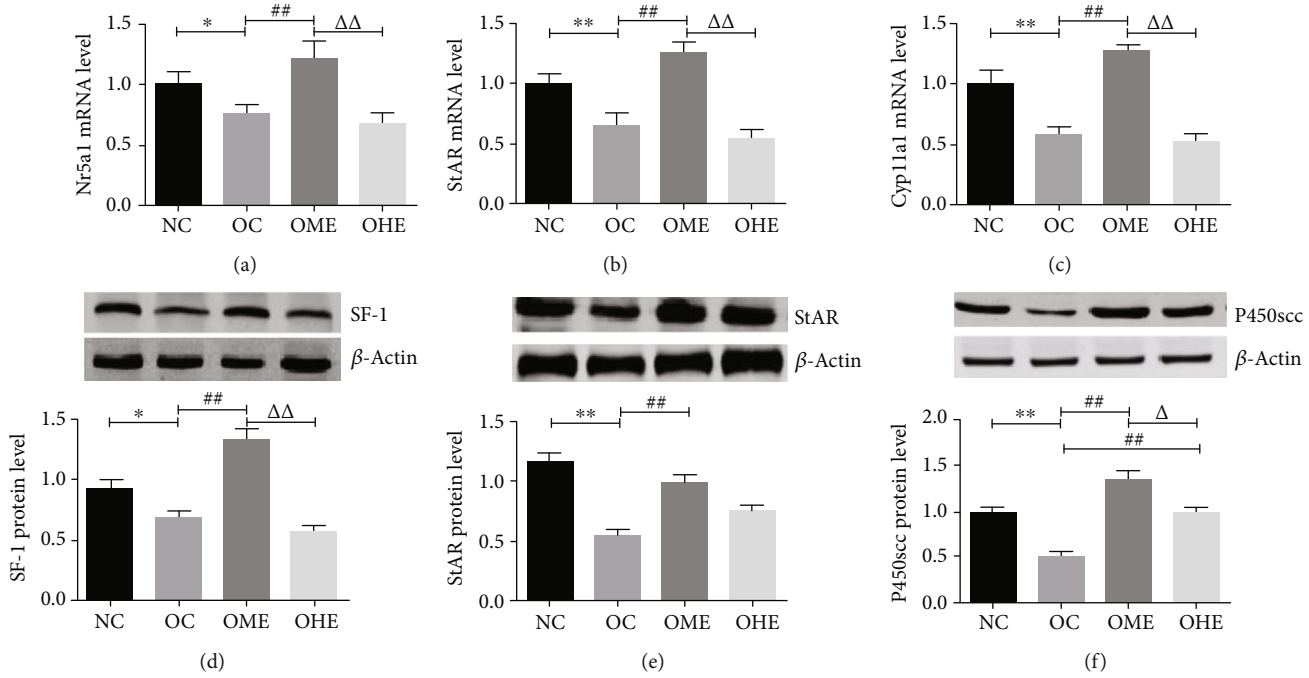

Figure 7: Influence of obesity and exercise on mRNA and protein expression of SF-1, StAR, and P450 in the testicular tissue. SF-1: steroidogenic factor-1; StAR: steroidogenic acute regulatory protein; and P450scc: P450 side chain cleavage (Cyp11a1). Data are mean ± SE; NC: normal control; OC: obesity control; OME: obesity moderate exercise; OHE: obesity high exercise, vs. NC: $^{*}P < 0.05$, $^{**}P < 0.01$; vs. OC: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$; vs. OME: $^{\triangle}P < 0.05$, $^{\triangle\triangle}P < 0.01$.

causes excessive accumulation of lipids in the body. For instance, in the Leydig cells of obese mice, the number and volume of lipid droplets are increased [10], causing an increase in the substrate load in the mitochondria, promoting the production of ROS in the mitochondria, and reducing the activity of SOD and GSH-PX [31]. Free radicals, when accumulated in excess, attack the unsaturated fatty acids (PUFA) in the membrane of mitochondria and endoplasmic reticulum, producing a large amount of MDA [33, 34]. The toxicity of MDA induces a decrease in the cholesterol synthesis, cholesterol transfer, and steroid synthesis capabilities of the endoplasmic reticulum, ultimately resulting in reduced testosterone synthesis and spermatogenesis [10, 35]. This association is corroborated by our result that the mRNA and protein levels of SF-1, StAR, and P450 in the testis tissue, along with the serum testosterone levels, were significantly lower in the OC group than in the NC group. Similarly, the sperm membrane surface and DNA molecules are rich in unsaturated fatty acids [35] that are also prone to ROS attacks, which generates large amounts of lipid peroxidation products. The lipid peroxidation products can harm the membrane integrity, fluidity, and permeability, as well as damage DNA structures and accelerate cell apoptosis, thereby resulting in increased defective sperm counts and reduced sperm motility [17]. These negative effects influence sperm capacitation and the acrosome reaction, thereby affecting the fertilization ability of the sperms [36]. Our experiment showed that the sperm quality parameters decreased and the apoptosis increased in the OC group. Therefore, obesity-induced fat accumulation in the testicular tissue triggered oxidative stress, inhibited testosterone synthesis and spermatogenesis, and reduced sperm quality, thereby negatively affecting obese male reproductive function.

Obesity-induced oxidative stress causes dysregulated expression of inflammation-related adipokines in the adipose tissue [29], which is promoted by the inflammatory signal transcription factor NF-κB that plays a key role in oxidative stress-induced dysregulation of adipokine expression and is recognized as a major mediator of oxidative stress-induced signal transduction in adipose cells [37, 38]. Studies have shown that ROS can activate IκB kinase, which promotes the degradation of IκB proteins. This results in the release of NF-κB dimers that translocate into the nucleus and control the gene transcription of certain proinflammatory cytokines (IL-1β, IL-6, TNF-α, and IL-8) [39]. Addition of the antioxidant *N*-acetyl cysteine has shown to impair NF-κB activation and inhibit TNF-α [40]. However, other studies have reported that proinflammatory cytokines, such as IL-1β and TNF-α, can inhibit the gene expression of testosterone synthases StAR, 3β-HSD, and P450c17 via the activation of NF-κB, which results in decreased testosterone synthesis within Leydig cells [13, 14]. Based on these findings, it was concluded that cytokines, such as TNF-α and IL-1β, simultaneously act as the downstream targets and stimulants of NF-κB, thereby activating NF-κB and further causing a continuous and magnified inflammatory response [41, 42]. In this study, we observed that obesity induced an increase in oxidative stress in the testicular tissue, which simultaneously increased the mRNA and protein levels of NF-κB, TNF-α, and IL-1; decreased the mRNA and protein levels of the

anti-inflammatory cytokine IL-10; and decreased the mRNA and protein levels of key testosterone synthases SF-1, StAR, and P450. Therefore, a long-term high-fat diet induced the production of large amounts of ROS in the testes, activating NF-κB, triggering the inflammatory response, and inhibiting testosterone biosynthesis [16, 17], which may be one of the main mechanisms for decreased serum testosterone levels in obese male mice.

In addition to reducing the mass of white adipose tissue (WAT), exercise training can also reduce oxidative stress in these tissues. Farias et al. [43] found that exercise training induced a reduction in the expression of the NAPDH oxidase NOX2 in the WAT and increased the enzyme activity of Mn-SOD, thereby reducing oxidative damage [43, 44]. However, very few studies have examined the effect of exercise on oxidative stress in the testicular tissue. In this study, the MDA, H_2O_2, NOS, and NO levels were significantly reduced in the testicular tissues of obese mice, whereas the T-AOC levels and activities and the mRNA expression of SOD, GSH, and catalase were significantly increased after 8 weeks of moderate-load exercise intervention. These results are consistent with the increased testosterone levels and improved sperm quality achieved by moderate-load exercise. However, these effects were not observed after high-load exercise; this may be related to the exercise load, which is closely associated with oxidative stress. It has been established that the oxygen demand increases during exercise and the oxygen consumption in the skeletal muscles increases by more than 100-fold compared to sedentary conditions; moreover, under exercise conditions, the free radical levels also increase [21]. On the other hand, the increases in free radicals can stimulate increased antioxidant enzyme activities, thereby preventing cell damage caused by excessive free radical production [22]. The effect of this positive adaptive response on the male reproductive function is typically manifested as a significant increase in the serum testosterone level and the quality, count, and DNA integrity of the sperm [45]. However, excessive exercise load leads to the production of large amounts of free radicals that exceed the body's antioxidative capacity; this excess of free radicals can induce damage to the male reproductive function. In addition, studies have shown that the testicular tissues of male rats subjected to strenuous exercise exhibited increased oxidative stress levels, decreased antioxidant enzyme activities, decreased levels of the key steroidogenic enzymes, and decreased testosterone synthesis and spermatogenesis, indicating a correlation between strenuous exercise-induced oxidative stress and reproductive dysfunction [23, 46]. In this study, oxidative stress, testosterone synthase expression, serum testosterone levels, sperm quality, and sperm apoptosis rate in the OHE group were not effectively improved compared with those in the OC group. Thus, oxidative stress induced by high-load exercise may have offset the protective effects of fat reduction against oxidative stress. Nevertheless, the molecular mechanisms underlying this hypothesis are not clear. Next, to find further evidence for this hypothesis, we measured the expression of cytokines related to the inflammatory response.

Interestingly, the expression analysis of testicular tissue in obese mice revealed that moderate-load exercise was associated with decreased mRNA and protein levels of NF-κB, IL-1β, and TNF-α, along with increased mRNA and protein levels of the anti-inflammatory cytokine IL-10. These observations are consistent with the changes in oxidative stress markers, mRNA and protein expression of the key testosterone synthesis enzymes (SF-1, StAR, and P450), serum testosterone level, and sperm quality parameters. Our findings in the OME group were similar to the results described in a study by Zhao et al. [45], which demonstrated that early-life or lifelong appropriate exercise effectively alleviated age-induced oxidative damage in the testes and downregulated the expression of the proinflammatory cytokines IL-1β and TNF-α and the inflammatory signaling pathway component NF-κB but increased the levels of the anti-inflammatory cytokine IL-10, enhancing testosterone biosynthesis, serum testosterone levels, and sperm quality parameters. In addition, previous *in vitro* studies had established that TNF-α and IL-1β could inhibit testosterone synthesis in rats by inhibiting the mRNA and protein expression of P450scc [47], 17 α-hydroxylase/17,20-lyase (P450c17), and 3β-hydroxysteroid dehydrogenase in rat Leydig cells [48]. Conversely, IL-10 can inhibit the synthesis of the proinflammatory cytokines TNF-α, IL-1α, and IL-1β [45]. Based on these previous findings and the outcomes in this study, it is suggested that long-term moderate-load exercise can inhibit the expression of proinflammatory cytokines by reducing oxidative stress and simultaneously promoting testosterone synthesis by enhancing anti-inflammatory cytokine expression.

In this study, we found that long-term high-load exercise did not significantly improve the mRNA and protein expression of NF-κB and proinflammatory cytokines in the testicular tissues of obese mice, which was consistent with our data for the oxidative stress, the testosterone synthesis factors SF-1, StAR, and P450, and the sperm quality. However, another study showed that high-load exercise training increased the levels of IL-1β, IL-6, IL-8, and TNF-α in the seminal plasma [49]. An earlier study in adult males also showed that after lipopolysaccharide stimulation, long-term high-load exercise training reduced the numbers of blood monocytes, neutrophils, and dendritic cells, along with decreases in the synthesis of IL-1β, IL-6, TNF-α, and macrophage inflammatory protein-1β [50]. The discrepancies between our results and those of previous studies may be related to the differences in the study subjects. Because of ectopic lipid deposition, the testicular tissues of obese male mice are in a state of high oxidative stress and high inflammatory response [10]. Although high-load exercise can reduce whole-body lipid deposition and ectopic lipid deposition, thereby reducing energy overload in the mitochondria and alleviating excessive ROS production [51], the high-load exercise can also increase the synthesis of free radicals [52], offsetting these positive effects in obese subjects. A limitation of this study was that the relationship between oxidative stress and inflammation in reproduction was not confirmed *in vivo* by injection of an antioxidant. Assessing this relationship will provide a theoretical basis to develop treatments for improving the reproductive function in men with obesity. This aspect will be further explored.

5. Conclusions

Long-term high-fat diet induces obesity, causing excessive ectopic deposition of lipids, triggering oxidative stress in the testis tissue, possibly triggering inflammatory response via NF-κB, and reducing testosterone biosynthesis and sperm quality. Moderate-load exercise can be effective in lowering body fat, alleviating obesity-induced, high oxidative stress in the testis tissue, downregulating the expression of NF-κB and proinflammatory cytokines, increasing the testosterone biosynthesis, and improving the sperm quality. Although the high-load exercise is better in reducing body fat, it has a negligible effect on reversing the high oxidative stress, the inflammatory response in the testis tissue, the testosterone biosynthesis, and the sperm quality in obese male mice. Overall, different exercise regiments may have different effects on the male reproductive function caused by obesity through the inhibition/stimulation of oxidative-stress inflammatory response.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

Authors' Contributions

Xuejie Yi, Donghui Tang, and Shicheng Cao conceived and designed the study. Haining Gao and Tie Ma conducted experiments; Tao Li, Tingting Yao, and Jing Li analyzed data. Tao Li prepared figures. Xuejie Yi and Bo Chang drafted the manuscript. All authors have read and approved the manuscript.

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Research Article

Effects of Diets Enriched in Linseed and Fish Oil on the Expression Pattern of Toll-Like Receptors 4 and Proinflammatory Cytokines on Gonadal Axis and Reproductive Organs in Rabbit Buck

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Infections of the genital tract can perturb the fertility in humans and animals. Pathogen recognition and activation of innate immunity onset through the pattern recognition receptor activation, such as Toll-like receptor 4 (TLR4), leading to the production of proinflammatory cytokines and mediators. TLR4 is expressed both on leukocytes and nonimmune cells. Rabbit TLR4 shows great similarity to its human counterpart. Moreover, the TLR4 signalling pathway could be modulated by long-chain polyunsaturated fatty acids (LC-PUFA). The objectives of this study were (i) to determine the expression levels of TLR4 and proinflammatory cytokines in the reproductive hypothalamic-gonadal axis of the male rabbit and (ii) to evaluate if the n-3 PUFA-enriched diets can modify their expression levels in the tissues and LC-PUFA profiles in seminal plasma. Fifteen rabbit bucks ($n = 5$/experimental group) were fed with different diets: commercial standard (group C), rich in extruded linseed (10%, group L), and in fish oil (3%, group FO) for 110 days. TLR4, TNF-α, and IL-1β mRNA were ubiquitously expressed throughout the hypothalamic-gonadal axis. However, TLR4 mRNA expression was lower in the hypothalamus than the epididymis ($P < 0.01$), seminal vesicles ($P < 0.01$), and pituitary gland ($P < 0.05$). Dietary enrichment in PUFA did not modify the gene expression profile nor the histological characteristics of the tissues. Conversely in seminal plasma, rabbits fed with L and FO had lower n-6 ($P < 0.05$), LC-PUFA n-6 ($P < 0.05$), and n-6/n-3 ratio ($P < 0.05$) but higher n-3 ($P < 0.001$) and LC-PUFA n-3 ($P < 0.01$) compared to the control group. Our study builds a map of the gene expression of TRL4 and proinflammatory cytokines in the reproductive hypothalamic-gonadal axis of the male rabbit, fundamental step for understanding the immune defence mechanisms. Diets enriched in LC-PUFA did not affect basal gene expression but modulated sperm fatty acid composition. Finally, rabbit may be an excellent animal model to study the relationship between inflammation and infertility, and the nutritional modulation of immune functions.

1. Introduction

Male infertility is increasing in the last decades in both human and animals [1–3]. Infertility can be due to genetic and nongenetic factors. Nongenetic factors consist of andrological problems such as varicocele and cryptorchidism as well as immunological diseases including antisperm antibodies in serum and ejaculation [1]. Nongenetic factors also include the nutrition that can negatively affect the fertility impairing the functions of the gonadal axis both at central and testicular levels [1, 4–6]. On the other hand, some nutrients such as PUFAs, minerals, and vitamins seem to improve the reproductive functions through several potential mechanisms of action [6–9]. For example, it is now known that the

diets rich in long-chain PUFAs (≥20 C, LC-PUFAs) improve semen quality by modifying the fatty acid profile of plasma, seminal fluid, and sperm [3, 10–14].

Moreover, other nongenetic factors that can induce hypofertility and infertility include infections and inflammation of the genital tract [2, 15]. According to the World Health Organization [16], they are among the main causes for male infertility. In livestock farms such as rabbitries, clinical and subclinical infections of genital apparatus are not only a welfare issue but also a source of considerable economic losses due to reduced productivity, culling rate, purchase of drugs for treatments, and veterinarian consultations [2, 6, 17]. The genital infections in rabbit, but also in humans and other animal species, are commonly caused by Gram-negative bacteria such as *Pasteurella multocida*, *Escherichia coli*, *Pseudomonas* spp., and *Salmonella* spp. [15, 18–20]. The understanding of the body's defence mechanisms against bacteria could help in developing new strategies to treat infections and thus reduce infertility problems by using alternative therapeutic agents to antibiotics [20–22].

In mammals, invading pathogens are detected by innate immune system by pattern recognition receptors, among which Toll-like receptors (TLRs) are the most characterized. In particular, the TLR4 recognizes the lipopolysaccharides (LPS) of Gram-negative bacteria [21, 23, 24]. The activation of TLR4 after LPS binding stimulates an intracellular signalling cascade resulting in the activation of NF-κB transcription factor that promotes the expression of immune response genes including those encoding proinflammatory cytokines and other mediators of inflammation [25–28]. Although the activation of TLR4 has a critical role in controlling the spread of invading pathogens in the genital tract, their overactivation may be dangerous for the host [17, 24, 29]. Indeed, TLR4 hyperactivation is involved in the development of several inflammatory pathologies and chronic diseases of the male genital tract. TLR4 has been localized in the human epididymis and prostate, where it can activate the inflammatory response and ultimately leads to temporary or persistent infertility [20, 29, 30]. However, its role in the switch from physiological to pathological condition of the genital tract, especially in the male, requires further studies [20, 24]. Moreover, relatively little is known about its expression and distribution in genital apparatus of the rabbit, although it is not only a livestock species but also a pet and an excellent animal model to study immunological aspects of the reproductive disorders [2, 21, 31–34]. Indeed, the rabbit TLR4 is more similar to the human one compared to mouse and rat [35].

Among the strategies to prevent and manage the infections of the genital tract, the use of some natural components in the diet, called functional foods or nutraceuticals, provides an attractive alternative to pharmacologic interventions. Indeed, experimental and epidemiological studies have shown the LC-PUFAs, in addition to their direct effects on sperm fatty acid profiles, have also anti-inflammatory properties [14, 36, 37]. In most of these studies, fish oil was used as source of eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) [6, 14, 36, 37]. Nevertheless, LC-PUFAs can be also obtained indirectly from other sources like the linseed. In fact, linseed contains α-linolenic acid (ALA, 18:3n-3) which can be endogenously converted into EPA and DHA [14, 33, 36]. Mechanisms underlying the anti-inflammatory actions of PUFAs not only include the inhibition of the production of eicosanoids, like prostaglandins and leukotrienes, but also of proinflammatory cytokines such as TNF-α, IL-1β, IL-6, and IL-8 [37–40]. However, to our knowledge, no studies have investigated if n-3 PUFAs affect the inflammatory response of the male genital tract by modulating the TLR4 signalling pathway.

The first aim of this study was to elucidate the expression profiles of TLR4 and the proinflammatory cytokines TNF-α and IL-1β in the gonadal axis of male rabbit. Then, it investigated whether diet rich in different sources of n-3 PUFAs, extruded linseed and fish oil, affected their basal expression levels and fatty acid profile in seminal plasma.

2. Materials and Methods

2.1. Animals. Fifteen healthy New White Zealand bucks trained for semen collection, of the same body weight (4.5 ± 0.50 kg) and age (6 months old), were kept individually in flat deck cages and housed under controlled conditions: constant temperature of $21 \pm 1^{\circ}$C, relative humidity of $55 \pm 10\%$, and a continuous photoperiod of 16L:8D. Fresh water was always available *ad libitum*.

2.2. Experimental Design. Rabbits were randomly assigned to one of three groups (5 animals/group): C group was fed with commercial standard diet (lacking linseed or fish oil), L group with diet rich in extruded linseed (10%), and FO group with diet rich in fish oil (3.5% NORDIC NATURALS omega-3®) [14, 33]. Vitamin E (200 mg/kg feed, synthetic alpha-tocopheryl acetate) was included in all diets. Tables 1 and 2 show formulation and proximate analysis of the diets. The rabbits received each 130 g/d of feed for 110 days and then they were slaughtered according to the standard procedure practised in commercial abattoirs. Before slaughter, semen samples were collected following procedures previously reported [11] and immediately transferred to the laboratory. This study was conducted in accordance with the Guiding Principles in the Use of Animals and approved by the Animal Ethics Monitoring Committee of the University of Siena (CEL AOUS); Authorisation no. 265/2018-PR, ISO-PRO 7DF19.23.

2.3. Fatty Acid Profile of Sperm. The total lipid extraction from the feed and semen was performed according to the method of Folch et al. [42], and the esterification was carried out following the procedure of Christie [43]. The transmethylation procedure was conducted using eicosenoic acid methyl esters (Sigma Chemical Co.) as internal standard. The recovery rates of the internal standard were $95 \pm 1\%$ and $89 \pm 4\%$ for feed and semen, respectively.

The fatty acid composition was determined using a Varian gas chromatograph (CP-3800) equipped with a flame ionisation detector and a capillary column of 100 m length $\times 0.25$ mm $\times 0.2$ μm film (Supelco, Bellefonte, PA, USA).

Table 1: Formulation and chemical composition of control (C) and enriched diets with extruded linseed (L) or fish oil (FO).

	Unit	Diet		
		C	L	FO
Ingredients				
Dehydrated alfalfa meal	g/kg	300	380	380
Soybean meal 44%	g/kg	150	100	150
Barley meal	g/kg	410	310	335
Wheat bran	g/kg	52	52	52
Soybean oil	g/kg	30	—	—
Extruded flaxseed	g/kg	—	100	—
Fish oil*	g/kg	—	—	35
Beet molasses	g/kg	20	10	10
Calcium carbonate	g/kg	7	7	7
Calcium diphosphate	g/kg	13.5	13.5	13.5
Salt	g/kg	7	7	7
DL-methionine	g/kg	0.5	0.5	0.5
Vitamin-mineral premix†	g/kg	10	10	10
Analytical data				
Crude protein	g/kg	175	174	175
Ether extract	g/kg	480	472	425
Crude fiber	g/kg	124	137	130
Ash	g/kg	89	84	90
Digestible energy§	MJ/kg f.m.	10.6	10.7	10.9

*NORDIC NATURALS omega-3®: purified deep sea fish oil (from anchovies and sardines) containing EPA 330 mg/100 g, DHA 220 mg/100 g, other n-3 LC-PUFA 140 mg/100 g+α-tocopherol for preservation. †Per kg diet: vitamin A 11.000 IU; vitamin D_3 2000 IU; vitamin B_1 2.5 mg; vitamin B_2 4 mg; vitamin B_6 1.25 mg; vitamin B_{12} 0.01 mg; alpha-tocopheryl acetate 200 mg; biotin 0.06 mg; vitamin K 2.5 mg; niacin 15 mg; folic acid 0.30 mg; D-pantothenic acid 10 mg; choline 600 mg; Mn 60 mg; Fe 50 mg; Zn 15 mg; I 0.5 mg; Co 0.5 mg; §Maertens et al. 1988 [41].

Table 2: Main fatty acid composition (expressed as percentage of total fatty acids) of diets.

Fatty acids	Diets			Pooled SE
	C	L	FO	
SFA	19.80_a	15.40_a	38.10_b	1.82
MUFA	17.40	15.80	14.50	0.87
PUFA	62.80_a	68.80_a	47.40_b	5.12
C18:2n-6, LA	50.45_b	22.30_a	20.50_a	2.11
C18:3n-3, ALA	11.15_a	45.80_b	18.50_a	1.42
C20:5n-3, EPA	—	—	3.50	0.21
C22:6n-3, DHA	—	—	4.20	0.28
LC-PUFAn-3	—	—	10.50	1.00
n-6	51.45_b	22.80_a	21.00_a	2.35
n-3	11.35_a	46.00_c	26.40_b	1.55
n-6/n-3	4.53_b	0.50_a	0.80_a	0.01

SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; LA: linoleic acid; ALA: α-linolenic acid; LC-PUFA: long-chain PUFA; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid. Values followed by the same subscript letter in each row do not differ significantly ($P \leq 0.05$).

Helium was used as the carrier gas with a flow of 2 ml/min. The split ratio was 1 : 80.

Individual FAME was identified by comparing the relative retention times of peaks in the sample with those of standard mixture (FAME Mix Supelco; 4 : 0 to 24 : 0) plus cis-9, cis-12 C18:2; cis-9, cis-12, cis-15 C18:3; cis-9, cis-12, cis-15 C18:3 (all from Sigma-Aldrich).

2.4. Tissue Collection. The genital tract, hypothalamus, and pituitary were collected at the slaughterhouse. The male reproductive organs were immediately excised and subdivided into the testicles, epididymis, seminal vesicles, and prostate. Moreover, the brain and pituitary glands were promptly removed from each animal. The pituitary glands were bisected through the medial sagittal plane into 2 symmetrical parts. The brains were sectioned to isolate the diencephalic portions. Samples of organs were collected for histological and polymerase chain reaction (PCR) evaluation. The TLR4 and proinflammatory cytokine mRNA levels in the various organs of the reproductive tract and gonadal axis were evaluated using real-time PCR.

2.5. Histological Assay of Tissues. Samples from each rabbit of the testicle, epididymis, seminal vesicles, prostate, pituitary gland, and hypothalamus were collected. Tissues were fixed in 10% neutral buffered formalin, paraffin-embedded (FFPE), cut into 5 μm sections, which were routinely stained with hematoxylin and eosin and examined by light microscopy.

2.6. RNA Extraction. Total RNA was extracted from formalin-fixed paraffin-embedded samples using the RNeasy FFPE Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Finally, the RNA concentration and quality were determined using a spectrophotometer (BioPhotometer, Eppendorf, Hamburg, Germany) at 260/280 nm wavelength. The samples were stored at -20°C.

2.7. Reverse Transcription and Real-Time PCR. Total RNA (1 μg) from each sample was reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. The cDNA obtained from each sample was used as a template for Sybr Green dye-based (SYBR Green PCR Master Mix, Applied Biosystems, Foster City, CA, USA) Real-Time PCR in an optimized 20 μl reaction volume in MicroAmp optical 96-well plates, as previously described [44]. Rabbit specific primers were purchased from Bio-Rad (Hercules, CA, USA). Their details are listed in Table S1.

A duplicate no-template control (NTC) was also included in each plate. Real-time quantitative PCR was carried out in the QuantStudio 3 Real-Time PCR Systems (Applied Biosystems, Foster City, CA, USA) at the following thermal cycle conditions: 2 minutes at 50°C, 10 minutes at 95°C followed by 50 cycles of 15 seconds at 95°C, and 1 minute at 60°C. Quantitation was determined after the application of an algorithm to the data analyzed by the software of the QuantStudio 3 Real-Time PCR Systems (Applied Biosystems, Foster City, CA, USA).

The expression of target genes (IL-1β, TNF-α, TLR4) was normalized using the calculated actin beta (housekeeping gene) cDNA expression (mean) of the same sample and run. The relative quantification of each gene was calculated using the formula $2^{-\Delta Ct}$ where $\Delta Ct = Ct$ (target gene)–Ct (housekeeping gene), where Ct (threshold cycle) values were the mean of two test replicates. The obtained values were multiplied by 10,000 in order to obtain the test arbitrary units.

2.8. Statistical Analysis. Diagnostic graphs and the Shapiro-Wilk test were used to verify the assumptions of the parametric tests and identify outliers. Data on sperm fatty acid composition were analyzed by the one-way analysis of variance (ANOVA), followed by multiple comparisons with Sidak adjustment. Results were presented as means and pooled standard errors (SE). When the assumptions were not met (gene expression, Table S2), values were log-transformed and a nonparametrical approach was chosen. Group differences were analyzed by using the Friedman tests treating the tissues as a within-subjects factor. Instead, differences between tissues were evaluated by the Kruskal-Wallis test using Dunn's test to carried out multiple comparisons. Data were expressed as a median value with interquartile range.

Statistical analyses were performed using SPSS 25.0 software (IBM, SPSS Inc., Chicago, IL, USA) and GraphPad Prism version 6.0 (San Diego, CA, USA). Statistical significance was set at $P \leq 0.05$.

3. Results

3.1. Histological Tissues. Histological evaluation of sampled organs did not show differences between the 3 groups. Individual cell degeneration in glandular tissues or cystic degeneration of *pars intermedia* of the pituitary glands was noted randomly distributed in the groups (Figures 1 and 2).

3.2. TLR4 and Cytokine Expression Profile in the Gonadal Axis and Effect of Diet Enrichment with n-3 PUFA. Differential expression of TLR4 along the gonadal axis was found in male rabbit ($P < 0.05$), although the prostate and testis were excluded from statistical analysis as they were detectable in an insufficient number of samples. In particular, TLR4 mRNA expression was lower in the hypothalamus than epididymis ($P < 0.01$), vesicle ($P < 0.01$), and pituitary gland ($P < 0.05$; Figure 3). Conversely, dietary treatment did not affect the expression of TLR4 ($P > 0.1$; Table 3).

There were no differences in TNF-α and IL-1β mRNA expression values either between tissues ($P > 0.1$; Figure 3) or groups ($P > 0.1$; Table 3).

3.3. Sperm Fatty Acid Composition. The diets altered fatty acid composition of the sperm (Table 4). Compared with the control group, rabbits fed with L and FO had lower n-6 ($P < 0.05$), LC-PUFA n-6 ($P < 0.05$), and n-6/n-3 ratio ($P < 0.05$). Conversely, the content of n-3 ($P < 0.001$) and LC-PUFA n-3 ($P < 0.01$) increased in the following order: the control < LI < FO group, mainly due to the DHA (22:6n-3) amount.

4. Discussion

There is a considerable body of evidence to support the fact that local and systemic infective and inflammatory disease may cause transient or permanent infertility in both humans and animals [2, 16, 20, 24].

Infections and inflammation can affect different sites of the male reproductive system, although the pathological modifications in sperm function are most probably due to direct effects of inflammatory mediators in the testis and accessory glands which induce damages, in particular, at seminiferous epithelium and vascular structures [20, 22, 45]. Moreover, there is evidence that bacterial LPS, proinflammatory cytokines, ROS, and nitric oxide may also determine infertility perturbing the secretion of hormones related to reproduction, acting at different levels of the gonadal axis [46–48]. The mechanisms of protection against the microbial agents are essential to maintain the male reproductive physiology. TLRs are a part of innate immune system and represent the link between innate and adaptive immunities. For this reason, they play a critical role in the activation of immune responses against infections [35]. TLRs have been characterized in different tissues and organs in human and different animal species [21, 24, 28]. However, studies about their expression and distribution in genital apparatus of the male are sparse and, as far as we know, this is the first study that tries to point out the map and the distribution of TLR4 and some proinflammatory cytokines in genital tract and gonadal axis on rabbit buck.

Our findings provide evidence that the mRNA encoding TLR4 is present in the testis, epididymis, seminal vesicles, prostate, pituitary, and hypothalamus of male rabbit although at different levels. In fact, TLR4 mRNA was not found in the testis and prostate of all samples analyzed and showed weak expression in the hypothalamus. Conversely, it was predominantly expressed in the epididymis and seminal vesicles.

Differences in TLR4 expression throughout the gonadal axis and genital tract suggest a tissue-specific immune surveillance analogous to that hypothesized for humans. In fact in humans, it is low in the prostate and testis, as well as in the brain [21, 35, 49]. Conversely, it could play an important role in defending against *Chlamydia* and *E. coli* infections in the epididymis [20, 22, 30] while seminal vesicles have been poorly studied.

Some authors also pointed out that TLR4 expression in the genital tract is different between the species, but these differences, as well as those between tissues, could simply be due to the lack of tools for analysis or lack of information [21]. Indeed, as mentioned above, TLR4 have been identified and localized in several tissues of male reproductive tract of rodents [22, 24, 35, 50] but information about nonhuman primates, swine, and dog is scarce [35]. Really, expression of TLR4 mRNA in the human epididymis has just been documented by Saeidi et al. [30] while it has only recently been located in the epididymal duct, vas deferens, and sperm of cats by immunohistochemistry and western blotting [51].

In rabbit, high levels of TLR4 expression have been found in the lung and bone marrow [28, 35]. Even if moderately, it

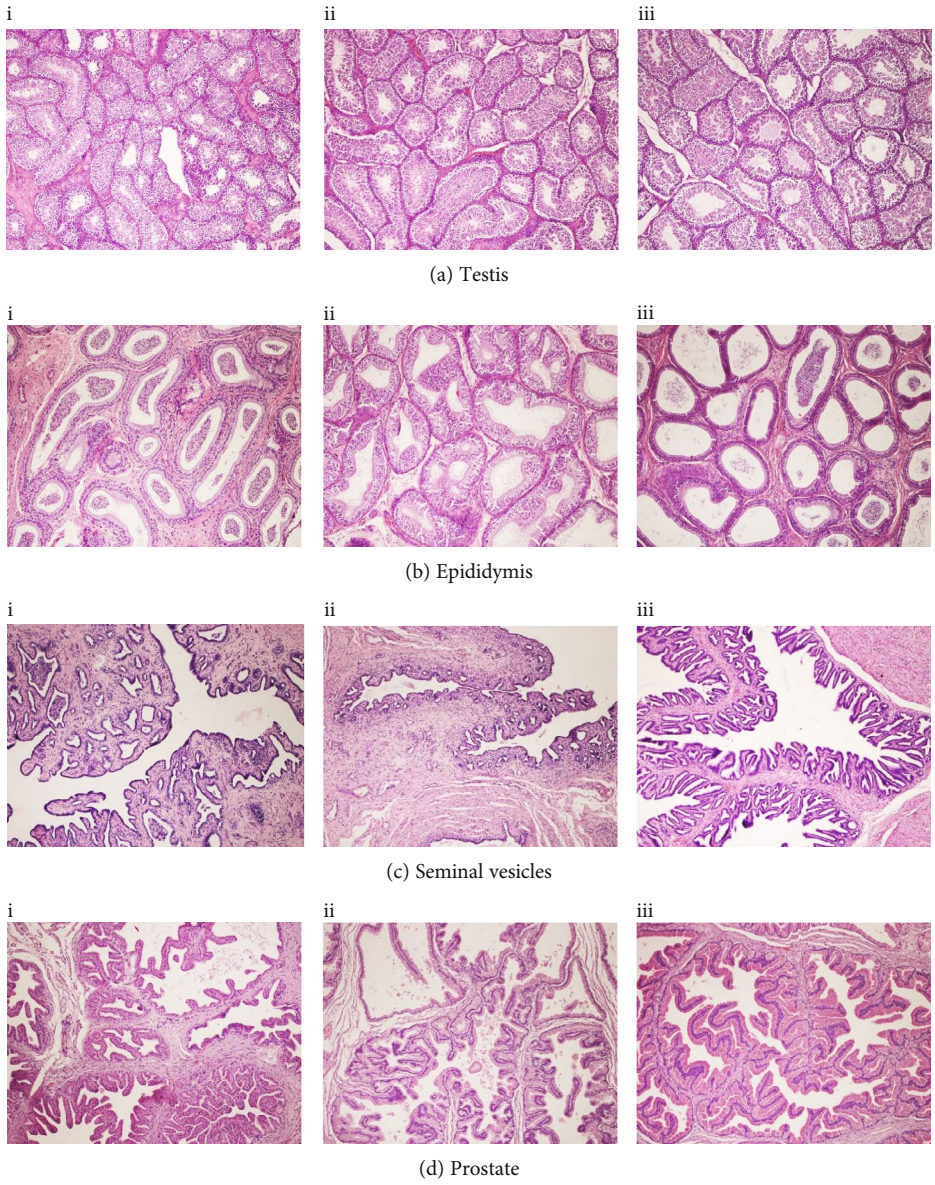

Figure 1: Seminiferous tubules of the testicles (a), tubules of the epididymis lined by ciliated tall columnar epithelium variably containing spermatozoa (b), glandular architecture of the seminal vesicles (c), and the prostate (d) from rabbit fed with control diet (C group, i), diet rich in linseed (L group, ii), or fish oil (FO group, iii). HE, 100x.

has been also detected in the kidney, spleen, heart, liver, and thymus [35]. Collodel et al. [8] showed that the TLR-4 was expressed in the testis and epididymis, but, to our knowledge, this study is the first that investigates the expression in the whole male reproductive hypothalamic-gonadal axis of rabbit. It is interesting to note that rabbit is a better animal model for the study of TLR4 compared to rodents, sharing a 72% amino acid similarity with humans [35].

Although a differential spatial distribution was observed, the ubiquitous expression of TLR4 supports the idea that the male reproductive tract is an aseptic site in an active surveillance state and that TLR4 plays a primary role in the immune response to an infection. Rabbit genital infections are mainly caused by Gram-negative bacteria such as *Pasteurella multocida*, *Escherichia coli*, *Pseudomonas* spp., and *Salmonella* spp. as a result of incorrect artificial insemination practices and poor environmental hygiene [15, 19]. TLR4 in complex with some accessory molecules such as CD14 and MD-2 can bind LPS, the major component of the outer membrane of Gram-negative bacteria [21, 23, 27]. Binding of LPS to TLR4 triggers the intracellular transduction events leading to the activation of different transcription factors. In particular, two different pathways can be activated: MyD88-dependent and MyD88-independent. The first pathway leads to the activation of NF-κB that in turn promotes the expression of proinflammatory cytokines such as IL-1β, TNF-α, and IL-6. MyD88-independent pathway, through the involvement of TRAM and TRIF adapter molecules, leads to the activation of IRF3 and the following expression of IFNs [27, 28, 35]. The activation of TLR4 also stimulates the production of

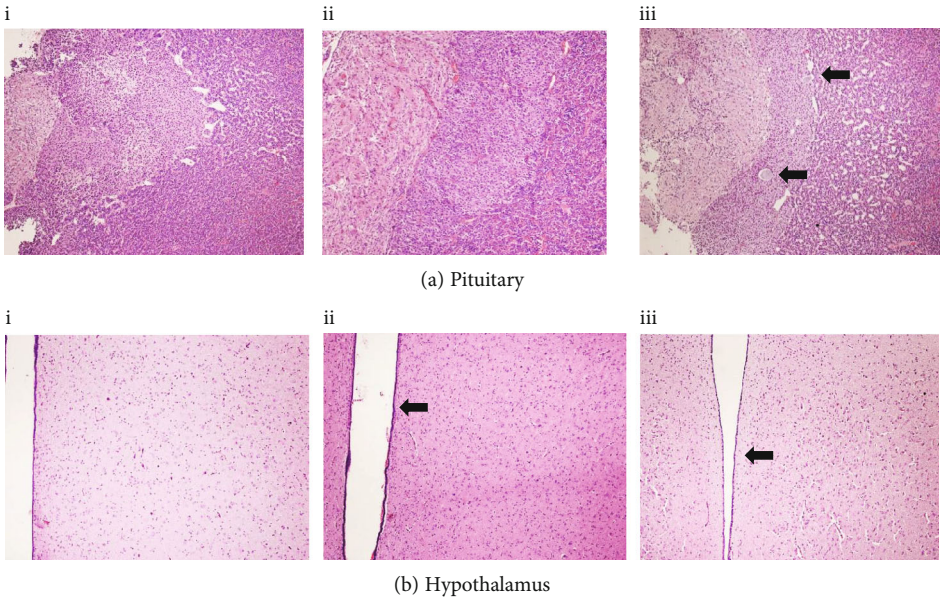

Figure 2: (a) Pituitary gland. Note the presence of multiple small cysts in the *pars intermedia* (arrows). (b) Hypothalamic areas where some nuclei are shown. The third ventricle is identified by the arrow. Rabbit fed with control diet (C group, i), diet rich in linseed (L group, ii), or fish oil (FO group, iii). HE, 100x.

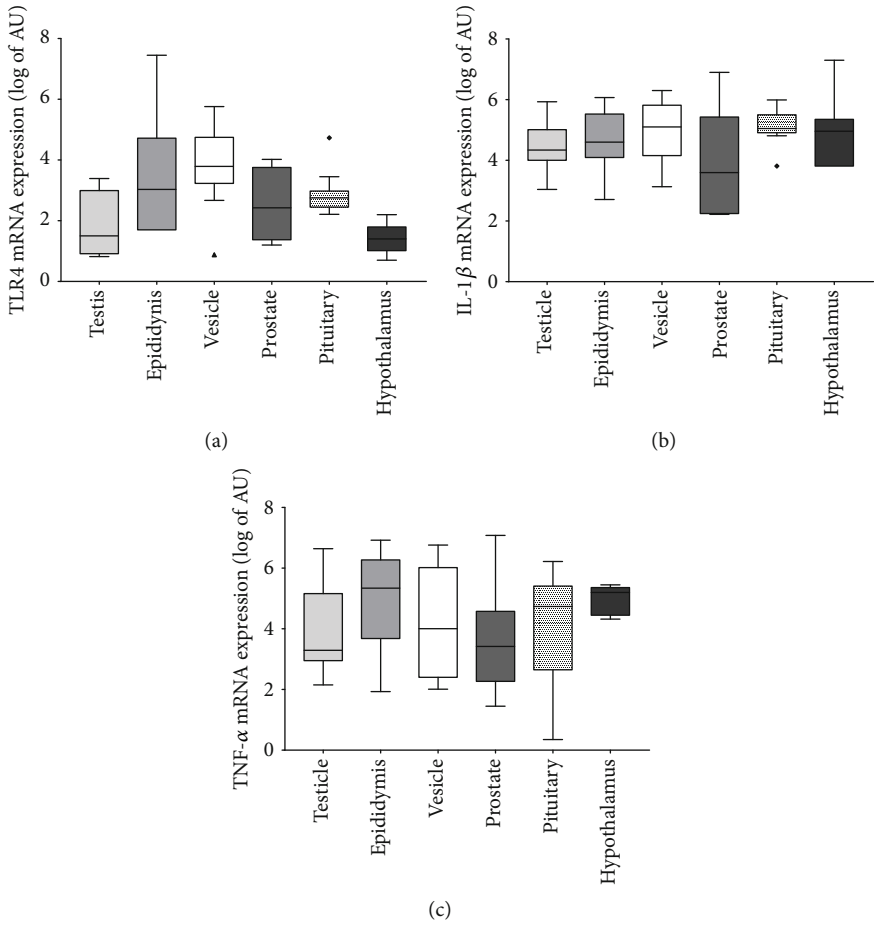

Figure 3: mRNA expression of TLR4 (a), IL-1β (b), and TNF-α (c) according to the tissues (regardless of the group). Values are log-transformed arbitrary units (AU). The symbols outside the box plot are outliers.

Table 3: Marginal medians and interquartile range of the normalized expression values of TLR4, IL-1β, and TNF-α according to group (regardless of the tissue). C: control diet; L: diet rich in extruded linseed; FO: diet rich in fish oil.

Gene target	Group			P value*
	C	L	FO	
TLR4	2.67 (1.55-3.81)	3.03 (1.70-4.73)	2.59 (1.50-2.96)	0.653
IL-1β	4.26 (3.59-5.13)	4.93 (4.34-5.16)	5.19 (3.95-5.68)	0.273
TNF-α	3.35 (2.85-4.65)	4.49 (3.34-5.34)	5.23 (3.29-6.06)	0.653

*From Friedman test.

Table 4: Polyunsaturated fatty acid composition (expressed as percentage of total fatty acids) of sperm. C: control diet; L: diet rich in extruded linseed; FO: diet rich in fish oil.

Fatty acids	Experimental diets			Pooled SE	P value
	C	L	FO		
C18:2n-6, LA	7.2_a	3.8_b	5.7_b	2.09	0.033
C20:4n-6, ARA	1.5_a	0.7_b	0.7_b	0.09	0.026
C22:5n-6	26.2_a	17.9_b	12.7_b	3.92	0.021
n-6	34.9_a	22.4_b	19.1_b	3.85	0.017
C18:3n-3, LA	0.2_a	1.3_c	0.5_b	0.11	0.020
C20:5n-3, EPA	0.2_a	0.5_a	5.3_b	0.10	0.011
C22:6n-3, DHA	0.3_a	2.4_b	7.6_c	1.25	<0.001
n-3	0.7_c	4.2_b	13.4_a	0.21	<0.001
n6/n-3	49.9_a	5.4_b	2.1_b	15.8	0.021
LC-PUFA n-3	0.5_c	2.9_b	12.9_a	0.26	0.005
LC-PUFA n-6	27.7_a	18.4_b	14.4_c	1.19	0.032

SE: standard error. P value: significance value of F ratio (ANOVA test which compares the three group means). Values in the same row not sharing the same subscript are significantly different ($P \leq 0.05$; multiple comparisons with Sidak correction).

other mediators of inflammation including ROS, nitric oxide, and antimicrobial factors such as defensins and heat shock proteins [25, 26, 47].

Our study demonstrated the ubiquitous expression of the proinflammatory cytokines IL-1β and TNF-α through the reproductive hypothalamic-gonadal axis of male rabbit without, moreover, highlighting differences between tissues.

Although the activation of TLR4, and the expression of IL-1β and TNF-α, provides an essential host immune defence mechanism, they are also involved in different aspects of infertility. Local production of the cytokines, in particular IL-1β and TNF-α, affects the blood supply of the testis, may inhibit Leydig cell steroidogenesis, and in turn affects spermatogenesis [24, 45, 46]. Thus, inflammatory mediators have a direct effect on the seminiferous epithelium and testicular vasculature, thus compromising the fertility. Moreover, they may contribute to the detrimental effects of epididymitis, prostatitis, and, probably, of prostate cancer upon male fertility [20, 24, 29]. Finally, ROS produced by immune and nonimmune cells during the inflammatory reaction can determine an oxidative damage of spermatozoa, lipid, and protein peroxidation, as well as DNA fragmentation, resulting in the loss of sperm functions [6, 13, 48]. Furthermore, there is a considerable body of evidence suggesting that local and systemic infections and inflammation may also act on the gonadal axis [52]. This alteration is mainly due to a reduction in the secretion of important hormones related to reproduction. It is evident that bacterial lipopolysaccharides, proinflammatory cytokines, and other inflammatory mediators such as ROS and nitric oxide can impair the hormonal secretion at different levels of the brain-pituitary-Leydig cell axis [45, 46].

In the last decades, the interest in the nutrition as a modifiable factor in immune function has growing. In particular, several indirect evidences suggest that n-3 PUFAs can modulate the immune response. For example, the ratio between n-6 to n-3 PUFAs in the diet is related to an increased risk of inflammatory chronic disease incidence and benefits of n-3 PUFA have been demonstrated in rheumatoid arthritis, inflammatory bowel disease, and asthma [36, 37]. In addition to their role as precursors of eicosanoids, n-3 PUFA have been demonstrated to modulate the inflammatory response attenuating the activation of the TLR4 signalling and inhibiting the expression of IL-1β, TNF-α, IL-6, NF-κB, and reactive oxygen and nitrogen species [37–40].

In light of these considerations, this study for the first time examined the effects of n-3 PUFA-enriched diets in the TLR4 signalling pathway of the male genital tract of the rabbit. The animals did not show any clinical signs throughout the period of the treatment and, both histological evaluation and quantification of gene expression, did not reveal differences on gonadal axis and genital tract due to PUFA enrichment.

The anti-inflammatory effects of LC-PUFA on the reproductive system have previously been evaluated in females with conflicting results depending on the tissue, species, and experimental design. DHA treatment attenuates inflammatory responses modulating the TLR4 signalling pathway in mammary tissues of saws [53], but incubation with LC-PUFA did not modify proinflammatory cytokine response in human placental cells [54]. LC-PUFA supplementation during pregnancy reduced inflammation in placental tissue of mice after bacterial induction of intrauterine infection [55] and in human adipose tissue and trophoblast cells [56]. TLR4 plays a key role in determining these anti-inflammatory effects [55, 56]. Our finding did not show an effect of diets enriched in LC-PUFA on the basal expression of TLR4 and cytokines through the hypothalamic-gonadal axis of male rabbit, but further studies could assess their levels after LPS stimulation.

Conversely, as expected, sperm fatty acid composition was altered by dietary treatment. Increased n-3 and LC-PUFA n-3 were found in rabbits supplemented with linseed

and fish oil while their n-6/n-3 ratio was considerably reduced. These findings confirm that sperm fatty acid profile of the rabbit is very sensitive to the diet rich in n-3 PUFAs [11]. Moreover, they suggest that n-3 PUFAs have beneficial effects on male fertility thanks to their impacts on the sperm structure.

Indeed, the type of PUFAs has strong effects on maintaining the integrity and fluidity of the sperm membrane, on membrane protein and ion channel function, on regulation of nuclear receptors, and therefore on the morphology, motility, and acrosome reaction of the sperm [3, 10, 11]. Furthermore, it has been suggested that the changes of PUFA composition on sperm by dietary fatty acids may also induce changes in metabolism and enzyme potential of germinal cells that influence the fertility [5].

However, in the present study, no lineal relationship exists between n-3 PUFA ingestion and their profile in the sperm: it seems that n-3 PUFAs from fish oil were more effective with respect to those provided by the linseed. This is justifiable by the fatty acid composition of rabbit sperm: membrane phospholipids were constituted by 43% of LC-PUFAs, and then they incorporate more easily EPA, DHA, and especially the docosapentaenoic acids from n-6 series (22:5n-6) with respect to their precursors LA and ALA (n-6 and n-3, respectively) [14].

Finally, PUFAs may influence the secretion of LH, FSH, and testosterone, although not all studies agree [7, 10, 12]. Improvement of membrane fluidity, motility, and fertility linked to dietary enrichment in PUFA and then to changes in sperm composition have been widely documented in humans and in several animal species [3, 7, 10–13].

However, the manipulation of the diet not always coincides with an improvement of sperm parameters and fertility [6, 13]. Different experimental procedures in terms of time and dose of administration might explain these responses. It is very important to note that an excessive fluidity can damage the plasma membrane leading to a decrease in the cell signal transduction with detrimental effects on the sperm [6, 12]. Moreover, PUFA are subjected to the attack of ROS that induces the lipid peroxidation of plasma membrane eventually resulting in the impairment of membrane fluidity, permeability, function of ion channels and receptors, and then to sperm functions [6, 11, 13]. In our study, an overnutritional amount of vitamin E was added to avert this danger [11, 13, 14]. Moreover, flaxseed also contains lignan, which is a kind of phytoestrogens that can act as an antioxidant important to increase the stability of PUFA in sperm and seminal plasma and, at the same time, reduce the production and the effects of ROS [11, 33]. Then, n-3 PUFAs on one hand can counteract oxidative effects of ROS but at same time, when excessive, can themselves be sources of ROS. In other words then, the dietary enrichment in n-3-PUFAs may improve semen quality given that its quantity is controlled so to avoid lipid peroxidation.

Indeed, two main limitations should be pointed out in the current study which could be subject to future research: (i) to investigate the effect of diets with different levels of PUFAs and, consequently, also different fatty acid compositions of sperm, and (ii) to investigate whether the diets enriched in PUFAs influence the expression of TLR4 and cytokines through the hypothalamic-gonadal axis of male rabbit after a proinflammatory challenge.

5. Conclusions

In this study, we pointed out, for the first time, the map of distribution of TLR4 and proinflammatory cytokines such as IL-1β and TNF-α in the reproductive organs and gonadal axis of the male rabbit. This knowledge may be important to understand the primary immune response sites to an invading pathogen agent in the reproductive tract and the mechanisms by which the consequent inflammation can induce infertility.

Our results showed that the diets rich in n-3 PUFAs do not affect the basal expression of TLR4 and cytokines in the genital tract. Future directions exploring their effects on TLR4 signalling pathway after LPS stimulation to eventually implement specific nutritional protocols, particularly in the setting of inflammatory diseases, are needed.

However, the reduced n-6/n-3 ratio on seminal plasma of rabbits supplemented with linseed or fish oil indicates that n-3 PUFAs could improve reproductive functions by modifying the sperm lipid composition.

Finally, our data suggest that the rabbit could be a useful animal model to understand the relationship between inflammation and infertility as well as to understand the mechanisms through which PUFAs may influence the male fertility.

Data Availability

The data used to support the findings of this study are included within the article and the supplementary information file. Additional information or data sets are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Supplementary Materials

Supplementary 1. Table S1: primer list.

Supplementary 2. Table S2: mRNA expression of TLR4, IL-1β, and TNF-α (arbitrary units, untransformed data).

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Research Article

Antioxidant Effect of the *Castanea sativa* Mill. Leaf Extract on Oxidative Stress Induced upon Human Spermatozoa

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This study was aimed at evaluating *in vitro* the effects of a 75% *v*/*v* ethanolic extract of leaves of *Castanea sativa* Mill. (var. Bastarda Rossa, Mount Amiata, Tuscany, Italy) on ejaculated human sperm. Total polyphenols and total flavonoids contained in the extract were determined by a colorimetric assay and HPLC-DAD. The DPPH test and electrochemistry were utilized to study the antioxidant activity of the extract. Swim-up-selected sperm from 8 healthy men were treated with the *C. sativa* leaf extract at different dilutions (1 : 100, 1 : 200, and 1 : 500), and sperm motility was assessed following the WHO guidelines. Swim-up-selected spermatozoa were incubated with 100 μM H_2O_2 to induce lipid peroxidation (LPO) and with H_2O_2 and the leaf extract (1 : 100, 1 : 200, and 1 : 500) to test the antioxidant activity of the extract. The levels of LPO were determined by measuring malondialdehyde (MDA) concentrations. The treated samples were also analyzed by transmission electron microscopy (TEM) for ultrastructural evaluation. The chemical analysis showed that one-third *ca.* of the polyphenols in the *C. sativa* extract were made up of flavonoids, with hyperoside present in high concentration. A good antioxidant activity was demonstrated by both the DPPH test and electrochemical analysis. The *C. sativa* leaf extract did not decrease sperm motility at all tested dilutions. Treatment with H_2O_2 alone caused a significant increment in MDA levels ($P = 0.006993$), while the treatment with H_2O_2 plus *C. sativa* extract diluted to 1 : 100 and 1 : 200 significantly reduced MDA levels ($P = 0.01476$ and $P = 0.01571$, respectively), with respect to H_2O_2 alone. TEM analysis confirmed the protective effect of the extract on damage induced by LPO, in particular that occurring at the plasma membrane level. The *C. sativa* leaf extract could be used in human and farm animal protocols for gamete handling, such as techniques of assisted reproduction and cryopreservation of semen, all conditions in which oxidative stress is exacerbated.

1. Introduction

Castanea sativa Mill. (*C. sativa*) is a very long-lived tree, belonging to the *Fagaceae* family present in South Europe and Asia. The fruit chestnut is a healthy food and has valuable nutritional qualities. It is noteworthy that when harvesting chestnuts, a great deal of waste products is obtained, such as leaves and inner and outer shells. Therefore, it could be important to try to use also the waste material to find substances that can be utilized in industrial, cosmetic, and nutraceutical fields and other areas, encouraging recycling and a sustainable economy [1].

Extracts of this plant have displayed a clear biological activity; in particular, they have shown cardioprotective [2], antihelminthic, antibacterial, and antiviral effects [3] and neuroprotective activity [4]. Observations recently made by Budriesi et al. [5] in high-fat diet rats suggested that the chestnut extract has the potential to be used in medicine as a dietary supplement in the treatment of obesity complications.

For a long time, chestnut leaf infusions have been used to treat rheumatism, cough, and diarrhea [6]. They also exert calming and sedative action and are an excellent remedy in the treatment of cold and cases of respiratory problems.

Recent studies highlighted the presence of high concentrations of phenolic compounds endowed with antioxidant activity in the chestnut waste material extract [1]. Leaves are rich in tannins (6%-8% of leaf dry weight) including ellagic acid, as well as flavonoids (0.1-0.3%) such as rutin, hesperidin, quercetin, apigenin, morin, galangin, and kaempferol [3, 7]. Cinnamic derivatives, such as chlorogenic acid, are also present [7]. It is known that these compounds are involved in the defense against the oxidative damage and are able to protect the main targets of reactive oxygen species (ROS), which are DNA, proteins, and lipids [8]. The scavenging activity of these compounds is due to the presence of hydroxyl groups [9].

Therefore, *C. sativa* leaf extracts are used in different applications. For example, they are able to protect from oxidative damage the DNA of pancreatic β-cells treated *in vitro* with the diabetogenic agent streptozotocin [10]. Quave et al. [11] reported that *C. sativa* leaf extracts rich in pentacyclic triterpenes, such as ursene and oleanane derivatives, blocked the *Staphylococcus aureus* pathogenic activity and virulence. In addition, leaf phenolic extracts of the Italian product Marrone di Roccadaspide (Campania region, Italy) showed a protective effect against UVB-induced damage on human keratinocytes [12].

It is known that oxidative stress results from alterations in the complicated balance between reactive oxygen species (ROS) generation and elimination. The presence of physiological levels of ROS supports some main functions of sperm, such as motility, capacitation, acrosome reaction, zona pellucida binding, and oocyte fusion [13]. However, an uncontrolled ROS production and/or the defects in the balance between ROS concentration and the antioxidant scavenging system affect the normal sperm function damaging sperm membranes, DNA, and proteins [14]. In particular, sperm membranes are rich in polyunsaturated fatty acids (PUFAs), and they are vulnerable to ROS that causes lipid peroxidation (LPO) [15].

Sperm and seminal plasma are endowed with enzymatic and nonenzymatic antioxidant compounds able to scavenge ROS [16]. However, semen laboratory processing such as centrifugation, cryopreservation, and, in general, semen handling leads to an increase in ROS production [17, 18]. A strategy to resolve this problem encompasses the supplementation of used media with antioxidants. With this purpose, many *in vitro* studies reported the scavenging ability of antioxidant compounds against oxidative stress induced to human sperm [19–23].

The aim of this study was to characterize the antioxidant potential of the extract of *Castanea sativa* Mill. (Bastarda Rossa) leaves and to evaluate its effect on oxidative stress induced upon human spermatozoa. The chemical analysis, performed by high-performance liquid chromatography-diode array detection (HPLC-DAD), enables the evaluation of the flavonoid and polyphenol content. The antiradical capacity was tested by the DPPH assay and electrochemical measurements. The effects of the *C. sativa* leaf extract were then assayed upon ejaculated human sperm in *in vitro* experiments. The possible toxicity of this extract was assessed by determining its effect at different dilutions on the sperm motility. The antioxidant ability of the extract against LPO [induced by hydrogen peroxide (H_2O_2)] was evaluated by measuring the levels of malondialdehyde (MDA) in human swim-up-selected sperm treated with H_2O_2 alone and H_2O_2 plus *C. sativa* extract. The morphological alterations induced by H_2O_2 upon spermatozoa and the protective effect of the extract were analyzed also by transmission electron microscopy (TEM).

2. Materials and Methods

2.1. C. sativa Extract Preparation. *Castanea sativa* Mill. var. Bastarda Rossa leaves were harvested in Arcidosso (Mount Amiata, Tuscany, Italy) in August, during their full development stage. Collection was performed in the morning, and leaves were manually chosen when they are intact and healthy and have no signs of microbial infection. Herbal material was dried to constant weight at 35°C.

The extraction procedure was accomplished using the automatic extractor Naviglio Estrattore® (Atlas Filtri, Padova, Italy) and validated and was very effective in obtaining an optimal yield of polyphenols from herbal drugs [24]. Briefly, dried *C. sativa* leaves were crushed and transferred into a 50 μm extraction bag up to complete filling. The bag was placed in the extraction chamber that was filled with solvent of extraction (ethanol 75% *v*/*v*). Naviglio Estrattore® parameters were set as follows: static phase: 8 min/cycle, dynamic phase: 18 laps/cycle, and total cycles of extraction: 100. At the end of the extraction procedure, the extract was adjusted to a final drug : extract ratio 1 : 10.

2.2. Chemical Analysis. Total polyphenol and flavonoid content of the ethanolic extract (CEE) was examined using spectrophotometric methods reported in Biagi et al. [24]. In detail, total polyphenols were determined by the colorimetric method of Folin-Ciocalteu: 0.1 ml of each extract was added to 2.9 ml of distilled water and 0.5 ml of Folin-Ciocalteu reactive (1 : 10 *v*/*v*) to distilled water.

After 30 s of shaking, 1 ml of Na_2CO_3 15% *w*/*w* was added to distilled water. After incubation at room temperature for 120 min, the absorbance at 700 nm was read using a SAFAS UV-MC2 instrument (Monte Carlo, Monaco). The polyphenol quantification was calculated by means of interpolation of the calibration curve constructed using gallic acid (Sigma-Aldrich, Milan, Italy).

Total flavonoid content of extracts was determined reading the absorbance at 353 nm of the extract diluted to 100-fold, according to Sosa et al. [25], and constructing a calibration curve using hyperoside as the standard (Sigma-Aldrich, Milan, Italy). All reagents were purchased from Sigma-Aldrich (Milan, Italy). A HPLC-DAD analysis was carried out to investigate the polyphenolic fraction of CEE and to identify the main chemical constituents. A Shimadzu Prominence LC 2030 3D instrument equipped with a

Bondapak® C18 column (10 mm, 125 Å, 3.9 mm × 300 mm column) (Waters Corporation, USA) was used.

Water+0.1% *v*/*v* formic acid (A) and acetonitrile+0.1% *v*/*v* formic acid (B) were used as mobile phases. The following program was applied: B from 10% at 0 min to 25% at 20 min and then B 50% at 35 min; the flow was set at 0.8 ml/min. Chromatograms were recorded at 280 nm and 340 nm.

Analyses were performed using 10 μl of CEE; hyperoside and quercetin (Sigma-Aldrich, Milan, Italy) were used as external standards. Calibration curves were established using reference standards ranging from 0.008 mg/ml to 0.5 mg/ml. The correlation coefficient (R^2) of each curve was >0.99.

2.3. Antiradical Capacity: DPPH Assay. The antiradical capacity of CEE was tested by means of the validated DPPH (2,2-diphenyl-1-picrylhydrazyl) test. All reagents were purchased from Sigma-Aldrich, Milan, Italy. The DPPH solution was prepared in methanol at a concentration of 1×10^{-4} M. CEE was tested in seven 1 : 2 serial dilutions (ethanol 75% *v*/*v*), ascorbic acid, used as the reference, was dissolved in pure water, and hyperoside and quercetin were dissolved in ethanol 75% *v*/*v* and diluted in order to obtain a range of concentrations from 0.031 to 2 mg/ml. All the samples were mixed with the DPPH solution (1 : 19), transferred into 1 cm path length cuvettes, and incubated for 30 min at room temperature in the dark. Water or ethanol 75% *v*/*v* in DPPH (1 : 19) was used as the positive control. The inhibition of DPPH was calculated according to the following formula:

$$\%\text{inhibition} = \left(\frac{\text{Abs}_c - \text{Abs}_e}{\text{Abs}_c}\right) \times 100, \quad (1)$$

where Abs_c is the absorbance of the positive control and Abs_e is the absorbance of the tested samples.

IC_{50} was calculated by constructing the curve of inhibition values for each tested concentration (in the linear range 10-75%) [22].

2.4. Electrochemical Measurements. Electrochemical experiments (cyclic voltammetry and differential pulse voltammetry (DPV)) were carried out to obtain information about redox properties of CEE as well as also of its main flavonoids such as hyperoside and quercetin. The electrochemical analyzer BAS100A (Bioanalytical Systems Inc., West Lafayette, USA) connected to a conventional three-electrode cell consisting of a glassy carbon working electrode, a platinum wire as an auxiliary electrode, and an Ag/AgCl reference electrode was used. The reported potentials were referred at a scan rate of 20 mV/s. Measures were acquired at room temperature under an ultrapure nitrogen inert atmosphere to prevent both the reactivity of the examined samples with the atmospheric oxygen and the dissolved oxygen reduction process. This analysis was carried out as follows:

(i) 1 ml of ethanolic solution of hyperoside 0.1 mg/ml was diluted in 5 ml of NaCl 0.1 M

(ii) 1 ml of ethanolic solution of quercetin 0.1 mg/ml was diluted in 5 ml of NaCl 0.1 M

(iii) 1 ml of ethanolic solution of CEE was diluted in 5 ml of NaCl 0.1 M

2.5. Semen Samples. Semen samples from 8 healthy men (aged 24-30 years) were collected by masturbation after 3–5 days of sexual abstinence. They were analyzed after liquefaction for 30 min at 37°C. Volume, pH, concentration, and motility were evaluated according to the World Health Organization guidelines [26]. All men provided an informed written consent before the inclusion in this study. The informed consent described the aims of the research and was approved by the Ethics Committee of Azienda Ospedaliera Universitaria Senese (CEAOUS).

2.6. Swim-Up to Select Motile Sperm. A swim-up technique was used to obtain the motile sperm fraction from 8 different ejaculates: 0.5 ml of each semen sample was placed in a sterile conical centrifuge tube and gently layered with 0.5 ml of Biggers-Whitten-Whittingham (BWW) medium [26]. The tubes, inclined at a 45° angle, were incubated for 45 min at 37°C and 5% CO_2. Then, 0.5 ml of the uppermost medium that contains motile sperm fraction was collected and used for the experiments.

2.7. Determination of Sperm Motility after C. sativa Leaf Extract Treatment. Different dilutions of *C. sativa* leaf extracts in BWW medium (1 : 100, 1 : 200, and 1 : 500) were added to swim-up-selected sperm. Mixtures were incubated at 37°C and 5% CO_2 for 1 h. Sperm motility was evaluated using a Burker counting chamber. Spermatozoa were categorized in different grades of motility (sperm with progressive and nonprogressive motility and immotile sperm) [26]. Aliquots of the selected sperm treated in the same conditions but without the *C. sativa* leaf extract were used as controls. All experiments were carried out eight times, and results were reported as mean values ± standard deviation (SD) and median.

2.8. LPO Induction and C. sativa Leaf Extract Treatment. Each sample of swim-up-selected human sperm was separated into aliquots, which were composed of sperm alone, sperm treated with 100 μM H_2O_2 and the leaf extract diluted to 1 : 100, 1 : 200, and 1 : 500 in BWW medium, and sperm treated with 100 μM H_2O_2 alone. Aliquots were incubated at 37°C and 5% CO_2 for 1 h. After incubation, the aliquots were centrifuged at 200 g to separate BWW and spermatozoa. The supernatant was stored at -80°C until levels of MDA were assessed.

2.9. Malondialdehyde (MDA) Level Assessment. The extent of LPO was estimated assessing free MDA concentrations according to Shara et al. [27]. After thawing, 500 μl of each sample was added to 500 μl of 0.04 M Tris(hydroxymethyl) methylamine (pH 7.4) and 0.01% butyl hydroxytoluene in acetonitrile (1 : 1 *v*/*v*) to avoid the artificial oxidation of polyunsaturated free fatty acids during the assay. The samples were centrifuged at 3000 g at 4°C for 15 min. The supernatant was used for MDA analysis after precolumn derivatization with 2,4-dinitrophenylhydrazine. The MDA-hydrazone was quantified by isocratic reversed-phase HPLC (Waters 600 E

Table 1: The quantification of total polyphenols expressed as gallic acid and total flavonoids expressed as hyperoside (mg/l of extract).

Sample	Total polyphenols expressed as gallic acid (mg/l)	Total flavonoids expressed as hyperoside (mg/l)
CEE	3475.23 ± 144.05	1081.83 ± 66.55

System Controller HPLC equipped with a Waters Dual λ 2487 detector, Milford, MA, USA) with UV detection at 307 nm. MDA values were reported as nmol/ml.

2.10. Transmission Electron Microscopy (TEM). Human sperm treated with 100 μM H_2O_2 and the *C. sativa* leaf extract diluted to 1 : 200 in BWW and with 100 μM H_2O_2 alone were processed for TEM. Sperm samples were fixed in cold Karnovsky fixative at 4°C for 2 h; then, they were washed in 0.1 mol/l cacodylate buffer (pH 7.2) for 12 h, postfixed in 1% buffered osmium tetroxide for 1 h at 4°C, dehydrated, and embedded in Epon Araldite. Ultrathin sections were cut with a Supernova Ultramicrotome (Reichert Jung, Vienna, Austria), mounted on copper grids, stained with uranyl acetate and lead citrate, and observed and photographed with a Philips CM12 TEM (Philips Scientific, Eindhoven, the Netherlands; Centro di Microscopie Elettroniche "Laura Bonzi," ICCOM, Consiglio Nazionale delle Ricerche (CNR), Via Madonna del Piano, 10, Firenze, Italy).

At least 300 sperm sections were analyzed for each sample, and the anomalies related to the acrosome, the chromatin, the axoneme, and the plasma membrane were quantified. These experiments were performed three times.

2.11. Statistical Analysis. The statistical elaboration was performed with software R for statistical computing, open source for Windows, version 3.3.1 [R Core Team (2016). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/]. The comparisons of sperm motility percentages and MDA levels in samples treated with 100 μM H_2O_2 and *C. sativa* leaf extracts at different dilutions were calculated by the Kruskal–Wallis test. When a statistically significant difference was found among the groups, the Wilcoxon rank sum test was then used between pairs of groups. The comparisons between the percentages of sperm organelle alterations after incubation with H_2O_2 and the *C. sativa* leaf extract diluted to 1 : 200 were calculated by the Kruskal–Wallis test. $P < 0.05$ was considered significant.

3. Results

3.1. Chemical Analysis of C. sativa leaf extract. The Folin-Ciocalteu colorimetric assay and direct spectrophotometry enabled the quantification of the total polyphenols and total flavonoids in CEE, respectively. Table 1 shows the quantifications of total polyphenols expressed as gallic acid equivalents (mg/l of extract) and total flavonoids expressed as hyperoside (mg/l of extract). Results showed that about one-third of the polyphenols in the extract are flavonoid type; it was plausible to refer the remaining polyphenolic portion to phenolic acids, hydroxycinnamic derivatives, and tannins.

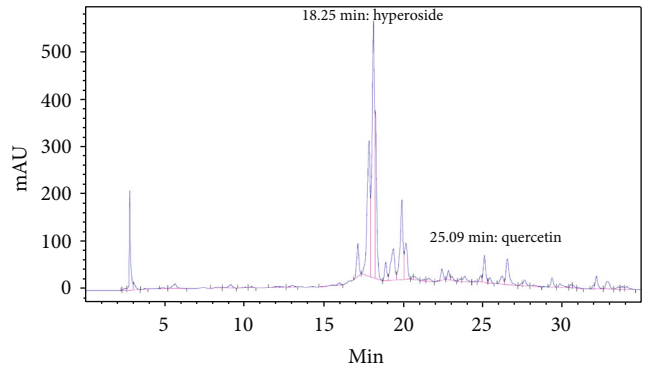


Figure 1: DAD spectrum of CEE recorded at 340 nm. The main CEE monomeric polyphenols were represented by quercetin glycosides, such as hyperoside (RT = 18.25 min). Quercetin is also present in the extract, but in low concentration (RT = 25.09 min).

HPLC-DAD analysis recorded 5 main constituents in CEE at the following retention times (RT): 17.85 min, 18.13 min, 18.25 min, 19.31 min, and 19.91 min (Figure 1). With the exception of the constituent at RT 17.85 min, all the other major constituents were found to be flavonoids, showing a characteristic UV profile of this class of metabolites.

From the comparison with the standards and the literature, it was possible to assign the peak at 18.25 min to the hyperoside (Figure 1), while the peak at 18.13 min is with high probability an isomer of the hyperoside, plausibly isoquercitrin. Quercetin is also present in the extract, but in low concentration (RT = 25.09 min, 6.30 mg/l). The main nonflavonoid constituent can be assigned to the catechin tannin class, but it was not identifiable not being referable to the used reference standards. In CEE, the concentration of hyperoside was 75.28 mg/l.

3.2. Antiradical Capacity: DPPH Assay. The DPPH assay was carried out on CEE to measure its antiradical capacity. Free radical inhibition values were linearly correlated with CEE concentration in the concentration range 0.05%-0.2%, while at higher concentrations, the typical maximal inhibition plateau was obtained.

From the construction of the correlation line between concentration and activity in the linear zone, it was possible to calculate the IC_{50} (inhibitor concentration that reduces the activity by 50%) of CEE, which was found to be 0.081% (0.72 mg/ml). Results were validated using ascorbic acid as the reference $IC_{50} < 10$ μg/ml [28]. The CEE phytocomplex exerted a synergistic effect compared to the main single compounds. Indeed, repeating the DPPH test on hyperoside and quercetin, we found that the best IC_{50} value was exerted by quercetin 0.01 mg/ml, while the IC_{50} value of hyperoside was 0.06 mg/ml; these values were higher than those which one would expect considering the presence of these compounds at the IC_{50} value of CEE (when CEE diluted at 0.081% contains 0.06 mg/l of hyperoside and 0.005 mg/l of quercetin).

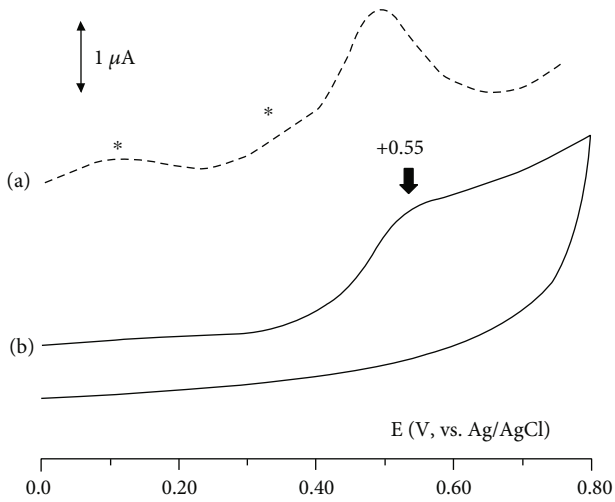


Figure 2: Differential pulse voltammogram (DPV) (a) and cyclic voltammogram (b) recorded for CEE 1 : 5 in water solution on a glassy carbon electrode. Scan rate: 20 mV/s. An anodic process (oxidation) at +0.55 V is recorded by differential pulse voltammetry and cyclic voltammetry methods. Other oxidation peaks were detected in DPV (asterisks).

3.3. Electrochemical Measurements. Cyclic voltammograms recorded in the solution containing quercetin showed a quasireversible oxidation process with an anodic peak at +0.33 V. The same profile was recorded in the solution containing hyperoside, but the anodic peak potential was reached at +0.44 V (data not shown in figures). More complicated redox behaviour was recorded in the extract solution. Indeed, the cyclic voltammogram of the CEE solution (Figure 2, curve b arrow) showed a main irreversible oxidation process at +0.55 V; however, the differential pulse voltammetry, analysis more sensitive than cyclic voltammetry, highlighted that CEE undergoes two other cathodic processes with peak potentials around +0.15 V and +0.40 V (Figure 2, curve a asterisks). It is interesting to note that both peak values are lower than that measured in hyperoside solution (+0.44 V) supporting the highest antioxidant activity recorded for CEE.

3.4. Effect of the C. sativa Leaf Extract on Sperm Motility. The effect of the *C. sativa* leaf extract on motility of swim-up-selected sperm was assessed. The seminal parameters of the used samples ranged from the 10th to 50th centile reported in the WHO guidelines [26]. Results showed that the *C. sativa* leaf extract used at the dilutions of 1 : 100, 1 : 200, and 1 : 500 did not have an effect on sperm motility (Table 2).

3.5. Effect of the C. sativa Leaf Extract on Induced LPO in Sperm Samples: MDA Evaluation. Swim-up-selected sperm were treated with H_2O_2 to induce LPO and with both H_2O_2 and *C. sativa* leaf extract diluted to 1 : 100, 1 : 200, and 1 : 500 in order to test the potential scavenging activity of this compound. MDA levels were measured in the supernatant, and the results are shown in Figure 3. The level of MDA significantly increases after H_2O_2 treatment (37.21 ± 12.24 nmol/ml) with respect to control (12.73 ± 3.98 nmol/ml, $P = 0.006993$). The MDA levels in samples incubated with H_2O_2 plus *C. sativa* leaf extract diluted to 1 : 100 (14.33 ± 5.00 nmol/ml, $P = 0.01476$) and 1 : 200 (13.65 ± 4.81, $P = 0.01571$) were significantly lower than those measured in the sample treated with H_2O_2 alone and were similar to the levels measured in the control specimens. No significant difference was found between MDA levels in sperm treated with both H_2O_2 and *C. sativa* leaf extract diluted to 1 : 500 (21.33 ± 5.81 nmol/ml) and those in sperm treated with H_2O_2 alone.

3.6. Effect of the C. sativa Leaf Extract on Induced LPO in Sperm Samples: Ultramorphological Evaluation. For TEM experiments, we used the dilution of 1 : 200 as it represented the lowest concentration that exhibited the highest antioxidant power out of the tested dilutions. The damage observed in sperm incubated with H_2O_2 consisted of broken plasma membranes, reacted and absent acrosomes, chromatin with altered texture (Figure 4(a)), and flagella with altered periaxonemal and axonemal cytoskeletal elements. The *C. sativa* leaf extract diluted to 1 : 200 showed a protective activity towards the considered alterations induced by LPO (Figure 4(b)). In particular, the percentages of sperm with a broken plasma membrane (85.67 ± 2.96 vs. 20 ± 2.89, $P < 0.05$), with the absent acrosome (81.66 ± 4.41 vs. 31.66 ± 2.08, $P < 0.05$), with altered chromatin (64.33 ± 2.96 vs. 35 ± 2.88, $P < 0.05$), and with anomalies in axonemal and periaxonemal structures (52.67 ± 1.45 vs. 28.33 ± 1.67, $P < 0.05$) were significantly reduced in samples treated with the *C. sativa* leaf extract (Figure 5).

4. Discussion

In this research, we characterized a 75% *v*/*v* ethanolic leaf extract of a typical Tuscan chestnut variety: "Bastarda Rossa" from Mount Amiata. *C. sativa* leaves are the least exploited by-product of this species, despite the fact that they are rich in phenolic compounds, which are endowed with antibacterial and DNA-protective activities and are efficient in the prevention and treatment of oxidative stress-mediated diseases such as photoaging [29].

A 70% *v*/*v* ethanolic extract of *C. sativa* leaves from Mirandela (Portugal) was investigated and resulted to be very effective in *in vitro* scavenging and antioxidant tests. In a cutaneous irritation test, performed in 20 healthy people, it resulted to be well tolerated and free from side effects [30]. Barreira et al. [31], in a comparative study, reported that antioxidant potential of chestnut leaves was higher than that of almond leaf extracts.

Chemical analysis conducted in this work showed that CEE contains a concentration of total polyphenols (3475.23 mg/l) higher than that of other well-studied antioxidant natural products such as red wine [32] and green and black teas from *Camellia sinensis* Kuntze leaves [33]. Interestingly, a large part of these polyphenols is represented by monomeric flavonoids; in particular, hyperoside and quercetin glycosides were the most abundant constituents. Recently, great attention has been focused on hyperoside for its effective antioxidant capacity in an innovative model of oxidative stress using the yeast *Saccharomyces cerevisiae* [34].

Table 2: Mean ± standard deviation and median (values in italics) of progressive sperm motility % evaluated in controls and samples treated with 1 : 100, 1 : 200, and 1: 500 *C. sativa* leaf extracts.

Sperm parameter	Control	*C. sativa* 1 : 100	*C. sativa* 1 : 200	*C. sativa* 1 : 500	Kruskal–Wallis test
Progressive sperm motility %	68.37 ± 10.97 *69*	68.5 ± 13.41 *69*	68 ± 11.93 *68*	68.25 ± 11.41 *73*	$P = 0.9731$

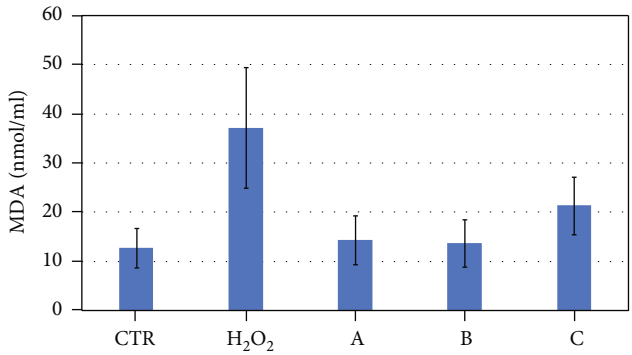


Figure 3: Mean values and standard error of MDA levels measured in swim-up-selected samples treated as follows: controls (CTR), aliquots treated with H_2O_2, and specimens treated with H_2O_2+*C. sativa* leaf extract 1 : 100 (A), H_2O_2+*C. sativa* leaf extract 1 : 200 (B), and H_2O_2+*C. sativa* leaf extract 1 : 500 (C). Kruskal–Wallis test: $P = 0.02913$. H_2O_2 vs. CTR: $P = 0.006993$. H_2O_2 vs. A: $P = 0.01476$. H_2O_2 vs. B: $P = 0.01571$.

Hyperoside also showed cytoprotective action against H_2O_2-induced cell damage in Chinese hamster lung fibroblast cells, exerting intracellular scavenging activity [35]. In addition, quercetin and its main glycosides, such as rutin and isoquercitrin, are well-known antioxidant molecules [36].

By monitoring the qualitative profile of the recorded chromatogram and according to Cerulli et al. [12] who studied the methanol extract of the leaves of *C. sativa* var. "Marrone di Roccadaspide," it is plausible to refer that phenolic acids, hydroxycinnamic derivatives, and tannins are also present in the CEE. To determine the antioxidant capacity of the chestnut leaf extract, we employed either the classic and validated DPPH test or the cyclic voltammetry, an interesting, but least exploited, technique. CEE exerted a good antiradicalic capacity in the DPPH test, showing to be effective even diluted to over 1000-fold. The IC_{50} value of the extract is comparable with that of a polyphenol-enriched red wine [37]. In spite of its simplicity, the qualitative conventional electroanalysis has a prominent role in the assessment of antioxidants in food and biological samples and in the evaluation of their antioxidant activity *in vitro*. The relationship between the sample voltammogram and its *in vitro* antioxidant activity was defined by its oxidation potential: peaks with low values of oxidation potential were associated with high antioxidant activity while peaks with the high values were associated with low antioxidant capacities. The high sensitivity of DPV enables pointing out processes with low peak current, which is difficult to highlight with cyclic voltammetry. In our extract, these peaks were present at peak potential values of +0.15 to +0.40 V and +0.55 V, with the last one evident also in the cyclic voltammogram. All these potential peaks are associated with a good antioxidant activity according to the electrochemical behaviour typical of flavonoids [38].

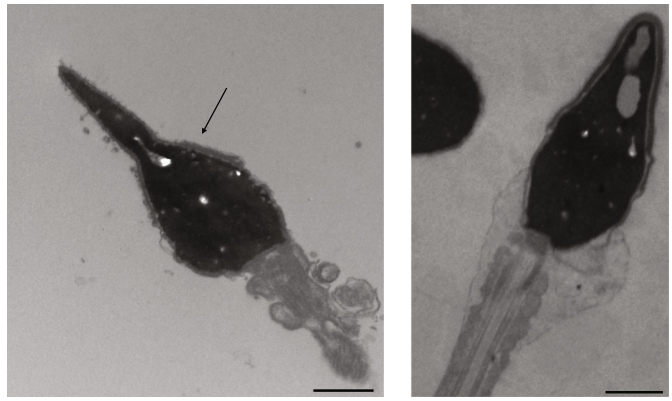
In particular, the peaks located at more cathodic potential values than quercetin and hyperoside indicate that the chemical composition of the extract includes minor components which are responsible for the antioxidant activity of CEE.

These findings related to the redox characteristics of the chestnut extract implemented those obtained in the evaluation of antiradicalic capacity with DPPH and highlighted the antioxidant characteristics of the extract phytocomplex.

The experimental protocol performed in this work confirmed that the chestnut leaf extract of Bastarda Rossa is worthy to be investigated for its interesting biological characteristics. For this reason, we decided to explore the ability of the *C. sativa* leaf extract to prevent LPO induced *in vitro* by H_2O_2 on swim-up-selected human sperm. Our final purpose was to find out natural substances that could be used as antioxidant supplementations in fertilization culture media.

It is known that semen processing for assisted reproduction technologies and cryopreservation requires protocols that include centrifugation and incubation of spermatozoa in culture media and that these procedures could exacerbate oxidative stress and induce damage to sperm cells [39] due to ROS generation. Spermatozoa, particularly human ones, are vulnerable to ROS attack. ROS may cause LPO of sperm membranes given that they are particularly rich in unsaturated fatty acids. The LPO process leads to a loss of membrane integrity, an increase in its permeability, inactivation of cellular enzymes, DNA damage, and cell apoptosis. This problem is not only related to semen handling, since it is estimated that semen samples from 25% *ca.* of infertile men present elevated levels of ROS and, often, decreased antioxidant capacity [40]. The oral supplementation of antioxidant substances may improve semen parameters and oxidant/-antioxidant status in infertile males [41]. Therefore, it seems reasonable to support the treatment of male infertility with substances able to neutralize ROS. In this particular field, the study of natural compounds endowed with antioxidant properties is appealing, either from the point of view of male infertility treatment or for the development of new strategies for the supplementation *in vitro* of media used for semen handling.

We have previously demonstrated the scavenging activity of some natural compounds, such as resveratrol, quercetin, rutin, naringenin, epicatechin, and Propolfenol®, a phytocomplex rich in European propolis and catechins, against LPO induced *in vitro* to human spermatozoa [20–22, 42]. Recently, Kedechi et al. [39] demonstrated the antioxidant



(a) (b)

Figure 4: TEM micrographs of human sperm treated with H_2O_2 without the *C. sativa* leaf extract (a) and H_2O_2 with the *C. sativa* leaf extract diluted to 1 : 200 (b). In (a), a spermatozoon with the reacted acrosome (arrow) and altered chromatin texture, particularly evident along the edge of the cell, is shown. The membrane is broken. In (b), a normal spermatozoon with integer structures is shown. Bars: 1 μM.

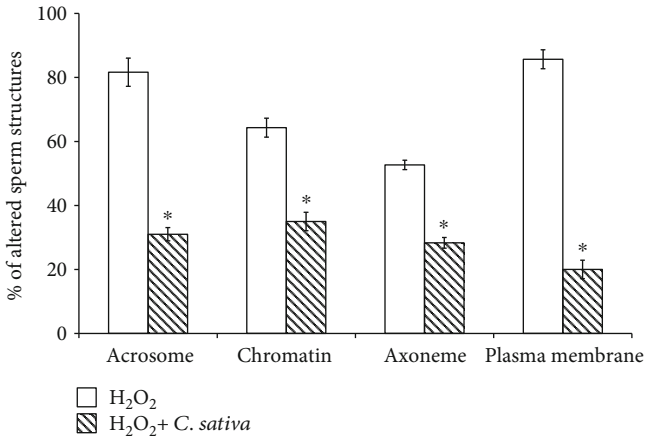


Figure 5: Mean values and standard errors of percentages of altered sperm structures analyzed by TEM in samples treated as follows: aliquots treated with H_2O_2 and specimens treated with H_2O_2 and *C. sativa* leaf extract 1 : 200. H_2O_2 vs. H_2O_2+*C. sativa*: $P < 0.05$.

effect of hydroxytyrosol, the main phenolic compound of virgin oil, during sperm centrifugation. In addition, in a mouse model, it was found that the supplementation of media with antioxidants during all stages of the *in vitro* fertilization procedures and cultures could play a beneficial effect on assisted reproduction and may preserve embryo viability [43].

The results of this study demonstrated that the *C. sativa* leaf extract used at dilutions of 1 : 100, 1 : 200, and 1 : 500 did not show any toxicity on sperm motility. These results are different from those obtained by some of our group using purified polyphenols [20, 21], which, at high concentration, decreased sperm motility and viability and damaged DNA, as we observed using liposomes loaded with 100 μM quercetin [42].

It seems that a combination of natural antioxidant compounds is more tolerated than single purified compounds and that the phytocomplex exerts a synergistic action, at least in this kind of *in vitro* experiments, as it was observed also when we used Propolfenol® in similar protocols [22]. Actually, the multitarget features and aspecific mechanisms are the rationale for the use of phytocomplexes in modern phytotherapy. The *C. sativa* leaf extract, at dilutions of 1 : 100 and 1 : 200, showed a scavenging activity on H_2O_2-induced LPO, restoring similar levels of MDA measured in the control samples. To visualize the protective activity of the extract, a deep analysis of the single sperm organelles was performed using TEM that is a useful method to explore the morphological variation of human sperm treated *in vitro* with different compounds [20, 21]. In general, TEM is one of the methods to study the different organelles characterizing sperm defects that can influence the fertilizing potential [44, 45]. In this research, we demonstrated that the *C. sativa* leaf extract diluted to 1 : 200 was powerful in protecting sperm against severe oxidative damage induced by H_2O_2. The extract was able to defend sperm membranes and the acrosomes, both structures particularly affected by H_2O_2 treatment.

5. Conclusions

The leaf extract of *Castanea sativa* Mill. var. Bastarda Rossa (Mount Amiata, Italy) is particularly rich in polyphenols and flavonoids and shows scavenging properties against oxidative stress induced by H_2O_2 to human ejaculated sperm. This extract could be used in protocols of gamete handling such as techniques of assisted reproduction and cryopreservation of semen, all conditions in which oxidative stress is exacerbated. We are aware that further studies are needed to confirm the antioxidant ability of the *C. sativa* leaf extract. Should such activity be corroborated, these studies could be extended to the field of farming animals, in which the cryopreservation procedures are particularly common.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the reported research.

Authors' Contributions

All the authors gave substantial contributions to the research design and drafting of the paper or its critical revision and approved the submitted version. Marco Biagi designed the analytical protocol of *Castanea sativa*, critically analyzed the results, and wrote the paper. Daria Noto, in particular, did the following: semen analysis, swim-up method, and treatment of sperm with *Castanea sativa*. Maddalena Corsini performed electrochemistry. Giulia Baini performed chemical analyses and DPPH. Daniela Cerretani, in particular, did the following: determination of MDA. Giorgio Cappellucci performed sampling and DPPH. Elena Moretti, in particular, contributed to the research design and performed TEM analysis, paper writing, data interpretation, and semen analysis.

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Research Article

Effect of Dietary *n*-3 Source on Rabbit Male Reproduction

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In the last two decades, the human sperm count linearly decreased in Western countries. Health problems, lifestyle, pollutants, and dietary behaviours are considered as the main risk factors, and the unbalance of dietary *n*-6/*n*-3 fatty acids is one of the most relevant. The aim of the present research is to study the effect of different dietary sources of *n*-3 polyunsaturated fatty acids (PUFA) on reproductive traits using rabbit buck as the animal model. Fifteen rabbit bucks were assigned to three experimental groups: the control group, the FLAX group fed 10% extruded flaxseed, and the FISH group fed 3.5% fish oil for 110 days (50-day adaptation and 60-day experimental periods). Semen samples were collected weekly, whereas blood was collected every two weeks for the analytical determination of semen traits, oxidative status, fatty acid profiles, isoprostanes, neuroprostanes, and the immunocytochemistry of docosahexaenoic acid (DHA) and eicosapentaenoic (EPA) acid. At the end of the trial, the rabbits were killed and the testes were removed and stored for the analysis of fatty acid profile and immunocytochemistry. Results showed that dietary administration of *n*-3 PUFA improved the track speed of the sperm and increased the *n*-3 long-chain PUFA mainly confined in the sperm tail. Seminal plasma increased the thiobarbituric reactive substances (TBARs) by three times in the groups fed supplemental *n*-3, whereas the F_2-isoprotanes (F_2-IsoPs) and F_4-neuroprostanes (F_4-NeuroPs) were lower and higher, respectively, in both supplemented groups than in the control. The testes and sperm showed a higher DHA and EPA distribution in rabbits from the *n*-3 supplemented groups compared with the control. In conclusion, supplemental dietary *n*-3 PUFA improved sperm motion traits and resulted in an enrichment of membrane fatty acid in the sperm and testes of the rabbits. However, such an increased amount of PUFA negatively affected the sperm oxidative status, which was mainly correlated with the generation of F_4-NeuroPs with respect to F_2-IsoPs. Accordingly, the latter cannot be considered a good marker of oxidation when diets rich in *n*-3 PUFA are provided.

1. Introduction

In the last two decades, the sperm count has been progressively and linearly decreasing [1]; accordingly, in the next 30 years, a dramatic decrease in the fertility rate is expected. Many factors can reduce male fertility: chronic health problems, environmental pollutants, stress, and lifestyle, including dietary habits. Lipids play a crucial role in the structure and function of cells; among them polyunsaturated fatty acids (PUFA) represent about 30% to 50% of total fatty acids (FA) in the membrane of mammal spermatozoa. The body of literature has reported that the FA profile of the sperm membrane is different in men with asthenozoospermia compared with normospermic men [2].

Long-chain (LC, ≥20C) PUFA result from elongations and desaturations of essential FA: linoleic acid (LA, 18 : 2*n*-6) and *α*-linolenic acid (ALA, 18 : 3*n*-3). These FA are known to affect membrane behaviour and flexibility [3] and are eicosanoid precursors [4]. Testes and sperm have a characteristic lipid composition that is highly enriched in LC PUFA, predominantly docosapentaenoic acid (DPA*n*-6, 22 : 5*n*-6) in rats and other rodents and docosahexaenoic acid (DHA, 22 : 6*n*-3) in humans [5]. PUFA accumulate in mammalian testes during puberty and are essential for sperm maturation, motility, and acrosome reaction [6]. They are incorporated into maturing germ cells by lysophosphatidic acid acyltransferase 3 [7]. An improper FA profile modifies the function of Sertoli cells as spermatogenesis supporters, influencing germ cell apoptosis [8]. Furthermore, during epididymal maturation, the lipid composition of the sperm membrane is remodelled and the saturation of FA increases from *caput* to the *cauda* epididymis, while the proportion of PUFA remains similar [9].

The current Western diet has an abundance of *n*-6 with respect to *n*-3 PUFA and the *n*-6/*n*-3 ratio is two to four times (10-20 : 1) higher than the requirement [10]. Dietary plans with different *n*-6/*n*-3 ratios may affect the sperm FA profile, physiology, and DNA integrity of the sperm and testicular cell subpopulations. Sperm PUFA are highly susceptible to the oxidative process, due to the high degree of unsaturation. Such oxidative damage is triggered by the insufficient protection exerted by antioxidants, which are mainly enclosed in seminal plasma [11]. Agarwal et al. [12] reported that 20–88% of subfertile men had a high presence of reactive oxygen species (ROS) in the semen.

n-6 PUFA derivatives resulting from arachidonic acid (ARA, 20 : 4*n*-6) have prothrombotic and proaggregatory properties, whereas *n*-3 metabolites resulting from eicosapentaenoic acid (EPA, 20 : 5*n*-3) and docosahexaenoic acid (DHA, 22 : 6*n*-3) have anti-inflammatory, antiproliferative, and antiatherosclerotic activities [13, 14]. In this view, isoprostanes have recently been identified as markers of *in vivo* and *ex vivo* oxidative damage [15]. Considering that isoprostanes are formed by the esterification of membrane phospholipids, the particular lipid composition of the sperm cell membrane may be one preferential source of their production. Higher levels of F_2-isoprostanes (F_2-IsoPs) originating from ARA have been detected in the semen of infertile patients with varicocele than in control and idiopathic infertile men [16] indicating that F_2-IsoPs could be considered a marker of testis inflammation. Moreover, F_2-IsoP synthesis appears to be affected by PUFA dietary intake [17] and exogenous administration of *n*-3 PUFA has been shown to reduce F_2-IsoPs in patients with Rett syndrome [18]. Esmaeili et al. [19] stated that a high intake of *n*-3 PUFA enhances sperm characteristics in a dose/time-dependent manner. Furthermore, Martinez-Soto et al. [20] showed that the *n*-6/*n*-3 PUFA ratio was lower in sperm of fertile men than in infertile patients. On the other hand, the *n*-6/*n*-3 PUFA ratio could be associated with the production of a different class of isoprostanes, namely F_4-neuroprostanes (F_4-NeuroPs), in that they are nonenzymatic oxidised products from DHA [21].

In humans, diet is difficult to standardise, thus many researchers use an *in vitro* approach, which evaluates the effect on isolated cells or tissue and does not consider the effect on spermatogenesis. The rabbit is an excellent model of reproductive functions because mature sperm can be easily and continuously collected with an artificial vagina in longitudinal studies [22], and it is particularly interesting for studying sperm alterations due to infection and/or inflammation [23, 24].

Accordingly, this paper aims to study the effect of different *n*-3 PUFA dietary sources on semen quality, using rabbit buck as the animal model. Spermatogenesis, semen parameters, and the lipid profile have been investigated. One diet was enriched with flaxseed, which has a very high ALA content, whereas fish oil diet directly supplies ALA derivatives (EPA, DPA*n*-3, and DHA).

2. Materials and Methods

2.1. Animals and Experimental Design. Thirty New Zealand White rabbit bucks aged 140 days were trained for semen collection for about 30 days. During the training period, the libido (defined as the time between the introduction of a female rabbit and ejaculation) and several sperm traits (volume, concentration, motility rate, and vitality) of each rabbit buck were recorded in order to form homogenous groups.

Fifteen rabbits were selected and divided into three experimental groups ($n = 5$/group) and fed different diets (Table 1):

(i) The control group was fed ad libitum with the standard diet

(ii) The FLAX group was fed a standard diet, which was supplemented with 10% of extruded flaxseed

(iii) The FISH group was fed a standard diet, which contained 3.5% of fish oil (Nordic Naturals Omega-3®)

In Figure 1, the experimental design is reported. The dietary protocol involved 50 days of adaptation during which the rabbits were only monitored for semen collection and a subsequent 60 days (a full spermatogenic cycle) during which sperm determinations were evaluated.

This study was conducted in accordance with the Guiding Principles in the Use of Animals and approved by the Animal Ethics Monitoring Committee of the University of Siena (CEL AOUS; authorization no. 265/2018-PR, ISO-PRO 7DF19.23).

2.2. Sampling of Semen, Blood, and Reproductive Organs. Semen samples were collected weekly from each rabbit buck (Figure 1), one week before the start of the trial and during the experimental period, producing a total of ten collections.

Semen samples were collected by means of an artificial vagina heated to 38°C with water and immediately transferred to the laboratory [25]. The evaluations of sperm

Table 1: Formulation and proximate analysis of the control and *n*-3-enriched diets.

Ingredients (g/kg)	Control	FLAX	FISH
Dehydrated alfalfa meal	300	380	380
Soybean meal 44%	150	100	150
Barley meal	410	310	335
Wheat bran	52	52	52
Soybean oil	30	—	—
Extruded flaxseed	—	100	—
Fish oil*	—	—	35
Beet molasses	20	10	10
Calcium carbonate	7	7	7
Calcium diphosphate	13.5	13.5	13.5
Salt	7	7	7
DL-methionine	0.5	0.5	0.5
Vitamin-mineral premix†	10	10	10
Crude protein	175	174	175
Ether extract	480	472	425
Crude Fiber	124	137	130
Ash	89	84	90

*Nordic Naturals Omega-3®=purified deep sea fish oil (from anchovies and sardines) containing EPA—330 mg/100 g, DHA—220 mg/100 g, and other *n*-3 LC PUFA—140 mg/100 g+α-tocopherol for preservation. †Per kg diet: vitamin A—11.000 IU; vitamin D_3—2000 IU; vitamin B_1—2.5 mg; vitamin B_2—4 mg; vitamin B_6—1.25 mg; vitamin B_{12}—0.01 mg; alpha-tocopheryl acetate—200 mg; biotine—0.06 mg; vitamin K—2.5 mg; niacin—15 mg; folic acid—0.30 mg; D-pantothenic acid—10 mg; choline—600 mg; Mn—60 mg; Fe—50 mg; Zn—15 mg; I—0.5 mg; Co—0.5 mg.

quality were immediately performed on raw samples, as described later. The semen samples were centrifuged at 200 × g for 15 mins; seminal fluid was recovered for the determination of F_2-IsoPs and F_4-NeuroPs (butylated hydroxytoluene (BHT) was added at a final concentration of 90 μM). The sperm cells were divided into three aliquots. One aliquot was processed for immunocytochemistry, and the other two aliquots of 10^8 spermatozoa/mL were stored at -80°C for the evaluation of the oxidative status and FA profile.

Every two weeks, blood samples (2 mL) were taken from the auricular marginal vein using a 2.5 mL syringe fitted with a butterfly needle, after the local application of an anaesthetic cream (EMLA®). Serum was obtained from blood samples coagulated at room temperature for 2 hrs, and then the collection tubes were rimmed and refrigerated at 4°C for 24 hrs before analysis. Plasma was obtained from blood samples collected in tubes containing Na_2-EDTA and immediately centrifuged at 2,500 × g for 15 mins at 4°C. For the determination of plasma F_2-IsoPs and F_4-NeuroPs, BHT was added (90 μM, final concentration).

At the end of the trial (110 days), the rabbits were killed and their testes were accurately removed; a part was fixed for immunocytochemistry, and another part was sampled in sterile tubes and stored at -80°C for the evaluation of the FA profile.

2.3. Sperm Quality Assessment. After collection, semen was immediately subjected to analyses to determine the following sperm traits:

(i) Volume (mL), which was determined by graduated tubes

(ii) Sperm concentration (number of sperm × 10^6/mL), which was measured by means of a Thoma-Zeiss cell counting chamber with a 40x objective

(iii) Kinetic characteristics, which were analysed by a Computer-Assisted Semen Analyzer (model ISAS®4.0, Valencia, Spain) after appropriate dilution (1/20) with a modified Tyrode's albumin lactate pyruvate buffer [26] at pH 7.4 and 296 mOsm/kg. This system consisted of a negative phase-contrast optic system (Olympus CH-2) equipped with a CCD Sony camera. The set-up parameters were previously established, and the acquisition rate was set at 100 Hz [27]. For each sample, two drops and six microscopic fields were analysed for a total of 300 spermatozoa. Numerous sperm motion parameters were recorded, but only the motility rate (percentage of motile sperm/total sperm) and track speed (μm/s, the sum of the incremental distances moved by the sperm in each frame along the sampled path divided by time) were reported

2.4. Oxidative Status of Seminal Plasma and Blood Plasma. The extent of sperm lipid peroxidation (thiobarbituric reactive substances (TBARs) was assessed by measuring malondialdehyde (MDA) along with other substances that are reactive to 2-thiobarbituric acid (TBA), as reported by Mourvaki et al. [26]. The molar extinction coefficient of MDA was 1.56×10^5 1/M ∗ cm. The results were expressed as nmol MDA/mL.

Lipid peroxidation was evaluated in the plasma using a spectrophotometer (set at 532 nm, Shimadzu Corporation UV-2550, Kyoto, Japan), which measured the absorbance of TBARs and a 1,1,3,3-tetraethoxypropane calibration curve in sodium acetate buffer (pH = 3.5) [28]. The results were expressed as nmol of MDA/mL of plasma.

2.5. Determination of the Levels of Free F_2-IsoPs and F_4-NeuroPs. The levels of free F_2-IsoPs and F_4-NeuroPs were determined by gas chromatography/negative-ion chemical ionisation tandem mass spectrometry (GC/NICI-MS/MS).

After thawing, the plasma and seminal samples were treated with a volume of acidified water (pH 3) and spiked with a tetradeuterated derivative of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$-d_4; 500 pg), as internal standard. Subsequently, solid phase extraction procedures were carried out according to a previously reported methodology [29]. Briefly, each sample (plasma or seminal plasma) was applied to an octadecylsilane (C_{18}) cartridge and the eluate was transferred to an aminopropyl (NH_2) cartridge to collect isoprostanes. All the final eluates were derivatised to convert the carboxyl group of the F_2-IsoPs or $PGF_{2\alpha}$-d_4 into pentafluorobenzyl ester and the hydroxyl group into trimethylsilyl ethers, as previously

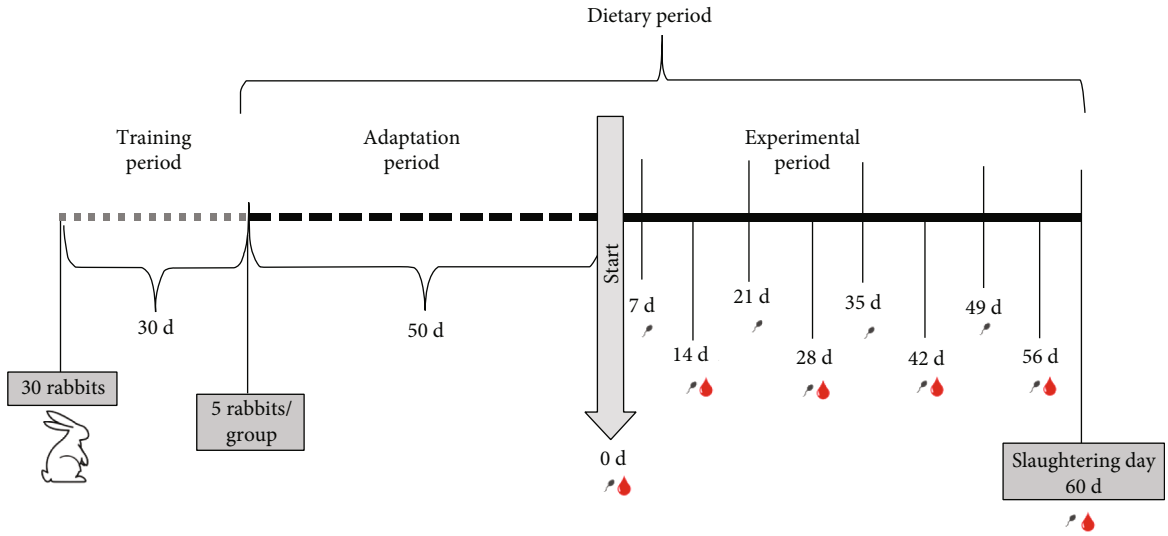


Figure 1: Experimental design of the trial. The gray line shows the training period during which the animals were trained for semen collection, and the seminal traits were analysed in order to create three homogeneous groups. The dashed black line shows the adaptation period during which the animals were fed with three different diets. The solid black line shows the experimental period during which the semen and blood samples were collected, and several traits were analysed.

reported [29]. The derivatised F_2-IsoPs and $PGF_{2\alpha}$-d_4 were analysed by GC/NICI-MS/MS. The ions that were determined were the product ions at m/z 299 and m/z 303, derived from the $[M\text{-}181]^-$ precursor ions of 8-iso-$PGF_{2\alpha}$, also referred to as 15-F_{2t}-IsoP (m/z 569) and $PGF_{2\alpha}$-d_4 (m/z 573), respectively [30].

With reference to F_4-NeuroPs, the mass ions that were determined were the product ions at m/z 323 and m/z303, derived from the $[M\text{-}181]^-$ precursor ions of 10-F_{4t}NeuroPs, considered as the most represented F_4-NeuroPs (m/z 593) [21] and $PGF_{2\alpha}$-d_4 (m/z 573), respectively.

2.6. Fatty Acid Profiles of the Sperm and Testis. The lipid extraction from the raw semen and testis was performed according to the method of Folch et al. [31], and the esterification was carried out following the procedure of Christie [32]. The transmethylation procedure was conducted using eicosenoic acid methyl esters (Sigma-Aldrich) as internal standard. The recovery rates of the internal standard were 89 ± 4% and 83 ± 3% in the semen and the testis, respectively.

The FA composition was determined using a Varian gas-chromatograph (CP-3800) equipped with a flame ionisation detector and a capillary column of 100 m length × 0.25 mm × 0.2 μm film (Supelco, Bellefonte, PA, USA). Helium was used as the carrier gas with a flow of 0.6 mL/min. The split ratio was 1 : 20. The oven temperature was programmed as reported by Mattioli et al. [33]. Individual FAME were identified by comparing the relative retention times of peaks in the sample with those of a standard mixture (FAME Mix Supelco; 4 : 0 to 24 : 0) plus *cis*-9 *cis*-12 C18 : 2; *cis*-9 *cis*-12 *cis*-15 C18 : 3; and *cis*-9 *cis*-12 *cis*-15 C18 : 3 (all from Sigma-Aldrich). The FA were expressed as % of total FA. The average amount of each FA was used to calculate the sum of the total saturated fatty acid (SFA), monounsaturated fatty acid (MUFA), and PUFA.

To evaluate the efficiency of the metabolising precursors (LA and ALA) into LC PUFA, the ratio LC PUFA/precursors was calculated for both *n*-3 and *n*-6 PUFA [34].

2.7. Testosterone Evaluation in Blood Plasma. The testosterone concentration in the rabbit serum was performed by radioimmunoassay assay (RIA). The kit that was used was the Testosterone (^{125}I) RIA KIT (Ref: RK-61M Institute of Isotopes Co. Ltd., Bucharest). This assay is based on the competition between unlabelled testosterone and a fixed quantity of ^{125}I-labelled testosterone for a limited number of binding sites on a testosterone-specific antibody. This allows the reaction of a fixed amount of tracer and antibody with different amounts of unlabelled ligand, the amount of tracer bound by the antibody being inversely proportional to the concentration of unlabelled ligand. Upon the addition of a magnetisable immunosorbent, the antigen-antibody complex is bound on solid particles, which are then separated by either magnetic sedimentation or centrifugation. Counting the radioactivity of the solid phase enables the construction of a standard curve and samples to be quantitated. This method showed the following cross-reactions: 100% testosterone, 35% 5α-dihydrotestosterone, 0.8 5β-dihydrotestosterone, 0.01 17β-estradiol, and 0.01 cortisol. The percentage of interference was calculated using the formula of Abraham: $X/Y * 100$, where X and Y were, respectively, the weight of the substance to be determined and the weight of the interfering substance, so as to reduce the binding capacity by 50%. The sensitivity was the lowest dose of testosterone that was 5% lower than the initial binding capacity.

2.8. Immunohistochemical Analysis

2.8.1. Testicular Tissue. The testes of the rabbit bucks that were fed the control and *n*-3-enriched diets were cut into

Table 2: FA profile of the control and *n*-3-enriched diets.

	Control (% of total FA)	FLAX (% of total FA)	FISH (% of total FA)	Pooled SE
SFA	19.80[a]	15.40[a]	38.10[b]	1.82
MUFA	17.40	15.80	14.50	0.87
PUFA	62.80[a]	68.80[a]	47.40[b]	5.12
LA	50.45[b]	22.30[a]	20.50[a]	2.11
ALA	11.15[a]	45.80[b]	18.50[a]	1.42
LC PUFA*n*-3	—	—	10.50	1.00
EPA	—	—	3.50	0.21
DHA	—	—	4.20	0.28
n-6	51.45[b]	22.80[a]	21.00[a]	2.35
n-3	11.35[a]	46.00[c]	26.40[b]	1.55
n-6/*n*-3	4.53[b]	0.50[a]	0.80[a]	0.01

a, b, and c on the same line means $P \leq 0.05$. Legend: SFA—saturated fatty acids; MUFA—monounsaturated fatty acids; PUFA—polyunsaturated fatty acids; LA—linoleic acid; ALA—α-linolenic acid; LC PUFA—long-chain PUFA; EPA—eicosapentaenoic acid; DHA—docosahexaenoic acid.

small blocks and treated with 10% buffered formalin for 24 hrs at 4°C and were then washed in water for 1 h. After fixation, the tissues were dehydrated in a series of ethanol (50%, 75%, 95%, and 100%) and cleared with xylene. The specimens were treated with three infiltrations of molten paraffin at 60°C for 1 h and then solidified at room temperature. The obtained blocks were sectioned using a Leica RM2125 RTS microtome (Leica Biosystem, Germany); sections (4 μm) were collected in glass slides and stained using the hematoxylin-eosin method for routine histology. The paraffin sections from the testicular tissue of the control and treated rabbits were deparaffinised with xylene and then treated in a series of ethanol concentrations (100%, 90%, 80%, and 70%) for 5 mins and, finally, in water to rehydrate the tissue. For antigen retrieval, the sections were washed and treated with heat-induced epitope retrieval 1 (HIER 1) buffer (10 mM sodium citrate) at pH 6 for 20 mins at 95°C. Specimens were treated overnight at 4°C with rabbit anti-DHA and FITC-linked rabbit anti-EPA polyclonal antibodies (MyBioSource Inc., San Diego, CA, USA) at a dilution of 1 : 40.

After three washes for 10 mins in phosphate-buffered saline (PBS), the slides treated with anti-DHA or EPA and were incubated with goat anti-rabbit FITC-conjugated antibody (Sigma-Aldrich, Milan, Italy), diluted 1 : 100 for 1 h at room temperature. The slides were washed with PBS three times and mounted with 1,4-diazabicyclo(2.2.2)octane (DABCO, Sigma-Aldrich, Milan, Italy).

2.8.2. Ejaculate Sperm. Briefly, washed, smeared sperm [35] were incubated with the same polyclonal antibodies (DHA and EPA).

All the samples were observed under a Leica DMI6000 microscope (Leica Microsystems, Germany) with a 63x objective, and the images were acquired using a Leica AF6500 Integrated System for Imaging and Analysis (Leica Microsystems, Germany). In detail, the images were obtained with HCX PL FLUOTAR 63x/1.25 oil objective; filters for TRIC and FITC were selected. The micrographs were not modified with image elaboration software.

The specificity of the antibodies, guaranteed in the datasheets of both antibodies, was also evaluated by omitting the primary antibody.

2.9. Statistical Evaluations. All the traits (semen volume, concentration, kinetics, oxidative traits, testosterone, and isoprostanes) had repeated values and were analysed with a mixed model to evaluate the fixed effect of diet (control, flaxseed, and fish oil) [36] and the random effect of rabbit buck over time. LSmeans and pooled SE were reported. The Bonferroni correction was applied for multiple comparisons. The significance was set at $P < 0.05$.

3. Results

The FA profile of *n*-3 supplemented diets (Table 2) was richer in *n*-3 (46.00 and 26.40% vs. 11.35% in FLAX, FISH, and control, respectively) and lower in *n*-6 (mainly LA) compared with that in the control. The *n*-6/*n*-3 ratio was about the same in both the *n*-3 supplemented groups (0.50 and 0.80 in FLAX and FISH, respectively) and much lower than that in the control (4.53).

The TBARs of blood plasma were not significantly ($P > 0.05$) increased by dietary *n*-3 (Table 3). On the contrary, seminal plasma had about three times more TBARs in the groups fed supplemental *n*-3.

Conversely, F_2-IsoPs in the blood plasma of *n*-3-enriched diets decreased by about 28% and 38%, respectively, in FLAX and FISH groups (Table 4) and the seminal plasma had the same tendency (-39% and -35%). On the contrary, the levels of F_4-NeuroPs both in blood and in seminal plasma were double the levels observed in the control group.

The sperm kinetic traits (motility rate and track speed) significantly improved in bucks fed with *n*-3 sources (Table 5).

The *n*-3 PUFA source also affected the FA profile of the testes (Table 6) and the sperm (Table 7), increasing the DHA in the sperm from the FLAX and FISH groups by 10 and 30 times, respectively, in comparison with the control group. Almost the same tendency was shown in testes with

Table 3: TBARs in the blood and seminal plasma of rabbit bucks fed the control or *n*-3-enriched diets.

	Blood plasma (nmol MDA/mL)	Seminal plasma (nmol MDA/mL)
Control	42.12	3.38[a]
FLAX	48.74	10.66[b]
FISH	45.59	11.66[b]
Pooled SE	3.55	0.87

a and b on the same line column means $P \leq 0.05$.

Table 4: Isoprostanes (F_2-IsoPs) and neuroprostanes (F_4-NeuroPs) in the blood and seminal plasma of the rabbit bucks fed the control or *n*-3-enriched diets.

	F_2-IsoPs (pg/mL)		F_4-NeuroPs (pg/mL)	
	Blood plasma	Seminal plasma	Blood plasma	Seminal plasma
Control	150.21[b]	124.08[b]	8.52[a]	14.54[a]
FLAX	108.75[a]	75.78[a]	16.85[b]	25.04[b]
FISH	92.28[a]	80.01[a]	17.77[b]	27.51[b]
Pooled SE	8.05	7.22	1.45	1.83

a and b on the same column means $P \leq 0.05$.

a concomitant reduction of *n*-6 LC PUFA (mainly DPA*n*-6, 22:5*n*-6) when dietary *n*-3 was added.

In Table 8, the metabolic indexes of the testes and sperm of the different dietary groups are compared. These indexes (e.g., *n*-6 LC/LA and *n*-3 LC/ALA) roughly estimate the anabolic ability of the precursors (LA and ALA) to produce long-chain derivatives (ARA, DPA*n*-6, EPA, DPA*n*-3, and DHA). All these indexes were affected by dietary PUFA; in particular, the testes of *n*-3-enriched groups showed lower *n*-6 LC PUFA production and higher *n*-3 LC PUFA, with respect to the control.

Figure 2 shows the blood testosterone trend, which was significantly affected by time (age of bucks) and dietary groups. The effect of time, which is common in all the groups, is probably related to the maturation of the animals.

Regarding the effect of diet only, the FLAX group showed higher testosterone concentration with respect to the others. The group fed with fish oil showed slight differences over time and was almost similar to the control.

Immunofluorescent staining was performed on the paraffin-embedded testis tissue of the rabbit bucks fed control or *n*-3-enriched diets. As shown in Figure 3, we observed that DHA was strongly expressed in the tubules and the interstitial tissue of testis from the *n*-3-enriched groups (Figures 3(b) and 3(c)) compared with the control (Figure 3(a)).

The labelling of EPA (Figures 4(a)–4(c)) was evident in interstitial tissue; it was weak in the seminiferous tubules of the control rabbits (Figure 4(a)), whereas it was very bright (FLAX, Figure 4(b); FISH, Figure 4(c)) in all stages of the spermatogenic process of the rabbit bucks fed *n*-3 enriched diets.

Ejaculated sperm from rabbit bucks fed control and *n*-3 enriched diets (FLAX and FISH) were treated with anti-DHA and EPA antibodies. In the sperm from the control rabbits, both DHA and EPA showed a weak fluorescent staining localised in the middle piece of the tail (Figures 5(a) and 5(d)); in the sperm from the treated groups, the fluorescent staining was strongly evident in the entire tail and often in the postacrosome region (FLAX, Figures 5(b) and 5(e); FISH, Figures 5(c) and 5(f)).

4. Discussion

As expected, the FA profile of the testes and sperm was affected by both dietary *n*-3 supplementations. Feeding male rabbits with fish oil resulted in the accumulation of *n*-3 LC PUFA in the testes and sperm. Flaxseed administration mainly increased ALA, but it also confirmed a certain LC PUFA synthesis and the ability of animals (liver and testis) to elongate and desaturate ALA [26, 37]. In agreement with our previous research [33], we demonstrated the ability of the reproductive tissues (e.g., ovary) to efficiently synthesize and accumulate *n*-3 LC PUFA. Similarly, in pig testes an increase in *n*-3 LC PUFA content (eleven-fold and two-fold more for EPA and DHA, respectively) was reported with dietary tuna oil administration [38], partially due to the higher fatty acid 2 (FADS2) gene expression.

The LC PUFA deposition in the sperm membrane probably contributes to the improvement of membrane fluidity and the relative motion traits of cells. Indeed, the track speed of the *n*-3 PUFA groups increased by about 30%. As a result, the tracing of dietary PUFA in testes and sperm is particularly interesting. Both diet supplementations may be able to increase DHA and EPA in two ways: being directly provided by fish oil or by flax, which provides their precursor.

An increased presence of DHA and EPA in the testes was also confirmed using the immunofluorescence technique. The DHA signal appeared to have increased in the interstitial tissue, putatively in the Leydig cells, germ cells, and Sertoli cells of rabbits belonging to both *n*-3 supplemented groups. In the testis, phospholipid DHA promotes sperm membrane structural changes that are required for a regular spermatogenesis [39]. Moreover, the presence of certain FA in Sertoli cells positively influences spermatogenesis for the significant role played by Sertoli cells during sperm maturation [40].

In a recent *in vitro* study, *n*-3 PUFA have been classified as protective for Sertoli cells [41]. EPA was mainly localised in the interstitial tissue of the control rabbits, but it was more abundant in the testes from the *n*-3 enriched groups, both in the interstitial tissue and germ cells. It is known that Leydig cells, producing testosterone, play a major role in spermatogenesis, and the enrichment of LC PUFA in the interstitial tissue of testes in *n*-3 PUFA groups may indicate a good status of Leydig cells. However, the secretory activity (e.g., testosterone) of these cells was affected in a different way by the *n*-3 dietary sources. Flaxseed, being one of the richest sources of phytoestrogens (lignans), which act as hormone-like compounds, may increase sex-hormone-binding-globulin synthesis by consequently stimulating testosterone production [42]. Qi et al. [43] have suggested that dietary

Table 5: Seminal traits of the rabbit bucks fed control or *n*-3-enriched diets.

	Control	FLAX	FISH	Pooled SE
Volume (mL)	0.61	0.57	0.55	0.04
Sperm concentration (10^6/mL)	255.31	280.10	248.32	18.53
Motility rate (%)	63.03[a]	77.22[b]	77.55[b]	5.40
Track speed (μm/sec)	180.91[a]	239.94[b]	228.72[b]	15.54

a and b on the same line means $P \leq 0.05$.

Table 6: Testes FA profile of the rabbit bucks fed the control or *n*-3-enriched diets.

	% of total evaluated FA	Control	FLAX	FISH	Pooled SE
Saturated fatty acid (SFA)	Miristic (C14 : 0)	2.50	2.80	1.80	1.35
	Palmitic (C16 : 0)	25.78	26.19	26.81	2.02
	Stearic (C18 : 0)	11.14	10.00	10.27	1.91
	SFA	39.43	38.99	38.88	2.90
Monounsaturated fatty acid (MUFA)	Palmitoleic (C16 : 1)	2.58	2.94	4.47	0.22
	Oleic (C18 : 1)	16.36	17.89	19.49	1.55
	MUFA	18.95	20.83	23.97	1.85
n-6 polyunsaturated acids (PUFA)	Linoleic (LA) (C18 : 2*n*-6)	11.85	13.37	14.01	2.01
	Eicosatrienoic (C20 : 3*n*-6)	5.87	5.39	3.67	0.08
	Arachidonic (ARA) (C20 : 4*n*-6)	8.46	5.87	6.27	0.04
	Adrenic (C22 : 4*n*-6)	2.32	1.60	1.18	0.24
	All-*cis* 4,7,10,13,16-docosapentaenoic (DPA*n*-6) (C22 : 5*n*-6)	10.04[b]	5.88[a]	7.01[ab]	3.34
	n-6 PUFA	38.53[b]	32.11[a]	32.15[a]	0.22
n-3 polyunsaturated acids (PUFA)	α-Linolenic (ALA) (C18 : 3*n*-3)	2.03[a]	7.02[b]	3.00[a]	0.05
	Eicosapentaenoic (EPA) (C20 : 5*n*-3)	0.01	0.05	0.04	0.09
	All-*cis*-7,10,13,16,19-docosapentaenoic (DPA*n*-3) (C22 : 5*n*-3)	0.01	0.05	0.09	1.50
	Docosahexaenoic (DHA) (C22 : 6*n*-3)	0.07[a]	0.83[b]	1.46[b]	0.10
	n-3 PUFA	2.13[a]	7.91[b]	4.58[b]	3.85
	n-6/*n*-3	18.13[c]	4.06[a]	7.02[b]	25.2

a, b, and c on the same line means $P \leq 0.05$.

flaxseed improves semen quality by increasing the testosterone hormone secretion, which may be related to higher StAR and P450scc mRNA and SF-1 expression. Moreover, Li et al. [44] showed that dietary linseed oil supplemented during peripuberty stimulates steroidogenesis and testis development in rams.

On the other hand, our results agree with Castellano et al. [38], who reported that the dietary supplementation of EPA and DHA partly reduced steroidogenesis. This may be due to an inhibition of prostaglandin release from ARA [38] and/or a reduction of the gene expression implicated in steroidogenic pathways [45].

Furthermore, the sperm from the control rabbits showed the EPA and DHA were localised in the sperm midpiece, whereas in the sperm from the *n*-3 supplemented groups, these FA increased and were distributed throughout the entire sperm tail. This localisation may be associated with an improvement in the sperm plasma membrane fluidity and an increase of the kinetic traits [46]. Mourvaki et al. [26] showed that dietary DHA in rabbit sperm is mainly incorporated into the midpiece and this enhancement is positively associated with sperm movement. In humans, other researchers [47] have reported that a higher intake of *n*-3 PUFA was positively correlated with sperm morphology, total sperm count, and sperm cell density, and that EPA has a positive influence on eel sperm performance [48]. Our data indicate that the membrane of the sperm head partly incorporates EPA and DHA, and this enrichment may influence two fundamental steps in the fertilisation process, such as acrosome reaction and membrane fusion [49]. It is known that changes in the sperm FA profile, essential for fertilisation, occur during cauda epididymal maturation [50]; however, this aspect has been not investigated here.

As already stated, PUFA are very susceptible to free radical attack, and oxidation increases as the number of double bonds increases. Therefore, the oxidisability of PUFA is correlated with the number of methylene groups located between two bonds and increases approximately two-fold for each additional methylene group [51].

Accordingly, although the diets had a high level of antioxidants (200 mg/kg vitamin E vs. the standard recommendation of 50 mg/kg) [52, 53], the increase of PUFA in

Table 7: Sperm FA profile of the rabbit bucks fed the control or *n*-3-enriched diets.

	% of total evaluated FA	Control	FLAX	FISH	Pooled SE
Saturated fatty acid (SFA)	Miristic (C14 : 0)	2.70[a]	4.00[b]	4.20[b]	1.35
	Palmitic (C16 : 0)	23.10	25.80	24.65	2.03
	Stearic (C18 : 0)	22.90[b]	23.90[b]	17.75[a]	1.90
	SFA	48.70[a]	53.70[b]	46.60[a]	2.89
Monounsaturated fatty acid (MUFA)	Palmitoleic (C16 : 1)	0.85	1.05	0.90	0.11
	Oleic (C18 : 1)	15.75[a]	17.50[ab]	20.50[b]	0.97
	MUFA	16.60[a]	19.55[ab]	21.40[b]	0.99
n-6 polyunsaturated acids (PUFA)	Linoleic (LA) (C18 : 2*n*-6)	7.15[b]	3.89[a]	4.65[a]	2.10
	Eicosatrienoic (C20 : 3*n*-6)	0.65[a]	1.72[b]	0.55[a]	0.08
	Arachidonic (ARA) (C20 : 4*n*-6)	1.55[b]	0.65[a]	0.70[a]	0.04
	Adrenic (C22 : 4*n*-6)	0.30	0.21	0.31	0.03
	All-*cis*-4,7,10,13,16-docosapentaenoic (DPA*n*-6) (C22 : 5*n*-6)	24.20[b]	15.88a	12.80[a]	3.48
	n-6 PUFA	33.85[b]	22.35[ab]	19.01[a]	3.85
n-3 polyunsaturated acids (PUFA)	*α*-Linolenic (ALA) (C18 : 3*n*-3)	0.20[a]	1.29[c]	0.45[b]	0.10
	Eicosapentaenoic (EPA) (C20 : 5*n*-3)	0.15[a]	0.45[a]	4.25[b]	0.10
	All-*cis*-7,10,13,16,19-socosapentaenoic (DPA*n*-3) (C22 : 5*n*-3)	0.10[a]	0.31[a]	1.65[b]	0.09
	Docosahexaenoic (DHA) (C22 : 6*n*-3)	0.25[a]	2.25[b]	6.65[c]	1.35
	n-3 PUFA	0.70[a]	4.20[b]	13.00[c]	0.20
	n-6/*n*-3	48.36[b]	5.32[a]	1.46[a]	3.88

a, b, and c on the same line means $P \leq 0.05$.

Table 8: Metabolic indexes of testes and sperm in the rabbit bucks fed the control or *n*-3-enriched diets.

	Control	FLAX	FISH	Pooled SE
Testes				
n-6 LC PUFA	26.70[b]	18.74[a]	18.14[a]	1.02
n-3 LC PUFA	0.09[a]	0.89[b]	1.58[b]	0.15
n-6 LC/LA	2.25[b]	1.40[a]	1.29[a]	0.11
n-3 LC/ALA	0.04[a]	0.13[ab]	0.53[b]	0.08
Sperm				
n-6 LC PUFA	26.78[b]	19.46[a]	14.36[a]	1.15
n-3 LC PUFA	0.50[a]	2.91[b]	12.55[b]	0.38
n-6 LC PUFA/LA	3.75	4.75	3.09	0.27
n-3 LC PUFA/ALA	2.50[a]	2.26[a]	27.89[b]	1.10

a, b, and c on the same line means $P \leq 0.05$. Legend: ALA—*α*-linolenic acid; LA—linoleic acid; LC PUFA—long-chain PUFA.

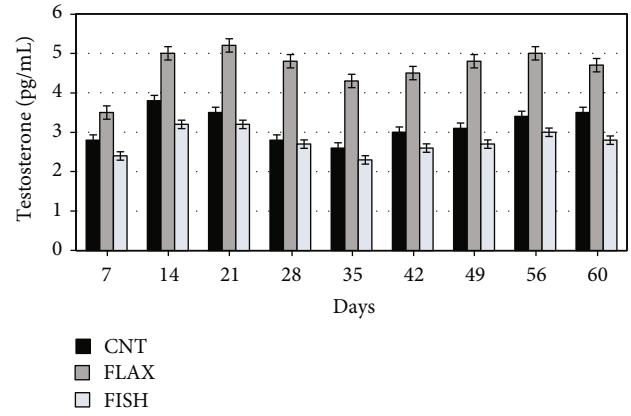
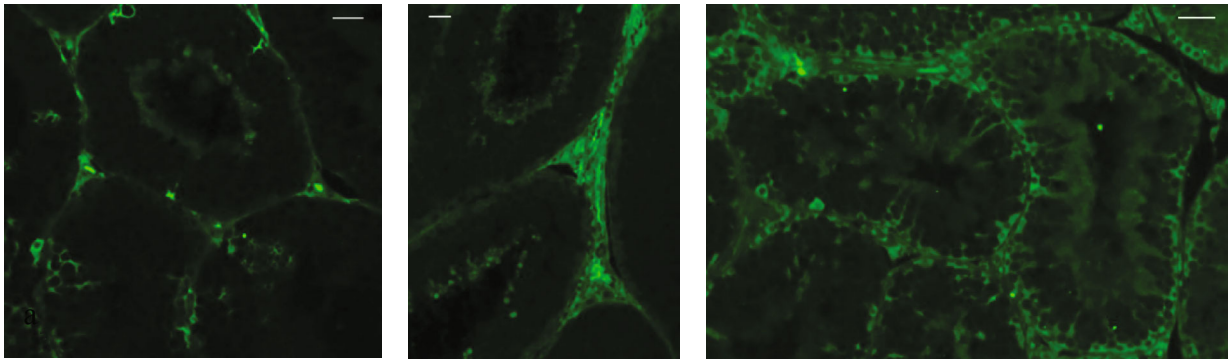


Figure 2: Effect of experimental diets on the testosterone (pg/mL) concentration in blood. The black bar shows the control (C), the dark gray bar shows the FLAX group, and the light gray bar shows the FISH group.

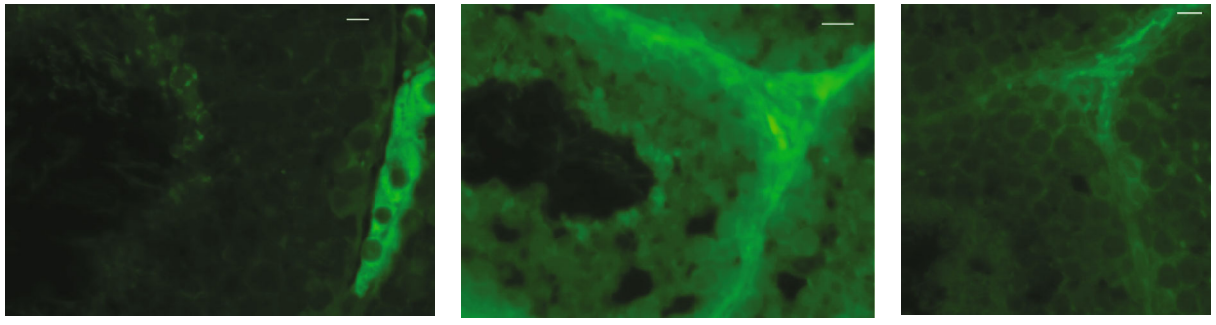
tissues and cells was accompanied by a reduction in the oxidative stability. In the present study, the extent of the TBARs was assessed by the MDA levels; it is conceivable that the supplementation of *n*-3 PUFA, increasing the substrate, directly influences the amount of MDA. It should be emphasised that the seminal plasma appeared more susceptible to peroxidation than the blood. Tissues exhibit different susceptibility to oxidative stress; the male reproductive system, similar to the central nervous system, may be particularly vulnerable to oxidative damage due to the limited efficiency of the antioxidant system and abundant PUFA content that is highly susceptible to lipid peroxidation [54].

At the same time, the isoprostanes (F_2-IsoPs and F_4-NeuroPs) did not appear to be strictly correlated with the overall oxidative status in both the blood and the semen. In particular, F_2-IsoPs, which are considered the "gold standard" for measuring oxidative stress in the whole body [55], were higher in the control group and the F_4-NeuroPs were lower when compared with the groups supplemented with *n*-3 PUFA. F_2-IsoPs decreased by up to 40% suggesting that *n*-3 effectively decreases the proinflammatory isoprostanes derived from arachidonate. At the same time, F_4-NeuroPs, deriving from DHA, increased by almost the same amount.



(a) (b) (c)

Figure 3: Immunolocalisation of DHA in testicular tissue from rabbit bucks fed control (a) and *n*-3 enriched diets ((b) FLAX; (c) FISH). A faint fluorescent stain in the germ cells and interstitial tissue (Leydig cells) is shown in (a). A high-labelled intensity in the interstitial tissue and germ cells is evident in (b) and (c). In (c), the signal is also present in the Sertoli cells. Bars: 30 μm (a); 20 μm (b); 40 μm (c).



(a) (b) (c)

Figure 4: Immunolocalisation of EPA in testicular tissue from rabbit bucks fed control (a) and *n*-3 enriched diets ((b) FLAX; (c) FISH). (a) EPA labelling is evident in interstitial tissue, and a faint fluorescent stain in germ cells is also shown in the control testis. A high-labelled intensity in the interstitial tissue and in germ cells is evident in FLAX (b) and FISH (c). Bars: 15 μm (a and c); 10 μm (b).

This investigation suggests that the F_2-IsoP level should be interpreted with caution as a marker of peroxidation in *n*-3 rich tissues (e.g., the reproductive system and the nervous system) or when that is induced by dietary *n*-3 PUFA. In such circumstances, it cannot be concluded that low F_2-IsoPs indicate no oxidative stress, as the reduction of F_2-IsoPs after the supplementation of *n*-3 PUFA might occur through several nonenzyme unidentified pathways and be accompanied by a comparable increase of F_4-NeuroP. Such an increase in neuroprostanes could be related to the peculiar localisation of the F_4-NeuroP precursor fatty acid (i.e., DHA). DHA, unlike ARA, is widely concentrated in the brain and testis, with relatively low levels in other organs [56]. Thus, in neural tissues, F_4-NeuroPs are the most abundant products of an oxidative nonenzyme pathway [57] and their quantification provides a highly selective index for *in vivo* neuronal oxidative damage.

To our knowledge, no other research has shown that F_4-NeuroPs in seminal plasma are affected by diet and were at low levels in animals fed with control diets (high LA and low *n*-3 PUFA). When additional *n*-3 PUFA are furnished, in *n*-3 rich tissues, these molecules increased and could act as specific biomarkers of *n*-3 peroxidation [58, 59]. Furthermore, other molecules resulting from the nonenzyme oxidation of PUFA could be also generated: i.e., F_3-IsoPs from EPA and E_1-IsoPs from ALA. In this regard, Roberts and Milne [60] found that with *n*-3 dietary supplementation, the brain level of IsoPs (F_3-IsoPs) produced from the oxidation of EPA significantly exceeds that of the F_2-IsoPs generated from ARA because EPA is more easily oxidisable (one more double bond than ARA).

Gao et al. [61] confirm that *n*-3 PUFA supplementation decreased F_2-IsoPs (up to 64%) in the heart tissue and led to the formation of F_3-isoprostanes, proving that *n*-3 effectively decreases levels of proinflammatory F_2-IsoPs formed from arachidonate. Such an observation is crucial because F_2-IsoPs are generally considered as proinflammatory molecules associated with the pathophysiological effect of oxidant stress. It is thus interesting to realise that the mechanism by which *n*-3 LC PUFA prevents certain diseases resides in its ability to decrease F_2-IsoP generation [60].

Syta-Krzyzanowska et al. [62] showed that in cerebral tissues, where the level of DHA is high (similarly to testes), a pathological situation (e.g., aneurysm) leads to the enhancement of lipid peroxidation with an increase in plasma F_2-IsoPs and F_4-NeuroPs (more than three-fold and eleven-fold, respectively). Related work [63] suggested that the metabolism of LC PUFA yields oxidised bioactive compounds that

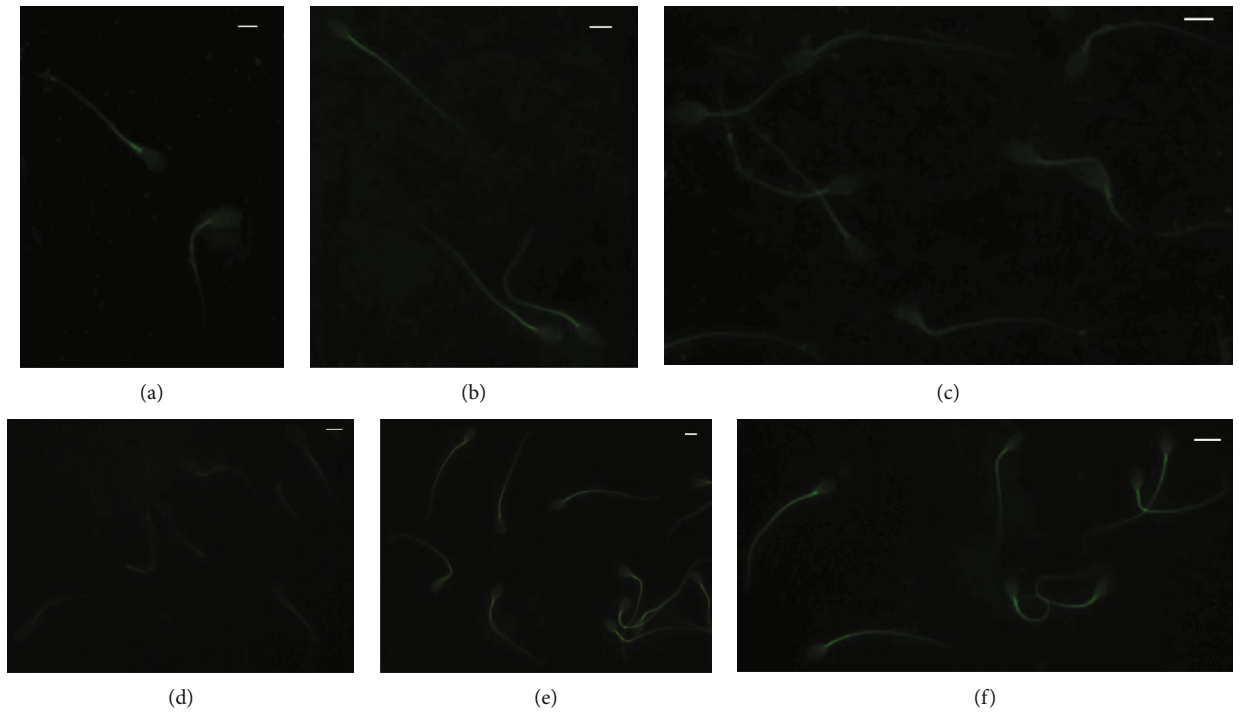


Figure 5: Spermatozoa from rabbit bucks fed control (a, d) and *n*-3 enriched diets ((b and e) FLAX; (c and f) FISH) treated with anti-DHA (a–c) and EPA (d–f) antibodies. A fluorescent stain was evident at the midpiece level in the control (a, d), and a high-labelled intensity stain along the sperm tail and, frequently, in the basal portion of the head was shown in the sperm from the treated groups ((b and e) FLAX; (c and f) FISH). Bars: 4 μm (a and b); 5 μm (c, d, and e); 6 μm (f).

mediate its effects. Serhan and Chiang [63] described novel anti-inflammatory hydroxylated EPA metabolites (termed E-series resolvins) deriving from the enzymatic-mediated oxidation of EPA. These findings have led to considerable interest in determining other oxidation products of EPA that may mediate the anti-inflammatory effects of this FA. It is conceivable that the increased sperm motility could be related to an increase of proresolving mediators (derived from DHA and EPA) or the biological activity of nonenzymatic *n*-3 PUFA metabolites (i.e., F_4-NeuroPs). However, the role and effect of these classes of compounds are still not entirely known.

5. Conclusions

The dietary administration of *n*-3 PUFA resulted in an enrichment of DHA and EPA in rabbit sperm and testes and indicated that the rabbit is a suitable model for the study of the spermatogenic process. Clearly, the fish oil diet supplemented with LC PUFA influenced the LC PUFA composition of testes and sperm more than the flax diet, which furnished the precursor. The *n*-3 supplementation improved the sperm motility rate and track speed in male rabbits; however, such an increased amount of PUFA negatively affected the sperm oxidative status, which could not be revealed with F_2-IsoP evaluations, because it is mainly derived from ARA (*n*-6 PUFA) metabolism. Therefore, F_2-IsoPs cannot be considered a good marker of oxidation when a diet rich in *n*-3 PUFA is provided. Furthermore, such oxidative thrust was mainly generated in the tissues with a higher concentration of *n*-3 PUFA, as demonstrated by the increased amount of F_4-NeuroPs. Further studies are in progress to test the effect of *n*-3 dietary supplementation on other rabbit tissues that are mainly involved in the *n*-3 PUFA-metabolic pathways (liver and brain).

Data Availability

The raw data (fatty acids and isoprostanes) used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Authors' Contributions

C. C. performed the conceptualization and project administration and wrote the original paper; S. M. performed the semen sampling, evaluated some semen traits (motility and sperm capacitation pattern) and fatty acid profiles, and wrote the original paper; C. S. performed the isoprostane determinations and conceptualization and wrote the original paper; E. C. performed animal care and evaluated semen traits and oxidative status; D. N. performed animal care, F_2-IsoP determination, and immunochemistry technique; E. M. performed the conceptualization, data interpretation, and immunochemistry analysis; A. D. B. performed the conceptualization, data interpretation, and statistical analysis; G. B. performed testes

immunocytochemistry experiments and image acquisition; T. D. performed F_4-NeuroP determinations and isoprostane data interpretation; C. D. F. performed F_4-NeuroP determinations and isoprostane data interpretation; G. C. performed immunocytochemical analysis, conceptualization, and project design and wrote the original paper.

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Review Article

Dietary Cholesterol and Lipid Overload: Impact on Male Fertility

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Lipid metabolic disorders due to poor eating habits are on the rise in both developed and developing countries, with a negative impact of the "Western diet" on sperm count and quality. Dietary lipid imbalance can involve cholesterol, fatty acids, or both, under different pathophysiological conditions grouped under the term dyslipidemia. The general feature of dyslipidemia is the development of systemic oxidative stress, a well-known deleterious factor for the quality of male gametes and associated with infertility. Sperm are particularly rich in polyunsaturated fatty acids (PUFA), an important characteristic associated with normal sperm physiology and reproductive outcomes, but also targets of choice for oxidative thrust. This review focuses on the effects of dietary cholesterol or different fatty acid overload on sperm composition and function in both animals and humans. The links between oxidative stress induced by dyslipidemia and sperm dysfunction are then discussed, including possible preventive or therapeutic strategies to preserve gamete quality, longevity when stored in cryobanking, and male fertility.

1. Introduction

In mammals, the formation of sperm able to fertilize is a multistep process consisting of the production of gametes in the testicles and their subsequent maturation. These posttesticular events begin in the epididymis [1] and continue in the female genital tract, allowing a small number of selected male gametes to finally reach the oocyte for the ultimate purpose of fertilization [2, 3]. Many sperm molecular components and/or properties are modified by the posttesticular maturation events (reviewed in [4–6]). A remarkable feature of these modifications is that they are all dependent on indirect mechanisms, *i.e.*, interactions/exchanges between sperm cells and their environment. Indeed, sperm cells are "silent" cells due to a very high DNA/nucleus compaction that does not allow gene transcription. In addition, the final step of morphological differentiation of sperm cells during spermiogenesis leads to the exclusion of most if not all cytosolic organelles, thus limiting the cells' ability to support translation and protein synthesis. Ultimately, this deprives sperm of any adaptive response to stress. Sperm cells are therefore very sensitive to the composition of their environment. As pathological situations lead to changes in the composition of the milieu in which sperm evolve, they can induce sperm dysfunction and male sterility. The epididymal territory is important to consider in this regard, as this organ is highly irrigated by both blood and lymphatic vessels, thus exposing cells to systemic influences that are themselves dependent on environmental conditions [7]. The composition of the epididymal fluid, responsible for sperm maturation, is the result of selective filtration of blood components through the epididymal epithelium. The composition of the blood and the integrity of the epididymal epithelium [8] are therefore two important parameters that can affect male fertility. It has been demonstrated in rodents that the cell junctions maintaining the integrity of the epididymal epithelium were targets of toxins of different origins [8], possibly altering the normal posttesticular maturation process of sperm.

Dyslipidemia is a term referring to a group of different blood lipid imbalances such as the frequent pure hypercholesterolemia (30% of the cases), hypertriglyceridemia, combined hyperlipidemia, or isolated decrease of HDL-cholesterol [9]. The prevalence of dyslipidemia is about 45% among men in Western countries, as reported in Canada [10] and France

[9]. Dyslipidemia is associated with different pathophysiological conditions such as metabolic syndrome, obesity, and cardiovascular diseases [11]. The negative impact of lipid metabolism disorders on fertility is now recognized, but there is a clear lack of knowledge about the underlying molecular mechanisms involved. This article proposes to examine the impact of dietary fat overload (cholesterol and other fats) on male fertility, with a particular focus on the links between dietary fat overload and oxidative stress.

2. Dietary Cholesterol and Male Infertility

2.1. Animal Models. The study of the relationships between dietary cholesterol intake and fertility is limited in men, so some information has been reported using animal models. Rabbits are interesting models because, unlike rodents, they are sensitive to a cholesterol-enriched diet and their lipid metabolism is closer to that of humans than that of mice. In this respect, it is one of the best models to study alterations in lipoprotein metabolism and atherosclerosis, a frequent cardiovascular consequence of dyslipidemia [12, 13]. The classic model consists in feeding rabbits a diet containing up to 2% cholesterol, which triggers a significant increase in plasma cholesterol levels and the lipoproteins β-VLDL (very low-density lipoprotein) derived from the liver, that are highly atherogenic. In the 1990s, two studies from the same group reported that rabbits fed a cholesterol-enriched diet (0.5%) showed (i) a significant decrease in the ability of sperm to undergo acrosomal reaction [14] and (ii) a significant increase in the concentration of filipin-sterol complexes in the plasma membrane of the acrosomal region, only in *cauda* epididymis sperm [15]. These two reports have shown that an overload of dietary cholesterol causes alteration of the acrosomal lipid domains when sperm pass through epididymal maturation. Plasma cholesterol levels were high when rabbits were fed a high cholesterol diet (HCD), but no differences were observed in seminal plasma cholesterol levels, sperm cholesterol levels, or even in the ratio of sperm cholesterol to phospholipids in *cauda* epididymis sperm. However, the molecular mechanisms behind these changes are still completely unknown.

A series of more recent publications using rabbits fed with HCD confirmed the previous results and further explored the phenotype of the spermatozoa. The animals were fed with a 0.05% cholesterol-enriched diet leading to dyslipidemia as the total cholesterol level in the serum was significantly increased. Under these conditions, the authors also found an increase in filipin-sterol complexes in the sperm acrosome region, associated with an increased percentage of morphologically abnormal sperm, reduced total motility, reduced ability to undergo normal capacitation (measured by tyrosine protein phosphorylation), and therefore, a reduced percentage of progesterone-induced acrosome-reacted spermatozoa [16]. In this study, the total sperm cholesterol content was increased when the rabbits were fed with the HCD. The authors showed in a complementary study that all the modified parameters described in rabbits fed with the HCD could be restored when they received a food supplement containing 7% olive oil [17]. The same group characterized HCD-fed rabbits as having abnormalities during the spermiogenesis process. These include a defective manchette, a temporary microtubular-based structure responsible for sperm elongation, which caused abnormal acrosome and nucleus development and inaccurate tail implantation [18]. These defects were shown to be due to an abnormal interaction between the manchette-acrosome complex and the membrane microdomains. Here, the authors demonstrated that a dietary intake of 7% olive oil combined with HCD could restore a normal phenotype [19]. Unfortunately, the authors did not propose any hypothesis on how olive oil dietary supplementation could act to restore a normal phenotype. The action of olive oil on sperm and testicles of hypercholesterolemic rabbits may be partly related to its ability to preserve the functional capacities of the membranes, probably due to the specific properties of the oleic acid contained in the oil [20]. In addition, olive oil also acts as an antioxidant due to its polyphenol content [21], which may be involved in limiting lipoperoxidative events as discussed below.

Data from other research groups, using rabbits as an animal model, provided additional information on the suspected causes of male fertility decline due to hypercholesterolemia, bringing forward a disruption of the integrity of the blood-testis barrier in 2% HCD-fed animals [22]. In a model obtained by feeding male rabbits a high-fat diet containing 0.5% cholesterol (and 4% peanut oil), severe dyslipidemia combining hypercholesterolemia, hypertriglyceridemia, and an increase in blood pressure were obtained, a situation very typical of metabolic syndrome [23]. In this study, sperm parameters were affected and a significant decrease in normal morphology, progressive motility, and total motility were observed in animals with the metabolic syndrome. The sperm cells were capacitation-deficient as measured by their ability to trigger the progesterone-induced acrosomal reaction. Finally, the cholesterol content of sperm cells had increased significantly in dyslipidemic animals.

Overall, hypercholesterolemia in rabbits modifies sperm morphology and function in combination with changes in plasma membrane composition and dynamics. These alterations appear to be due to testicular and epididymal dysfunctions, with changes in the membrane lipids more likely due to epididymal maturation defects.

Although rabbits are the gold standard for studying foodborne hypercholesterolemia, studies have also been conducted in rodents. A number of transgenic mice strains have been used to study the molecular regulation of intermediates in the cholesterol metabolism, whether or not associated with dietary intake. Mice were also used to study male infertility related to diet-induced obesity (for recent examples, read [24, 25]); however, this is out of the scope of this review. In our group, we developed a diet-induced posttesticular infertility model triggered by feeding 3-month-old male Liver-X-Receptor knockout mice (*Lxr* α; β-/-) for 4 weeks with a cholesterol-enriched diet (1.25%). This model underlined that the epididymis is very sensitive to circulating factors that can interfere with normal gamete maturation. Dietary cholesterol overload (well managed by wild-type mice) led in the transgenic mouse strain to sperm abnormalities

similar to those described in hypercholesterolemic rabbits including morphological changes, decreased motility, and capacitation failure associated with abnormal sperm plasma membrane lipid composition resulting in dynamic dysfunctions [26, 27].

The impact of dietary cholesterol overload on male fertility has also been studied in rats. Male rats fed for 120 days with a hypercholesterolemic diet showed a significant reduction in secondary spermatocytes and spermatids associated with increased plasma LDL levels and the development of aortic atherosclerosis [28]. A diet enriched with cholesterol (400 mg/kg body weight) administered for 60 days to albino rats resulted in a significant reduction in sperm motility, epididymal epithelial cell height (both in *caput* and *cauda* epididymal regions), and seminal tubule and Leydig cell nucleus diameters associated with increased plasma cholesterol and triglycerides. A decreased number of implanted fetuses in females mated with hypercholesterolemic males was also reported [29]. The deleterious effects of oxidized LDL (oxLDL) on male reproductive function were demonstrated by comparing a high cholesterol diet (2%) to a high oxidized-cholesterol diet in male Wistar rats for 14 weeks. The most adverse impact was obtained with a diet high in oxidized cholesterol, which resulted in a very significant increase in circulating oxLDLs and a significant decrease in sperm count, motility, morphology, and viability compared to control and cholesterol-fed animals [30]. This study underlines the additional effect of dyslipidemia and oxidative stress, a point that will be further developed.

An overload of dietary cholesterol has a very negative impact on the reproductive function of male mice and rats, in accordance with the data obtained on rabbits.

2.2. Data in Humans and Clinical Management. The possible relationship between dietary cholesterol and semen characteristics is difficult to assess in humans. As infertility clinicians only see patients when dyslipidemia is established, it is therefore impossible to compare in the same individuals semen parameters in the predyslipidemia state. Nevertheless, data are available on pathophysiological conditions involving dyslipidemia, such as obesity and metabolic syndrome, but these clinical situations are not strictly specific to cholesterol overload. In these situations, it has however been clearly demonstrated that adverse effects on male fertility exist (examined by [31–33]). The data available in men regarding the relationship between plasma cholesterol levels and semen parameters are mainly from studies conducted to investigate the effect of cholesterol-lowering treatments. The effects of pravastatin (inhibitor of hydroxymethylglutaryl coenzyme A reductase, a rate-limiting enzyme in cholesterol biosynthesis) or cholestyramine (sequestering bile acids) were compared on semen parameters in 14 hypercholesterolemic men with high LDL plasma levels [34]. Both treatments surprisingly caused a decrease in sperm motility at 6 and 12 months, which was probably the result of a decrease in total cholesterol and LDL levels, not a specific side effect of one of the treatments. The decrease in LDL cholesterol can also affect the maturation of epididymal sperm, as sperm acquire motility during this process, a point that has not been studied by the authors. In another study, the same group found no evidence of clinically significant effects of simvastatin or pravastatin on multiple sperm quality parameters as well as on gonadal testosterone production or testosterone reserve [35]. Other studies reported that statins administered to hypercholesterolemic men had no significant effect on sperm parameters. For example, simvastatin has been shown to have no effect on sperm quality in terms of motility, concentration, viability, and morphology after 14 weeks of treatment [36]. This was also the case for the long-term effects of pravastatin evaluated in eight hypercholesterolemic patients [37]. These data are not consistent with the data obtained in rats since one study reported that concomitant administration of alpha-tocopherol and simvastatin to male hypercholesterolemic rats improved their reproductive efficacy and provided additional protection against fertility loss induced by hypercholesterolemia [38]. This may indicate that it is not possible to reproduce the nutritional conditions of animal models in humans, or that the number of subjects included in these studies is too small, or, else, that human testes and epididymides may be less sensitive than animal tissues to dietary cholesterol overload.

Links between hypercholesterolemia and male sperm parameters are rare, so comparative and prospective studies with a large number of men are definitely needed. The evaluation should be expanded to include, in addition to the usual sperm parameters, capacitation tests, fertilization biomarkers, and in vitro fertilization (IVF) data. Only such studies could provide a better understanding of the effect of hypercholesterolemia on human male fertility.

3. Lipid Overload and Oxidative Stress: Links, Consequences, and Clinical Management

Sperm cells are very sensitive to lipid peroxidation because of their high content of polyunsaturated fatty acids (PUFA), particularly docosahexaenoic acid (DHA, C-22:6*n*-3, a fatty acid containing six double bonds [39]). The modification of the fatty acid composition of sperm has been linked to sperm dysfunction and fertility disorders in many studies. For example, when human sperm were separated on a discontinuous Percoll gradient, the DHA content was significantly different in the sperm of all fractions [40], more mature sperm containing 2.5 times less DHA than slightly less mature cells. Even though the net sperm DHA content decreases in relation with sperm maturity, it is important to mention that DHA remains the major PUFA of sperm cells, and which content is systematically lower in sperm from infertile men [41]. This decrease in DHA is part of a decrease in global sperm PUFA levels associated with an increase in the *n*-6/*n*-3 ratio in sperm cells of oligo- and/or asthenozoospermic men, suggesting a link between fatty acid composition and infertility [41]. This causal relationship was established in a report showing that defective human sperm cells contained high levels of nonesterified unsaturated fatty acids that promoted the generation of reactive oxygen species (ROS) by their mitochondria, thus creating oxidative stress and a concomitant loss of functional competence [42]. Taking all these elements into account, it is therefore very likely that diet-

induced lipid changes modify the composition and functions of sperm cells and promote oxidative damage. In support of this, a recent study described a negative association between body mass index (BMI), DHA, and palmitic acid levels in semen [43].

3.1. Dietary Lipids Modify Sperm Composition and Quality

3.1.1. Animals. Dietary lipid supplementation has several objectives related to male fertility: (i) to improve male reproductive fertility and (ii) to promote sperm resistance to freeze-thaw procedures widely used in artificial insemination (AI). There is a fairly large amount of literature on these different topics, sometimes using *in vitro* supplementation rather than *in vivo* dietary intake. We will focus here on data on dietary intake and showing the consequences on the lipid composition of sperm.

Improving sperm quality has long been a subject of interest for the reproduction of agronomic species such as birds and cattle. A positive influence of dietary fatty acid supplementation on sperm composition was demonstrated in roosters with the observation that the proportion of *n*-3 fatty acids in sperm was increased (and inversely that of *n*-6 fatty acids was lower) when the males were fed salmon oil rather than corn oil. This observation was associated with higher fertility rates [44]. In consequence, the *n*-6/*n*-3 ratio appears to be an important parameter associated with rooster fertility, higher ratios being related to lower fertility. In addition, roosters fed a control diet or a diet containing corn oil, fish oil, or flaxseed oil showed different changes that were associated with the peculiar fatty acid composition of each lipid source (for example, flaxseed has the lowest *n*-6/*n*-3 ratio). However, it seems that other factor(s) can affect the sperm fatty acid composition as the lowest *n*-6/*n*-3 semen ratio was obtained with fish oil, but not with flaxseed [45]. Unfortunately, no fertility data was available in this study. Several reports deal with the impact of dietary fatty acid supplementation on sperm quality and reproductive performance in ageing roosters. Although various dietary supplements have been used, the consensual result of these studies is that *n*-3 polyunsaturated fatty acids have the strongest effect on the reproductive performance of ageing roosters with regard to AI [46, 47]. Overall, from these rooster studies, there is a clear relationship between dietary lipid supplementation and sperm fatty acid composition [46–48], with an increase in DHA systematically linked to improved reproductive performance [46, 47]. These data are consistent with the fact that the DHA content was found associated with optimal sperm maturation and ability to fertilize in human sperm, as already mentioned above.

Dietary supplementation is also widely used in large domestic mammals to improve their fertility. Bovine reproduction worldwide is mainly carried out using AI with frozen semen, which makes the fertilizing capacity of frozen-thawed sperm an economic challenge. Studies comparing the impact of different dietary fatty acid supplements on fresh and frozen-thawed bovine sperm are available. It appears that DHA-enriched oils or the use of nutraceuticals have systematically improved the quality of fresh semen after several weeks, at least regarding the classical motility parameter [49–51]. When the fatty acid composition of sperm was assessed, an increase in *n*-3 fatty acids was observed after DHA-rich oil supplementation [51, 52], with at least in one of these studies, a parallel increase in *n*-3 fatty acid plasma concentrations. In frozen-thawed sperm, analyzed from the same bulls, the results were not always consistent, with some studies showing no improvement in sperm quality [49], others showing a positive effect on motility parameters [51], or the percentage of sperm with intact acrosome [52]. A study reported that the lipid composition of sperm improved after the freeze-thaw procedure, with an increase in the proportion of *n*-3 PUFA in sperm from fish oil-supplemented bulls compared to control animals, in association with improved sperm quality assessed by viability, progressive motility, and morphology [50]. In all these studies, no data were available on the reproductive performance of the bulls. Another limitation of these studies is that they were conducted on relatively small numbers, since the number of bulls *per* group was generally between 6 and 15. With the exception of one study where the presence of intact acrosomes, membrane fluidity, and ROS generation were assessed by flow cytometry [52], only "classical" parameters were assessed such as motility (subjective or using CASA), viability, and sperm morphology giving no clues as to the real beneficial effect of these supplementation in terms of reproductive performance.

In laboratory models such as rodents, the impact of dietary lipids on sperm and/or reproductive performance was also examined. Male Wistar rats fed for three successive generations on a semipurified diet in which fat was provided *via* hydrogenated vegetable fat showed a reduced litter size and a decrease in the percentage of morphologically normal spermatozoa [53]. In rats too, the effects of the *n*-3/*n*-6 PUFA ratio on male reproductive performance were studied by feeding male Sprague-Dawley rats for 2 months with diets containing different *n*-3/*n*-6 ratios (ranging from 0.13, 0.40, 0.85, and 1.52 to 2.85) which ratios were obtained by mixing adequate amounts of linseed oil and soybean oil. It appears that sperm concentration, normal sperm head morphology, and motility were highest at a *n*-3/*n*-6 ratio of 1.52, in association with higher litter size and birth weight [54]. These data are consistent with the beneficial effects of *n*-3 fatty acid consumption on male reproductive capacity. They also show that an appropriate dietary ratio of *n*-3/*n*-6 PUFA is necessary for optimal male reproductive function. Unfortunately, this last study does not mention the *n*-3/*n*-6 ratio of sperm, which could have strengthened the link between dietary lipids and sperm composition, as suggested by other authors. In another study, Ferramosca *et al.* tested the hypothesis that supplementation of a high-fat diet with different sources of PUFA could improve rats' metabolic and reproductive parameters. By using either olive oil (as a source of monounsaturated fatty acids (MUFA)) or krill oil (for *n*-3 PUFA), they showed that olive oil partially neutralized the negative effects of a high-fat diet on sperm quality, increasing gamete mobility, reducing oxidative stress (lipoperoxidation), and slightly improving mitochondria function [55]. These data are in accordance with beneficial effects of olive oil

demonstrated on spermatozoa from hypercholesterolemic rabbits, as described above [17, 19]. In mice, data on dietary lipid supplementation and its effect on sperm function are rare. Mice were mainly used to evaluate the effects of gene invalidation on fertility, sometimes in combination with dietary supplementation to restore a phenotype or to induce a pathophysiological situation. For example, in an acrylamide-induced oxidative stress model, the addition of corn oil or pork fat to the standard diet has been shown to have potentiating effects on the negative impacts observed on sperm cells and epididymal tissue. The addition of either of these two supplements resulted in a significant decrease in sperm concentration, mobility, and viability compared to mice solely exposed to acrylamide [56]. This decrease in sperm quality was accompanied by a significant increase in lipoperoxidation (as measured by the malondialdehyde (MDA) content of the sperm) and carbonylation of sperm proteins, as well as a significant decrease in primary antioxidant enzyme activities (superoxide dismutase (SOD) and glutathione peroxidase (GPx)). This study brings forward the potentiating effect of dietary lipids when an oxidative stress state exists. Finally, the importance of dietary fatty acids in male fertility was demonstrated by a study on mice invalidated for the enzyme delta-6 desaturase, the first rate-limiting enzyme in PUFA synthesis (*n*-3 and *n*-6 PUFA). Males of this strain showed infertility and spermatogenesis arrest at late spermiogenesis. Dietary supplementation with 0.2% DHA was sufficient to restore spermatogenesis and fertility [57]. Although the underlying molecular mechanisms explaining this phenotype and its rescue have not been described, DHA emerged as a central fatty acid in male fertility, as already mentioned earlier in this review.

Dietary fatty acid intake has significant effects on gamete composition and male reproductive capacity. It is particularly important to maintain an n-3/n-6 ratio in an optimal range by modulating the n-3 intake.

3.1.2. Humans. The relationship between dietary fat and sperm quality has also been studied in men. In a study where diet was assessed using a dietary frequency questionnaire, the higher total fat intake was negatively correlated with total sperm count and concentration [58]. Dietary intake of saturated fat was the cause of this negative correlation; conversely, intake of *n*-3 fatty acids showed a positive correlation with normal sperm morphology. Dietary fats were weakly associated with sperm or seminal fatty acid levels, and there were only modest correlations between sperm, seminal fatty acid composition, and sperm quality. However, the authors reported that levels of saturated fatty acids in semen and seminal plasma were negatively correlated with sperm concentration and motility. This is in agreement with previous work showing higher concentrations of saturated fatty acids in the sperm of asthenozoospermic [41, 59] and oligozoospermic males compared to normozoospermic subjects [41]. Consistent with these data and animal data, Safarinejad *et al.* showed that *n*-3 PUFA blood and sperm levels were higher in fertile men than in infertile men with oligoasthenozoospermia. In these groups, the *n*-6/*n*-3 ratio of PUFA was significantly higher in the serum of infertile men [60]. Recently, the "FERTINUTS" study analyzed in healthy men aged 18-35 years the impact of a 60 g/day nut supplementation (30 g nut, 15 g almond, and 15 g hazelnut) on sperm DNA fragmentation; ROS production; chromosome X, Y, and 18 abnormalities; total DNA methylation; and microRNA content [61]. Walnuts contain about 50% fat, most of which is MUFA, with the exception of hazelnuts, where PUFA are the main fatty acids. After 14 weeks of nut supplementation, the authors reported several improvements over the control group in terms of sperm count, vitality, total motility, progressive motility, and morphology. Of all the other parameters evaluated, only the sperm DNA fragmentation was reduced in the "nuts" group. The authors concluded that "only a reduction in DNA fragmentation after nut consumption could explain these beneficial effects," a point that seems questionable because it could be a collateral benefit of the absorption of antioxidant molecules such as vitamin E (as shown in supplementary table 7 of reference [61]). One of the limitations of this particular study is that dietary intake was only assessed by means of a questionnaire, and even if blood lipids were measured, there was no significant variation between the two groups for HDL, LDL, VLDL, total cholesterol, and triglycerides. In addition, fatty acids, and in particular *n*-3 and *n*-6, were not measured in blood or sperm, thus limiting the mechanistic relevance of nut supplementation to the fatty acid composition of sperm. However, eating nuts seems to be beneficial for human sperm because another study had previously reported improvements in sperm vitality, motility, and morphology when men took 75 g/d of walnuts for 12 weeks [62].

Clinical trials have also been initiated to investigate the potential effect of dietary PUFA on sperm quality in infertile patients. In a randomized, double-blind, placebo-controlled study analyzing the impact of 500 mg/day of DHA for 10 weeks, Martinez-Soto *et al.* showed no effect on traditional sperm parameters or lipid composition of the sperm membrane [63]. Interestingly, they however reported a significant decrease in the proportion of sperm with DNA damage in the DHA group as measured with the TUNEL assay using flow cytometry. They have no mechanistic explanation for this observation. Another study reported a positive effect of DHA supplementation on progressive sperm motility after 3 months with 0.5 or 1 g DHA/day [64]. A surprising result of this study was a significant increase in ROS sperm production in asthenozoospermic patients. This increase was however not accompanied by an increase in sperm lipoperoxidation, which led the authors to propose that DHA supplementation provokes a "positive" oxidative stress. Most recently, a review based on literature search tools examined the influence of DHA or EPA dietary supplementation, alone or with micronutrients, on sperm parameters in infertile and control men [65]. This analysis extracted three publications for a total of 147 infertile patients and 143 fertile controls, revealing a positive influence of *n*-3 fatty acids on total sperm motility and seminal plasma DHA concentration, without modification of sperm content.

Overall, it appears that there is an influence of n-3 dietary fatty acids on sperm quality, which is not systematically associated with changes in the fatty acid profile of sperm.

Mechanistic studies will be needed to understand the relative importance of testicular and posttesticular compartments in molecular changes associated with dietary fatty acid supplementation. Potential molecular regulators involved in the observed effects of n-3 PUFA supplementation are presently being examined [66].

3.2. Oxidative Stress and Clinical Management. Spermatozoa are one of the body's richest cells in PUFA, which play a pivotal role in the regulation of their function, but this property also makes them very sensitive to oxidative stress. It is of the utmost importance that PUFA, typical of the plasma membrane of mammalian sperm and particularly sensitive to oxidation, are protected against lipoperoxidation. If this protection is not sufficient, the aldehydes resulting from the lipoperoxidation of the sperm plasma membrane will create a vicious circle of ROS amplification that will be very detrimental to the sperm structures and functions [67]. Although mature sperm cells are very susceptible to oxidative damage, paradoxically, sperm use ROS to complete their posttesticular maturation, particularly for optimal condensation of the sperm nucleus, a critical process that determines the level of integrity of paternal DNA [68]. In addition, sperm cells need ROS to complete major steps in the fertilization process, such as capacitation [69] and acrosomal reaction ([70] and reviewed by [71]). Oxidative stress is a characteristic of lipid metabolic disorders such as obesity and is related to subfertility (discussed in [72]). PUFA-based dietary fatty acid supplementation may be a good approach to overcoming sperm disorders, but it is pertinent to combine it with antioxidant supplementation to be more effective.

3.2.1. Data on Animals. As mentioned above, dietary fatty acid supplementation can modify the PUFA composition of sperm cells, as shown in the work on roosters [44–46]. In some studies, the most important benefits observed on sperm parameters and fertility were obtained when dietary supplementation included vitamin E (Vit E) to protect sperm from lipoperoxidation [46, 47]. Combinations of canola oil, canola/fish oil or flaxseed oil, and 200 mg/kg Vit E were the most effective in improving AI results and sperm quality. The protective effects of Vit E supplementation have also been demonstrated in rats with improved sperm concentration and progressive motility [73]. Vitamin E supplementation has also been tested in bulls, but mainly in *in vitro* studies. It could improve sperm motility, membrane integrity, and oxidative stress after freezing and thawing of sperm when added as a complex with methyl-beta-cyclodextrin, a carrier in the cryopreservation medium [74]. Another study reported a protective effect on membrane lipids and better freezing of the bull's gametes when Vit E was added to the sperm extender [75]. Finally, a summary of the effects of *n*-3 PUFA dietary supplementation on rabbit reproductive parameters (male and female), with or without appropriate antioxidant protection (200 mg/kg vitamin E), has recently been published [76]. This study highlights the need for antioxidant supplementation in addition to dietary intake of *n*-3 PUFA to achieve beneficial reproductive effects in many species.

Oxidative stress is harmful to male fertility in vivo and is a real problem for the preservation of sperm quality and DNA integrity in frozen samples used for AI. Although the benefits of different additives such as vitamin E have been reported, further studies are needed to improve the effectiveness of semen-freezing media.

3.2.2. Data on Humans. In men, structural and/or functional defects of sperm cells are due to multifactorial causes. Genetic, environmental, or lifestyle factors alone or in combination are at the roots of most infertility situations. Oxidative stress that alters sperm structures and functions is very often involved regardless of the cause of infertility. Oxidative damages to the sperm nucleus and in particular to paternal DNA have been detected in more than 60% of men visiting *in vitro* fertilization (IVF) centers, testifying of its prevalence [77]. Prevalence which is further increased in patients with idiopathic infertility, in whom this percentage rises to 80% [78]. Oxidative stress disrupts the integrity of sperm DNA but also damages the proteins and lipids in their plasma membranes, thus altering their fertility potential. To mitigate this damage, the use of antioxidant therapy has been suggested in the literature for many years [79–81] but there is still no consensus on its true clinical relevance due to the lack of adequate and rigorous clinical trials.

The most studied pathology combining lipid disorders, oxidative stress, and male infertility is obesity. In male mice, systemic oxidative stress induced by obesity was correlated with oxidative alterations in sperm DNA (as demonstrated by the TUNEL assay) and decreased fertility (measured *in vitro*) in males [82]. Paternal obesity or exposure to a high-fat diet also negatively affects the reproductive and metabolic health of the offspring, involving the alteration of sperm epigenetic marks such as DNA methylation and the sperm miRNA content. The results obtained in animal models have paved the way for the study of the effects of obesity on human sperm cells [83, 84]. Studies in men report that obesity causes a systemic inflammatory response that has negative consequences on sperm parameters and quality (reviewed in [85]). The inflammatory state induces the recruitment of white blood cells into male reproductive organs and/or seminal plasma, which leads to increased exposure of sperm to ROS and alterations in their genetic integrity. The pathophysiological situation of obesity is complex to analyze because it involves hormonal, inflammatory, and physical causes, all of which have a negative influence on male fertility. The underlying molecular dysfunctions are not well characterized, but more and more publications emphasize the importance of seriously addressing this pandemic, as the epigenetic legacy is now established [85–87].

Clinical management of male infertility should include good characterization of sperm abnormalities and special attention to paternal DNA damage to limit transgenerational effects. In addition, a better individual characterization would make it possible to propose appropriate therapies. In the case of obesity, it is known that a well-balanced diet associated with physical activity can improve sperm quality [88], but to date no data are available regarding the restoration of epigenetic markings. Limiting the oxidative stress

Table 1: Summary of the main results reported in this review, showing the associated references.

Lipids involved	Main results and associated references			
	Animal models	Refs.	Humans	Refs.
Cholesterol	*Rabbits* fed a cholesterol-enriched diet (0.5%) present multiple sperm dysfunctions of epididymal origin due to membrane lipid microdomain alterations. High plasma cholesterol levels, but no changes in seminal plasma or sperm cholesterol levels.	[14, 15]	Studies on hypocholesterolemic compounds (statins, cholestyramine) gave heterogeneous results concerning sperm parameters.	[34–38]
	Rabbits fed with 0.05% cholesterol diet showed the same dysfunctions but with increased sperm cholesterol contents. A dietary supplementation with 7% olive oil restored normal characteristics.	[16, 17]		
	Sperm parameters were affected in *rabbits* with a dietary-triggered metabolic syndrome, deficiency in capacitation associated with an increase in sperm cholesterol levels.	[23]		
	Dietary cholesterol-induced dyslipidemia provokes posttesticular infertility in LXR-deficient *mice*.	[26, 27]		
	Cholesterol-enriched-diet altered sperm functions in *rats*.	[29]		
Fatty acids	Dietary supplementation with *n*-3 polyunsaturated fatty acids modifies the *n*-6/*n*-3 ratio and ameliorates sperm quality, fertility ratio, and reproductive performance of ageing *roosters* (artificial insemination). Increased amounts of DHA are associated with these improvements.	[44–47]	Nut consumption for several weeks improves sperm count, vitality, total motility, progressive motility, morphology, and sperm DNA fragmentation compared to the control group.	[32, 61]
	Dietary supplementation with DHA-enriched oils improves fresh and cryopreserved *bovine* sperm quality, in association with higher *n*-3 PUFA contents (for cryopreserved samples).	[49–52]	Dietary supplementation with 500 mg/day DHA for 10 weeks has no effect on sperm parameters or sperm membrane lipid composition.	[64]
	The *n*-3/*n*-6 ratio of dietary oils ameliorates reproductive outcomes of male *rats*.	[54]	A review based on literature search tools showed positive influence of dietary supplementation with *n*-3 fatty acids on total sperm motility and seminal plasma DHA concentration without modification of sperm content.	[65]
	Dietary supplementation with 0.2% DHA restores the infertility due to spermatogenesis arrest in D6-desaturase invalidated *mice*.	[57]		
Links with oxidative stress	The most important benefits observed on *rooster* sperm parameters and fertility were obtained when dietary supplementation included vitamin E to protect sperm from lipoperoxidation.	[46, 47]	Obesity causes a systemic inflammatory response that has negative consequences on sperm parameters and quality, associated with an increased exposure of sperm to ROS.	[85]
	Vitamin E supplementation is also efficient in *rats* and *in vitro* on *bull* spermatozoa.	[73, 74]	Diet (*n*-3 fatty acids from fish oil and nuts) and antioxidant supplementation have been shown to be effective in limiting oxidative damage to sperm.	[90, 91]

encountered in obesity as in many other diseases seems to be a good strategy to improve sperm quality [77, 89]. However, to date, there is no consensus in infertility clinics, and various antioxidant treatments without scientific justification are given on a totally empirical basis. Each treatment must be adapted to the abnormalities of the individual's sperm, but in the absence of a detailed evaluation, clinicians are powerless to make a rational therapeutic choice. To this day, the evaluation of human fertility capacities is still based on WHO standards that only concern sperm concentration and morphology, which parameters are unanimously recognized as poorly predictive. Diet and supplementation have been shown to be effective in limiting oxidative damage to sperm, and the increased intake of different nutrients such as fruits and vegetables, selenium, zinc, *n*-3 fatty acids (mainly from fish and nuts), coenzyme Q10, and carnitines,

alone or in combination have all been positively related to sperm quality [90, 91]. In this situation of lack of consensus and appropriate scientific and clinical data, antioxidant supplementation is going wild and could have deleterious effects for some patients. To date, there are more than 120 commercially available antioxidant formulations worldwide that claim to improve male fertility. None of them, except one [92], is supported by convincing scientific and clinical data. With regard to the particular profile of the sperm plasma membrane rich in PUFA, vitamin E supplementation should be the standard of excellence. However, some of these commercially available formulations do not even contain vitamin E, and for those that do, it is synthetic vitamin E, while nature has endowed mammalian cells with 8 natural isoforms of vitamin E, all of which are important to some extent. Knowing that there is only one cell membrane transporter for these vitamin E isoforms, it is easy to imagine what can happen when only one of the 8 is brought in excess. Preclinical data as well as rigorous, placebo-controlled, double-blind, multicenter clinical trials are absolutely necessary. We are confident that, in the case of established lipid disorders, individual testing for damage caused by the oxidation of sperm DNA will provide clinicians with the opportunity to propose intelligent and adapted therapeutic approaches that could avoid the use of assisted reproductive technologies. We also recommend that in situations of unexplained idiopathic infertility, especially in young men, the assessment of systemic lipid status should be part of the systematic male fertility examination.

Diet can have a negative or positive effect on human fertility, as is the case for other major health problems (cardiovascular diseases, cancer, neurodegenerative pathologies, etc.). More research is needed to understand how diet can influence sperm parameters and fertility. A critical challenge will be to decipher the mechanistic actions of food supplements on male gametes in order to use them judiciously and in adapted pathophysiological situations. This is particularly important given the potential consequences that diet can have on the health of the offspring via epigenetic modifications.

4. Conclusion

Dietary fats can influence the lipid composition of sperm and have harmful or beneficial consequences on male fertility (see Table 1 for an overview). This issue is of economic interest to the reproductive capacities of agricultural species such as birds and livestock. Dietary supplementation using a combination of *n*-3 PUFA (DHA) with antioxidant Vit E has shown the most beneficial effects to date. It is clear that further studies will be needed to understand the molecular mechanisms underlying *n*-3 PUFA supplementation. Dietary lipids are also of primary importance in human pathological situations, where their deleterious impact is associated with the concomitant onset of oxidative stress and sperm DNA damage. The increasing proportion of dyslipidemic men in the world raises a public health issue and a future challenge for infertility treatments because metabolic diseases are now considered as hereditary diseases *via* epigenetic mechanisms.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Metformin Ameliorates Testicular Damage in Male Mice with Streptozotocin-Induced Type 1 Diabetes through the PK2/PKR Pathway

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Approximately 90% of male diabetes mellitus patients have varying degrees of testicular dysfunction. The molecular mechanism underlying diabetes-induced testicular damage has not been thoroughly elucidated. In this research, we sought to determine the influence of metformin (Met) on diabetes-induced testicular injury and the mechanism involved with a focus on testicular dysfunction, apoptosis, autophagy, and prokineticin 2 (PK2) signalling. In our study, C57BL/6J mice were randomly divided into the normal control group, the diabetes group, and the Met-treated group. Streptozotocin (50 $mg \cdot kg^{-1} \cdot d^{-1}$) was injected intraperitoneally into the mice for 5 days in a row to induce type 1 diabetes, which was diagnosed by a blood glucose level ≥ 16.7 mmol/L after 7 days. The experimental animals were orally administered Met (250 $mg \cdot kg^{-1} \cdot d^{-1}$) for 16 weeks. Properties of testicular function, including sperm motility and the total concentration of epididymal sperm, were assessed. Changes in testicular structure, such as the blood-testis barrier, histological pathology, and organelles, were observed. The levels of apoptosis and expression of related proteins, such as Bax and Bcl-2, were measured. Moreover, autophagy-related proteins, including Beclin-1, p62, and LC3B, as well as the PK2/PKR pathway, which consists of PK2, PKR1, PKR2, AKT, and GSK3β, were analysed. Upon the induction of diabetes, reproductive capacity was significantly impaired and a disordered arrangement of testicular seminiferous tubules and destroyed organelles in spermatogenic cells was observed. Met administration preserved testicular function and structure. In addition, in mice with diabetes, the levels of PK2, PKR2, p-Akt, and p-GSK3β were significantly decreased at different times, while that of PKR1 was markedly increased, and these changes were normalized by Met. Furthermore, diabetic mice showed increased apoptosis and decreased autophagy in the testes, the effects of which were nullified by Met. These results suggest that Met rescues diabetes-induced testicular damage by attenuating apoptosis and inducing autophagy. This effect is likely mediated by the PK2/PKR/AKT/GSK3β signalling pathway.

1. Introduction

Diabetes mellitus is a disorder of glucose metabolism caused by an absolute or relative insufficiency of insulin secretion. According to a prediction by the International Diabetes Federation [1], the number of diabetic patients will increase to 693 million in 2045, accounting for approximately 10% of the global population. Recently, researchers have elucidated that testicular dysfunction occurs in a startling number of diabetes cases and that testicular dysfunction has become a

prevalent complication of diabetes. Approximately 94.4% of diabetes cases are associated with hypotestosteronaemia, which can lead to diabetic erectile dysfunction [2], and the incidence of sexual and reproductive dysfunction in diabetic patients is 5-10 times higher than that in nondiabetic patients [3, 4]. The major pathological manifestations of diabetes-induced testicular dysfunction include low testosterone levels and compromised reproductive function, which may be attributed to apoptosis and autophagy and may engender hypogonadism. Nevertheless, the precise molecular mechanism by which testicular dysfunction is caused by diabetes remains unclear, and no specific medicines are available for treatment.

Currently, numerous medicines are available for diabetes mellitus, and metformin (Met) is an effective hypoglycaemic drug that can effectively control blood glucose by reducing the absorption of sugar in the intestinal tract, enhancing the intracellular transport of glucose, and inhibiting the production of glycogen in the liver. During its more than 60 years of clinical application, Met has been used not only to control blood glucose levels in patients with diabetes but also to prevent diabetes complications. It has a curative effect on diabetic cardiomyopathy [5] and diabetic retinopathy [6]. Although Met has some effect on other complications caused by diabetes mellitus, it has not been reported for the treatment of testicular dysfunction and the mechanisms of Met in testicular tissue remain unexplored.

Prokineticin 2 (PK2), also called Bombina variegata 8 (Bv8), is a secreted low-molecular-weight protein extracted from the skin secretions of toads [7]. Previous studies have demonstrated that PK2 plays a role in a variety of biological processes, including nerve growth, angiogenesis, the immune response, and inflammation [8–10]. In addition, there is increasing evidence that PK2 plays a role in regulating gonadotropin-releasing hormone (GnRH) for gonadal development, as knockout of the PK2 gene was delayed and deformed in the testes of male mice, leading to spermatogenesis deficiency [8, 11, 12]. Prokineticin receptors (PKRs) are specific G protein-coupled receptors (GPCRs) that comprise two functional complexes, namely, PKR1 and PKR2, which are responsible for the biological effects induced by PK2 in effector cells. Previous studies have shown that PKR2 is more important in the regulation of testicular growth than PKR1 [13]. Although PK2/PKRs play an indispensable role in the reproductive system, there are no reports about their role and mechanism in reproductive injury caused by diabetes mellitus. Accordingly, this study attempted to evaluate the role of Met in diabetes-induced testicular damage, reproductive dysfunction, apoptosis, autophagy, and the PK2/PKR pathway.

2. Materials and Methods

2.1. Experimental Animals. SPF C57BL/6J male mice (18-22 g, 5-6 weeks) were obtained from the Hubei Laboratory Animal Research Center and housed at a temperature of 22 ± 2°C and a moisture content of 40% under a 12 h light/dark cycle. All experimental subjects were treated according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85–23, revised 1996). All experimental procedures were approved by the Institutional Animal Care and Use Committee of Hubei University of Science and Technology.

2.2. Induction of Experimental Diabetes. A week after adaptive feeding, mice were randomly divided into 2 groups: the control group ($n = 60$) and the diabetes group (DM, $n = 100$). The DM mice were intraperitoneally injected with streptozotocin (STZ, 50 $mg \cdot kg^{-1} \cdot d^{-1}$ in 0.1 mol/L citric acid buffer, pH 4.5) for 5 days in a row [14]. The mice in the control group were injected with an equal amount of citric acid-sodium citrate buffer solution. Random blood glucose was monitored within seven days after STZ injection; the experimental animals with a blood glucose level ≥ 16.7 mmol/L were diagnosed as diabetic, and their blood glucose was monitored weekly [14]. At appropriate time points (2 months, 3 months, 4 months, and 5 months) after the diagnosis of diabetes, the mice were euthanized under anaesthesia, and the testes were quickly removed and stored in Bouin's fixative fluid for histomorphological examination. The other samples were stored at -80°C for other experiments. The epididymis was collected to determine reproductive function.

2.3. Treatment with Met. Mice were randomly divided into the normal control group ($n = 20$), the DM group ($n = 25$), and the Met treatment group (DM+Met, $n = 25$). The diabetes model was established according to a previously described method. In the Met treatment group, mice that were diagnosed with diabetes after STZ injection were orally administered Met hydrochloride solution (250 $mg \cdot kg^{-1} \cdot d^{-1}$) in potable water for 4 months. Mice in the other groups were administered an equal volume of water.

2.4. Biotin Tracer Studies. The skin and fascia of anaesthetized mice were incised to expose the testicular tissue. A total of 50 μL of EZ-link Sulfo-NHS-LC-Biotin (50 mg/mL) was injected under the tunica albuginea using a microinjection needle. Thirty minutes after the skin was sutured, the mice were sacrificed, and their testicles were removed and fixed in a fixative solution. The tissue was embedded in paraffin overnight and sectioned. The tissues were dewaxed in xylene and hydrated in graded ethanol solutions. Antigen retrieval was performed by incubating the sections in 5% skim milk in PBS containing 0.01% Triton X-100 for 15 min. An Alexa Fluor 568-conjugated streptavidin solution was diluted with PBS at a 1 : 200 ratio and added to the sections. The sections were incubated at 37°C for 2 h in a dark room and then washed 3 times with PBS. The sections were incubated with DAPI-containing antifluorescence quenching tablets and observed under a fluorescence microscope.

2.5. Measurement of Reproductive Capacity. The cauda epididymes of the mice were placed in physiological saline at 37°C and homogenized. The numbers of active and inactive sperm in 5 visual fields were counted, and the sperm activity rate (active sperm number/total sperm number × 100%) was calculated. The sperm suspension was placed in physiological saline at 37°C and dried on a cell counting plate after blending. The concentration of sperm in the five central squares

was counted, and the average was multiplied by 10^9 to obtain the number of sperm per litre of semen, as previously described [15].

2.6. Histopathological Examination. Testicular tissue was soaked in Bouin's fixative solution for 12 h, embedded in paraffin, and sectioned into 4 μm thick slices. After HE staining, changes in the tissue structure were observed under a light microscope. The testicular tissue was immobilized with an electron microscopy fixative and embedded with an acetone-812 embedding agent. The organelle structure of the testicular tissue was observed by transmission electron microscopy.

2.7. Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End-Labelling (TUNEL) Assay. A TUNEL assay was performed according to the instructions of the TUNEL kit (Roche Applied Science, USA). Paraffin sections were dewaxed in xylene, rehydrated in a 100%~70% ethanol gradient, and sequentially incubated in 10 μg/mL proteinase K at 37°C for 30 min for antigen retrieval, in 1% Triton X-100 at room temperature for 20 min, in TDT-enzyme and fluorescein-labelled dUTP in proportion at 37°C for 2 h, in 10% serum for 30 min at room temperature, and, finally, in a 10% hydrogen peroxide-methanol solution for 15 min. After 30 min of incubation with converter-POD (HRP-labelled fluorescein antibody), TUNEL-positive cells (green under a microscope) were randomly observed under a fluorescence microscope (Leica, USA). The sections were washed three times with PBS after each step.

2.8. Immunohistochemical Determination of Protein Expression. The samples were incubated with polyclonal primary antibodies at 4°C for 12 h, washed three times with PBS, and then incubated with a biotinylated horse antimouse immunoglobulin (IgG) solution for 1 h. The samples were rinsed with PBS three more times and incubated with freshly prepared 3,3′-diaminobenzidine (DAB) solution for colour development. Finally, the sections were sealed with neutral resin and observed under an optical microscope.

2.9. Real-Time Reverse Transcription Polymerase Chain Reaction (RT-qPCR). TRIzol reagent was used to extract total RNA from the testicular tissues, and 1 μL of RNA from each sample was reverse transcribed into cDNA with the following PCR programme: 95°C for 10 min followed by 40 cycles of 95°C for 15 s and annealing at 60°C for 60 s. After the reaction was completed, the reaction product was separated by agarose gel electrophoresis, and the results were analysed by the $\Delta\Delta$CT method. Table 1 provides the nucleotide sequences of the primers used in this research.

2.10. Western Blot Analysis. The testicular tissues were weighed, and approximately 50 mg of tissue was added to RIPA buffer (Cell Signaling Technology, USA) that included protease inhibitors and phosphatase inhibitors. The protein concentration was determined by the BCA protein assay (Beyotime, China). Equivalent amounts of protein were loaded onto gels and separated by SDS-PAGE. Then, the following antibodies were used for Western blotting: Beclin-1, LC3B, protein kinase B (AKT), phosphor-AKT, glycogen synthase kinase-3beta (GSK3β), phosphor-GSK3β, Bax, Bcl-2 (1 : 1000, Cell Signaling Technology, USA), p62 (1 : 500, Wanleibio, CN), PK2 (1 : 1000, Abcam, USA), and PKR1 and PKR2 (1 : 2000, Santa Cruz Biotechnology, USA). The samples were incubated with the appropriate secondary antibodies for 1 h at room temperature. The blots were analysed with an ECL kit (Meilunbio, CN).

Table 1: Primer sequences for RT-qPCR.

Gene	Primer	Sequence
Bcl-2	Forward	5′-TGACTTCTCTCGTCGCTACCGT-3′
	Reverse	5′-CCTGAAGAGTTCCTCCACCACC-3′
Bax	Forward	5′-GCCTTTTTGCTACAGGGTTTCAT-3′
	Reverse	5′-TATTGCTGTCCAGTTCATCTCCA-3′
PCNA	Forward	5′-GTCGGGTGAATTTGCACGTA-3′
	Reverse	5′-CTCTATGGTTACCGCCTCCTC-3′
PK2	Forward	5′-CTCTATGGTTACCGCCTCCTC-3′
	Reverse	5′-GAAAGAAGTCCTTAAACACGCCA-3′
PKR1	Forward	5′-GAAAGAAGTCCTTAAACACGCCA-3′
	Reverse	5′-GACAGTCACAAAGCAGAGCGTA-3′
PKR2	Forward	5′-CTACTTCCTCTTCGTCTTCGGG-3′
	Reverse	5′-AGAAGTCTCGCACTATGGTAAAGC-′
β-Actin	Forward	5′-GTGACGTTGACATCCGTAAAGA-3′
	Reverse	5′-GTAACAGTCCGCCTAGAAGCAC-3′

2.11. Statistical Analysis. The data are presented as the mean ± SEM of replicated experiments. Analysis was performed by one-way analysis of variance (ANOVA). Differences with P values < 0.05 were considered statistically significant.

3. Results

3.1. General State and Reproductive Potential of Mice over Time. The diabetic mice showed the typical symptoms of diabetes, specifically polydipsia, polyuria, polyphagia, and weight loss. As demonstrated in Table 2, compared with the control group, the diabetic mice had dull, rough, and disorderly hair. In the DM group, body weight was significantly reduced, and a hyperglycaemic status was maintained for a long time (Table 2). In terms of reproductive function, although the total sperm count in the testes of mice in the early stage of diabetes was low compared to that in testes of mice in the normal control group, it was still at a sufficient level to maintain vitality. As the disease progressed, the reproductive capacity of the diabetic mice gradually decreased, and the sperm motility and total sperm count also significantly decreased continuously (Table 2) ($P < 0.05$ vs. the DM group).

Table 2: Metabolic abnormalities and reproductive capacity in diabetic mice.

Group	TW (mg)	BW (g)	TW/BW (mg/g)	Blood glucose (mmol/L)	Sperm vitality (%)	Sperm count ($*10^9$/L)
2M control	173 ± 5	29.2 ± 1.0	6.0 ± 0.3	7.2 ± 0.3	61.7 ± 4	1.86 ± 0.07
2M DM	131 ± 7*	17.3 ± 0.4*	7.7 ± 0.5*	27.4 ± 0.2*	63.4 ± 6	0.70 ± 0.10*
3M control	185 ± 4	30.7 ± 0.4	6.0 ± 0.1	6.7 ± 0.6	51.4 ± 2	1.86 ± 0.13
3M DM	158 ± 5*	21.3 ± 0.5*	7.4 ± 0.2*	27.3 ± 0.3*	33.6 ± 3*	0.51 ± 0.06*
4M control	195 ± 3	30.8 ± 0.4	6.3 ± 0.1	6.2 ± 0.4	51.6 ± 5	1.51 ± 0.06
4M DM	162 ± 4*	20.0 ± 0.4*	8.1 ± 0.2*	26.0 ± 1.3*	27.8 ± 2*	0.28 ± 0.08*
5M control	183 ± 5	31.0 ± 0.4	5.5 ± 0.4	7.0 ± 0.2	47.2 ± 2	1.46 ± 0.07
5M DM	167 ± 4*	21.6 ± 0.4*	7.8 ± 0.2*	26.0 ± 0.4*	25.4 ± 2*	0.27 ± 0.03*

TW: testis weight; BW: body weight; TW/BW: testis weight/body weight. The data are the means ± SEM; *$P < 0.05$ vs. the control group; $n = 9 – 15$.

3.2. Diabetes Destroys Normal Testicular Physiological Structure in Mice. The physiological changes in the mice are shown in Figure 1. In the control group, spermatogenic cells at all levels of the spermatogenic epithelium were arranged in an orderly manner, as most of the spermatogenic epithelia had 6-7 layers, and numerous spermatozoa were found in the lumen of the seminiferous tubule. In contrast, the atrophy of and damage to the seminiferous tubules in the testes of diabetic mice resulted in a marked decrease in the number of cell layers in the seminiferous epithelia, with most having 3-4 or fewer layers, and spermatogenesis was significantly reduced in the seminiferous tubules when spermatogenic cells were lost. As the disease progressed, the damage gradually worsened. Within five months, the basic morphology of the testicular seminiferous tubules of the diabetic mice was completely destroyed and normal reproductive function was completely lost.

3.3. Expression of PK2/PKRs in the Testicular Tissue of Diabetic Mice. In previous reports, PK2/PKR2 were found to be important regulatory proteins for the normal physiological function of testicular tissue in mice. Therefore, we speculated that the PK2/PKR signalling pathway affects diabetic mice. As shown in Figure 2, the expression of PK2 and PKR2 was downregulated in diabetic mice compared with control mice. PKR1 levels were downregulated at 2 and 3 months and upregulated at 4 and 5 months in diabetic mice (Figure 2). AKT/GSK3β is the downstream target of PK2/PKRs. In our study, when the PK2/PKR2 protein expression was inhibited, AKT/GSK3β was also expressed at lower levels (Figure 3).

3.4. Expression of AKT/GSK3β in the Testicular Tissues of Diabetic Mice. It is known that the AKT/GSK3β pathway is associated with glycogen synthesis, cell growth, and survival. To further elucidate the potential mechanism underlying diabetes-induced testicular damage, we determined the effects of the AKT/GSK3β signalling pathway on testicular damage. As shown in Figure 3, a significant decrease in the phosphorylation of AKT and GSK3β was found in testis tissue of the DM group compared with the control group (Figure 3).

3.5. Met Improves the General State and Reproductive Capacity of Diabetic Mice. To improve the quality of life and reproductive ability of diabetic mice, we treated diabetic mice with Met for four months. After treatment, the hair of the diabetic mice gradually regained its lustre, the body weights of the mice increased, and the blood glucose levels were also well controlled (Table 3). Furthermore, the sperm vitalities and sperm numbers of the diabetic mice were increased significantly, which enhanced their reproductive potential (Table 3). Compared with the DM mice, the mice treated with Met had better reproductive potential. There were significant differences between the two groups ($P < 0.05$).

3.6. Met Improves Histomorphological Damage to Testicular Tissue in Diabetic Mice. Similarly, in mice treated with Met, the morphology of the seminiferous tubules in the testes was similar to that in the control group. In the Met treatment group, the damage to the spermatogenic epithelium was significantly alleviated, the number of spermatogenic cell layers was noticeably increased, the cell arrangement was neat, and spermatogenesis was observed (Figure 4(c)). In the biotin tracer study, EZ-link Sulfo-NHS-LC-Biotin was distributed outside of the seminiferous tubules in the control group, but in the diabetic mice, biotin crossed the Sertoli cell gap. Met was able to repair the blood-testis barrier and prevent this damage (Figure 4(b)).

Transmission electron microscopy elucidated that the spermatogenic cells were orderly arranged at all levels of testicular tissue in the control group (Figure 4(d), I). In the DM group, the spermatogenic cells exhibited a disordered arrangement, and vacuolar degeneration or disintegration in the cytoplasm, unclear nucleoli in the nucleus, obvious mitochondrial oedema, massive chromatin aggregation, and widespread breakdown of intercellular bridges were observed (Figure 4(d), II). However, Met treatment normalized the alterations in the mitochondria, which exhibited slight oedema, and chromatin still accumulated (Figure 4(d), III).

3.7. Met Prevents Diabetes Mellitus-Induced Testicular Apoptosis. A TUNEL assay kit was used to detect the number of TUNEL-positive cells. The number of apoptotic cells was elevated in DM mice compared to control mice. Compared with untreated diabetic mice, diabetic mice treated with

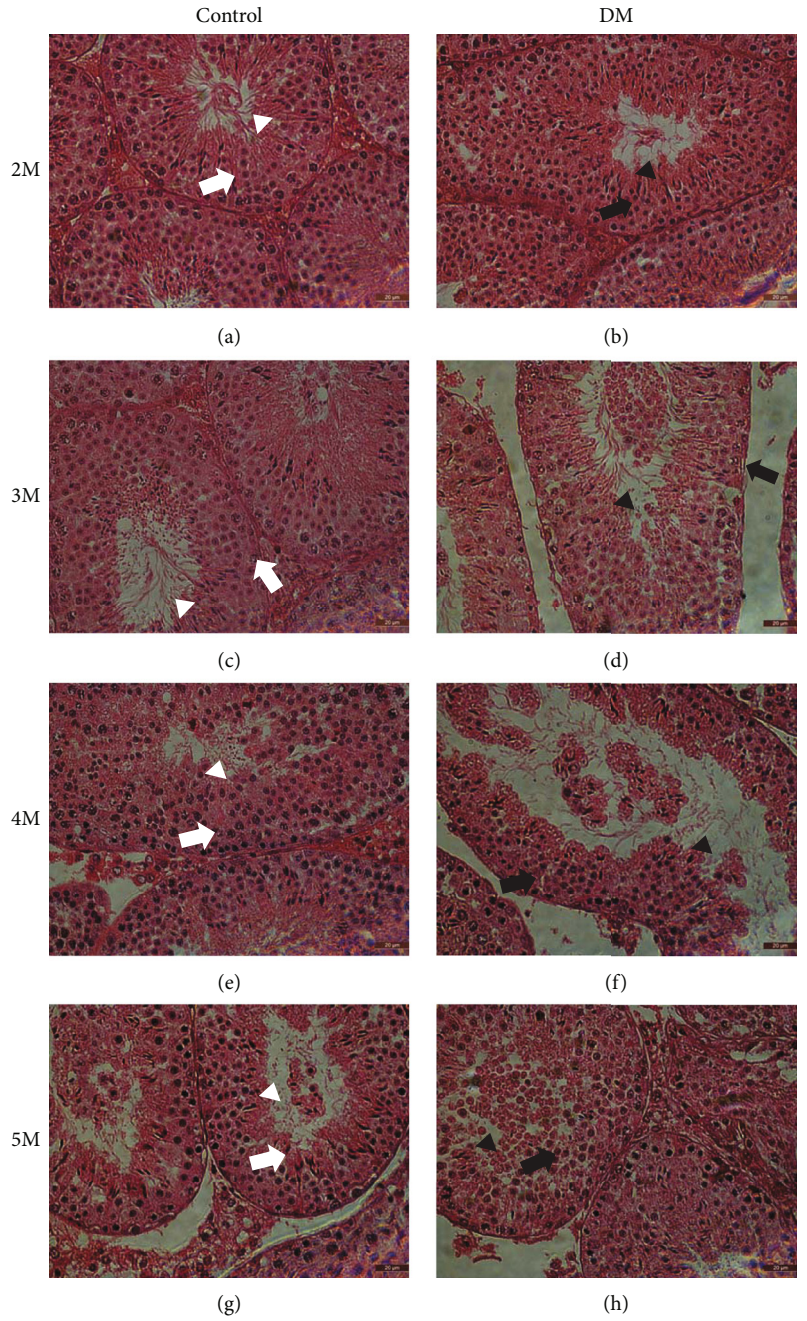

Figure 1: Histomorphological damage in diabetic mice. (a–h) HE staining of testes sections at 2 months, 3 months, 4 months, and 5 months. The white arrowhead shows a normal spermatogenic epithelium, the white triangle shows spermatogenesis, the black arrowhead shows fewer spermatogenic cell layers in the DM group than in the control group, and the black triangle shows sparse spermatogenesis in the DM group. Magnification = 400x; scale bar = 20 μm; $n = 4 - 5$.

Met exhibited significantly inhibited diabetes mellitus-induced testicular apoptosis (Figure 5(a)).

It is well known that Bax and Bcl-2 are regulatory markers of apoptosis. Proliferating cell nuclear antigen (PCNA) is a sliding clamp for the DNA polymerase complex. In addition to having roles in DNA repair and DNA methylation, PCNA has been implicated in apoptosis. As demonstrated in Figure 5, the expression of Bcl-2 and PCNA was decreased, but Bax expression was increased in the testes of the DM group. Met treatment markedly reduced the ratio of Bax/Bcl-2 and increased PCNA expression in diabetic mice (Figure 5).

3.8. Met Activates Autophagy in Testicular Cells in Diabetic Mice. To evaluate the effect of autophagy in testicular injury in diabetes mellitus, we selected the autophagy-related proteins Beclin-1, p62, and light chain 3B (LC3B) to observe changes in autophagy levels in diabetic mice after Met treatment. The data showed that Met effectively activated autophagy in the testicular tissue of mice (Figure 6).

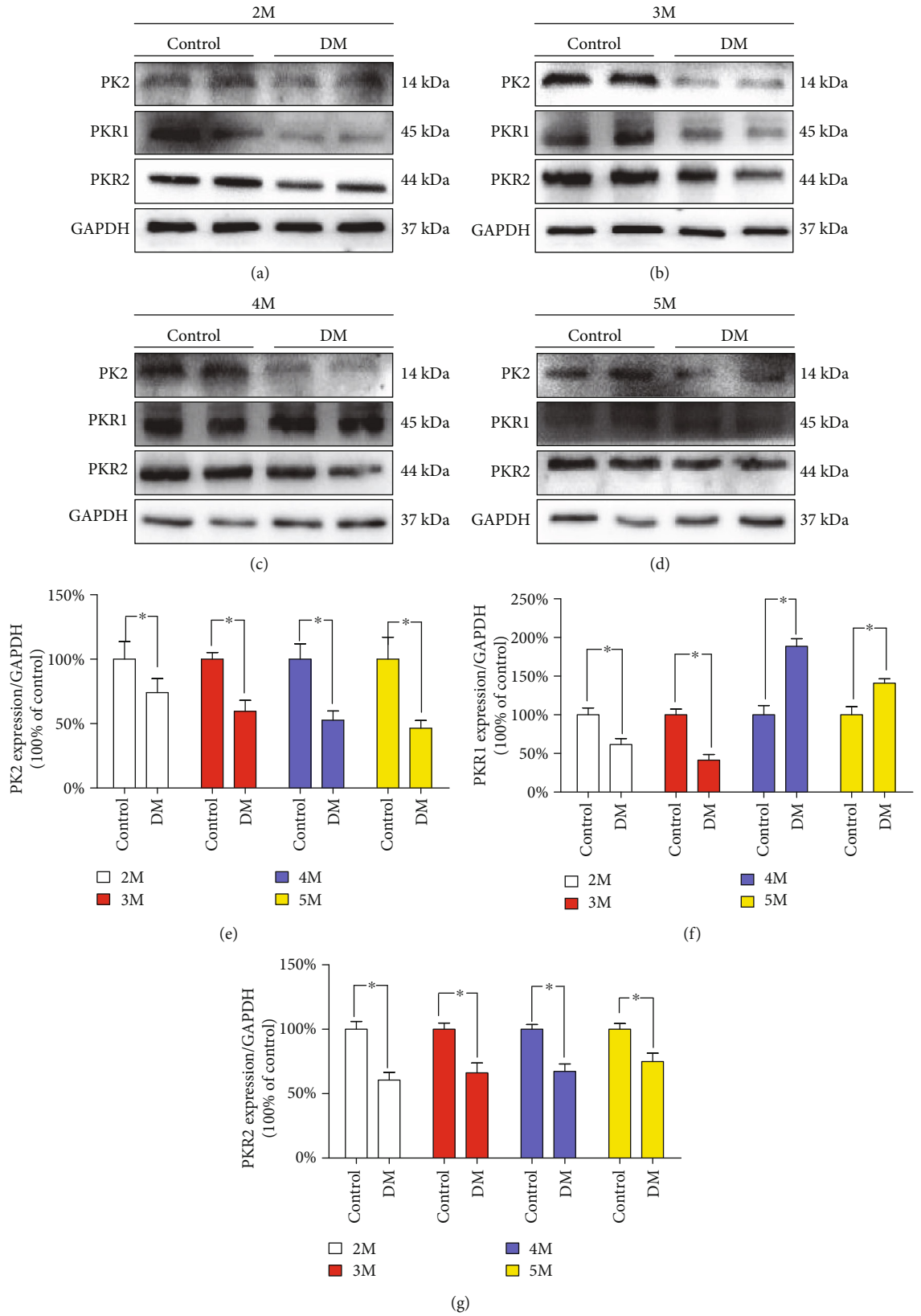

Figure 2: Testicular expression of the PK2/PKR proteins in the diabetic mouse growth process. (a) Typical protein expression of PK2, PKR1, and PKR2 at 2 months. (b) Typical protein expression of PK2, PKR1, and PKR2 at 3 months. (c) Typical protein expression of PK2, PKR1, and PKR2 at 4 months. (d) Typical protein expression of PK2, PKR1, and PKR2 at 5 months. (e) Quantification of PK2 protein expression at different time points. (f) Quantification of PKR1 protein expression at different time points. (g) Quantification of PKR2 protein expression at different time points. $n = 4 - 6$ per group. The values are expressed as the mean ± SEM. $^*P < 0.05$ vs. the control group.

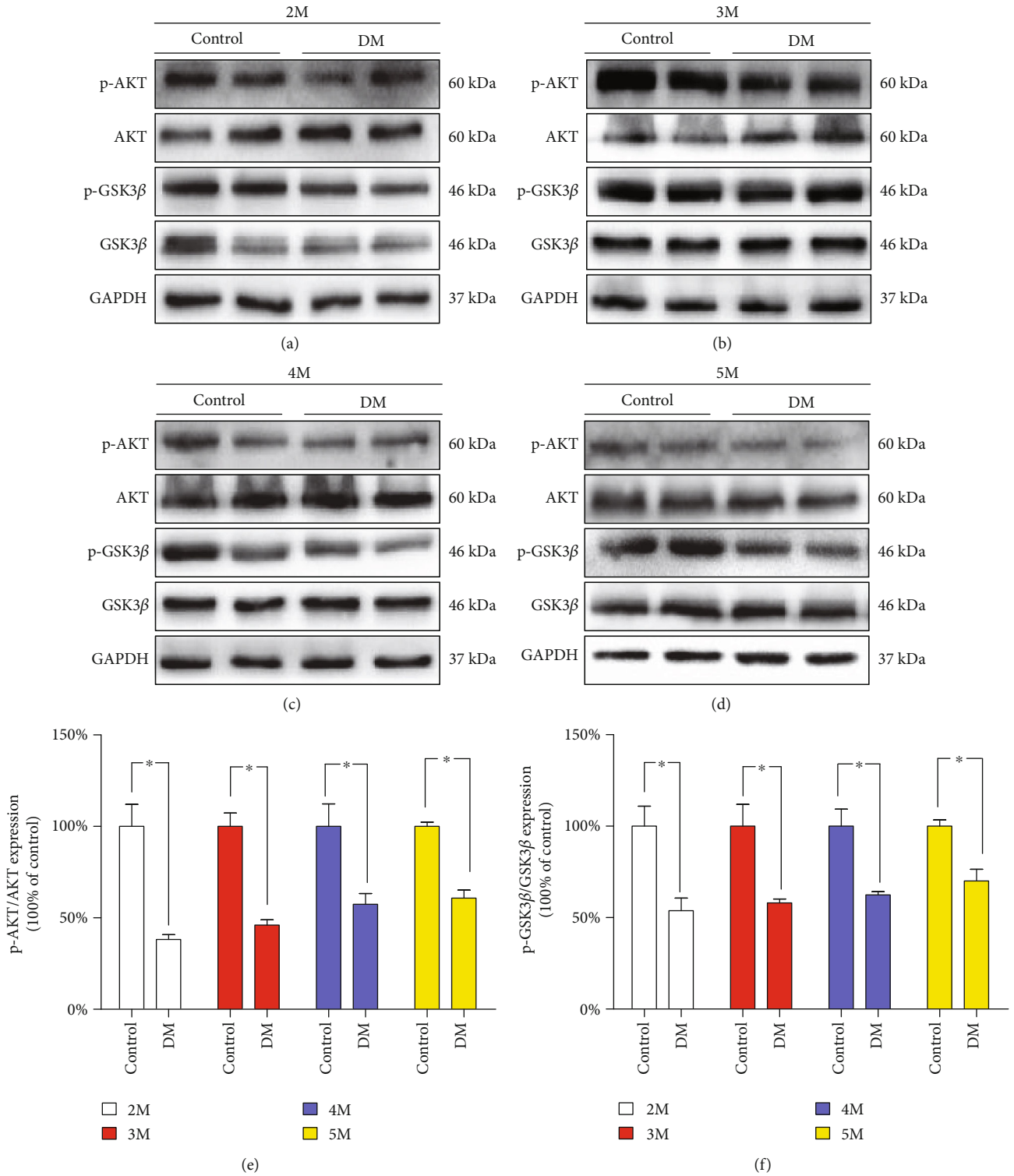

Figure 3: Testicular expression of the AKT/GSK3β proteins during the diabetic mouse growth process. (a) Typical protein expression of p-AKT, AKT, p-GSK3β, and GSK3β at 2 months. (b) Typical protein expression of p-AKT, AKT, p-GSK3β, and GSK3β at 3 months. (c) Typical protein expression of p-AKT, AKT, p-GSK3β, and GSK3β at 4 months. (d) Typical protein expression of p-AKT, AKT, p-GSK3β, and GSK3β at 5 months. (e) Quantification of p-AKT/AKT expression at different time points. (f) Quantification of p-GSK3β/GSK3β expression at different time points. $n = 4 - 6$ per group. The values are presented as the mean ± SEM. $^*P < 0.05$ vs. the control group.

3.9. Met Activates the PK2/PKR2 Pathway in Mouse Testes. In the above experiments, we proved that PK2/PKRs play an important role in the series of changes that occur during diabetic testicular injury. To evaluate the significance of Met, we analysed the levels of PK2/PKRs in testicular tissues by RT-qPCR, immunohistochemistry, and Western

Table 3: Met prevents metabolic abnormalities and maintains reproductive capacity.

Group	TW (mg)	BW (g)	TW/BW (mg/g)	Blood glucose (mmol/L)	Sperm vitality (%)	Sperm count ($*10^9$/L)
Control	197 ± 7	31.6 ± 0.6	6.2 ± 0.2	6.3 ± 0.3	53.6 ± 2.0	1.75 ± 0.02
DM	158 ± 6*	19.3 ± 0.5*	8.2 ± 0.3*	24.1 ± 1.4*	26.7 ± 1.4*	0.30 ± 0.06*
DM+Met	173 ± 9	28.0 ± 0.4*#	6.2 ± 0.3#	17.1 ± 1.6*#	43.1 ± 4.6*#	0.54 ± 0.05*#

TW: testis weight; BW: body weight; TW/BW: testis weight/body weight. The data are the means ± SEM; $^*P < 0.05$ vs. the control group; $^\#P < 0.05$ vs. the DM group; $n = 10 – 15$.

blotting. In contrast to those in the control group, the levels of PK2 and PKR2 were significantly decreased and the levels of PKR1 were increased in the testicular tissues of diabetic mice. Met supplementation significantly reversed these changes (Figure 7).

3.10. Met Activates the AKT/GSK3β Signalling Pathway. Akt is a crucial downstream element of the PK2/PKR2 pathway, and the rapamycin receptor in mammals is a key regulator of autophagy under the mediation of AKT. Diabetes significantly decreased the phosphorylation of both AKT and GSK3β in the testis, a change that was reversed by Met (Figure 8).

4. Discussion

The findings of our study indicate that the PK2/PKR pathway plays an irreplaceable role in diabetic testicular injury and that Met protects against STZ-induced testicular impairment, apoptosis, and autophagy by regulating PK2/PKRs and restores the phosphorylation of the AKT/GSK3β signalling pathway in the testes. Apoptosis and autophagy are caused by glucose toxicity-induced testicular injury in patients with diabetes [15, 16]. While the clinical management of testicular injury remains challenging, our study suggests that Met can act as a treatment for testicular complications in diabetes. Our data demonstrate a likely role of PK2/PKRs in the beneficial effects of Met on diabetes-induced testicular injury.

It is well known that diabetes plays a crucial role in the pathology of testicular dysfunction by leading to the atrophy of the seminiferous tubules and damage to spermatogenetic cells, which are regarded as morphological indices of spermatogenesis dysfunction [17]. We found that diabetic mice exhibited noticeable morphological alterations in their testes, including damage to the structure of the seminiferous tubules and the blood-testis barrier, and a reduced sperm function. Upon treatment with Met, these changes were reversed in our study.

Similar to other GPCRs, the binding of PK2 and PKRs is regulated by local effects and/or endocrine hormones and is coupled with G proteins to mediate multiple biological actions [18]. Accumulating evidence has demonstrated that the PK2 protein in testicular tissue exists in only primary spermatocytes, and PK2 plays an essential role in inflammation by binding to PKR1, thus increasing the expression of PKR1. Interestingly, as a consequence of PK2 and PKR2 deficiencies, GnRH fails to be secreted, which results in dysfunctional sexual development and fertility in both male and female mice [12, 19–21]. To our knowledge, this is the first study to validate the changes in PK2 expression in the testes of mice with diabetes. Furthermore, our study focused on the presence of PKRs in mouse testes following STZ administration, which indicates that the expression of PK2 is expressed at low levels and in different patterns in the testes of diabetic mice. At the same time, PKR2 was noted to be overtly decreased and continuously expressed at a low level, while PKR1 was expressed at a lower level during the initial stage and then increased during the end stage of diabetes mellitus, which may be the main contributor to the expression of PK2 in response to inflammation and defective angiogenesis in diabetes. In addition, Met attenuated testicular injury by downregulating PKR1 activity, upregulating PK2/PKR2, and alleviating these pathologic alterations. The analysis indicates that the effect of Met is involved in regulating PK2 signalling pathways.

Cumulative evidence suggests that PK2/PKRs participate in myocardial survival, angiogenesis, and the haematopoietic system through the AKT and STAT3 signalling pathways [19, 22]. When culturing testicular tissue cells in vitro, the AKT signalling pathway regulates functional anchoring junctions, especially in Sertoli cells and spermatogenic cells [23]. Recent reports have indicated that higher AKT phosphorylation is tightly associated with testicular injury in STZ-induced diabetic mice; thus, we speculated that the AKT signal transduction pathway in testicular tissue could be used to investigate its effect on diabetes-induced testicular dysfunction. Our data suggest that the AKT/GSK3β signalling pathway continues to be inhibited during testicular injury in diabetes and that Met effectively reverses the diabetes-induced inactivation of AKT. It is conceivable that the uninterrupted high expression of PK2/PKR2 during Met treatment is beneficial for the activation of the AKT pathway.

Autophagy is a self-clearing process in eukaryotic cells. Normal autophagy can help maintain the balance of cell growth in organisms, especially to protect against testicular injury caused by hyperglycaemia and hypoxia [24]. However, abnormal autophagy is believed to be involved in the pathogenesis of many related diseases. Insufficient or excessive autophagy leads to the degeneration of germ cells. At the same time, it can degrade organelles, such as mitochondria and the endoplasmic reticulum, to destroy the stability of the testes, affecting their growth and development as well as their normal physiological functions [25]. Hyperglycaemia affects the mammalian target of rapamycin complex 1 (mTORC1) and leads to the inactivation of autophagy-related gene 4 (ATG4) by oxidation, ultimately leading to the lipidization of autophagy-related gene 8 (ATG8) and

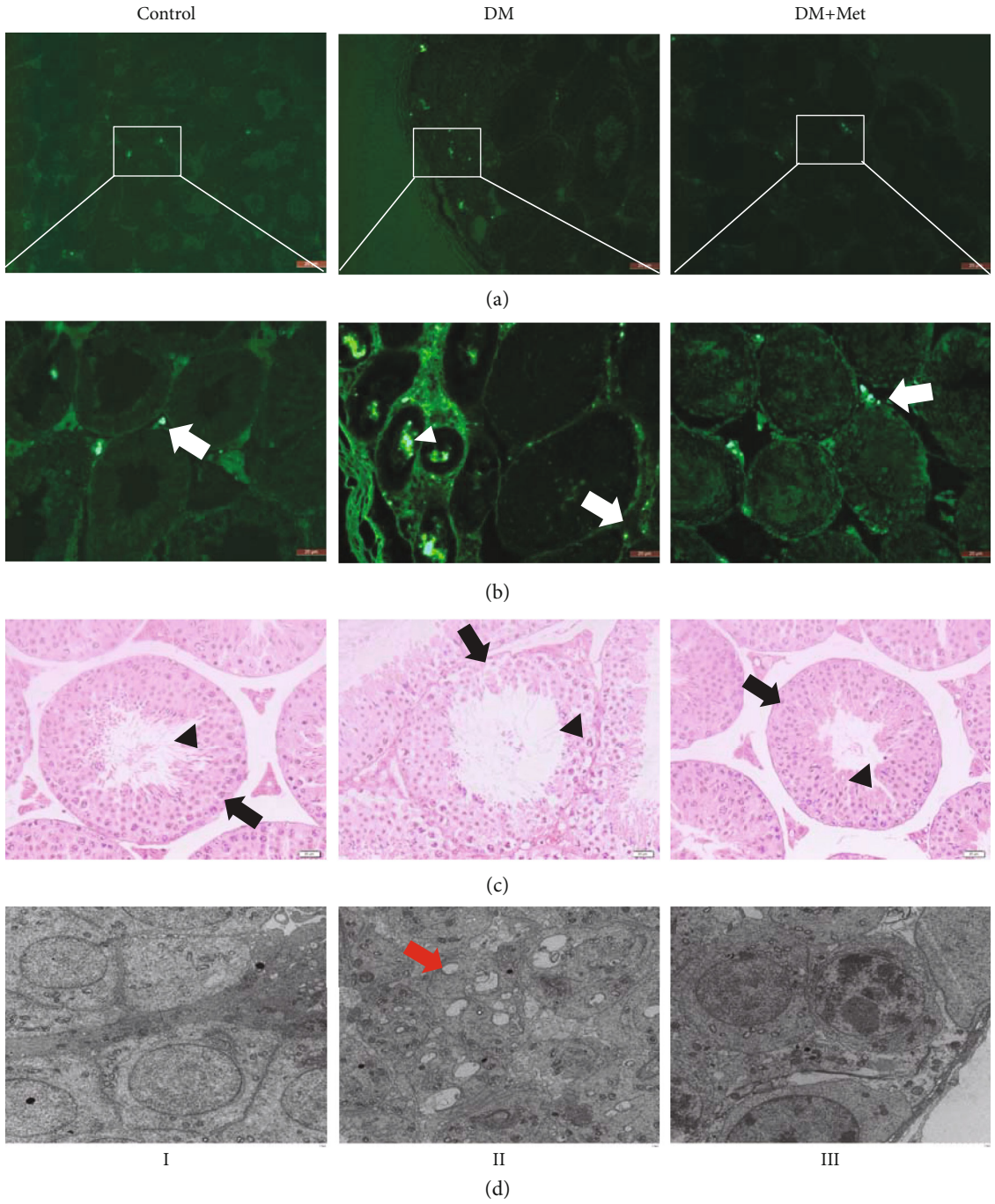

Figure 4: Histomorphological damage in diabetic mice. (a, b) Met prevented biotin from entering the seminiferous tubules. The white arrow indicates biotin between the tubules, and the white triangle indicates biotin in the seminiferous tubules. (a) Magnification = 100x; (b) magnification = 200x; scale bar = 20 μm; $n = 5$. (c) Met reversed the morphology of testicular tissue in mice, as determined by HE staining. The black arrowhead shows the spermatogenic cell layers, and the black triangle shows spermatogenesis. Magnification = 400x; scale bar = 20 μm; $n = 5$. (d) Met restored the organelle damage in testicular cells, and the red arrow indicates vacuolar degeneration or disintegration in the cytoplasm.

the formation of autophagy-related autophagosomes in the cell to induce autophagy [26]. The autophagy-related proteins Beclin-1, light chain 3B (LC3B), and ubiquitin-binding protein (p62/SQSTM1) are markers of autophagy activation in biological tissues. Beclin-1 and LC3B participate in the formation and elongation of autophagosomes, and p62, as a scaffold protein, binds ubiquitinated substrates and aids their aggregation and degradation by macroautophagy [27]. We noted suppressed autophagy, decreased Beclin-1 expression, a decreased LC3II-to-LC3I ratio, and increased p62 expression in diabetic mice, which corresponds to previous reports [28, 29]. Furthermore, we also

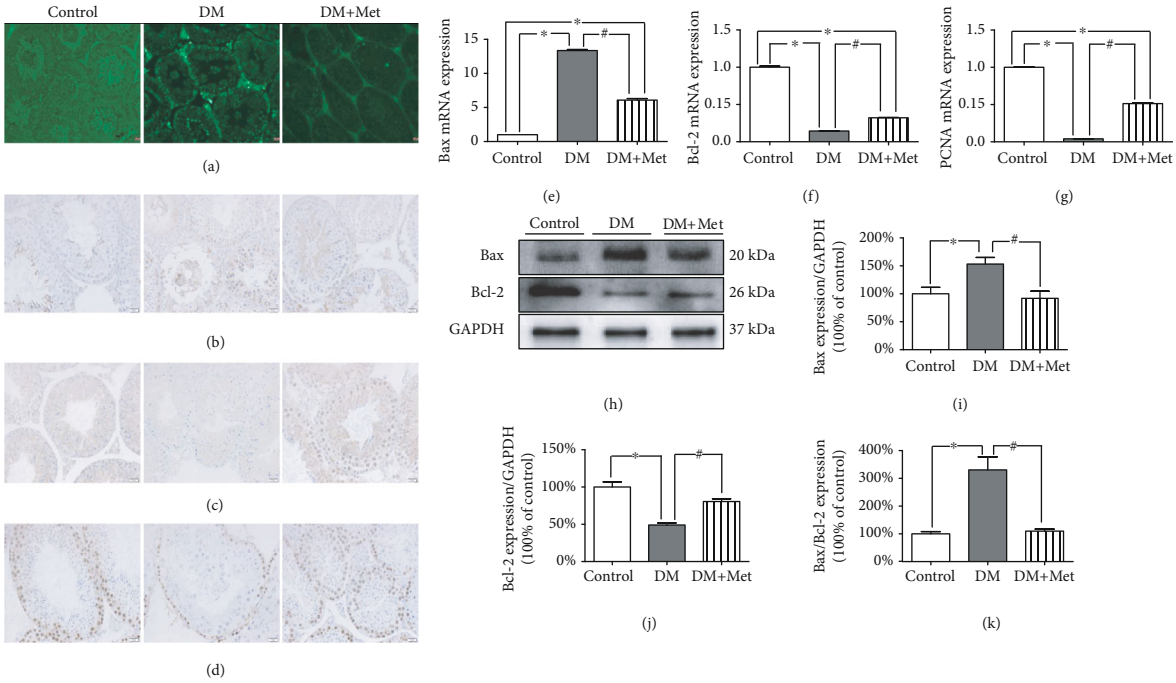

Figure 5: Met reduced diabetes mellitus-induced apoptosis in the testes. (a) A typical image of apoptotic cells stained by TUNEL (magnification = 100x; scale bar = 20 μm). (b) Typical immunohistochemical staining of Bax (magnification = 200x; scale bar = 20 μm). (c) Typical immunohistochemical staining of Bcl-2 (magnification = 200x; scale bar = 20 μm). (d) Typical immunohistochemical staining of PCNA (magnification = 200x; scale bar = 20 μm). (e) Quantification of Bax mRNA expression. (f) Quantification of Bcl-2 mRNA expression. (g) Quantification of PCNA mRNA expression. (h) A typical image of Bax and Bcl-2 proteins. (i) Quantification of Bax expression. (j) Quantification of Bcl-2 expression. (k) Quantification of the ratio of Bax/Bcl-2 expression. $n = 3 - 6$ per group. The values are presented as the mean ± SEM. $^{*}P < 0.05$ vs. the control group; $^{\#}P < 0.05$ vs. the DM group.

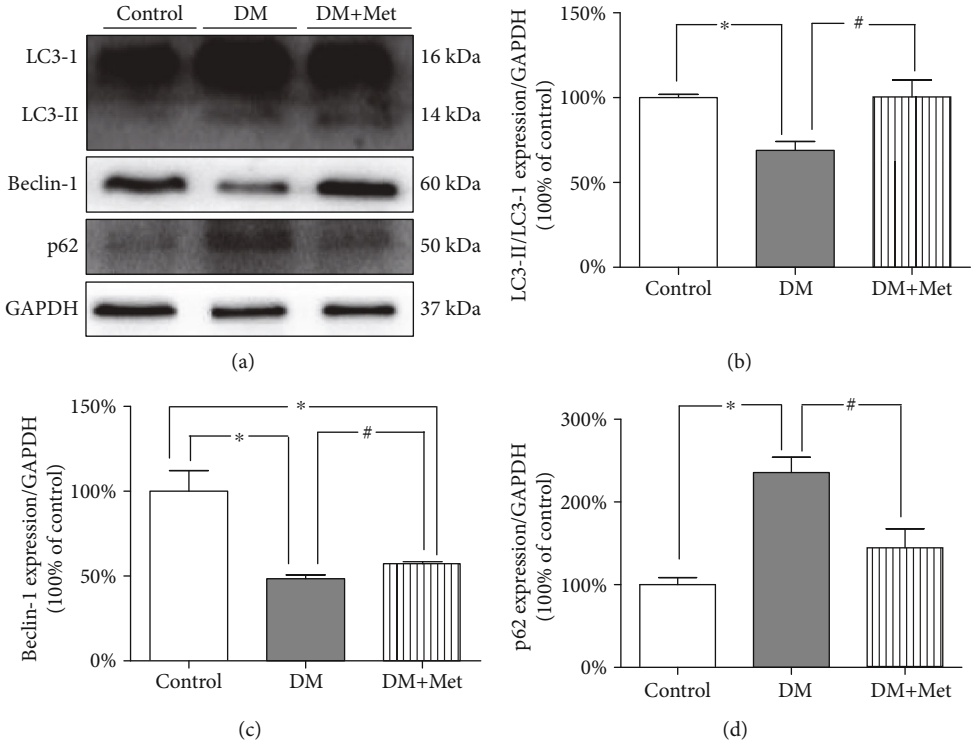

Figure 6: Met activated autophagy in the testes. (a) Typical protein expression of LC3B, Beclin-1, and p62. GAPDH served as the loading control. (b) Quantification of LC3B protein expression. (c) Quantification of Beclin-1 protein expression. (d) Quantification of p62 protein expression. $n = 4$ per group. The values are presented as the mean ± SEM. $^{*}P < 0.05$ vs. the control group; $^{\#}P < 0.05$ vs. the DM group.

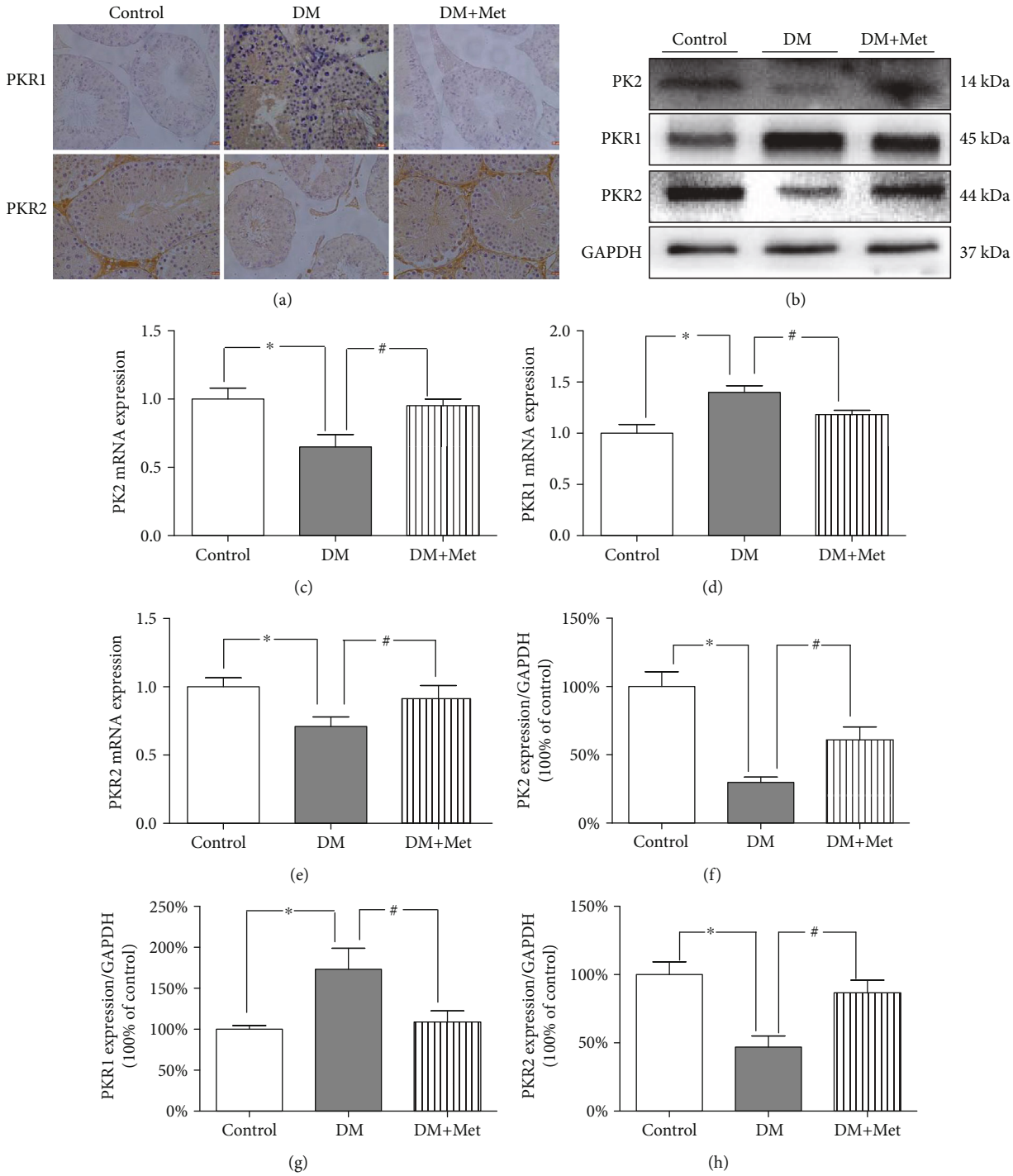

FIGURE 7: Met restores the protein expression of PK2/PKRs. (a) Typical immunohistochemical staining of PKRs (magnification = 400x; scale bar = 20 μm). (b) Typical expression of PK2 and PKRs. (c) Quantitative analysis of PK2 expression. (d) Quantitative analysis of PKR1 mRNA expression. (e) Quantitative analysis of PKR2 mRNA expression. (f) Quantification of PK2 protein expression. (g) Quantification of PKR1 protein expression. (h) Quantification of PKR2 protein expression. $n = 4 - 6$ per group. The values are presented as the mean ± SEM. $^{*}P < 0.05$ vs. the control group; $^{\#}P < 0.05$ vs. the DM group.

found that Met treatment induces the enhancement of the autophagy level in testicular tissue.

Apoptosis, which is jointly regulated by the apoptosis-inducing gene Bax and the apoptosis-inhibiting gene Bcl-2, is considered a major factor in the possible mechanism of testicular injury induced by diabetes. An imbalance between Bax/Bcl-2 can activate the downstream caspase signalling pathway to induce apoptosis, which ultimately leads to spermatogenesis dysfunction [30]. Evidence suggests a link between autophagy and apoptosis pathways. Autophagy can remove excess cell components to inhibit apoptosis, and activation of the apoptosis-related protein caspase

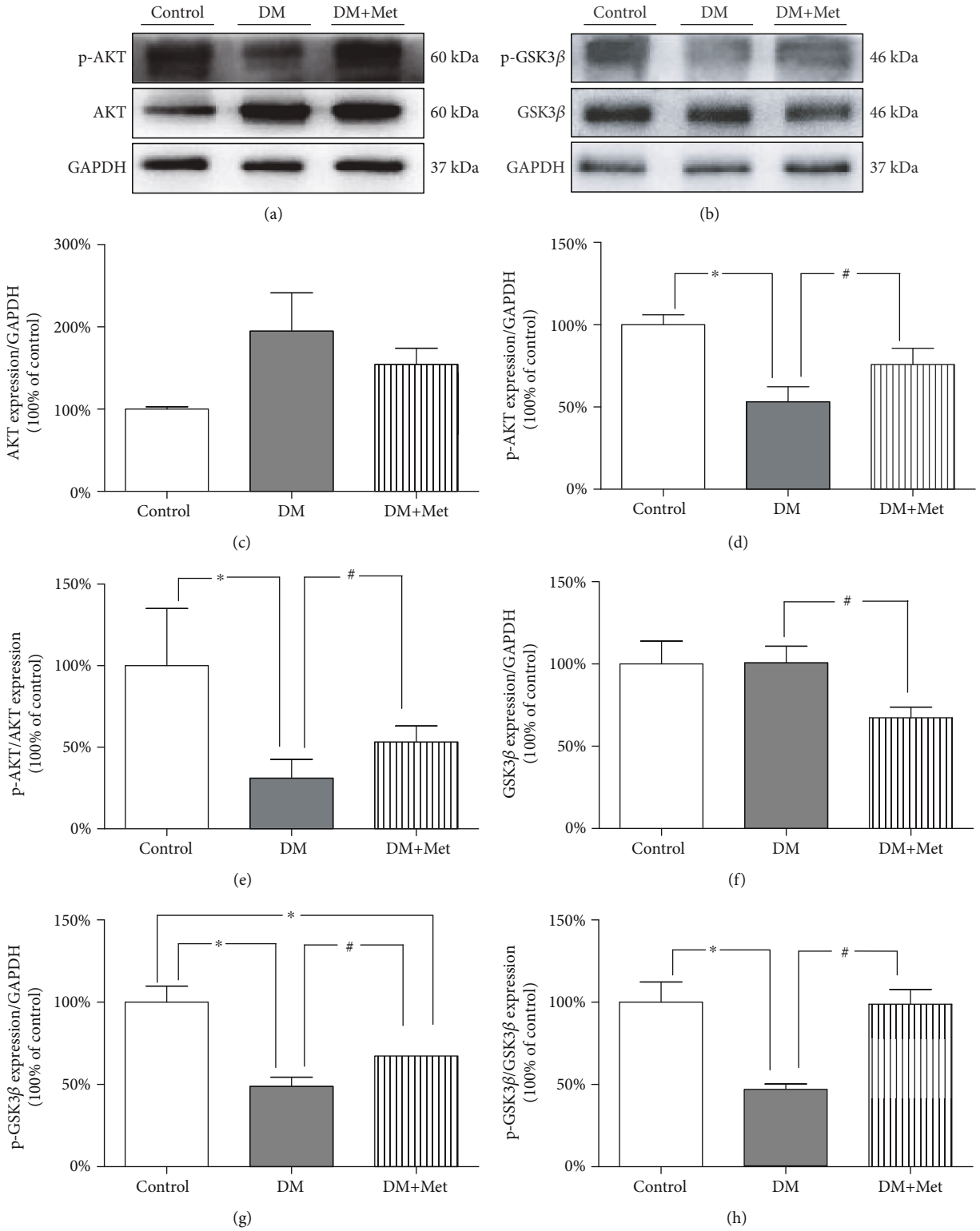

Figure 8: Met activates AKT/GSK3β protein expression. (a) Typical protein expression of p-AKT and AKT. (b) Typical protein expression of p-GSK3β and GSK3β proteins. (c) Quantification of AKT protein expression. (d) Quantification of p-AKT protein expression. (e) Quantification of p-AKT/AKT protein expression. (f) Quantification of GSK3β protein expression. (g) Quantification of p-GSK3β protein expression. (h) Quantification of p-GSK3β/GSK3β protein expression. $n = 4 - 6$ per group. The values are presented as the mean ± SEM. * $P < 0.05$ vs. the control group; $^{\#}P < 0.05$ vs. the DM group.

hinders the formation of autophagosomes [31]. Our study shows that the apoptosis of testicular tissue in diabetic mice is enhanced in parallel with increased Bax and decreased Bcl-2 and PCNA. When diabetic mice are treated with Met, the apoptosis of cells involved in the process of testicular injury is effectively inhibited. Therefore, our study supports a possible role for the PK2/PKR signalling cascade in the alteration of Met-elicited autophagic responses and the inhibition of apoptosis during diabetic toxicity.

In summary, our study strongly demonstrates that Met can regulate the PK2/PKR signalling pathway in testicular tissue, thereby eliciting AKT/GSK3β activity and regulating autophagic activity and apoptosis in testicular cells to protect the reproductive function of testicular tissue in mice. This study provides a new opportunity for the prevention and treatment of testicular reproductive injury, which is worthy of further clinical study.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors have no competing interests to declare.

Authors' Contributions

YNL, ZY, DBK, and YZZ contributed to the development of this study. WY and WLZ contributed to the analysis and interpretation of the data. WY and WLZ were responsible for critically revising the manuscript for important intellectual content.

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Research Article

Antioxidants, Dietary Fatty Acids, and Sperm: A Virtual Reality Applied Game for Scientific Dissemination

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Fatty acid (FA) profile appears to be critical to infertility, and the effects of dietary FAs on sperm FA content are a current focus of studies in the field of nutrition and reproduction. Starting from a validated "OXISTRESS" model in which modification of FA content results to influence reactive oxygen species, antioxidants, isoprostanes, cytokines, sperm kinetic, and acrosome reaction, we developed a virtual reality game where the player, in order to improve the health of some virtual spermatozoa, is called to take dietary choices and then discover their consequences on the main biological aspects. In the LabVR of the University of Siena, a team of VR environment designer and developer used Unity development engine to make the experience run on Oculus Quest and a wireless 6DOF (six degrees of freedom of movement in 3D space) VR Headset. In the game, the player is immersed in the epididymis and observes closer how dietary n-3 may change the sperm plasma membrane and consequently modify sperm traits. A simulation game in the virtual reality may represent a tool to give greater visibility to scientific data in the relevance of appropriate dietary habits in the human health.

1. Introduction

Understanding the molecular aspects of spermatogenesis would provide very interesting information on reproductive biology.

The phospholipid fraction of mammalian sperm membrane consists of about 50% of long chain-polyunsaturated fatty acids (LC-PUFAs), which influences many aspects of sperm physiology. LC-PUFAs play a key role in the sperm membrane fluidity and susceptibility to lipid peroxidation, sperm maturation, motility, and acrosome reaction [1].

Fatty acid (FA) profile appears to be critical to infertility, and the effects of dietary FAs on sperm FA content are a current focus of studies in the field of nutrition and reproduction [2]. Dietary FAs influence sperm FA profiles, and it seems that sperm FA contents are most sensitive to dietary n-3 PUFAs [3]. The potential application of a dietary strategy in relation to the improvement of FA profile of sperm and fertility has been investigated.

We are testing, using rabbit buck as an animal model, the effect of dietary plans with different n-6/n-3 PUFA ratio on semen quality.

It is widely recognized that several aspects affect the oxidative stress of semen such as infection, varicocele, environmental conditions, genetics, chronic inflammation, and diet.

Starting from experimental results in terms of FA profile, we developed and validated an OXISTRESS model (see Figure 1) in which modification of FA content results to influence reactive oxygen species, different index molecules (antioxidants, isoprostanes, and cytokines), sperm kinetic, and acrosome reaction.

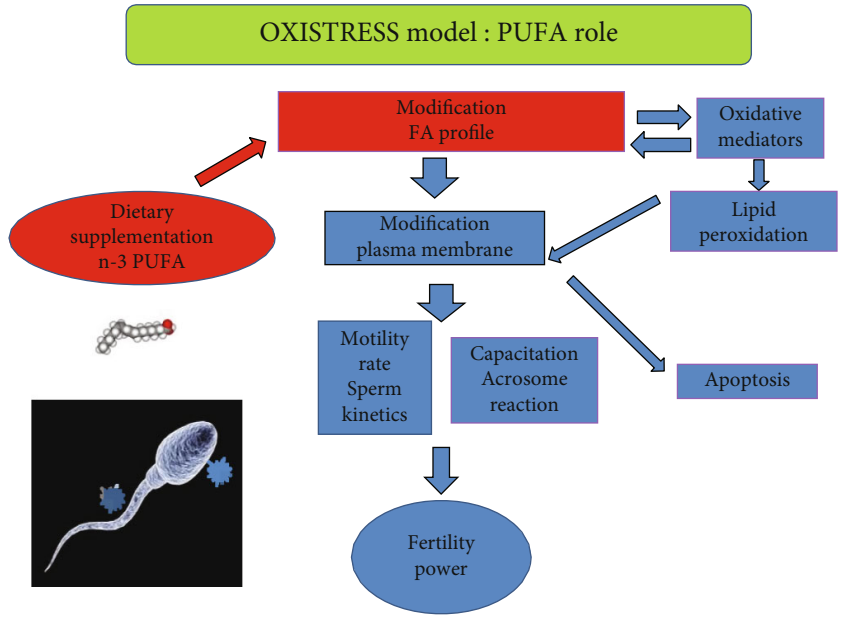

Figure 1: OXISTRESS model. Modification in fatty acid (FA) profile positively or negatively influences sperm function; n-3 supplementation positive influences sperm parameters.

Since it is difficult to imagine the composite effect of a dietary FA intervention in the sperm cell and to observe the effect on its function and to attract a broader community, we tried to find an alternative and effective way to reach a not specialized audience in order to maximize the benefit of the research with minimum delay.

Along with production of papers addressed towards the scientific community, we decided to design and develop, in collaboration with the laboratory of virtual reality (LabVR) of the University of Siena, a VR application which uses a plain language and appropriate information level to target an audience, which is not preengaged in the subject matter nor waiting for the output of the research.

The VR application is under development in the LabVR by a team of VR environment designer and developer, which use Unity development engine to make the experience run on Oculus Quest, a wireless 6DOF (Six degrees of freedom of movement in 3D space) VR Headset, released recently.

Digital applications and digital game environment have become important tools for education and training, and increasingly evidence-based theories are found about the educational benefits of computer games related to improvement of learning outcome thanks to value-adding features [4], improvement of general cognitive skills [4], and enhancement of motivation towards the learning subject [5, 6].

When games are compared to conventional media, there are substantially no results pointing that games are generally inferior to traditional instruction [4]; indeed, especially when we look at successful case studies in health [7] and food education [8], we can see "some reason to suspect that games can be as effective or more effective than traditional instruction for certain instructional domains and objectives" [4].

Virtual reality (VR) offers several advantages including a more realistic, lifelike environment that may allow subjects to "forget" they are being assessed, allowing a better participation and an increased generalization of learning [9]. It provides a simulated artificial environment in which one's actions determine sensory stimulation and is a valuable tool for investigating a wide spectrum of behaviors from sensorimotor interactions to spatial navigation and cognition [10].

In neuroscience, the VR system has been used to evaluate the patient's multimodal integration and to aid rehabilitation of cognitive abilities [11]. Specifically, in stroke rehabilitation, VR has been considered an important patient-centered tool because of its characteristics, such as contextualized environments and task-oriented training [12]. VR has been also used to treat various psychiatric disorders [13]. Recently, authors report that public speaking anxiety decreases within repeated VR training sessions [14] and VR offers promising results in sex therapy, particularly for the treatment of genital pain disorders in which anxiety plays a significant etiological role [15]. Regarding biology, a life sciences software company developed a VR visualization tool, called InViewR and ChimeraX; another molecular visualization tool for proteins was projected at the University of California [16].

These experiments show some different possibilities of VR application in repro field; however, we decided to move beyond the immersive visualization of microorganisms and to give priority to the interaction of the user with the dynamics of living systems.

2. OXISTRESS VR Applied Game

We aimed to build a compelling experience in which the players would discover the behaviors of the repro system subject of the research through the immersion and interaction within that system. We also wanted them to learn, during play, the effects of food-related habits on reproductive health.

In collaboration with the colleagues of the LabVR, we found a main problem to deal with: for the game to be effective as a learning tool, we would have needed to design a conflict (a problem to solve) which would have requested to the player the development of that precise skill that we wanted her to learn. For example, a "sperm race" would have been a useless activity since it would train the player on skills like spatial attention and hand-eye coordination which are not pertinent with our learning objectives. Accordingly, we imagined a game where the player, in order to improve the health of some virtual spermatozoa, is called to take dietary choices and then discover their consequences on the main biological aspects. In the game, the player is immersed in the epididymis and observes closer how dietary n-3 may change the sperm plasma membrane and consequently modify sperm traits.

Through the dietary choices, the user can modulate the quantity and the interaction of FAs, antioxidants, and ROS and is nudged to understand their role in the system in order to take informed choices which would be resonant and, at the end, easy to remember.

By this design, the relevance of a correct diet would have directly visualized and demonstrated in male reproduction but it leaves space for another issue: we know the dietary choices are just one of the many factors that determinate the health state of cell membranes. We also know that a game design rule is for the player to be engaged; the player must perceive the critical actions to progress in the game. Would the player feel a sense of agency if the choices would not have been decisive to reach the game goal?

To overcome this obstacle, we came to conceive a fictional scenario where player's choices have immediate and strongly exaggerated consequences and any other variable, even those being decisive in the real world, becomes irrelevant.

The sci-fi nature of the scenario (the control of sperm quality in a group of astronauts has to colonize a new planet, which is protected by a hyperconsequential force field) would have helped us to justify at a game level the focus on the sperm quality, which would be hard to be turned into an "epic goal" with a different narrative setting.

Here, we came to the last doubt we had to solve: are we going too far? Will this game be close enough to reality to be still useful for his dissemination scope?

To answer the question, we find and illuminate a quote from the pioneer environmental scientist Donella Meadow [17]: "Model utility depends not on whether its driving scenarios are realistic, but on whether it responds with a realistic pattern of behavior".

Concerning the player point of view, we are pretty convinced that as long as we provide a plausible conflict to deal with and the space for meaningful choices (for the concept of meaningful play see Salen and Zimmerman [18]), we can rely on the players' suspension of disbelief for expecting them to engage in the activity by taking its outcomes very seriously, as they usually do in any game.

In conclusion, this new approach represents a facilitation in dissemination of the paramount influence of nutrients in the sperm quality and consequently in male reproduction. By the same game, the role of nutrients could be emphasized in different physiological and pathological conditions and could also represent a model to implement with other effects (environmental, stress, pathological situation, smoke etc.).

A simulation game in the virtual reality may represent a tool to give greater visibility to scientific data in the relevance of appropriate dietary habits in the human health.

Data Availability

The data used to support the findings of this study are included within the supplementary information file.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

M. M., G. C., A. I., and V. S. designed the study; S. B., L. M. L., M. S., and V. S. helped in developing the virtual reality; C. C., S. M., D. N., G. C., E. M., and C. S. tested the animal model; G. C., V. S., C. S., and M. M. wrote the manuscript; M. M. and A. I. contributed in acquiring funds. All authors read and approved the final manuscript.

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Supplementary Materials

In the "Supplementary material," a detailed description of the game, to which the manuscript refers, is provided. The principles of the game design are reported and a map of the game is detailed, step by step. The described game is to be intended as a game-based learning. *(Supplementary Materials)*

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Research Article

Resistin in Human Seminal Plasma: Relationship with Lipid Peroxidation, CAT Activity, GSH/GSSG Ratio, and Semen Parameters

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Resistin is an adipokine involved in inflammation and able to induce the expression of other proinflammatory cytokines. It is known that, in human semen, resistin is correlated with inflammatory cytokines and sperm quality. The aim of this prospective study was to explore the potential relationship between resistin, lipid peroxidation (LPO), catalase (CAT) activity, and reduced and oxidized glutathione (GSH/GSSG) ratio in semen samples of infertile patients with leukocytospermia (no. 19), infertile patients with varicocele (no. 17), and fertile men (no. 17). Semen analysis was performed following the WHO guidelines, and sperm apoptosis and necrosis were evaluated with annexin V/propidium iodide assay. Seminal plasma samples were used to determine resistin levels by an immunological method, MDA concentration by a HPLC analysis with UV detection, GSH/GSSG ratio by an enzymatic method, CAT activity by a spectrophotometric method. The results showed that, in both groups of infertile patients, semen parameters were significantly reduced ($P < 0.001$) and sperm apoptosis and necrosis percentages were increased. Resistin levels were significantly higher in leukocytospermia and varicocele groups ($P < 0.001$ and $P < 0.01$, respectively) as well as MDA concentration ($P < 0.001$) compared to controls. The MDA level was also significantly increased in the leukocytospermia group versus the varicocele group ($P < 0.05$). The GSH/GSSG ratio was higher in fertile controls than the leukocytospermia group ($P < 0.05$) and the varicocele group ($P < 0.001$) and in the leukocytospermia group versus the varicocele group ($P < 0.05$). Both the leukocytospermia and varicocele groups showed increased values of CAT activities ($P < 0.001$) than controls. Briefly, the correlation between variables, calculated in the whole patient population, showed that resistin levels positively correlated with MDA levels, CAT activity, sperm apoptosis, and necrosis and negatively with sperm parameters and GSH/GSSG ratio. These results support an active role of resistin in an inflammatory process causing LPO, increase of CAT activity, and decrease of GSH/GSSG ratio in seminal plasma of infertile men vs. fertile controls.

1. Introduction

Adipokines, such as leptin, resistin, adiponectin, chemerin, omentin, and visfatin, play a critical role in the development of complications related to obesity and inflammatory conditions [1–3]. In addition, they are also involved in other functions of the organism including those pertinent to the gonadal and hypothalamic-pituitary axis, both in females and in males. Among these adipokines, we studied resistin, a cytokine that belongs to a family of low molecular weight cysteine-rich secretory proteins, synthesized by adipose tissue. It is well known that resistin regulates glucose metabolism in mammalians and that high levels of circulating resistin are responsible for insulin resistance [4]. Therefore, it is postulated that resistin represents a molecular link between obesity and type 2 diabetes [5]. Patel et al. [6] demonstrated that, in contrast to what it was observed in mouse, resistin was almost undetectable in human adipose

tissue. In addition, the analysis of the resistin gene expression in different human tissues revealed that macrophages, peripheral blood mononuclear cells, and bone marrow represent the main sources of resistin [7–10]. These studies demonstrated the role of resistin in inflammatory pathways since proinflammatory mediators are able to enhance resistin expression in peripheral mononuclear cells [11]. Similarly to the other adipokines, resistin is involved in both male and female reproductive functions [2]. With regard specifically to the male reproductive system, the expression of resistin is controlled by gonadotropins, indicating that this peptide plays a hormonal impact upon the testes [9]. Despite resistin was found in rat Leydig and Sertoli cells within the seminiferous tubules [12], resistin human testis localization has not been explored yet. A recent review dealing with adipokines in semen [13] reported that only three studies measured resistin levels in human seminal plasma. Some of our group found that semen resistin levels negatively correlated with sperm motility and viability and positively with sperm apoptosis and necrosis [14]. These data were not in accord with those reported by Kratzsch et al. [15] and Thomas et al. [16]. Despite these differences, the authors of both groups [14, 15] agree on the positive relationship between semen resistin concentrations and semen levels of proinflammatory mediators such as elastase, interleukin-6 (IL-6), and tumour necrosis factor-alpha (TNF-alpha) [14, 15]. Such observation may suggest a potential role of resistin as a marker of inflammation in human semen. Indeed, in the course of inflammatory events or under other pathological conditions, cytokine levels and reactive oxygen species (ROS) increase. In particular, ROS are necessary in several physiological steps such as development and maturation of spermatozoa, capacitation, acrosome reaction, and fertilization [17]. When, in seminal plasma, an imbalance between oxidants and antioxidants in favor of the antioxidants occurs [18], a disruption of redox signaling and control can interfere with normal sperm function causing membrane lipid peroxidation (LPO), DNA fragmentation, etc. [19, 20]. It was recently demonstrated that induced varicocele has a negative effect on rat spermatogenesis and increases oxidative stress leading to the production of sperm with damaged chromatin which reduces the fertility potential [21]. Since a relationship between resistin and oxidative stress in pathologies such as diabetes mellitus [3, 22] and fatty liver disease [1] was reported, we decided to study this subject in human semen. Thus, we determined resistin levels in seminal plasma of infertile patients affected by leukocytospermia or varicocele and in a group of fertile individuals. In the same samples, the LPO and redox imbalance were assessed by malondialdehyde (MDA) and antioxidant processes, such as those related to reduced and oxidized glutathione (GSH/GSSG) ratio and catalase (CAT) activity. In human semen, the relationship between these parameters was still unexplored; therefore, this research represents a new subject of study.

2. Materials and Methods

2.1. Patients and Controls. Among patients attending our laboratory for semen analysis, we selected 36 consecutive infertile patients (aged 25-35) with leukocytospermia or varicocele. Infertile patients did not obtain pregnancy after two years of unprotected sexual intercourses; the female factor infertility was excluded. 19 patients (aged 27-35) showed leukocytospermia and 17 patients (aged 25-32) varicocele. Leukocytospermia was determined during semen analysis following the WHO guidelines [23]. Varicocele was assessed by both physical examination and scrotal colour Doppler ultrasonography analysis performed in laboratories different from ours. For this study, we included patients with grade II and grade III varicocele.

All patients satisfied the following criteria: non-azoospermic men, 46, XY karyotype, BMI $< 25\,kg/m^2$. They showed normal levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone (T). Hormone concentrations were determined in serum by commercial kits (Beckman Coulter Access for FSH, LH, and testosterone). A normal range for FSH was 0.7-11.00 mU/ml (sensitivity 0.2 mUI/ml, intra- and interassay coefficient of variation < 10%), for LH was 0.8-8.0 mU/l (sensitivity 0.2 mUI/ml, intra- and interassay coefficient of variation < 10%), and for T was 2.7-10.9 mg/ml (sensitivity 0.1 ng/ml, intra- and interassay coefficient of variation < 10 %). Genitourinary infections were excluded by semen culture. Samples were seeded using a calibrated loop on agar plates, which were incubated overnight at 37°C in normal air with 5% CO_2. The microorganisms were identified by gram stain, oxidase, catalase, and other biochemical tests using BioMérieux products (BioMérieux, Florence, Italy). Semen cultures were positive when the number of colonies was $\geq 10^4$ CFU ml^{-1} in case of gram positive cocci and $\geq 10^5$ CFU ml^{-1} in case of gram negative rods. The selected patients which had no chronic diseases and did not receive medication, chemotherapy, and radiotherapy. None of subjects took oral antioxidant supplements for at least five months before the analysis. Subjects with a history of recreational drug use, alcohol consumption, and smoking habit were excluded from this study.

17 fertile men (aged 25-33) which fathered at least one child in the last 3 years represented the control group. These subjects were not affected by infections and anatomical and hormonal problems. Their semen parameters were higher than 25 percentiles as reported in the WHO guidelines [23].

Before the inclusion in this study, all patients and controls provided an informed written consent. The informed consent describes the aims of the research, and it is approved by the Institutional Review Board of Siena University.

2.2. Semen Analysis. The analysis of human semen samples was performed following the WHO guidelines [23]. Samples were collected by masturbation after 3-5 days of sexual abstinence and analysed after liquefaction for 30 min at 37°C. Semen volume, pH, sperm concentration, and motility were determined. The Papanicolaou (PAP) test modified for spermatozoa enabled to assess sperm morphology. Leukocytes in semen samples were identified by peroxidase stain, and a leukocyte concentration ≥ 1 million cell/ml was considered abnormal (WHO, [23]).

2.3. Detection of Sperm Apoptosis and Necrosis. To detect sperm apoptosis and necrosis, the Vybrant apoptosis assay (Invitrogen Ltd., Paisley, United Kingdom) based on fluorescein isothiocyanate- (FITC-) annexin V (AnV, green fluorescence) and propidium iodide (PI, red fluorescence) was used. AnV protein binds to negatively charged phospholipid phosphatidylserine, located in the inner leaflet of the plasma membrane, that during apoptosis is actively externalized to the outer leaflet of the plasma membrane. PI stains necrotic cell with broken membrane. The detailed procedure is described in Moretti et al. [14]. About 300 sperms for each sample were scored, and undamaged sperm (AnV negative, PI negative, not stained), apoptotic sperm (AnV positive, PI negative, green stained), and necrotic sperm (AnV negative, PI positive, red stained) were classified. Sperms showing both green and red signals were considered as necrotic since their membrane was damaged.

2.4. Resistin Assay. Resistin was determined in semen samples of the 53 participants to the study. 500 μl of each semen sample was collected 1 h after production and fractioned by centrifugation (200 g for 15 min). The supernatant, composed of seminal plasma without spermatozoa, was stored at -80°C until resistin levels were evaluated by enzyme-linked immunosorbent assay (ELISA) using resistin (human) ELISA kit (Phoenix Pharmaceutical, Inc., Burlingame, CA, USA), following the manufacturer's instructions. Briefly, samples were added to microtiter wells sensitized with anti-human resistin antibodies. Some wells contained known amounts of resistin, which are important to build the regression line. After incubation for 2 hours at room temperature, wells were washed. Then, wells were incubated with the biotinylated anti-human resistin detection antibody diluted 1 : 150 for 2 hours at room temperature. At the end of incubation, wells were washed and streptavidin-horseradish peroxidase solution was added into each well and incubated for 30 min at room temperature. After washing, the substrate solution provided in the kit was added into each well; finally, the reaction was stopped with 2N hydrochloric acid. The optical density was read at 450 nm using the iMark™ Microplate Absorbance Reader (Bio-Rad, Italy). The results were expressed in ng/ml.

2.5. Reduced and Oxidized Glutathione Evaluation. After thawing, 200 μl of seminal plasma without spermatozoa of each subject was diluted with an equal volume of 10% metaphosphoric acid. Specimens were centrifuged at 2000 g for 10 min at 0°C. Total glutathione (GSH) and oxidized glutathione (GSSG) were measured in the supernatant by a microassay method [24] and expressed in nmol per mg of protein. Each sample was determined in triplicate, and these indices were expressed as reduced (GSH) to oxidized glutathione (GSSG) ratio.

2.6. Catalase Activity Determination. After thawing, seminal plasma without spermatozoa of each subject was centrifuged at 4000 g for 15 min at 4°C. A microassay procedure was used [25] to determine the catalase (CAT) activity.

This method that requires 20 μl of seminal plasma depends on the reaction of CAT with methanol in the presence of an optimal concentration of hydrogen peroxide. The formaldehyde production was measured spectrophotometrically at 540 nm with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald, Sigma-Aldrich, Milan, Italy) as a chromogen. One unit of catalase activity was defined as the amount of enzyme that caused the formation of 1 nmol of formaldehyde per min at 25°C. The readings were made three times (20 μl each time). Results were expressed as nmol/min/mg of protein.

2.7. Protein Assay. Protein concentrations were determined according to Lowry et al., and the calibration curves were prepared with dry bovine serum albumin [26].

2.8. Malondialdehyde (MDA) Assessment. Free MDA levels were measured in seminal plasma without spermatozoa. After thawing, 500 μl of seminal plasma was added to 500 μl of tris-HCl 0.04 M and acetonitrile containing 0.1% butylated hydroxytoluene (BHT) to prevent artifact oxidation of polyunsaturated free fatty acids during the assay. The samples were centrifuged at 3000 g for 15 min. The supernatant was used for MDA HPLC analysis with UV detection after derivatization with 2·4 dinitrophenylhydrazine according the method of Shara et al. [27] with minor modifications. The samples were immediately stirred and were extracted with 5 ml of pentane; finally, the samples were dried by using nitrogen. A calibration with concentrations of MDA by 0.5 nmoli/ml to 10 nmoli/ml was used for MDA determinations. The MDA hydrazone was quantified by isocratic high-performance liquid chromatography using a Waters 600 E System Controller HPLC (Milford, MA, USA) equipped with a Waters Dual λ 2487 UV detector (Milford, MA, USA) set at 307 nm. To separate the hydrazone derivative, a 5 μ ultrasphere ODS column C18 (Beckman, San Ramon, CA, USA) was used at the flow rate of 0.8 ml/min with the acetonitrile (45%)-HCl 0.01 N (55%) as mobile phase. The concentrations of MDA were obtained by peak areas determined using an Agilent 3395 integrator (Agilent Technologies, USA). Each sample was assessed in triplicate, and the results are expressed in nmol of MDA per ml of seminal plasma.

2.9. Statistical Analysis. Statistical analysis was performed using the SPSS software package (version 19, SPSS Inc., Chicago, IL, USA). The Kolmogorov-Smirnov test was used in order to examine the normality of distribution of the variables investigated. Levene's test were used to verify the assumption of homoscedasticity of the variance of groups. One-way analysis of variance (ANOVA) was utilized to evaluate differences among the groups. When the assumption of homoscedasticity was respected, Tukey's test was used for multiple comparisons. The Welch test and Games-Howell post hoc test were used in condition of heteroskedasticity. The values of $P < 0.05$ were considered significant. The correlation between the investigated variables was assessed using Spearman's rank correlation coefficient (rho).

Table 1: Semen parameters (means ± standard error) of infertile men are classified into 2 groups according to clinical diagnoses and fertile controls.

Variables	Leukocytospermia L	Varicocele V	Fertile men F	HDS Tukey post hoc test (P value)	Games-Howell post hoc test (P value)
Volume (ml)	3.51 ± 0.26	3.83 ± 0.35	3.58 ± 0.24	—	—
Sperm/ml $\times 10^6$	56.07 ± 7.30	69.34 ± 7.15	166.86 ± 19.60	—	F *vs.* L*** F *vs.* V***
Motility %	30.52 ± 2.33	26.65 ± 2.84	51.76 ± 1.15	F *vs.* L*** F *vs.* V***	
Normal morphology %	8.74 ± 0.34	8.06 ± 0.46	17.06 ± 0.51	F *vs.* L*** F *vs.* V***	
Apoptosis %	13.63 ± 1.44	13.82 ± 1.69	7.47 ± 0.99	F *vs.* L** F *vs.* V**	
Necrosis %	20.10 ± 1.31	15.29 ± 2.15	10.64 ± 1.15	F *vs.* L***	

Legend: volume (ml); sperm/ml $\times 10^6$ (number of sperm/ml); motility % (percentage of rapid and slow progressive sperm motility); normal morphology % (percentage of sperm with normal morphology assessed with Papanicolaou staining); apoptosis % (percentage of sperm apoptosis assessed with AnV/PI assay); necrosis % (percentage of sperm necrosis assessed with AnV/PI assay). $^{**}P < 0.01$; $^{***}P < 0.001$.

Table 2: Resistin and stress oxidative parameters (means ± standard error) of infertile men classified into 2 groups according to clinical diagnoses and fertile controls.

Variables	Leukocytospermia L	Varicocele V	Fertile men F	HDS Tukey post hoc test (P value)	Games-Howell post hoc test (P value)
Resistin (ng/ml)	3.13 ± 0.46	2.66 ± 0.49	0.84 ± 0.25		F *vs.* L*** F *vs.* V**
MDA (nmoli/ml)	6.87± 1.25	3.56± 0.61	0.56± 0.05	—	F *vs* L*** F *vs.* V*** L *vs.* V*
GSH/GSSH	11.20 ± 0.97	8.12 ± 0.77	14.49 ± 0.66	F *vs.* L* F *vs.* V*** L *vs.* V*	
CAT (nmoli/min/mg of proteins)	9.61 ± 0.91	8.21 ± 0.73	3.02 ± 0.40	—	F *vs.* L*** F *vs.* V***

Legend: resistin (ng/ml); MDA (malondialdehyde; nmoli/ml); GSH/GSSG (reduced glutathione/oxidized glutathione; nmol/mg of protein); CAT (catalase activity; nmoli/min/mg of protein). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

3. Results

The semen variables of the 53 subjects grouped as infertile patients with leukocytospermia (no. 19), infertile patients with varicocele (no. 17), and fertile controls (no. 17) are shown in Table 1. The values of semen volume were similar in the three groups; the sperm concentration, the percentage of progressive motility, and the percentage of sperm with normal morphology were significantly reduced in both groups of infertile patients ($P < 0.001$) compared to those observed in the fertile group (Table 1). The percentage of apoptotic sperm was significantly higher in the leukocytospermia and varicocele groups ($P < 0.01$) than that detected in fertile men; the percentage of sperm necrosis was significantly increased in the leukocytospermia group ($P < 0.001$) versus fertile men (Table 1). Table 2 shows the comparisons of resistin level, MDA concentration, GSH/GSSG ratio, and CAT activity among the considered groups. Resistin values were significantly higher in the leukocytospermia group ($P < 0.001$) and the varicocele group ($P < 0.01$) than that measured in the control group. The MDA concentrations were significantly increased in both infertile groups ($P < 0.001$) compared to controls and also in the leukocytospermia group versus the varicocele group ($P < 0.05$). The GSH/GSSG ratio was significantly higher in fertile controls than the leukocytospermia group ($P < 0.05$) and the varicocele group ($P < 0.001$) and in the leukocytospermia group versus the varicocele group ($P < 0.05$). Both the leukocytospermia and varicocele groups showed higher values of CAT activities ($P < 0.001$) than that detected in the control group.

The correlations between all considered variables calculated in the 53 study participants are reported in Table 3. Sperm concentration, motility, and normal morphology were positively correlated to each other ($P < 0.001$). Sperm concentration positively correlated with GSH/GSSG ratio ($P < 0.001$) and negatively with necrosis ($P < 0.001$), resistin

Table 3: Correlations (rho Spearman's coefficient) between all considered variables in 53 individuals.

	Sperm/ml $\times 10^6$	Motility %	Normal morphology %	Apoptosis %	Necrosis %	Resistin	MDA	GSH/GSSG	CAT
Sperm/ml $\times 10^6$	**1**								
Motility %	**0.674*****	**1**							
Normal morphology %	**0.677*****	**0.733*****	**1**						
Apoptosis %	**-0.255**	**-0.444*****	**-0.450*****	**1**					
Necrosis %	**-0.432*****	**-0.506*****	**-0.455*****	**0.262***	**1**				
Resistin	**-0.312***	**-0.316***	**-0.445*****	**0.378****	**0.470*****	**1**			
MDA	**-0.355****	**-0.471*****	**-0.444*****	**0.318***	**0.413****	**0.416****	**1**		
GSH/GSSG	**0.463*****	**0.497*****	**0.591*****	**-0.169**	**-0.216**	**-0.293***	**-0.124**	**1**	
CAT	**-0.671*****	**-0.464*****	**-0.605*****	**0.390****	**0.330***	**0.453*****	**0.411****	**-0.507*****	**1**

Legend: sperm/ml $\times 10^6$ (number of sperm/ml); motility % (percentage of rapid and slow progressive sperm motility); normal morphology % (percentage of sperm with normal morphology assessed with Papanicolaou staining); apoptosis % (percentage of sperm apoptosis assessed with AnV/PI assay); necrosis % (percentage of sperm necrosis assessed with AnV/PI assay); resistin (ng/ml); MDA (malondialdehyde; nmoli/ml); GSH/GSSG (reduced glutathione/oxidized glutathione; nmoli/mg of protein); CAT (catalase activity; nmoli/min/mg of protein). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

($P < 0.05$), MDA concentration ($P < 0.01$), and CAT activity ($P < 0.001$). Sperm motility showed positive correlations with GSH/GSSG ($P < 0.001$) and negative correlations with apoptosis ($P < 0.001$), necrosis ($P < 0.001$), resistin ($P < 0.05$), MDA concentration ($P < 0.001$), and CAT activity ($P < 0.001$). The percentage of sperm with normal morphology showed a positive correlation with GSH/GSSG ratio ($P < 0.001$) and negative correlations with sperm apoptosis and necrosis ($P < 0.001$), resistin ($P < 0.001$), MDA concentration ($P < 0.001$), and CAT activity ($P < 0.001$). Sperm apoptosis was positively correlated with necrosis ($P < 0.05$), resistin ($P < 0.01$), MDA concentration ($P < 0.05$), and CAT activity ($P < 0.01$). Sperm necrosis displayed positive correlations with resistin ($P < 0.001$), MDA concentration ($P < 0.01$), and CAT activity ($P < 0.05$). Resistin was positively correlated with MDA concentration ($P < 0.01$) and CAT activity ($P < 0.001$) and negatively with GSH/GSSG ratio ($P < 0.05$). CAT activity was positively correlated with MDA concentration ($P < 0.01$) and negatively with GSH/GSSG ratio ($P < 0.001$).

4. Discussion

Here, we reported a relationship between resistin, LPO, and redox imbalance in human semen. Resistin is an adipokine able to trigger proinflammatory state in vitro and in vivo and to play a role in inducing the expression of other cytokines [11], so it may be considered an inflammatory biomarker [28]. Evidence for a crosstalk between adipokines, including resistin, inflammation, and redox imbalance, has been provided in several tissues and in different pathological conditions [1, 3, 29, 30].

Since in a previous research a significant relationship between resistin and proinflammatory cytokines was demonstrated in human semen samples [14], we decided to explore the potential relationships between resistin, LPO, CAT activity, and GSH/GSSG ratio in human semen. It is well known that oxidative stress plays a detrimental role on sperm function damaging membranes by LPO and causing mitochondrial and nuclear DNA fragmentation. Spermatozoa are vulnerable to ROS attack since their membranes are particularly rich in polyunsaturated fatty acids. LPO induces a loss of membrane properties such as membrane permeability and membrane potential and generally affects the cellular integrity [31]. LPO generates highly reactive lipid peroxidation products such as malondialdehyde (MDA), 4-hydroxynonenal, and isoprostanes, which all represent the best products to study such a process [32]. To protect sperm from the damages induced by oxidants, human semen is equipped with nonenzymatic and enzymatic antioxidants [33]. The reduced GSH is a nonenzymatic antioxidant able to react with lipid peroxides, protecting sperm plasma membranes [34]. The ratio GSH/GSSG was utilized in this study because it offers a simple representation of oxidative stress [35]. The enzyme CAT is able to decompose hydrogen peroxide to water and oxygen; thus, CAT is able to protect cells from oxidative damages caused by ROS [36].

Leukocytospermia and varicocele are both conditions associated with oxidative stress, a consequence of inflammatory situations [20, 37–41]. In addition, it is known that macrophages and neutrophils are both major sources of resistin involved in the inflammatory pathway [8, 11]; thus, leukocytospermia appears to be an interesting pathology to study. Likewise, it is reported that varicocele induces inflammation and, consequently, impairs spermatogenesis [42]. Recently, Ghandehari-Alavijeh et al. [43] suggested a relevant involvement of the hypoxia pathway in the etiology of varicocele leading to decrease sperm quality and DNA integrity. It is known that the presence of this pathology increases the levels of oxidants and reduces those of antioxidants [40].

In a previous paper, we demonstrated that semen resistin was increased in patients with leukocytospermia and smoking habit and also in infertile patients when we compare them with control subjects with unknown reproductive potential [14]. In this study, the patient's selection was more

stringent and we selected infertile patients with abacterial leukocytospermia, infertile patients with varicocele, and a group of fertile patients used as control.

The data from this research showed that infertile patients affected by leukocytospermia and varicocele had increased levels of resistin with respect to fertile men. The impairment of redox status of both pathologies is highlighted by the decrement of GSH/GSSG ratio and the increase of MDA in both groups of infertile patients. Fraczek et al. [38] reported a high MDA concentration in sperm lysates in the presence of leukocytes, and Ni et al. [44] observed an increased MDA concentration in normozoospermic men with clinical varicocele and in infertile astheno/oligozoospermic patients with clinical varicocele. It is noteworthy that, in our study, MDA was significantly increased in the leukocytospermia group compared to the varicocele group, indicating that leukocytes, known to be one of the major sources of ROS and to stimulate sperm to produce ROS [45], can induce the oxidative stress. The GSH/GSSG ratio is decreased in both groups of infertile patients, particularly in varicocele patients, where following the unsuccessful attempt to counterbalance the excess of ROS, redox imbalance is predominant. Semen CAT activity is increased in the presence of leukocytospermia and varicocele, suggesting a sort of "chronic oxidative stress." In some cases, enhanced ROS amounts cannot be neutralized even in the presence of increased expression of antioxidants and related enzymes. For this reason, enhanced ROS levels can be stabilized and lead to modifications of several cell components, perturbing homeostasis [46]. This observation fits with our results as the high MDA levels detected in the leukocytospermia and varicocele groups indicated that the increased CAT activity was not completely effective to counteract the excess of hydrogen peroxide which led to LPO and cell damage. Obviously, to draw final conclusions, additional redox indicators, other than CAT activity and GSH/GSSG ratio, should be evaluated at the same time.

It is well known that, in human semen, resistin is positively correlated with proinflammatory cytokines [14, 15]. In this research, the association between resistin, LPO, CAT activity, and GSH/GSSG ratio is a novel finding. Resistin was negatively correlated with semen parameters and GSH/GSSG ratio and positively with indices of sperm death, apoptosis, and necrosis and with MDA and CAT activities. These data confirm the relationship between resistin, inflammatory processes, and the relative redox imbalance. We then hypothesized that the presence of leukocytes in semen and varicocele could cause an increase in resistin (and other cytokines) levels concomitant with a redox imbalance able to influence semen quality: sperm motility was decreased and apoptosis was enhanced in both pathologies as also observed by other authors [47]. Sperm necrosis was particularly increased in the leukocytospermia group, in which the level of MDA was significantly higher than that observed in varicocele and fertile samples.

Very few data are available in the literature on the mechanism of action through which resistin can be involved in an inflammatory pathway in the human male reproductive system. Three receptors for resistin have been proposed: decorin, adenylyl cyclase-associated protein 1, and Toll-like receptor 4 (TLR4) [48]. Different experimental and clinical researches identified the TLR4 signaling pathways activated by resistin as the molecular mechanism that links insulin resistance and obesity [49]. Interestingly, TLR2 and TLR4 are both localized in the acrosomal and tail regions of human sperm [50]. Recently, Hagan et al. [51] found that TLR2 and TLR4 are both upregulated in the seminal plasma of patients with leukocytospermia. These data could represent the link between resistin and inflammation in human semen.

5. Conclusions

The results of this study indicate that resistin levels are increased in infertile patients with leukocytospermia and with varicocele and are correlated with impaired sperm quality, LPO, and semen redox imbalance. It should be interesting to test if antioxidant supplementation, in the presence of different pathologies, is able to influence the resistin level in the male reproductive system.

Data Availability

The data are reported in the paper.

Conflicts of Interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the reported research.

Authors' Contributions

All the authors gave substantial contributions to the research design, drafting of the paper, or revising it critically and approved the submitted version. Elena Moretti, in particular, did the following: research design, paper writing, data interpretation, and semen analysis. Lucia Micheli, in particular, did the following: determination of GSH, GSSG, and catalase and data interpretation. Daria Noto, in particular, did the following: semen analysis, determination of sperm apoptosis and necrosis, and determination of resistin. Andrea Menchiari, in particular, did the following: statistical analysis. Daniela Cerretani, in particular, did the following: research design, determination of MDA, and paper writing.

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Research Article

Resveratrol Improves Boar Sperm Quality via 5′AMP-Activated Protein Kinase Activation during Cryopreservation

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Mammalian sperm is highly susceptible to the reactive oxygen species (ROS) stress caused by biochemical and physical modifications during the cryopreservation process. 5′AMP-activated protein kinase (AMPK) is involved in regulating both cell metabolism and cellular redox status. The aim of the present study was to investigate whether the resveratrol protects boar sperm against ROS stress via activation of AMPK during cryopreservation. Boar sperm was diluted with the freezing medium supplemented with resveratrol at different concentrations (0, 25, 50, 75, 100, and 125 μM). It was observed that the addition of 50 μM resveratrol significantly improved the postthaw sperm progressive motility, membrane integrity, acrosome integrity, mitochondrial activity, glutathione (GSH) level, activities of enzymatic antioxidants (glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase), and the phosphorylation of AMPK. Meanwhile, the lipid peroxidation, ROS levels, and apoptosis of postthaw sperm were reduced in the presence of 50 μM resveratrol. Furthermore, when fresh boar sperm was incubated with the medium in the presence of 50 μM resveratrol and 30 μM Compound C (an AMPK inhibitor), the effects of the resveratrol were partly counteracted by the Compound C. These observations suggest that the resveratrol protects boar sperm via promoting AMPK phosphorylation. In conclusion, the addition of resveratrol to the freezing extenders protects boar sperm against ROS damage via promoting AMPK phosphorylation for decreasing the ROS production and improving the antioxidative defense system of postthaw sperm. These findings provide novel insights into understanding the mechanisms of resveratrol on how to protect boar sperm quality contrary to the ROS production during cryopreservation.

1. Introduction

The cryopreservation of sperm is one of the most essential assisted reproductive techniques in the livestock industry. This technique involves some physiochemical procedures including cooling, freezing, and thawing, which are known to produce excess ROS through an alteration in the sperm physical or chemical conditions that impair the homeostasis of sperm metabolism [1]. The removal of seminal plasma during the cryopreservation process is reported to reduce the antioxidant defenses in sperm [2–4], and thus, sperm becomes vulnerable to the oxidative stress [5]. Boar sperm plasma membrane is rich in polyunsaturated fatty acids with low cholesterol or phospholipid that are susceptible to lipid peroxidation by ROS attack *in vitro* [6]. The excessive ROS generated during the cryopreservation process is detrimental to sperm motility and fertilizing ability [7]. Numerous studies reported that the treatment with exogenous antioxidants is an effective strategy to resist oxidative stress and to improve sperm quality during cryopreservation [8–10].

Resveratrol (3,5,4′-trihydroxystilbene), a stilbenoid, is a natural polyphenol structurally similar to diethylstilbestrol

and estradiol [11]. Resveratrol was first identified as the principal active ingredient from the dried roots of *Polygonum cuspidatum*, a plant used in traditional Chinese medicine for responding to injury, stress, bacterial or fungal infection, UV irradiation and exposure to ozone [12, 13]. Previous studies showed that resveratrol is widely consumed in the Mediterranean diet in the form of grapes, peanuts, and red wine [14, 15]. Additionally, the resveratrol is the main reason for the "French paradox" that French people who had a low incidence of coronary heart diseases despite the consumption of a diet with high saturated fat [16]. In somatic cells, resveratrol has been regarded as an antioxidant due to its ability to reduce mitochondrial ROS production, scavenge superoxide radicals, and inhibit lipid peroxidation as well as regulate the expression of antioxidant cofactors and enzymes [17]. Furthermore, *in vivo* studies revealed that resveratrol plays a beneficial role against the diseases of aged people having impaired energy metabolism [15]. Adenosine triphosphate (ATP) production through the mitochondrial oxidative phosphorylation (OXPHOS) is essential to maintain motility tracks in boar sperm [18]. However, ROS generation is a natural by-product of mitochondrial OXPHOS [19, 20]. The ROS level in sperm is controlled by the balance between ROS generation and ROS scavenging by antioxidants [21]. Notably, AMPK is an energy sensor of cellular metabolism [22] that was observed to be activated by resveratrol in somatic cells *in vitro* [23, 24]. Additionally, our previous study has been identified that the AMPK regulates energy metabolism in goat sperm [25]. Therefore, it is hypothesized that the addition of resveratrol to the freezing extender may activate the AMPK in sperm. If the AMPK is activated by resveratrol in sperm, it will not only scavenge the ROS but also enhance the sperm antioxidative defense system.

Numerous studies have been reported that the addition of resveratrol to the freezing extenders improves sperm motility and mitochondrial activity in bulls [26] and reduces the DNA damage of postthaw human sperm [27]. Additionally, Gadani et al. reported that the addition of resveratrol to the thawing solution efficiently improved the penetration rate of boar sperm *in vitro* [28]. However, there is no report on the addition of resveratrol to the freezing extender of boar sperm. Moreover, the mechanism of how the resveratrol protects sperm against oxidative damages is still unclear. Therefore, this study was aimed at investigating the effect of resveratrol treatment on boar sperm during cryopreservation and at understanding the mechanism of how resveratrol protects boar sperm against ROS attack. In this study, we hypothesized that the resveratrol might protect the sperm against ROS-associated damages by reducing the ROS generation through the activation of AMPK and consequently enhance the sperm antioxidant systems during cryopreservation.

2. Experiment Design

Experiment I was designed to detect whether the addition of the resveratrol to the freezing extender could improve the quality of frozen-thawed boar sperm via examination of sperm progressive motility, membrane integrity, intact acrosome, mitochondrial activity, oxidative DNA damage, and lipid peroxidation. The ROS level, GSH content, activities of GPx, SOD, and catalase, and apoptosis as well as AMPK phosphorylation were also analyzed to reveal the mechanism in which resveratrol protects sperm via activating AMPK and how it enhances sperm's antioxidative defense system.

Experiment II was set up to study whether resveratrol could protect sperm during the process of cryopreservation from cooling, equilibration, freezing, and thawing and incubation of frozen-thawed sperm *in vitro*.

Experiment III was carried out to verify that the role of resveratrol in protecting boar sperm via activating AMPK against ROS stress *in vitro*. H_2O_2 was used to induce ROS damage; AMPK activator (5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranoside (AICAR)) and inhibitor (Compound C) were used to regulate AMPK. Specifically, as shown in Supplementary Figure 1, there were five treatment groups in Experiment III, where the Modena extender was supplemented with 200 μM H_2O_2 (1), with 200 μM H_2O_2 and 50 μM resveratrol (2), with 200 μM H_2O_2 and 2 mM AICAR (3), with 200 μM H_2O_2, 50 μM resveratrol, and 30 μM Compound C (4), and Modena extender without resveratrol, AICAR, Compound C, or H_2O_2 (5). Sperm progressive motility, acrosome integrity, membrane integrity, mitochondrial activity, lipid peroxidation, GSH level, ROS, and activities of GPx, SOD, and catalase, along with AMPK phosphorylation, were analyzed in those treatments.

3. Materials and Methods

3.1. Chemicals and Extenders. All chemicals and reagents were purchased from Sigma-Aldrich, China, unless specified otherwise.

The Modena solution was prepared in the laboratory which composed of 153 mM D-glucose, 26.7 mM trisodium citrate, 11.9 mM sodium hydrogen carbonate, 15.1 mM citric acid, 6.3 mM EDTA-2Na, 46.6 mM Tris, 1000 IU/mL penicillin G sodium salt (Solarbio, Beijing, China), 100 μg/mL polymyxin B, and 1 mg/mL streptomycin sesquisulfate (Solarbio, Beijing, China). Modena solution was used as the extender for liquid semen or thawing solution. The freezing extender (NSF) as described by Okazaki et al. [29] was used in this study with some modifications (mNSF). The first modification was done by adjusting the osmolarity to 400 mOsm/kg at a final concentration (mNSF1). The mNSF1 was further modified as mNSF2 by adding 1.5% (*v*/*v*, final concentrations: 0.75%) Orvus Es Paste (Miyazaki Chemical Sales Ltd., Tokyo, Japan) and 4% (*v*/*v*, final concentrations: 2%) glycerol.

In Experiment I, resveratrol was added to the mNSF1 at concentrations of 0, 25, 50, 75, 100, or 125 μM to determine the optimum concentration, which was used to the mNSF1, mNSF2, or Modena solution in the subsequent Experiments II and III.

3.2. Collection of Semen. Seven mature and fertile Duroc boars (aged 2 years) were used in the present study. All animals and experimental procedures were approved by the

Northwest A&F University Institutional Animal Care and Use Committee. The sperm-rich fraction was collected weekly from each boar with gloved-hand technique and filtered using a double gauze.

3.3. Semen Processing. According to Okazaki et al. [30], the semen was directly diluted with Modena solution ($v : v = 1 : 1$) and incubated for 2 h at 15°C. The semen was divided into 6 parts, centrifuged for 10 min at 700 ×g to remove the Modena solution. The sperm pellets were resuspended with mNSF1 (2.0×10^9 sperm/mL), added with different concentrations of resveratrol (0, 25, 50, 75, 100, or 125 μM), and slowly cooled from 15 to 5°C over 1.5 h. Subsequently, the sperm suspension was diluted in the same volume of mNSF2 and packed into a 0.5 mL plastic straw. The straws were placed in liquid nitrogen vapor for 10 min and plunged into it for storage. The straws were stored in the liquid nitrogen at least one week. The straws were thawed in water at 60°C for 8 s, and the frozen-thawed sperm was quickly diluted with 4.5 mL of thawing solution.

3.4. Evaluation of Sperm Motility, Membrane Integrity, and Acrosome Integrity. Sperm motility was measured using a computer-assisted sperm motility analysis (CASA) system (Integrated Semen Analysis System; Hview, Fuzhou, China). Briefly, 5 μL of semen was placed on an analyzer's Makler chamber and maintained at 37°C during the analysis. Three fields were selected for computer-assisted analysis [18].

Sperm membrane integrity and acrosome integrity were evaluated using a LIVE/DEAD Sperm Viability Kit (L7011; Thermo Fisher Scientific) and a fluorescein isothiocyanate-peanut agglutinin (FITC-PNA), respectively, according to Zhu et al. [8]. The stained sperm was monitored and photographed by an epifluorescence microscope (Nikon 80i; Tokyo, Japan) with a set of filters (200x).

3.5. Mitochondrial Activity. JC-1 Mitochondrial Membrane Potential Detection Kit (Beyotime Institute of Biotechnology, China) was used to analyze the changes in sperm mitochondrial activity ($\Delta\Psi$m) [25, 31]. There are two types of JC-1 in stained mitochondrial plasma; one is a monomer that emits green fluorescence in a low $\Delta\Psi$m, while the aggregates emit red fluorescence in a high $\Delta\Psi$m. Briefly, sperm samples (2×10^6/mL) were stained with 1x JC-1 at 37°C for 30 min. Fluorescence intensity of both mitochondrial JC-1 monomers (λex 514 nm, λem 529 nm) and aggregates (λex 585 nm, λem 590 nm) was detected using a monochromator microplate reader (Safire II, Tecan, Switzerland). The $\Delta\psi$m of sperm in each treatment group was calculated as the fluorescence ratio of red (aggregates) to green (monomer). Analyses were performed in triplicate ($n = 3$).

3.6. Lipid Peroxidation. The probe BODIPY 581/591C_{11} (Molecular Probes) was used to measure the sperm lipid peroxidation according to our previous study [32]. The intact probe fluoresces red when it is intercalated into the membrane, and it shifts to green after oxidative radical attacked. The staining samples were analyzed using a fluorescence microreader with an emission filter set to 635 nm for red and 535 nm for green. All experiments were carried out in triplicate.

3.7. Detection of Sperm Oxidative DNA Damage. The generation of the oxidized base adduct, 8-hydroxyguanosine (8-OHdG), was detected as a biomarker for oxidative DNA damage. The detection of 8-OHdG in postthaw boar sperm was according to our previous study [32]. Sperm samples were washed twice and resuspended in 500 μL PBS for flow cytometric analysis after 8-OHdG staining. BL2 (red fluorescence, long-pass dichroic filter 600 nm, bandpass filter 575 nm, detection width 560-590 nm) was used to detect the 8-OHdG level of postthaw sperm. A total of 20,000 sperm-specific events were evaluated. Data were processed by using the CellQuest program (BD Biosciences). The samples were also viewed and photographed using an epifluorescence microscope (80i; Nikon) with a set of filters (400x). Negative control with mouse IgG instead of the anti-8-OHdG antibody was included to ensure assay specificity.

3.8. Annexin V-FITC/PI Assay. Annexin V-FITC/PI apoptosis detection kit (Sigma-Aldrich, St. Louis, MO, USA) was used to assess sperm apoptosis according to the manufacturer's instruction with slight modifications. The postthaw sperm was centrifuged and washed thrice with PBS at 400 ×g for 5 min. The sperm was resuspended with 1x Annexin V-binding buffer at a concentration of 1×10^6 sperm/mL. A total of 5 μL Annexin V-FITC (AN) and 3 μL PI were then added to each aliquot of a 100 μL sample. The tubes were mixed gently and incubated at room temperature for 10 min in the dark. For the flow cytometric analysis, BL2 (red fluorescence, long-pass dichroic filter 600 nm, bandpass filter 575 nm, detection width 560-590 nm) and BL1 (green fluorescence, long-pass dichroic filter 550 nm, bandpass filter 525 nm, detection width 505-545 nm) were used to detect the stained sample. A total of 20,000 sperm-specific events were evaluated. Different labeling patterns of the stained sperm were also observed with a fluorescence microscope (80i; Nikon) at 400x magnification.

3.9. Detection of Sperm Intracellular ROS and GSH Level. Sperm ROS and GSH level were measured with Reactive Oxygen Species Assay Kit (Beyotime Institute of Biotechnology, China) and Cell Tracker Blue CMF_2HC Molecular Probes (Invitrogen Inc., Carlsbad, CA, USA), respectively [31]. The sperm samples were incubated for 30 min in the Modena extender containing DCFH-DA (10 μM) and Cell Tracker Blue (10 μM), respectively. The samples were washed by centrifugation at 800 ×g for 5 min to remove the unbound probe and analyzed with a microplate reader (Synergy HT, BioTek, USA) at 485 nm excitation and 535 nm emission for ROS and 371 nm excitation and 464 nm emission for GSH. The stained samples were also viewed and photographed under a fluorescence microscope (80i; Nikon).

3.10. Measurement of GPx, SOD, and Catalase Activities. Activities of sperm GPx, SOD, and catalase were analyzed by the glutathione peroxidase assay kit, total superoxide dismutase assay kit, and catalase assay kit, respectively

Table 1: Effects of different concentrations of resveratrol on postthaw sperm motility parameters.

Sperm parameters	Resveratrol (μM)					
	0	25	50	75	100	125
TM (%)	39.7 ± 3.5^{c}	38.6 ± 3.5^{c}	64.3 ± 2.2^{a}	60.7 ± 3.4^{a}	49.3 ± 3.3^{b}	44.7 ± 3.7^{bc}
PM (%)	24.7 ± 1.5^{c}	24.2 ± 2.5^{c}	45.5 ± 3.1^{a}	42.6 ± 3.0^{a}	33.5 ± 2.0^{b}	27.5 ± 6.8^{bc}
LIN (%)	23.4 ± 2^{d}	26.1 ± 1.6^{c}	33.5 ± 1.3^{a}	30.2 ± 1.0^{b}	30.3 ± 1.4^{b}	26.9 ± 0.9^{c}
VSL (μm/s)	20.9 ± 2.7^{a}	28.5 ± 4.1^{bc}	44.2 ± 1.9^{a}	41.4 ± 2.9^{a}	36.5 ± 2.1^{b}	33.0 ± 3.3^{b}
VCL (μm/s)	90.1 ± 5.4^{c}	103.7 ± 9.5^{bc}	133.1 ± 6.0^{a}	134.8 ± 5.1^{a}	124.5 ± 6.3^{b}	124.9 ± 7.0^{b}
VAP (μm/s)	36.9 ± 4.4^{d}	48.1 ± 5.4^{c}	69.4 ± 3.0^{a}	67.3 ± 3.6^{a}	60.0 ± 3.1^{b}	59.9 ± 5.9^{b}
BCF (Hz)	26.7 ± 0.9^{a}	28.3 ± 1.8^{a}	27.3 ± 0.5^{a}	27.9 ± 0.2^{a}	29.9 ± 2.9^{a}	28.4 ± 1.5^{a}

Values are expressed as mean ± SEM. Different letters within column indicate significant difference ($p < 0.05$). TM: total motility; PM: progressive motility; VCL: curvilinear velocity; VSL: straight-line velocity; VAP: average path velocity; BCF: beat-cross frequency; LIN: linearity (VSL/VCL).

(Beyotime Institute of Biotechnology, China), according to Zhu et al. [32, 33]. The sperm pellets were rinsed three times with PBS and resuspended, then lysed ultrasonically (20 kHz, 750 W, operating at 40%, on 3 s, off 5 s, 5 cycles) on ice and centrifuged at 12,000 ×g for 10 min at 4°C. The supernatants were used to analyze the GPx, SOD, and catalase activities according to the manufacturer's instruction.

3.11. Immunofluorescence. Postthaw sperm was fixed with 4% paraformaldehyde for 10 min at room temperature after washing in PBS. The sperm sample was spread onto the poly-L-lysine slides and air dried at room temperature. The samples were permeabilized with 0.5% Triton X-100 in PBS for 10 min. Nonspecific binding was blocked with PBS supplementation of 10% BSA for 30 min at room temperature. Samples were then incubated overnight at 4°C with anti-AMPK (1 : 100, CST). On the next day, the sperm were washed three times in PBS and incubated with the goat anti-rabbit (1 : 100, Santa Cruz Biotechnology) antibody for immunofluorescence labeling. Sperm was washed and counterstained with DAPI (CWBIO); fluorescent images were captured with fluorescence microscopy (80i, Nikon).

3.12. Western Blotting. Sperm total protein was extracted according to our previous study [25]; 25 μg of the extracted protein was added to each lane of a 12.5% polyacrylamide gradient gel, then transferred to the PVDF membrane. After being blocked with 5% BSA, the membrane was incubated with anti-α-tubulin (Santa Cruz, 1 : 1000), anti-AMPK (CST, 1 : 1000), anti-p-AMPK (CST, 1 : 1000), anti-p53 (CST, 1 : 1000), anti-cleaved caspase-3 (CST, 1 : 1000), anti-cleaved caspase-9 (CST, 1 : 1000), and anti-Parp-1 (CST, 1 : 1000) at 4°C overnight and incubated with the secondary antibody (CWBio, 1 : 2000) at room temperature for 1 h. Enhanced chemiluminescence (ECL) detection was performed by using the ECL™ Prime Western Blotting Detection Reagents (RPN2235, GE Bioscience) according to the manufacturer's specifications and appropriate exposure of blots to Fuji X-ray film (ChampChemi Top 610, China).

3.13. Statistical Analysis. All data were analyzed by one-way ANOVA, and Tukey's multiple comparison test was performed using SPSS version 19.0 for Windows (SPSS Inc., Chicago, IL). All values are presented as mean ± standard error of the mean (SEM). Differences with values of $p < 0.05$ considered to be statistically significant.

4. Result

4.1. Resveratrol Improved Sperm Motility Patterns, Membrane Integrity, and Acrosome Integrity during Cooling, Freezing, and Thawing Processes. Compared to the control, the values of postthaw sperm total motility (TM), progressive motility (PM), straight-line velocity (VSL), linearity (LIN), curvilinear velocity (VCL), and average path velocity (VAP) were significantly improved by the addition of resveratrol (from 50 to 125 μM). Meanwhile, the beat-cross frequency (BCF) was unchanged in all treatments (Table 1). Moreover, the membrane integrity and acrosome integrity of postthaw sperm were also significantly increased by resveratrol treatment with all doses from 50 to 125 μM (Figure 1(a)). Interestingly, the 50 μM dose of resveratrol showed the highest value in those parameters (Table 1 and Figure 1(a)).

To elucidate whether the resveratrol could improve the sperm quality at each step of cryopreservation and postthaw incubation, the boar sperm was exposed with 50 μM resveratrol during the cryopreservation processes and postthaw incubation. It was observed that the progressive motility, membrane integrity, and acrosome integrity were significantly increased in resveratrol-treated sperm during cooling from room temperature to 5°C and equilibration at 5°C for 30 min (Figure 1(b)) and also during the 2 h of postthaw incubation (Figures 1(c)–1(e)).

4.2. Resveratrol Reduced the Lipid Peroxidation and Oxidative DNA Damage and Increased the Mitochondrial Activity of Postthaw Sperm. The lipid peroxidation of postthaw sperm was significantly decreased in the treatments with 50, 75, 100, and 125 μM resveratrol; meanwhile, the 50 and 75 μM resveratrol treatments showed the lowest lipid peroxidation (Figure 2(a)). Interestingly, the mitochondrial activity was significantly increased by adding resveratrol, especially 50 μM to the freezing medium (Figure 2(b)). Moreover, the analysis of the oxidative DNA damage in postthaw sperm using 8-hydroxydeoxyguanosine (8-OHdG) staining

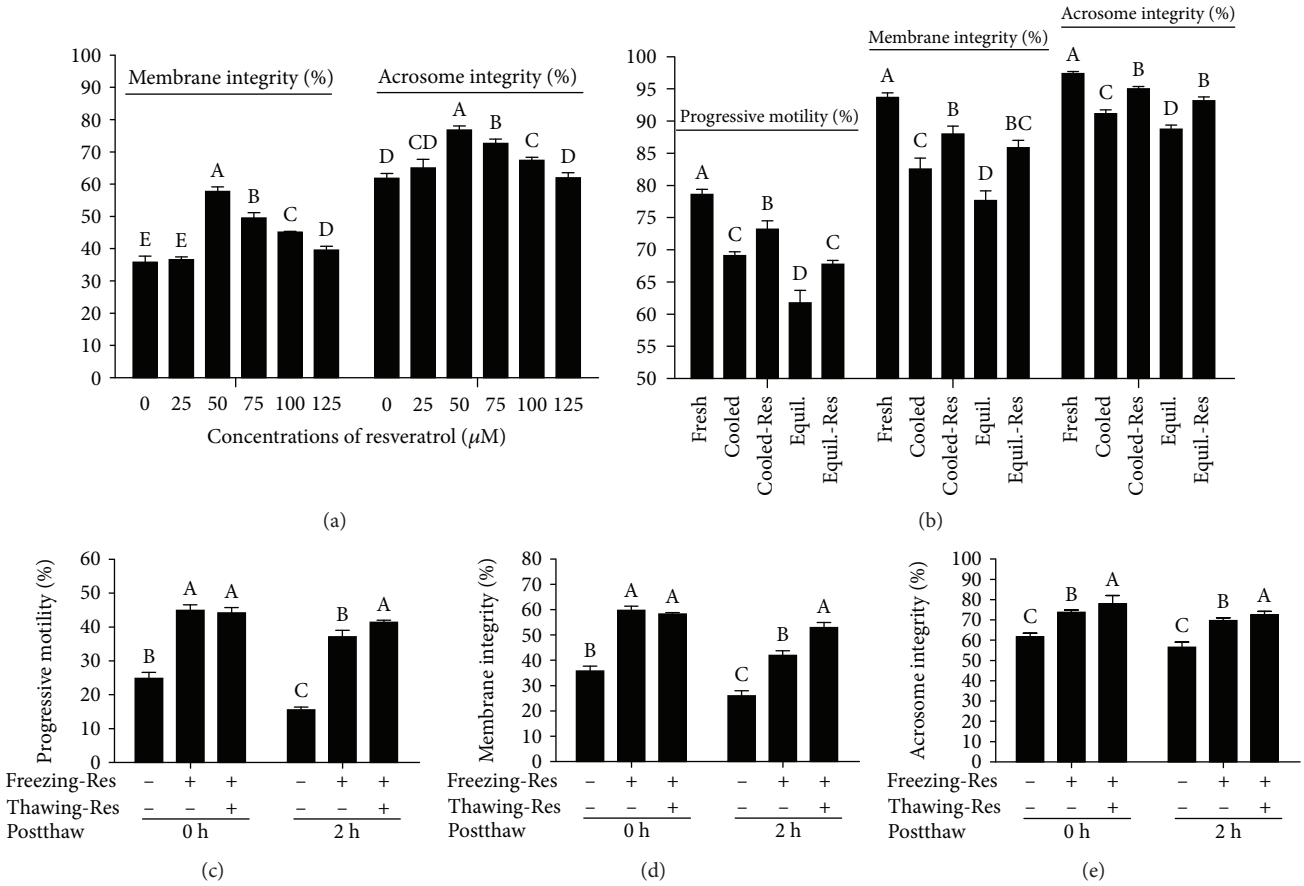

Figure 1: Effects of resveratrol on sperm progressive motility, membrane integrity, and acrosome integrity during cooling, freezing, and thawing incubation processes. Cooled: fresh sperm cooled from room temperature to 5°C. Cooled-Res: fresh sperm cooled from room temperature to 5°C with 50 μM resveratrol. Equil.: cooled sperm equilibrated for 30 min at 5°C. Equil.-Res: cooled sperm equilibrated for 30 min at 5°C with 50 μM resveratrol. Freezing-Res: freezing extender added with (+) or without (-) 50 μM resveratrol. Thawing-Res: thawing solution supplemented with (+) or without (-) 50 μM resveratrol. Data are the mean ± SEM ($n = 5$ independent replicates). Columns with different uppercase letters differ significantly ($p < 0.05$).

revealed that the level of 8-OHdG in postthaw sperm was significantly decreased with the addition of 50, 75, 100, and 125 μM resveratrol (Figures 3(a)–3(h)). In addition, the 8-OHdG was also detected in the head and midpiece of sperm, where the sperm nuclear DNA and mitochondrial DNA are located, respectively (Figure 3(i)).

4.3. Resveratrol Decreased the ROS Level, Increased the Activities of GPx, SOD, and CAT, and Maintained GSH Content of Postthaw Sperm. The ROS level of postthaw sperm was significantly decreased by the addition of resveratrol to the freezing medium, where the 50 μM resveratrol treatment showed the lowest ROS level (Figure 2(c)). The sperm with a high green fluorescence level indicated that the sperm was in a high level of intracellular ROS (Figure 2(e), red arrow), whereas the low green fluorescence indicated sperm with low intracellular ROS (Figure 2(e), yellow arrow). Moreover, the addition of 50 and 75 μM resveratrol significantly increased the GSH level, compared to the control (Figure 2(d)). It was observed that the GSH was distributed in the sperm head, midpiece, and tail, and higher blue fluorescence in the tail, midpiece, and postacrosomal sheath of the sperm head indicated that the GSH level was higher in those sections (Figure 2(e), white arrow).

The GPx, SOD, and catalase enzymes are components of the cellular antioxidative defense system. It was observed that the addition of resveratrol to the freezing medium increased the activities of GPx, SOD, and catalase in postthaw sperm (Figures 2(f)–2(h)). Additionally, the postthaw sperm treated with 50 μM resveratrol presented the highest value of those enzyme activities among all the treatments (Figures 2(f)–2(h)).

4.4. Resveratrol Promotes the AMPK Phosphorylation against the ROS Damage for Improving Sperm Quality. To investigate the mechanism of resveratrol on how to improve sperm quality, the AMPK phosphorylation of postthaw sperm was detected. The AMPK protein was localized in the acrosome and the midpiece of the flagellum in postthaw boar sperm (Figure 4(a)). The addition of 50, 75, 100, and 125 μM resveratrol significantly increased the Thr^{172}-AMPK phosphorylation of postthaw sperm; among all doses, the 50 μM treatments showed the highest value of Thr^{172}-AMPK (Figures 4(b)–4(d)). However, there is no significant change

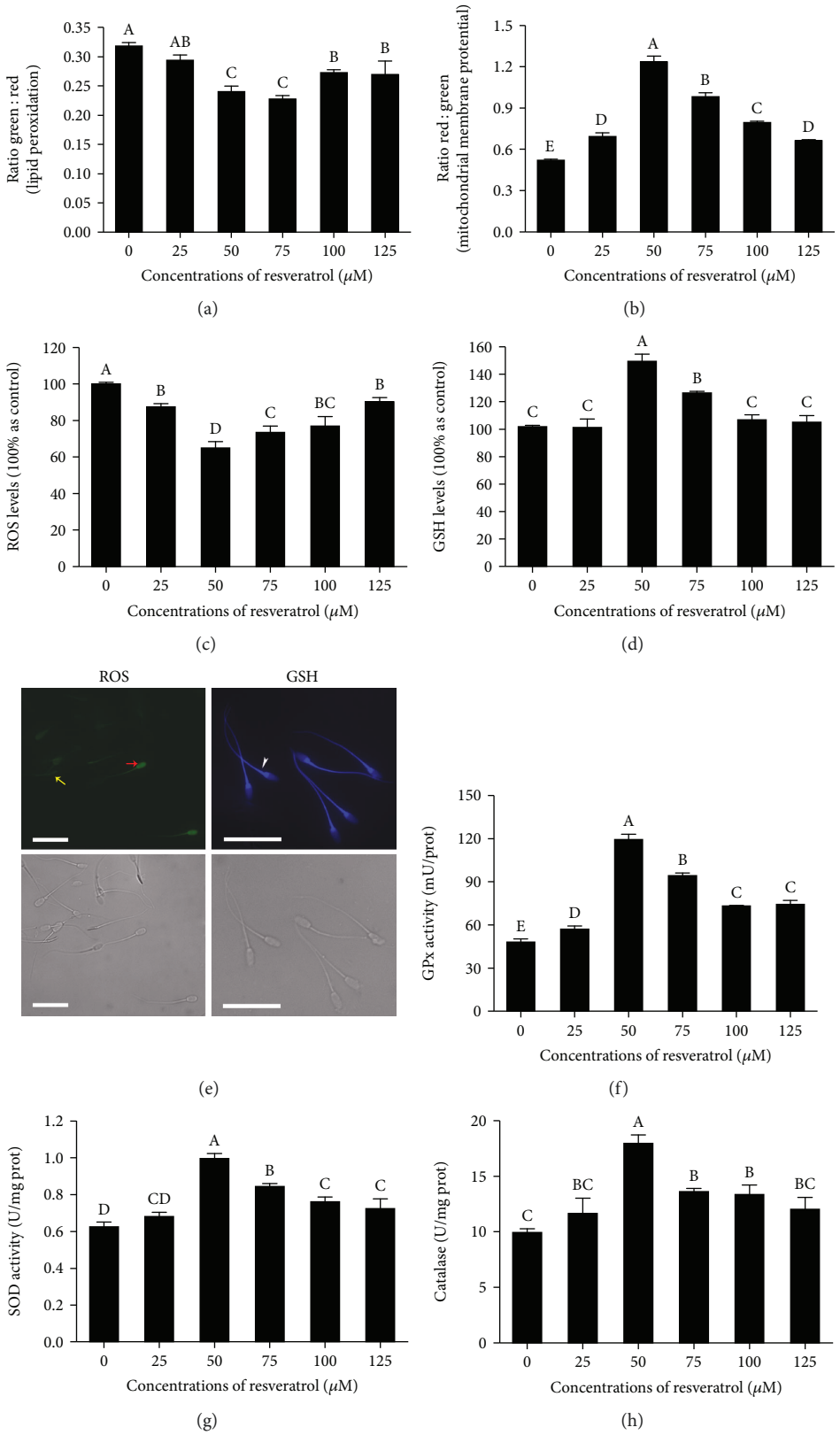

Figure 2: Effects of different concentrations of resveratrol on postthaw sperm lipid peroxidation (a), mitochondrial membrane potential (b), ROS level (c), GSH level (d), GPx activity (f), SOD activity (g), and catalase activity (h). Photomicrographs of the postthaw sperm stained with ROS and GSH probes, respectively (e): the red arrow indicates sperm with a high level of intracellular ROS (high green fluorescence level), the yellow arrow indicates sperm with a low intracellular ROS level (low green fluorescence level), and white arrow indicates the distribution of GSH in sperm. Bars = 30 μm.

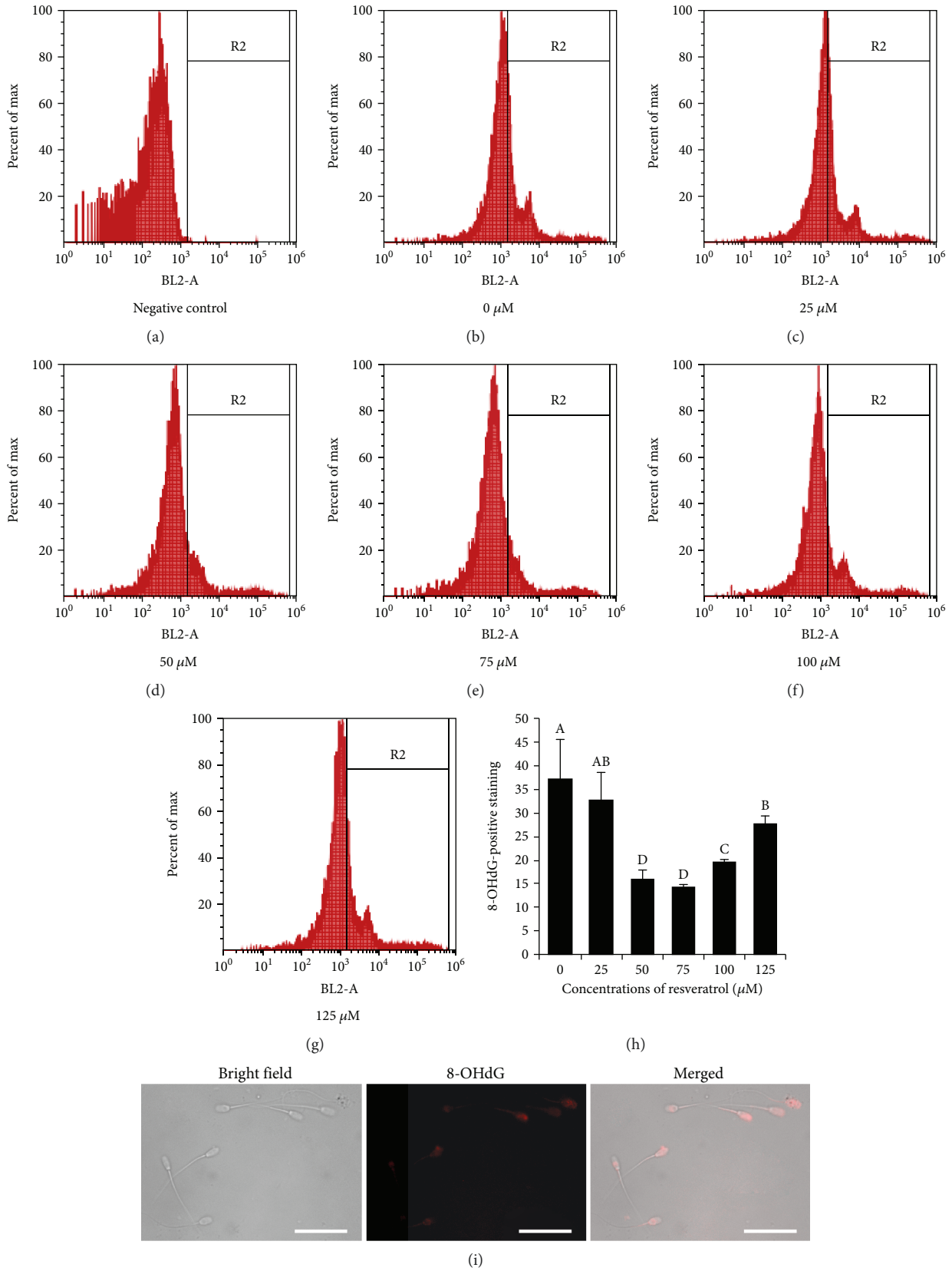

Figure 3: Effects of different concentrations of resveratrol on sperm oxidative DNA damage (b–h). Negative control (a). Photomicrographs of the postthaw sperm stained with 8-OHdG (i). Columns with different uppercase letters differ significantly ($p < 0.05$). Bars = 30 μm.

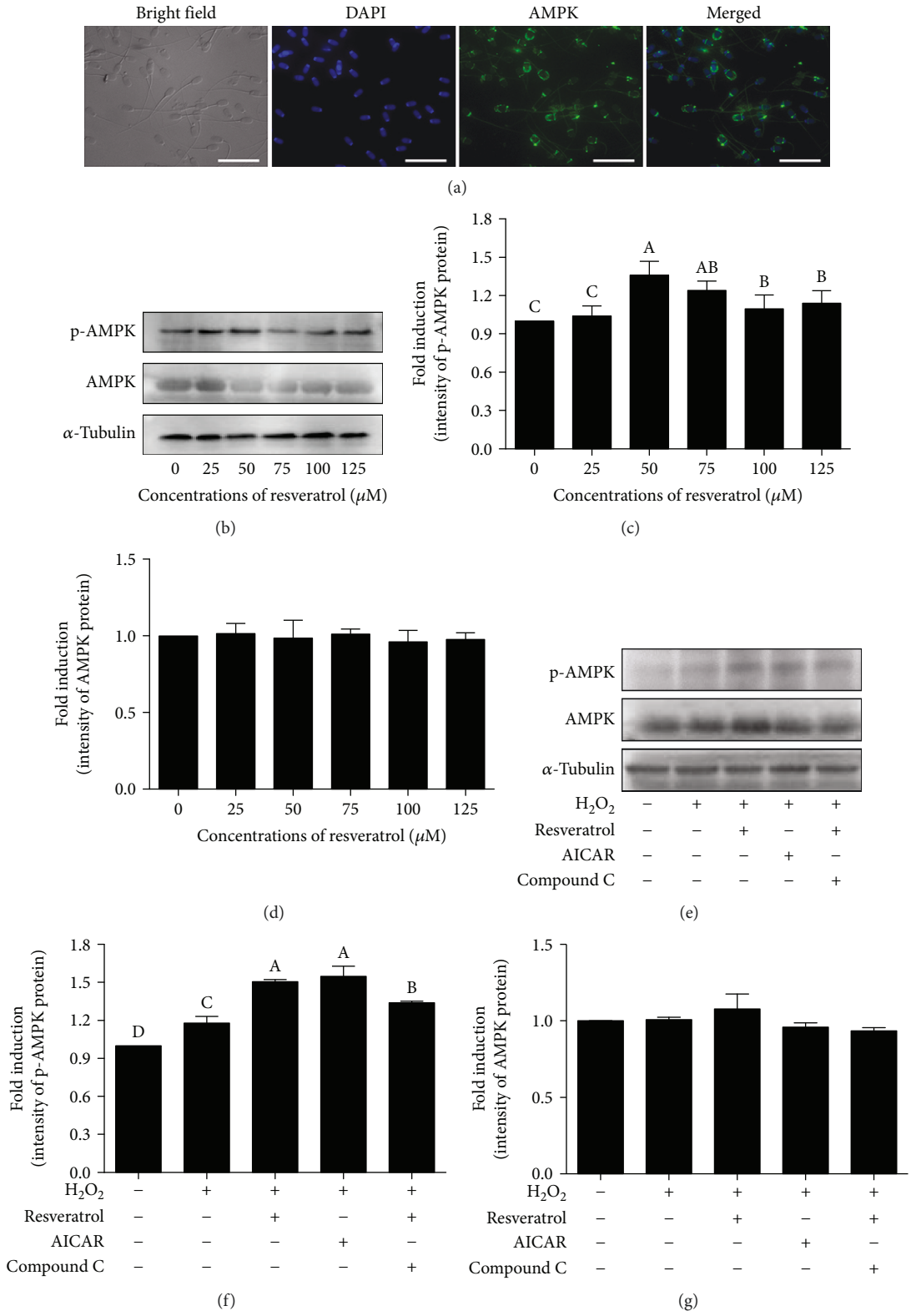

Figure 4: Location of AMPK in postthaw boar sperm was analyzed by immunofluorescence (a). Effects of different concentrations of resveratrol on postthaw boar sperm AMPK phosphorylation (b–d). (b) Western blotting image is showing the expression of the p-AMPK, AMPK, and α-tubulin of postthaw boar sperm. (c, d) Quantitative expression of the p-AMPK and AMPK over α-tubulin generated from western blotting (b). (e) Western blotting image is showing the expression of the p-AMPK, AMPK, and α-tubulin of sperm in the H_2O_2-induced oxidative stress model. (f, g) Quantitative expression of the p-AMPK and AMPK over α-tubulin generated from western blotting (e). Data are the mean ± SEM ($n = 3$ independent replicates). Columns with different uppercase letters differ significantly ($p < 0.05$).

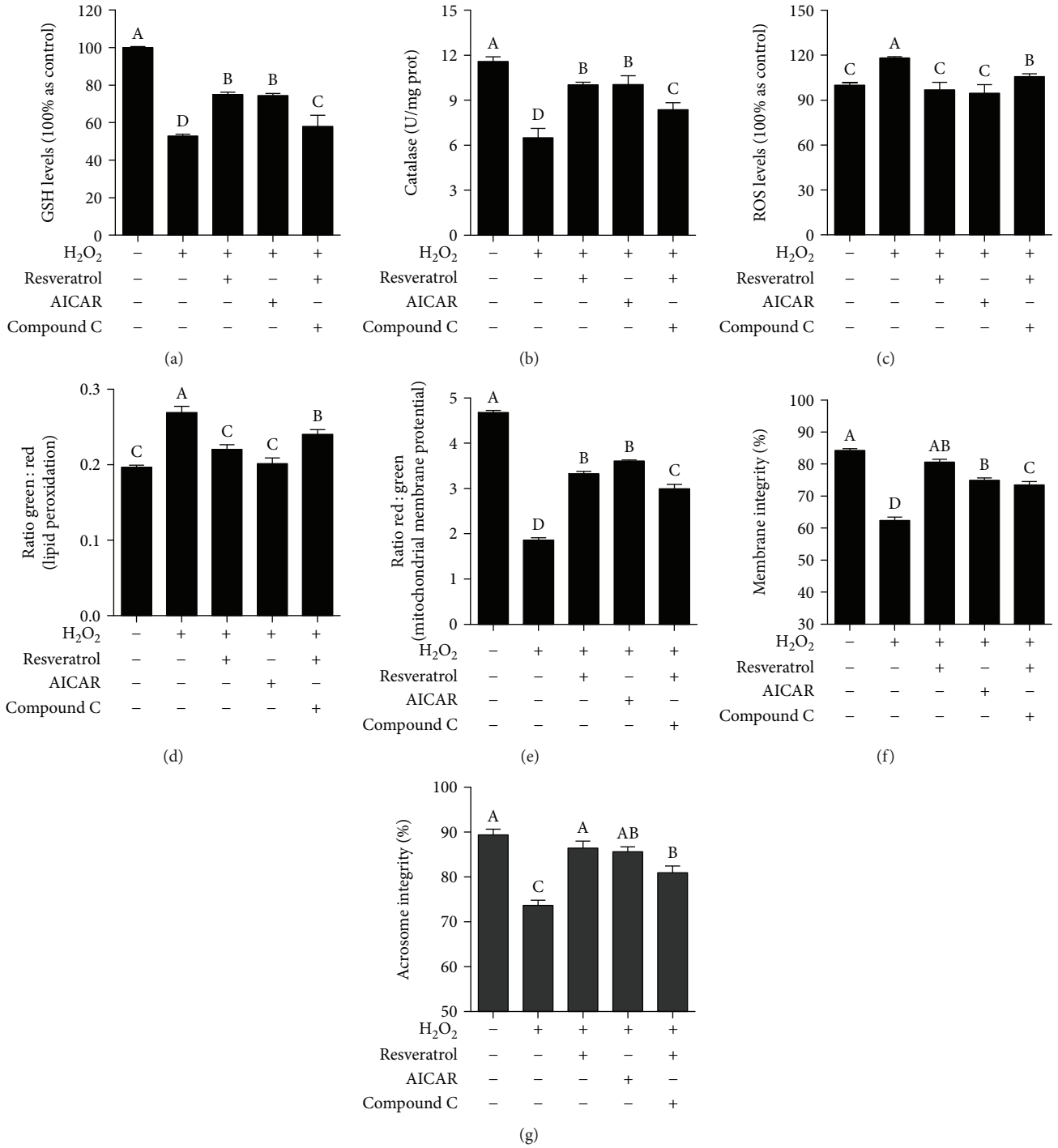

Figure 5: Effects of resveratrol, AMPK activator (AICAR), and inhibitor (Compound C) on the sperm GSH level (a), catalase activity (b), ROS level (c), lipid peroxidation (d), mitochondrial membrane potential (e), membrane integrity (f), and acrosome integrity (g) in the H_2O_2-induced oxidative stress model *in vitro*. Data are the mean ± SEM ($n = 3$ independent replicates). Columns with different uppercase letters differ significantly ($p < 0.05$).

in the total AMPK level among all the treatments (Figures 4(b)–4(d)). To investigate whether the resveratrol protects boar sperm via activating AMPK against ROS stress *in vitro*, we used H_2O_2 to induce the ROS damages, as well as the AMPK activator (5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranoside (AICAR)), and inhibitor (Compound C) was used to regulate AMPK. As showed in Figures 4(e)–4(g), the H_2O_2 treatment promoted the sperm AMPK phosphorylation compared to the control. It was also observed that the addition of either resveratrol or AICAR to the H_2O_2 treatment significantly increased the AMPK phosphorylation; however, the addition of Compound C counteracted

Table 2: Effects of resveratrol, AMPK activator (AICAR), and inhibitor (Compound C) on sperm motility parameters in the H_2O_2-induced oxidative stress model *in vitro*.

Sperm parameters	FS	H_2O_2	H_2O_2+R	H_2O_2+AICAR	H_2O_2+R+CC
TM (%)	80.9 ± 6.5^{a}	58.5 ± 9.6^{c}	64.3 ± 2.2^{b}	68.4 ± 8.8^{b}	65.5 ± 9.0^{b}
PM (%)	62.8 ± 5.2^{a}	38.9 ± 4.1^{d}	52.7 ± 2.0^{b}	53.3 ± 2.6^{b}	46.5 ± 5.9^{c}
LIN (%)	46.8 ± 2.5^{a}	37.7 ± 3.5^{c}	43.4 ± 1.4^{a}	43.6 ± 1.1^{a}	44.9 ± 3.4^{a}
VSL (μm/s)	80.3 ± 4.4^{a}	56.4 ± 8.8^{d}	73.2 ± 4.3^{b}	72.8 ± 6.1^{b}	$64.0.5 \pm 6.4^{c}$
VCL (μm/s)	171.9 ± 3.4^{a}	136.3 ± 14.2^{b}	169.3 ± 11.4^{a}	165.5 ± 11.0^{a}	167.9 ± 8.4^{a}
VAP (μm/s)	101.9 ± 1.9^{a}	82.0 ± 19.7^{c}	95.5 ± 5.6^{ab}	94.1 ± 5.3^{ab}	91.6 ± 4.2^{b}
BCF (Hz)	30.9 ± 1.0^{a}	30.9 ± 3.0^{a}	28.0 ± 2.7^{a}	30.9 ± 0.9^{a}	29.1 ± 2.0^{a}

Values are expressed as mean ± SEM. Different letters within the column indicate significant difference ($p < 0.05$). TM: total motility; PM: progressive motility; VCL: curvilinear velocity; VSL: straight-line velocity; VAP: average path velocity; BCF: beat-cross frequency; LIN: linearity (VSL/VCL); FS: fresh sperm; R: resveratrol; CC: Compound C.

the effect of resveratrol (Figures 4(e) and 4(f)). The total level of AMPK protein was observed to remain unchanged in all treatments (Figure 4(g)).

Moreover, the GSH content and catalase activity of sperm were observed to be significantly decreased when incubated with H_2O_2 compared to control, which were significantly improved by treatment with either resveratrol or AICAR (Figures 5(a) and 5(b)). However, the positive effects of resveratrol on the sperm antioxidative defense system were counteracted with the addition of Compound C (Figures 5(a) and 5(b)). The sperm ROS level and lipid peroxidation were also reduced in either resveratrol or AICAR treatment (Figures 5(c) and 5(d)). Furthermore, sperm parameters (such as sperm motility patterns, mitochondrial activity, membrane integrity, and acrosome integrity) were also improved by the treatment with resveratrol or AICAR compared to the H_2O_2 treatment (Table 2 and Figures 5(e)–5(g)).

4.5. Resveratrol Decreased the Postthaw Boar Sperm Apoptosis. As shown in Figure 6(a), the postthaw sperm stained with the Annexin V-FITC/PI assay kit were observed with following four subpopulations: live sperm (AN-/PI-; blue arrow), early apoptotic sperm (AN+/PI-; white arrow), late apoptotic sperm (AN+/PI+; yellow arrow), and nonviable necrotic sperm (AN-/PI+; black arrow). It was observed that the addition of resveratrol significantly decreased the apoptosis of postthaw sperm (Q2+Q3; Figures 6(b)–6(h)), and the 50 μM resveratrol treatment showed the lowest percentage of postthaw sperm with apoptosis (Figures 6(b)–6(h)). Moreover, in western blotting, it was observed that the expression of apoptotic factor proteins (Parp-1, cleaved caspase-3, cleaved caspase-9, and p53) was significantly decreased by resveratrol treatments at different concentrations (25 to 125 μM). Interestingly, the 50 μM showed the lowest expression of the aforementioned apoptotic factor proteins (Figures 7(a) and 7(b)).

5. Discussion

Cryopreservation of sperm is an efficient procedure for the management and preservation of male fertility in human and domestic animals [34, 35]. Sperm cryopreservation is routinely used in the cases of azoospermia patients or the patients susceptible to infertility due to therapeutic treatments for malignant diseases, who want to have babies using their sperm at a later time [34]. In domestic animals, sperm cryopreservation is also an extensively practiced technique to accelerate the rate of genetic improvement [35]. However, during the cryopreservation, sperm mitochondrial dysfunction occurs due to suffering from sudden temperature changes, ice formation, and osmotic stress [36]. It is well known that most of the ROS are generated as a by-product in the cellular mitochondrial oxidative phosphorylation of the energy pathway [19]. Moreover, the sperm ROS level was observed to be significantly increased during the cryopreservation process in our previous study [8]. The excess ROS reduced the fertilization by decreasing sperm quality [37]. In this study, the sperm quality was reduced with the increase of the ROS level during cooling, freezing, and thawing processes. The supplementation of resveratrol, a mitochondria-targeted antioxidant, significantly improved sperm progressive motility, membrane integrity, acrosome integrity, mitochondrial activity, and AMPK phosphorylation as well as the sperm antioxidative defense system and reduced the ROS level, lipid peroxidation, and sperm apoptosis, suggesting that the resveratrol is beneficial for improving the quality of postthaw boar sperm by activating the AMPK activity to reduce sperm apoptosis.

Cellular ROS homeostasis is controlled by the ROS generation and elimination in cells [38]. The mitochondria are the main ROS-generated site in sperm [39]. The emission of electrons from the mitochondrial electron transfer chain forms the ROS, which was reported to be increased with the mitochondrial dysfunction during cryopreservation [40, 41]. In addition, the cellular antioxidative systems (scavenging enzymes) are located in the cytoplasm and the sperm contain very few cytoplasms [42], indicating that sperm is susceptible to ROS damage. Cryopreservation also has been shown to diminish the sperm antioxidant enzyme activity [43, 44], suggesting that the ability of sperm to scavenge ROS is reduced during the cryopreservation process. The peroxidative damage induced by increased concentration of ROS is associated with the damage to the membrane integrity, acrosome integrity, and DNA stability as well as mitochondrial function [45], which lead to induced sperm apoptosis and ultimately reduced fertility [46]. Therefore,

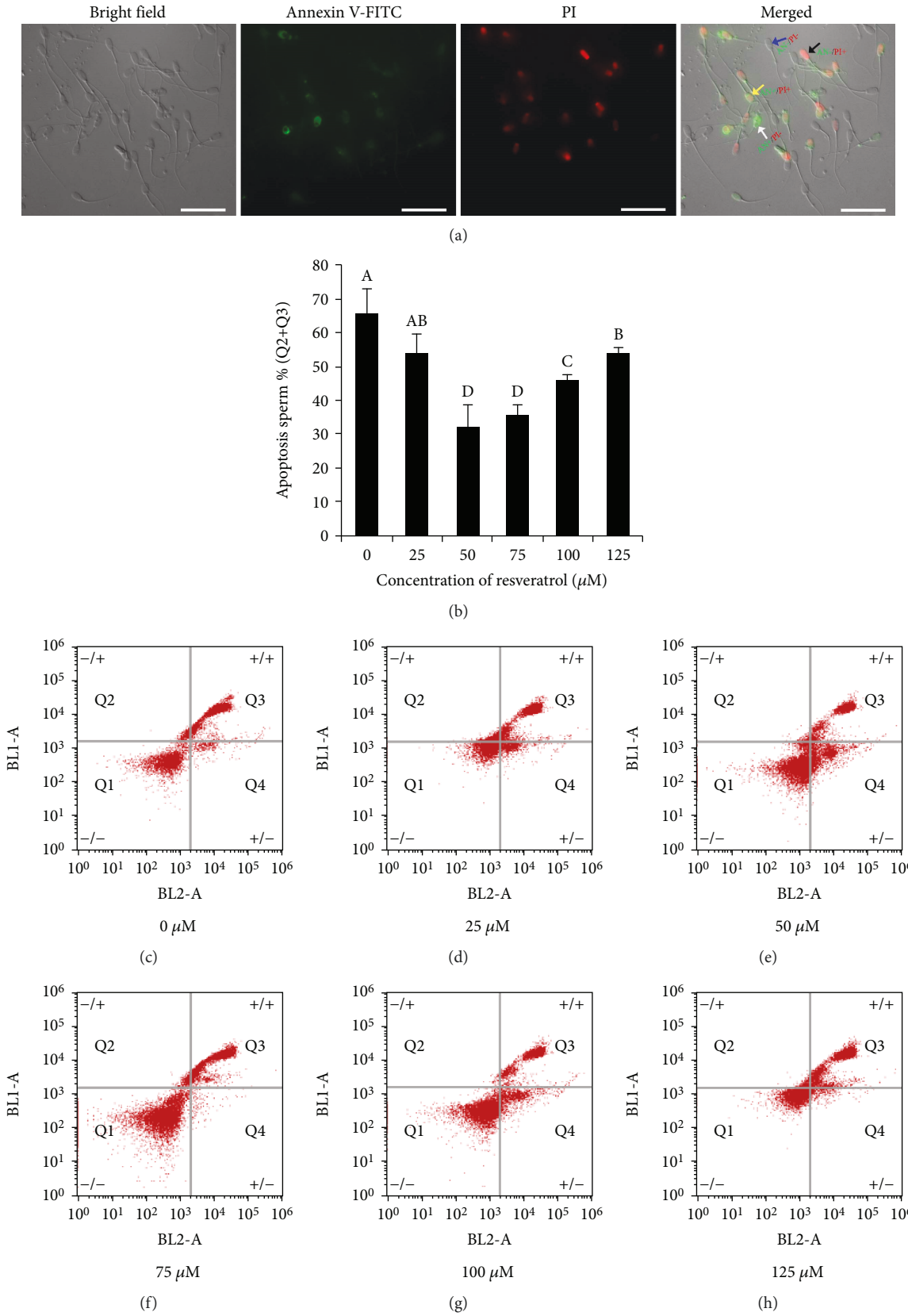

Figure 6: Photomicrographs of the postthaw sperm stained with the Annexin V-FITC/PI assay kit: live sperm (AN-/PI-; blue arrow), early apoptotic sperm (AN+/PI-; white arrow), late apoptotic sperm (AN+/PI+; yellow arrow), and nonviable necrotic sperm (AN-/PI+; black arrow) (a). Effects of different concentrations of resveratrol on postthaw sperm apoptosis (b–h). Data are the mean ± SEM ($n = 3$ independent replicates). Columns with different uppercase letters differ significantly ($p < 0.05$).

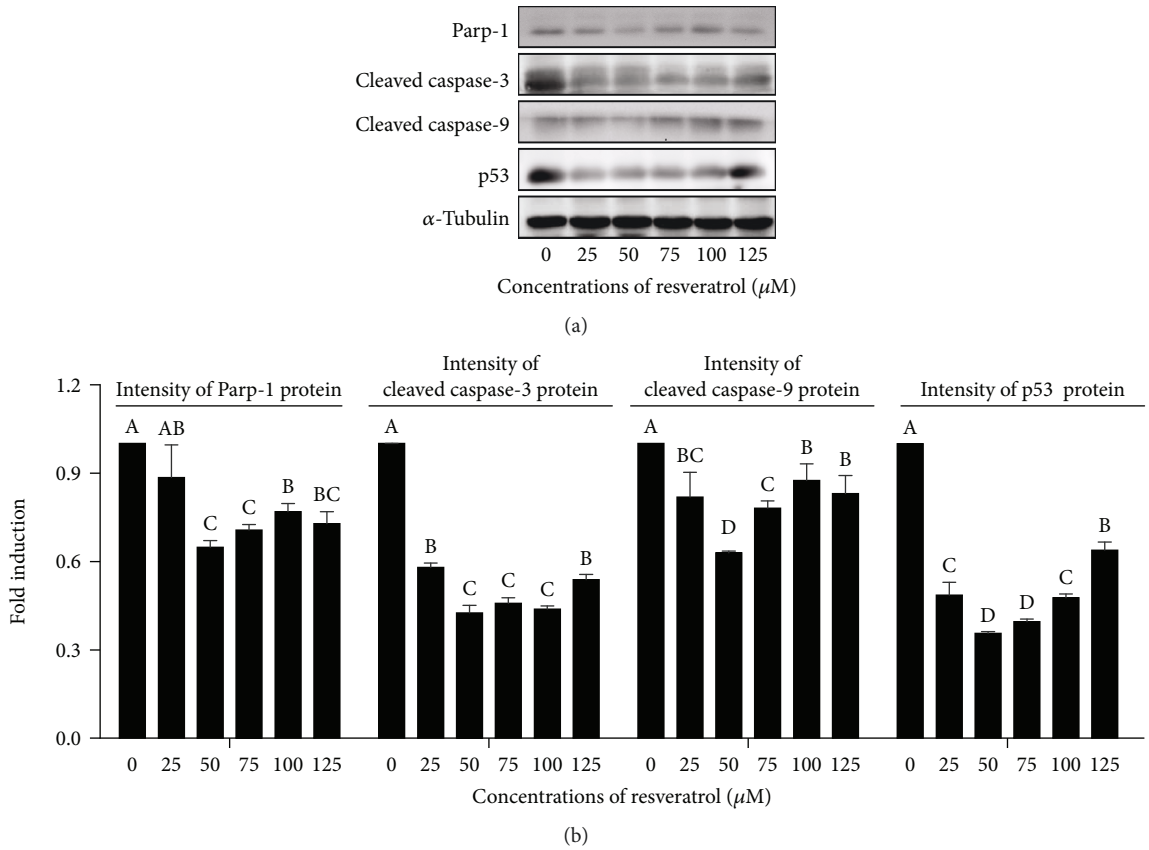

Figure 7: Western blotting image is showing the expression of apoptosis proteins in postthaw boar sperm (a). (b) Quantitative expression of the Parp-1, cleaved caspase-3, cleaved caspase-9, and p53 over α-tubulin generated from western blotting. Data are the mean ± SEM ($n = 3$ independent replicates). Columns with different uppercase letters differ significantly ($p < 0.05$).

reducing the ROS generation, as well as enhancing the ROS scavenging ability, is essential to minimize the damage of oxidative stress in sperm.

AMPK is a key kinase involved in regulating the cellular redox state by switching the metabolic pathway under the stressful conditions [47]. In somatic cells, AMPK could promote glucose uptake and glycolysis, facilitating antioxidant production [47]. Kukidome et al. reported that the activation of AMPK by AICAR reduced the hyperglycemia-induced mitochondrial ROS generation in human umbilical vein endothelial cells [48]. AMPK activation by metformin also suppressed ROS production in mouse Schwann cells [49]. Moreover, Kim et al. showed that AMPK activation could inhibit palmitate-induced apoptosis through suppression of ROS production in bovine aortic endothelial cells [50]. *In vivo* AMPK activation also decreased the ROS level in rat diabetic fibrosis, kidney tissues, and type 2 diabetes patients [51, 52]. Additionally, AMPK activation has been reported to increase the expression of antioxidant enzymes in monocytes-macrophages [53] and restore GSH depletion [52]. Furthermore, in chicken and rabbit sperm cryopreservation, AMPK activation by either AICAR or metformin significantly increased the SOD, GPx, and catalase activities of postthaw sperm, whereas the Compound C decreased those enzyme activities and increased the level of lipid peroxidation and ROS with negative effects on the sperm quality [31, 54]. Therefore, if the AMPK is activated during the cryopreservation, the quality of postthaw sperm will be improved through the AMPK regulating ROS generation and sperm antioxidative defense.

Notably, it has been reported that the AMPK was activated by the addition of resveratrol in liver steatosis of either different hepatic cell models *in vitro* or animal models (mice and rats) *in vivo* [55]. Moreover, the activation of AMPK by resveratrol in somatic cells was revealed through a variety of mechanisms in previous studies [56, 57]. In the present study, it was observed that the AMPK phosphorylation of postthaw boar sperm was significantly increased by the addition of 50 μM resveratrol to the freezing extender. The antioxidative defense (GSH level and activities of GPx, SOD, and catalase) in postthaw sperm was also increased with the treatment with 50 μM resveratrol, while the ROS level was decreased. Interestingly, in the H_2O_2-induced sperm ROS damage model, the positive effects of resveratrol were partly counteracted in the presence of the AMPK inhibitor (Compound C). These observations suggested that the addition of resveratrol could decrease ROS production and enhance the antioxidative defense of postthaw boar sperm by promoting AMPK phosphorylation.

Resveratrol, a mitochondria-targeted antioxidant, has been not only used as a therapy to human aging disease

in vivo but also used to improve male fertility *in vitro* and *in vivo* [58]. Bucak et al. reported that the addition of 1 mM resveratrol to the freezing medium significantly improved postthaw bull sperm motility and mitochondrial activity [26]. Moreover, the resveratrol also decreased the lipid peroxidation as well as DNA damage of postthaw sperm in bull [26] and human [27]. In the present study, it was also observed that the addition of 50 μM resveratrol improved postthaw boar sperm motility, membrane integrity, acrosome integrity, and DNA stability as well as mitochondrial activity. These observations coincided with the changes in the ROS level and antioxidative defense after treatment with resveratrol. The high concentration of ROS and fall of antioxidant enzymes led to cell apoptosis [59]. The addition of resveratrol significantly reduced sperm apoptosis in this study, suggesting that the resveratrol might decrease the postthaw sperm apoptosis via scavenging the excess ROS and enhancing the sperm antioxidative defense system. Therefore, the addition of resveratrol is beneficial for improving sperm quality during cryopreservation.

In conclusion, the ROS is induced in sperm during cryopreservation. The addition of resveratrol activates AMPK phosphorylation, which reduces the ROS production and enhances the sperm antioxidative defense system (such as the GSH level and activities of GPx, SOD, and catalase). Consequently, the ROS level was decreased, and thereby, the sperm motility, membrane integrity, and acrosome integrity as well as mitochondrial activity were increased while reducing the sperm apoptosis and DNA damage. This study using the boar sperm cryopreservation model helps us to understand whether and how the resveratrol improves sperm quality, which will contribute new insights to the human and animal reproductive field.

Abbreviations

AICAR:	5-Aminoimidazole-4-carboxamide-1-beta-4-ribofuranoside
AMPK:	5′AMP-activated protein kinase
ATP:	Adenosine triphosphate
ROS:	Reactive oxygen species
BCF:	Beat-cross frequency
CASA:	Computer-assisted sperm motility analysis
FITC-PNA:	Fluorescein isothiocyanate-peanut agglutinin
GPx:	Glutathione peroxidase
GSH:	Glutathione
LIN:	Linearity
NSF:	Freezing extender
OXPHOS:	Mitochondrial oxidative phosphorylation
PM:	Progressive motility
SEM:	Standard error of the mean
SOD:	Superoxide dismutase
TM:	Total motility
VAP:	Average path velocity
VSL:	Straight-line velocity
VCL:	Curvilinear velocity
$\Delta\Psi$m:	Mitochondrial activity
8-OHdG:	8-Hydroxyguanosine.

Data Availability

All data used to support the findings of this study are included in the article.

Conflicts of Interest

The authors have nothing to disclose.

Authors' Contributions

Zhendong Zhu and Wenxian Zeng conceived and designed the experiments. Zhendong Zhu and Rongnan Li performed the experiments. Zhendong Zhu and Xiaoteng Fan analyzed the data. Yinghua Lv, S. A. Masudul Hoque, De Wu, and Yi Zheng contributed reagents/materials/analysis tools. Zhendong Zhu wrote the manuscript.

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Supplementary Materials

Supplementary Figure 1 Group (1): Modena extender containing 200 μM H_2O_2. Group (2): Modena extender containing 200 μM H_2O_2 and 50 μM resveratrol. Group (3): Modena extender containing 200 μM H_2O_2 and 2 mM AICAR. Group (4): Modena extender containing 200 μM H_2O_2, 50 μM resveratrol, and 30 μM Compound C. Group (5): Modena extender without resveratrol, AICAR, Compound C, or H_2O_2. *(Supplementary Materials)*

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