

Review

Oxidative Stress as an Underlying Mechanism of Bacteria-Inflicted Damage to Male Gametes

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Abstract: Bacterial infestation of the male reproductive system with subsequent effects of bacteria on the structural integrity and functional activity of male gametes has become a significant factor in the etiology of male reproductive dysfunction. Bacteria may affect male fertility either by directly interacting with structures critical for sperm survival or indirectly by triggering a local immune response, leukocytospermia or reactive oxygen species (ROS) overproduction followed by oxidative stress development. This review aims to provide an overview of the currently available knowledge on bacteriospermia-associated sperm damage with a special emphasis on oxidative mechanisms underlying sperm deterioration caused by bacterial action. At the same time, we strive to summarize readily available alternatives to prevent or counteract alterations to spermatozoa caused by bacterial colonization of semen or by oxidative stress as an accompanying phenomenon of bacteriospermia.

Keywords: bacteriospermia; infection; sperm quality; oxidative stress; reactive oxygen species; subfertility; bacterial contamination



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1. Introduction

A global challenge of today's era lies in the understanding, prevention, and management of ever-increasing male reproductive dysfunction. Particularly in mammals, male sub- or infertility is a complex and multifactorial condition that may be caused by a multitude of factors, ranging from genetic causes and systemic ailments to environmental exposure or nutrition [1]. Within this vast array of etiologies, bacteriospermia—the presence of bacteria in semen—is thought to be the causative agent in around 15% of all cases of subfertile males [1,2]. In clinical practice, bacteriospermia is acknowledged when the bacterial count exceeds 1.000 colony-forming units (CFU)/mL of semen [3]. The primary reason for bacterial colonization of ejaculates lies in an acute or chronic infection of the urogenital tract including diseases such as urethritis, prostatitis, epididymitis or orchitis, subsequently compromising proper spermatogenesis and sperm maturation [4]. Alternatively, bacteria may invade the reproductive system from urinary tract infection [5], by hematogenous dissemination [6] or by sexual intercourse [7]. In certain cases, bacteriospermia may be caused by the overgrowth of natural microflora present in the urogenital tract even in clinically healthy subjects [8,9]. Semen may be also infested by bacteria from external sources, such as skin, urine, feces [10] as well as laboratory tools, equipment or media needed for semen collection and/or processing for assisted reproduction techniques (ARTs) [11].

Bacteria present in semen may affect male gametes through adhesion [12] and agglutination events, which will ultimately result in sperm immobilization and inability to reach and fertilize the oocyte [13]. The released bacterial toxins may have direct cytotoxic effects on the male gamete, leading to increased sperm cell death [14]. Furthermore, bacterial infection may trigger persistent inflammation and leukocytic infiltration accompanied by

the release of pro-inflammatory cytokines that will contribute to permanent damage of structures vital for sperm survival [9,15].

While the mechanisms of which bacteria may cause alterations to male reproductive cells are diverse, a convincing body of evidence indicates a notable involvement of reactive oxygen species (ROS) in the phenomena associated with bacteriospermia. As such, understanding the role of oxidative processes underlying bacteria-inflicted damage to spermatozoa may assist in the development of adequate intervention strategies to mitigate bacteriospermia-associated male subfertility.

2. Sources of ROS in Bacteriospermia

The involvement of ROS in male reproduction with beneficial as well as adverse effects has become indisputable in modern andrology. Carefully controlled physiological ROS levels play a crucial role in signaling pathways involved in sperm production and maturation, as well as during capacitation, hyperactivation, acrosome reaction and sperm-egg fusion. Nevertheless, ROS accumulation has been previously acknowledged to act either as a primary cause or an accompanying phenomenon in a vast array of male reproductive pathophysiology [16–19]. Correspondingly, earlier reports on bacteriospermia have frequently observed increased oxidative damage resulting from a shift in the prooxidant–antioxidant milieu in favor of prooxidants, favoring further ROS release and action [20–23].

By and large, three sources of ROS are most relevant to bacteriospermia: (1) immature, damaged or dead spermatozoa, (2) bacterial metabolism, and (3) activated leukocytes.

2.1. Spermatozoa

The process of sperm maturation involves a series of events that take place during the sperm transition from the seminiferous epithelium to the epididymis. These processes include structural remodeling, male germ cell differentiation, acquisition of the ability to move and to accomplish acrosome reaction, all of which are dependent on changes in the architecture and fluidity of the plasma membrane [24]. In morphologically normal spermatozoa, cytoplasmic deposits located in the midpiece are extruded to allow for subsequent cell elongation and nuclear condensation to occur during spermiogenesis [25] (Figure 1a).

If sperm maturation is not carried out properly, spermatozoa will retain the cytoplasm in the form of proximal or distal droplets, containing large amounts of cytoplasmic enzymes such as lactate dehydrogenase, superoxide dismutase (SOD) and glucose-6-phosphate dehydrogenase [24–26], all of which have been shown to be directly involved in the cellular oxidative balance reviewed by [27] (Figure 1b). In particular, glucose-6-phosphate dehydrogenase has been repeatedly associated with an increased risk for peroxidative damage, since it produces nicotinamide adenine dinucleotide phosphate (NADPH). Subsequently, ROS is generated from NADPH via the intramembrane-located NADPH oxidase 5 (NOX 5) [28], a prime enzyme responsible for ROS production in the acrosomal, equatorial or post-acrosomal regions of morphologically abnormal spermatozoa [29]. In addition, significant associations have been reported between ROS production and the stage of sperm development, with particularly high ROS levels being observed in immature spermatozoa [30]. Correspondingly, increased ROS production has been reported in patients suffering from teratozoospermia [31] or in subjects with a high occurrence of cytoplasmic droplets [25,26].

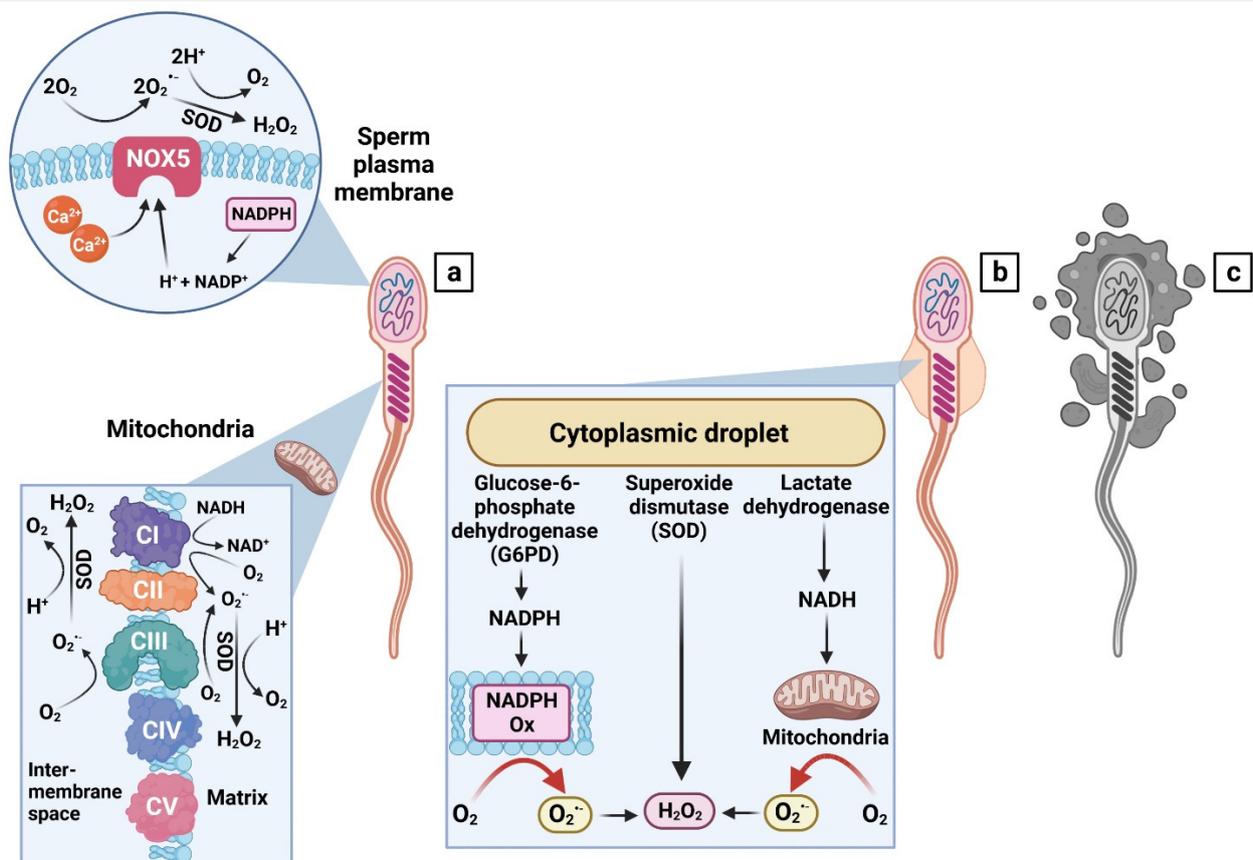


Figure 1. Sources of ROS in normal (a), immature (b) or dead (c) spermatozoa.

On the other hand, it has been suggested that apart from immature or morphologically abnormal sperm, increased amounts of damaged or dead male gametes may represent a threat to still viable and functional spermatozoa. The risks associated with the presence of dying spermatozoa lie in an increased release of intracellular content into their surroundings, containing large amounts of ROS, which may then attack the polyunsaturated fatty acids (PUFAs) located in the membrane of viable male gametes. Such compromised cells will then exhibit phosphatidylserine externalization, loss of membrane fluidity and an increased occurrence of DNA breaks, all of which will ultimately result in the activation of apoptotic or even necrotic cell death (Figure 1c). In this sense, cell necrosis represents a larger risk for the loss of viable spermatozoa, since any disturbance to intracellular homeostasis is succeeded by the rupture of membranous structures and a subsequent contamination of the extracellular space with toxic metabolites, sustaining the chain reaction of damage to other functional male gametes [32]. Moreover, an increased occurrence of dead spermatozoa has been suggested to trigger the immune system and to contribute to the formation of sperm granulomas. These abnormal structures consist of spermatozoa, macrophages, and other immune cells, eventually developing into a mass attached to the vas deferens, causing an obstruction and raising the intraluminal pressure [33].

- (a) Mitochondria are the primary source of ROS in structurally and functionally normal spermatozoa. Superoxide ($\text{O}_2^{\bullet-}$) is created by electron leakage within NADH dehydrogenase (complex I) and ubiquinol cytochrome C oxidoreductase (complex III). The radical is quickly scavenged by superoxide dismutase (SOD) to generate hydrogen peroxide (H_2O_2). Alternatively, $\text{O}_2^{\bullet-}$ and H_2O_2 are generated by the NADPH-oxidase isoform 5 (NOX5) located in the plasma membrane and are activated through NADPH and calcium ions (Ca^{2+}). Modified from [29]. CI—complex I, CII—complex II (succinyl-CoA synthetase).

- nate dehydrogenase), CIII—complex III, CIV—complex IV (cytochrome C oxidase), CV—complex V (ATP synthase).
- Immature or abnormal spermatozoa present with cytoplasmic droplets containing glucose-6-phosphate dehydrogenase (G6PD), which produces NADPH serving as a substrate for the creation of $O_2^{\bullet-}$ by NADPH oxidase systems (NADPH Ox). SOD present in the droplets catalyzes the conversion of $O_2^{\bullet-}$ to H_2O_2 . In the meantime, lactate dehydrogenase produces the reduced form of nicotinamide adenine dinucleotide (NADH), which then proceeds to be oxidized by the NADH dehydrogenase (complex I) in the mitochondria. Modified from [28].
 - In necrotic spermatozoa, the intracellular content including ROS will be released into the environment, subsequently contaminating the surrounding cells with toxic metabolites.

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2.2. Bacteria

The extent of ROS production by bacterial action is largely affected by the bacterial load, diversity, and metabolism of bacteria representing the seminal microflora [34]. In aerobic bacteria, more than 90% of cellular oxygen will be consumed by oxidative phosphorylation to produce ATP and water. Similarly to eukaryotic cells, a small portion of molecular oxygen captures 0.1%–1% of electrons present in the transport chain, continually forming superoxide radical ($O_2^{\bullet-}$) by NADPH oxidase, which is the primary source of ROS in bacteria [35] (Figure 2a).

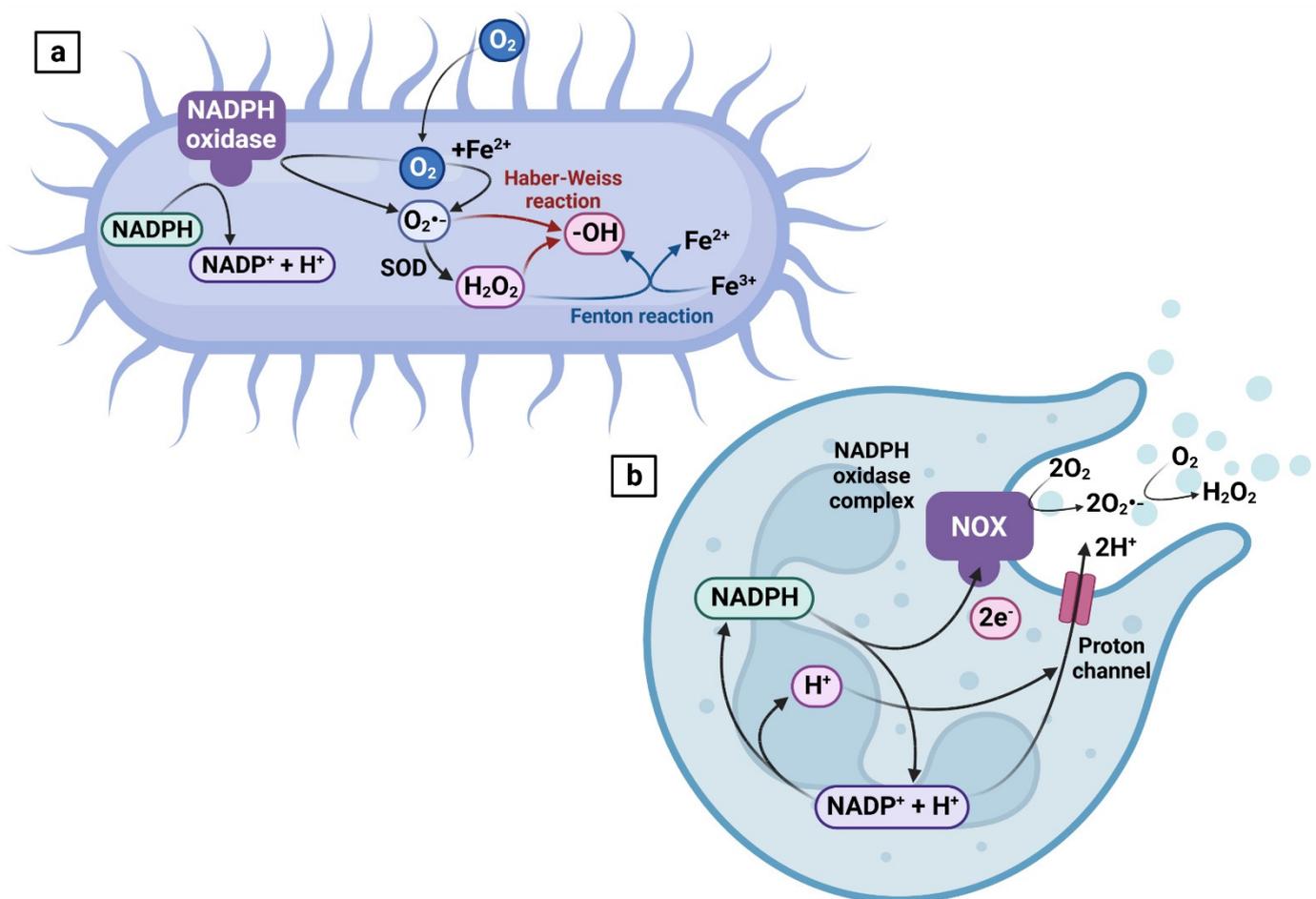


Figure 2. Sources of ROS in aerobic bacteria (a) and leukocytes (b).

While obligate anaerobes are not equipped to perform aerobic respiration, it has been speculated that these can deploy low-potential electron transfer pathways to produce ROS [36]. $O_2^{\bullet-}$ can be generated abiotically from the reaction of O_2 with ferrous ion-like metal ions [37,38]. Correspondingly to higher organisms, hydrogen peroxide (H_2O_2) in bacteria is generated as part of the dismutation of $O_2^{\bullet-}$ catalyzed by SOD [38] (Figure 2a). These two ROS are prevalent reactive intermediates produced by the bacterial cell, and the presence of antioxidant enzymes (such as SOD, catalase, thioredoxin or thiol peroxidase) in several bacterial species fortifies the evidence that bacteria themselves are capable of producing, regulating and sustaining certain levels of free radicals [39].

Additionally, $O_2^{\bullet-}$ as well as H_2O_2 have been observed to be actively released by numerous potentially uropathogenic bacteria [40,41]. As suggested by Fraczek et al. [22], additional ROS may subsequently cause significant damage to the sperm membranes, evidenced by increased malondialdehyde (MDA) levels in spermatozoa subjected to in vitro culture in the presence of *Campylobacter ureolyticus*, *Staphylococcus haemolyticus*, and *Escherichia coli*. Similar experiments of in vitro bacteriospermia designed by Ďuračka et al. [41] also revealed that selected *staphylococci* contributed to increased ROS levels in a sperm-bacteria co-culture, leading to decreased sperm motility, mitochondrial membrane potential and DNA integrity.

2.3. Leukocytes

Under physiological conditions, the primary source of ROS in semen is polymorphonuclear neutrophilic granulocytes, which are activated by an ongoing inflammatory process. The primary role of such peroxidase-positive leukocytes is to dispose of the pathogen by an increased release of ROS into the source of inflammation [42]. In this case, high amounts of $O_2^{\bullet-}$ and H_2O_2 are produced by a series of reactions referred to as respiratory burst. This phenomenon is mediated by the NADPH-oxidase complex, which is rapidly assembled following neutrophil activation. As discussed previously, NADPH oxidase produces $O_2^{\bullet-}$ similarly to NOX5 found in immature spermatozoa [28,29,43]. Subsequent dismutation catalyzed by SOD takes place to create H_2O_2 . Both ROS may then be released individually, or these may interact with products of other microbicidal systems to generate ozone, a hydroxyl radical or singlet oxygen [43] (Figure 2b). While the primary role of respiratory burst lies in an additional line of protection to the cells against a pathogen, excessive infiltration and inappropriate activation of leukocytes may result in the release of excessive ROS and a subsequent deterioration of sperm function, as repeatedly observed in subjects suffering from leukocytospermia [9,10,44,45].

- (a) $O_2^{\bullet-}$ is created by NADPH oxidase localized on the bacterial plasma membrane. The molecule is further processed by SOD to generate H_2O_2 . Additional ROS may be generated by the Fenton and Haber–Weiss reaction catalyzed by iron. Modified from [37].
- (b) Respiratory burst is activated by the NADPH-oxidase complex, which will produce $O_2^{\bullet-}$. Subsequently, SOD will dismutate the radical to generate H_2O_2 .

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3. Adhesion and Agglutination Events

The ability of bacteria to adhere to the cell surface is a crucial prerequisite for subsequent bacterial colonization of ejaculate since adhesion events may lead to alterations to the sperm morphology and motion behavior [46]. Furthermore, bacterial species attached to the sperm structures may release signaling molecules to attract other bacteria to form agglutinating complexes that will completely immobilize male gametes [47]. Further development of agglutination structures may then stimulate the release of extracellular polymeric substances and initiate biofilm formation [46,48]. Finally, successful bacterial adherence may stimulate the secretion of bacterial endotoxins with cytotoxic effects on sperm functional activity and fertilization ability [49].

3.1. Bacterial Adhesion

Bacteria accomplish adhesion events with polymeric fibers called “fimbriae” or “pilli” that serve to establish contact among the cellular surfaces and serve as virulence factors able to mediate the formation of bacterial aggregates by the recognition of receptors located on the host cell [49,50]. In the case of G^- bacteria, their pilli possess a high affinity to mannose receptors found in the sperm membrane [47,51]. Two major types of pilli have been studied in uropathogenic G^- bacteria, namely Type 1 fimbriae with a high affinity to mannose located in the sperm head [47] and a mannose-resistant *P fimbriae*, which recognizes α -D-galp1-4-9-D-galp (gal gal) located predominantly in the sperm tail [52].

With respect to G^+ bacteria (particularly *Lactobacillus* and *Corynebacterium*), the most common fimbriae involved in bacterial colonization is SpaCBA with a versatile ability to bind to host cells, mucin, and mucous collagen. It has been revealed that bacterial aggregation enabled by this pillus only affects sperm motility without any impact on the morphology or vitality of male reproductive cells [46,47].

Several factors play a role in bacterial adherence, particularly the physical properties of the bacterial cell wall, area of contact, charge distribution or hydrophobicity [46]. While the exact molecular pathways driving the adhesion events are not completely understood yet, pilot studies indicate at least a partial involvement of oxidative mechanisms in enabling proper bacterial anchoring into the sperm membrane.

Integrins are transmembrane receptors that are intricately involved in important cell functions, such as cell adhesion or migration by regulating direct cell-to-cell associations or their interplay with extracellular matrix (ECM) proteins [53]. In addition to somatic cells, integrins have also been reported on the sperm surface and are known to play an important role in sperm membrane remodeling, organization of sperm protein complexes and sperm-oocyte fusion [54]. Nevertheless, activation of integrins by their specific ligands opens a transmembrane link for the transmission of molecular signals or mechanical forces across the plasma membrane in both directions. Several integrin receptors may be exploited by pathogenic agents to enable first contact with the host cell [53]. Furthermore, a growing list of recent reports demonstrates that integrin engagement by ECM proteins activates several intracellular cascades accompanied by induced ROS production promoting tight cell adhesion and cytoskeleton organization [55].

Although the underlying molecular mechanism of integrin-initiated ROS production remains to be elucidated, it has been hypothesized that the interplay between integrins and ECM proteins triggers ROS production by promoting changes in the mitochondrial redox function and activation of several oxidoreductases including NADPH-oxidases, 5-lipoxygenase and cyclooxygenase-2 [56–58]. The GTPase Rac1 activated by integrin engagement is essential for the stimulation of all ROS-producing systems [56]. Conversely, there is evidence that ROS can also affect integrin-mediated inside-out signaling by inducing conformational changes required for the activation of integrins [58]. According to Taddei et al. [59] an immediate cell-to-cell interaction carried out by integrins induces structural changes to adjacent adhesion molecules that are accompanied by mitochondrial ROS in the early stage of adhesion while 5-lipoxygenase-derived ROS peak during later stages of cell attachment. Furthermore, Chiarugi et al. [57] proposed that modulation of integrin signaling and cell adhesion by ROS is partially mediated by upregulation of focal adhesion kinase through oxidative inhibition of low molecular weight protein tyrosine phosphatases. Since ROS are capable of interacting with a wide array of biomolecules, their impact on cellular signaling events during cell adhesion depends on the site of production [56] and on the modification of redox-sensitive molecules. As such, target molecules may include phosphatases and kinases, receptors, transcription factors, actin and actin-associated proteins [56,58–60].

Finally, a recent report by Stempel et al. [61] has unraveled that H_2O_2 and hypochlorite that are frequently used as disinfecting agents may induce adaptive signaling pathways in *Pseudomonas aeruginosa*, particularly by increasing intracellular levels of the second messenger c-di-GMP and diguanylate cyclase. This enzyme is pivotal in mediating the

motility and interactions of *Pseudomonas aeruginosa* with macrophages, and plays major roles in the biofilm formation typical of late stages of bacterial adhesion and aggregation. As such, it may be worth to assume that even extracellular oxidative agents traditionally used to counteract bacterial growth may in fact contribute to the initiation of bacterial adaptive mechanisms, which may further aggravate bacterial contamination of biological samples.

3.2. Sperm Agglutination

Sperm agglutination regarded as a direct result of bacterial adherence to the cell surface is defined as a phenomenon when previously motile spermatozoa stick to each other. Such compromised male gametes may be connected to each other either in a head-to-head, tail-to-tail, or mid-piece-to-tail pattern. Nonspecific agglutination may also occur by the adherence of either nonmotile sperm to each other or of motile sperm to cell debris, mucus, or other cell types [62]. The type of agglutination is by and large predetermined by the type of fimbrinae responsible for the initial bacterial adherence: while head-to-head agglutination is primarily caused by Type 1 fimbrinae, P-fimbriae are responsible for tail-to-tail agglutination. If the sample is infested by different bacterial types, a mixed agglutination may occur [63,64].

Events associated with sperm immobilization and an inherent high agglutination capacity of bacteria will ultimately lead to the formation of a complex structure called a biofilm. The unique architecture of biofilm provides a more favorable environment for bacteria to grow and reproduce, by creating niches occupied by bacterial cells that are covered by layers of extracellular polymeric molecules preventing the entry of antibacterial remedies [64,65]. The bacterial load within the biofilm is usually very high, promoting the spread of drug resistance phenotypes [65].

Two molecules released by bacteria play a pivotal role in the promotion of sperm agglutination and immobilization—the sperm agglutinating factor (SAF) and the sperm immobilizing factor (SIF). SAFs have been primarily detected in *Escherichia coli* [66] or *staphylococci* [67,68], and are able to block sperm motility, cause morphological abnormalities and act as spermicides at higher concentrations. The motility inhibition lies in the ability of SAFs to inhibit Mg^{2+} -dependent ATPase as well as to trigger membrane receptors responsible for the initiation of apoptosis [66–68].

SIFs are molecules previously isolated from *Escherichia coli* [69] and *Staphylococcus aureus* [70], which directly immobilize male gametes without causing their agglutination. These molecules also have a specific receptor on the sperm membrane, and besides inhibiting ATP synthesis, also promote a premature acrosome reaction by causing an ionic imbalance in the sperm cell [69]. Furthermore, it has been revealed that *Staphylococcus aureus*-derived SIFs may have a direct cytotoxic effect on the male reproductive cell [70]. Besides *Escherichia coli* and *staphylococci*, sperm agglutination or immobilization have also been reported in the presence of *Chlamydia trachomatis* [71], *Mycoplasma* [72] or *Trichomonas vaginalis* [73].

Agglutinated sperm present with a high proportion of morphological and acrosomal abnormalities. Since both SAFs and SIFs target primarily ATPase, sperm agglutination and/or immobilization may lead to a disruption in the mitochondrial activity necessary for sperm motion [62]. As suggested by Wang et al. [74], sperm agglutination favors thiol-rich, oxidative-stressed, and apoptotic or necrotic spermatozoa, which themselves become additional sources of ROS. Furthermore, copper-induced lipid peroxidation (LPO) has been implicated in the loss of cholesterol from the membranous structures with a subsequent membrane destabilization in agglutinated spermatozoa [75]. Consequently, rupture of the plasma and mitochondrial membranes of spermatozoa engulfed by bacterial aggregates may result in the release of cytochrome C and ROS, leading to cell death and a decreased semen quality [14].

4. Bacterial Toxins

In addition to detrimental effects induced by physical contact between male gametes and bacteria, damage to spermatozoa may be caused by an array of molecules that are being

synthesized and released by bacteria, including lipopolysaccharide (LPS) and hemolysins (Figure 3).

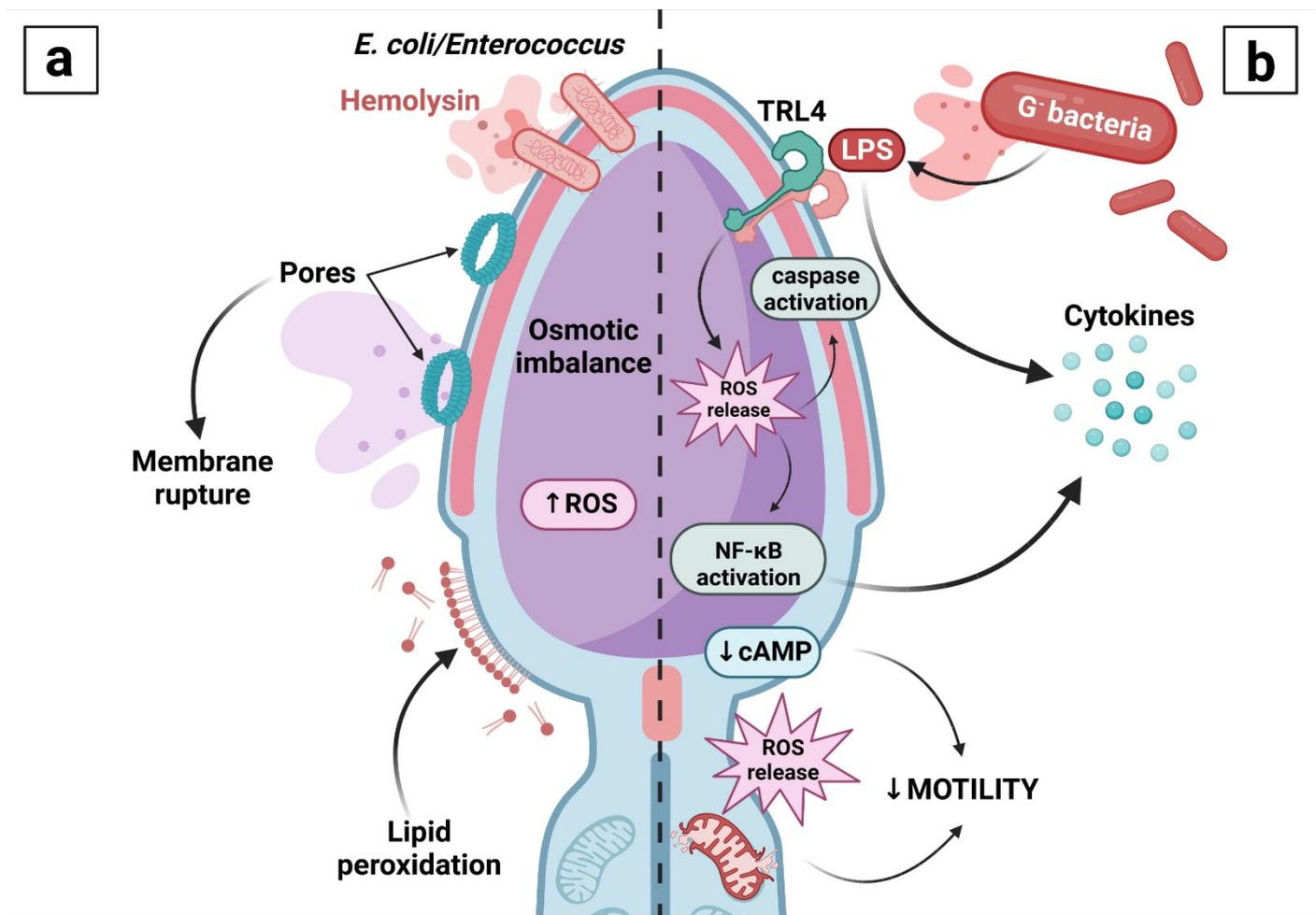


Figure 3. Damage to the sperm cell caused by (a) hemolysins or (b) lipopolysaccharide (LPS).

4.1. Lipopolysaccharide

LPS is a major component of the outer membrane of G^- bacteria [76]. The molecule is defined as a prototypical endotoxin, which triggers a significant immune response [77]. During bacteriospermia, LPS is primarily recognized by the Toll-like receptor 4 (TLR4) in the acrosomal and tail regions of spermatozoa [78], leading to the activation of nuclear factor- κ B to commence the transcription of downstream inflammatory factors [79]. LPS has been consistently linked to reprotoxicity [78,80,81] by affecting the expression patterns of pro-apoptotic genes [78,82], and by decreasing the levels of cyclic adenosine monophosphate (cAMP), Ca^{2+} , and the intensity of protein phosphorylation [80]. LPS is also a very potent prooxidant, exposure to which leads to increased levels of mitochondrial ROS and occurrence of cell death with a concomitant decrease in sperm motility [78,83]. He et al. [78] observed that LPS induces mitochondrial transcription factor A (TFAM) translocation, which activates mitochondrial DNA (mtDNA) replication and subsequent activation of the mitochondrial oxidative phosphorylation system (OXPHOS) in boar sperm. As a result, ROS production in the mitochondria is elevated, which leads to oxidative stress (OS), mitochondrial membrane LPO, and a decrease in sperm motility and viability [84]. In this sense, the activation of TLR4 and mitochondrial translocation of TFAM are considered to act as prime mechanisms underlying LPS action [78,85].

4.2. Hemolysins

Hemolysins are unique pore-forming toxins that cause alterations to the membrane integrity and thus contribute to sperm immobilization. *Escherichia coli* is the sole producer of α -hemolysin, which forms pores in the host cell membrane, ultimately leading to cellular lysis [86]. The molecule is receptor-independent, since it presents with a versatility to permeabilize lipid bilayers of different architectures and compositions, and subsequently disturbs the intracellular osmotic pressure by forming voltage-dependent ion channels [87]. As such, α -hemolysin is only released if the bacterium adheres to the sperm surface [46]. With respect to spermatozoa, hemolytic *Escherichia coli* immobilizes spermatozoa more quickly and effectively when compared with nonhemolytic strains. An important side effect of hemolysin toxicity lies in the accumulation of lipoxigenase products, increased release of ROS (particularly $O_2^{\bullet-}$) and reactive nitrogen species [87]. This disturbance of the oxidative milieu is accompanied by a decline in the antioxidant capacity and sperm mitochondrial membrane potential initiated by cellular rupture [88].

β -hemolysin is a toxin produced by *Enterococcus* that presents with the same mechanism of action as α -hemolysin and that exerts its toxic effects primarily on the sperm head, neck, and the middle segment of the tail. The head seems to be the primary target of the toxin, since a premature acrosome reaction and an increased release of hydrolytic enzymes are typical characteristics of urogenital infections caused by *enterococci* [89,90].

Finally, bacteriospermia is often accompanied by elevated levels of enzymes that are not per se toxic to male gametes but serve to create an optimal environment for the bacterial colonization of semen to proceed. These include but are not limited to proteases [91], lipases [92], coagulase or coagulation factors [93].

- (a) Since hemolysins are receptor-independent, a direct contact between the bacterium and the sperm cell is needed for their secretion. Once hemolysins are released, these will form pores in the plasma membrane, leading to the loss of its semipermeability and subsequent alterations to the intracellular osmotic balance. Hemolysins will additionally promote ROS overproduction, leading to peroxidation of the membrane lipids followed by membrane disintegration.
- (b) Once LPS is released, it will be recognized by the Toll-like receptor 4 (TLR4) located on the sperm surface. Its activation and a subsequent release of ROS will then trigger nuclear factor- κ B (NF- κ B) to promote inflammation, as well as caspases to initiate apoptosis. Meanwhile, LPS will activate the mitochondrial oxidative phosphorylation system, leading to increased ROS production by the mitochondria, peroxidation of mitochondrial lipids and a subsequent mitochondrial rupture. Initiation of the apoptotic process as well as mitochondrial dysfunction will then result in the loss of sperm motility.

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5. Leukocytospermia

Semen is a heterogeneous biological fluid containing an array of cells other than spermatozoa. Several components representing molecular immunity are found in ejaculates, such as immunoglobulins, chemokines, cytokines, or growth factors [94]. Furthermore, almost every semen specimen will contain white blood cells including granulocytes, lymphocytes, or macrophages, which represent the principal defense mechanism against foreign organisms [95]. Supraphysiological amounts of leukocytes is a condition defined as leukocytospermia, which according to the World Health Organization (WHO), is acknowledged if the concentration of peroxidase-positive white blood cells exceeds 1×10^6 /mL of semen [96]. This cut-off value is considered a potential indicator of an ongoing and often asymptomatic male genital infection [95], particularly if a bladder infection or urethritis have been previously excluded [97].

Symptomatic as well as “silent” urogenital infections accompanied by increased levels of leukocytes are frequently observed in clinical practice [98]. Nevertheless, a conclusive

interconnection among leukocytospermia and sub-fertility needs to be reinforced since some reports observed a direct correlation between increased concentrations of leukocytes and alterations to the sperm motion, morphology, or chromatin stability [99], while others excluded any impact of leukocytes on the fertilization potential of spermatozoa, particularly in the case of ARTs [100,101]. Despite this controversy, it is acknowledged that the presence of leukocytes in semen, regardless of their final concentration, is associated with ROS overproduction that may be of negative influence on certain sperm quality parameters [96].

Different mechanisms of leukocyte-inflicted damage to male gametes are known, out of which three may be considered as pre-dominant: (1) spermiphagy, (2) secretion of pro-inflammatory cytokines and (3) extracellular traps (ETs). Alterations to the sperm structure or function may occur individually or simultaneously, during isolated leukocytospermia or by leukocytospermia diagnosed concurrently with bacteriospermia [102].

Spermiphagy occurs when white blood cells, inherently programmed to detect and dispose of damaged or dead sperm, become overactivated and thus surround, engulf, and destroy even healthy male gametes [98,102,103]. The engulfment of spermatozoa by the cytoplasm of phagocytic cells is preceded by a direct contact of both cells and adhesion of leukocytes to the sperm head, midpiece, and flagellum, which is further reinforced by the secretion of proinflammatory cytokines [102,103].

According to Fraczek and Kurpisz [102], cytokines act within a network, where the toxicity of one cytokine may be modulated in the presence of other immune molecules. Among the most common pro-inflammatory cytokines, tumor necrosis factor (TNF) α released during inflammation and/or infection is most frequently reported to induce sperm cell death or to contribute to the loss of DNA integrity [104,105]. The cytotoxic behavior of TNF α may be further mediated via ROS or nitric oxide [106] that is inversely correlated with sperm motility. Within a vast and heterogeneous group of proinflammatory interleukins (ILs), IL-1b, IL-6, IL-8, IL-12, and IL-18 have been reported to be involved in inflammation-inflicted damage to spermatozoa in response to bacterial infestation of semen and a compromised motion behavior [18,105,107]. Similarly to TNF α , increased levels of ILs have been observed in subfertile subjects or in semen samples with ROS overload [8,42,100].

A recently observed immune response to the presence of bacteria is the creation of ETs by activated white blood cells. ETs represent complex 3D web-like scaffolds of DNA strands embedded with histones and other antimicrobial molecules, such as myeloperoxidase, lactoferrin, elastase, bactericidal permeability-increasing protein or cathepsin G [108,109]. Physical contact between a leukocyte and a sperm cell leads to fast activation of the white blood cell, releasing ET structures that will then engulf the male gamete, causing its immobilization. This phenomenon is aggravated by phagocytosis, degranulation, and cytokine release [110,111]. Recent studies have observed that ROS are intricately involved in most reaction cascades promoting the release of ETs. Evidence gathered from a variety of perspectives has shown that ET-osis involves ROS generation by NADPH oxidase. This event occurs either directly via molecular signaling that enables ET formation and release, or indirectly by affecting other factors that modulate the process. Oxidative mechanisms are likely to act as important players in the regulatory network that determines whether the process of ET release and activation will be beneficial or detrimental [112].

6. Oxidative Stress and its Impact on Sperm Function

Regardless of the causes for ROS overproduction, oxidative stress has become one of the leading contributors to alterations in the sperm architecture or functional behavior. Oxidative stress is defined as a phenomenon when oxidants “overpower” antioxidants, when fast chain reactions of peroxidation develop and when these processes exhibit pathological effects on the cell [113]. The impact of OS is proportional to the intensity of these events since the cell is capable of overcoming minor perturbations and returning to its original state.

The complex and intricate cellular structure of male gametes predisposes them to be particularly vulnerable to OS, since their plasma membranes are characterized by large quantities of PUFAs, while containing only minor concentrations of antioxidant molecules in their intracellular compartments [17]. Increased ROS generation has been associated with a decline in sperm motility [18] either through the ability of H₂O₂ to diffuse across the membranes and inhibit the activity of several enzymes crucial for the sperm movement [114] or through inhibition of phosphorylation of axonemal proteins and subsequent sperm immobilization [115]. Furthermore, the motility loss is highly correlated with LPO, suggesting that oxidation of lipid structures is a major cause for alterations in the sperm motion behavior [116]. LPO of the sperm membrane is considered to be the key mechanism of ROS-induced sperm damage leading to subfertility [116], since PUFAs involved in the maintenance of membrane fluidity and transmembrane communication are most susceptible to oxidative insults [96]. Sperm LPO is a self-propagating process during which alkoxy and peroxy lipid radicals are formed, and subsequently act on other lipids in the membrane until all of them have undergone peroxidative changes [19]. These domino reactions will ultimately contribute to oxidative disintegration of DNA and proteins through lipoperoxides [19,116].

Within the molecule of DNA, bases and phosphodiester backbones are prone to oxidative insults. While sperm DNA protects itself against ROS by its specific compact organization and by antioxidants in the seminal plasma, spermatozoa lack any DNA repair mechanisms and rely on the oocyte to accomplish any repair following fertilization [117]. Out of different types of DNA damage caused by ROS, deletions, base modifications, DNA cross-links, occurrence of base-free sites, and chromosomal rearrangements are among the most commonly found in infertile males [118]. OS has also been linked to high frequencies of single- and double-strand DNA breaks and gene mutations that have been reported to occur, especially during sperm production and maturation [117,118]. Furthermore, mutations in mtDNA, which may also be affected by ROS, may compromise mitochondrial energy metabolism and sperm motility *in vivo* [119].

Oxidative insults to proteins may lead to in site-specific amino acid modifications, changes to the electric charge, disintegration of the peptide chain, and alterations to the susceptibility to proteolysis. Sulphur-containing amino acids are ought to be extraordinarily susceptible to changes in the oxidative balance. Such disturbance of the structure and function of sperm proteins may ultimately result in a decreased sperm ability to penetrate the zona pellucida and to fuse with the ovum [120].

Finally, ROS may trigger a chain of reactions that will ultimately lead to apoptosis. Under normal circumstances, programmed cell death is a natural process that helps to dispose of abnormal germ cells. Nevertheless, high ROS levels may disrupt the inner and outer mitochondrial membrane, accompanied by cytochrome C release and activation of caspases responsible for the promotion of apoptosis [121]. In the case of bacteriospermia, previous reports hypothesize that the principal pro-apoptotic mechanism lies in the activity of bacterial endotoxins, such as LPS or porins to interact with Toll-like receptors 2 and 4 located on the sperm surface [122]. Exposure of male gametes to these toxins may lead to ROS overproduction and subsequent mitochondrial depolarization followed by activation of caspase 3-mediated cell death [123]. Furthermore, the immune system may play a significant role in sperm apoptosis through the cytokine network, since it has been observed that IL-1b, IL-6, IL-8 or IL-18 could activate the Fas/Fas ligand complex on the sperm membrane, and subsequently initiate caspase 8-driven apoptotic machinery [14].

7. Clinical Effects of Bacteriospermia on Semen Quality

The effects of bacterial presence in semen on the resulting sperm structural integrity and functional activity are complex and multivariable as previously reported in several studies on humans [3,8,10,102,124,125] as well as domestic animals [23,90,126–131]. By and large, these reports agree that bacteriospermia may lead to the loss of sperm membrane stability [23,102,126,127,132], acrosome rupture and morphological alterations to the sperm

head, mid-piece, and tail [23,48,102,127,133], and mitochondrial dysfunction accompanied by ATP depletion [23,126,127,133], all of which will ultimately result in the loss of sperm motility and a subsequent inability of spermatozoa to reach and fertilize the oocyte. A frequently observed phenomenon associated with the presence of bacteria in semen is sperm agglutination, DNA disintegration and early onset of cell death [14,41,126,127]. Furthermore, the presence of bacteria in semen has been frequently associated with the onset of leukocytospermia and increased levels of cytokines with cytotoxic properties [3,10,102]. Finally, preliminary reports have indicated that bacterial activity could modify the biochemical or physicochemical properties of semen, thus creating a less favorable environment for sperm survival [134,135]. Table 1 provides a summary of the most prominent effects of uropathogens on male gametes across currently available original papers published on the topic.

Table 1. Leading consensual in vivo and in vitro effects of the most frequent uropathogenic bacteria on semen quality and/or sperm structural integrity and functional activity.

Bacterium	In Vivo Observations	In Vitro Observations
<i>Escherichia coli</i>	<ul style="list-style-type: none"> ↓ sperm count [8,9,23,126,136,137] ↓ sperm motility [8–10,23,126,136–138] ↓ sperm viability [8,9,23,126,136] ↓ sperm morphology [9,136] ↓ acrosome integrity [8,23,126] ↑ DNA damage [8,9,23] ↓ chromatin integrity [8] ↑ leukocytospermia [8,9,23,44,126,137] ↑ concentration of cytokines [8,23,126] ↓ concentration of antibacterial proteins [8,126] 	<ul style="list-style-type: none"> ↓ sperm motility and viability [20,21,133,139] ↓ sperm morphology [139] ↓ acrosome integrity [140] ↑ caspase 3 and 7 activity [20]
<i>Klebsiella pneumoniae</i>	<ul style="list-style-type: none"> ↓ sperm count, motility, viability and acrosome integrity [126] ↓ mitochondrial activity [126] ↑ DNA damage [8,9,126] ↑ leukocytospermia and cytokine levels [126] ↓ concentration of antibacterial proteins [126] 	<ul style="list-style-type: none"> ↓ sperm motility [20,21,141] ↓ sperm viability [20,21] ↓ sperm morphology [21] ↑ caspase 3 and 7 activity [20]
<i>Pseudomonas aeruginosa</i>	<ul style="list-style-type: none"> ↓ sperm count, motility and morphology [128,142] ↓ sperm viability [122,128] 	<ul style="list-style-type: none"> ↓ sperm motility [143] ↓ sperm viability [20] ↑ caspase 3 and 7 activity [20]
<i>Enterococcus</i> spp.	<ul style="list-style-type: none"> ↓ sperm count, motility and acrosome integrity [8,9] ↓ sperm viability [8,9] ↓ sperm morphology [9] ↓ mitochondrial activity [8,9,126] ↑ sperm apoptosis and phospholipid scrambling [9] ↑ DNA damage [8,9,126] ↓ chromatin integrity [8,86,126] ↑ leukocytospermia and cytokine levels [8,9,126] ↓ concentration of antibacterial proteins [8,126] 	<ul style="list-style-type: none"> ↓ sperm motility and viability [20,90] ↓ acrosome integrity [90] ↓ mitochondrial activity [90] ↑ caspase 3 and 7 activity [20] ↓ chromatin integrity [90]
<i>Staphylococcus</i> spp.	<ul style="list-style-type: none"> ↓ sperm count and motility [8,9,23,127] ↓ sperm viability [8,9,127] ↓ sperm morphology [9] ↓ acrosome integrity [8,23,127] ↓ mitochondrial activity [8,9,23,127] ↑ sperm apoptosis and phospholipid scrambling [9] ↓ chromatin integrity [8,23,127] ↑ leukocytospermia [8,9,23,127] ↑ concentration of cytokines [8,23,127] ↓ concentration of antibacterial proteins [8] 	<ul style="list-style-type: none"> ↓ sperm motility and viability [41,133,139] ↓ sperm morphology [139] ↓ acrosome integrity [41] ↓ mitochondrial activity [41,133]

Table 1. Cont.

Bacterium	In Vivo Observations	In Vitro Observations
<i>Campylobacter ureolyticus</i>	↓ sperm count [9,137] ↓ sperm motility and viability [9] ↓ cell morphology [9,137] ↓ mitochondrial activity [9] ↑ sperm apoptosis, DNA damage and phospholipid scrambling [9] ↑ leukocytospermia [9,137]	N/A
<i>Ureaplasma urealyticum</i>	↓ sperm count and motility [9,144,145] ↓ sperm viability [9] ↓ sperm morphology [9,144] ↓ mitochondrial activity [9] ↑ sperm apoptosis, DNA damage and phospholipid scrambling [9] ↑ leukocytospermia [9]	↓ sperm motility and viability [133]
<i>Mycoplasma</i> spp.	↓ sperm count and motility [9,146–148] ↓ sperm morphology [9,146,148] ↓ sperm viability [9,146] ↑ sperm apoptosis, DNA damage and phospholipid scrambling [9] ↓ chromatin integrity [148] ↑ leukocytospermia [9,147] ↑ concentration of cytokines [147]	↓ sperm motility and morphology [149]
<i>Chlamydia trachomatis</i>	↓ sperm count, motility and morphology [148,150,151] ↓ sperm viability [151] ↓ chromatin integrity [148,150]	N/A

Recent studies on bacteriospermia have also provided a solid body of evidence, indicating a significant involvement of OS in bacteria-inflicted damage to male gametes. Interactions among bacteria and spermatozoa have been shown to trigger ROS overgeneration and subsequent oxidative insults, particularly to the membranous structures of the sperm cell, leading to the loss of sperm functionality in vivo or in vitro. Changes observed in the oxidative profile of semen and/or spermatozoa affected by the presence of bacteria are summarized in Table 2.

Table 2. Leading consensual in vivo and in vitro effects of the most frequent uropathogenic bacteria on oxidative characteristics of semen and/or spermatozoa.

Bacterium	In Vivo Observations	In Vitro Observations
<i>Escherichia coli</i>	↑ intracellular ROS production [23,126] ↑ superoxide production [9] ↓ antioxidant capacity [8,23,126] ↑ LPO [8,9,23,126] ↑ protein oxidation [23,126]	↑ intracellular ROS production [21,22] ↑ mitochondrial ROS production [20] ↑ LPO [22]
<i>Klebsiella pneumoniae</i>	↑ intracellular ROS production [126] ↓ antioxidant capacity [126] ↑ LPO and protein oxidation [126]	↑ mitochondrial ROS production [20] ↑ LPO [141]
<i>Pseudomonas aeruginosa</i>	N/A	↑ mitochondrial ROS production [20] ↑ LPO [20]

Table 2. Cont.

Bacterium	In Vivo Observations	In Vitro Observations
<i>Enterococcus</i> spp.	↑ intracellular ROS production [126] ↑ superoxide production [9] ↓ antioxidant capacity [8,126] ↑ LPO [8,9,126] ↑ protein oxidation [126]	↑ intracellular ROS production [90] ↑ mitochondrial ROS production [20]
<i>Staphylococcus</i> spp.	↑ superoxide production [9] ↓ antioxidant capacity [8,23,127] ↑ LPO [8,9,23,127] ↑ protein oxidation [23,127]	↑ intracellular ROS production [22,41] ↑ LPO [22]
<i>Campylobacter ureolyticus</i>	↑ superoxide production [9] ↑ LPO [9,137]	↑ intracellular ROS production [22] ↑ LPO [22]
<i>Ureaplasma urealyticum</i>	↑ superoxide production [9] ↑ LPO [9]	N/A
<i>Mycoplasma</i> spp.	↑ superoxide production [9] ↑ LPO [9]	N/A

8. Management of Bacteriospermia in Practice: Strategies, Options and Alternatives

8.1. Antibiotics

Current management of bacteriospermia both in vivo as well as in vitro is by and large dependent upon antibiotics. The primary mechanism of action of these substances is based on inducing cell death by interfering with primary structural targets or by corrupting target-specific processes within the respective bacterial cell. Nevertheless, it is now well known that these target-specific interactions trigger stress responses that induce redox-related physiological alterations resulting in the formation ROS, which will further contribute to bacterial damage and/or death [152].

If bacteriospermia is caused by chronic or acute bacterial infections, these will be generally treated using a broad spectrum of antibiotics, among which ciprofloxacin, norfloxacin, and ofloxacin are most recommended in clinical practice [153]. In the meantime, supplementation of streptomycin, penicillin, spectinomycin, and lincomycin to semen extenders and cryopreservation media is required by law to avoid any potential transmission of disease among recipients [154].

Despite an indisputable contribution of traditional antibiotics to the prevention of bacteria-inflicted damage to male gametes, several reports have indicated that the inherently toxic properties of antibiotics could at least partially affect the male gametes themselves [155]. It has been recently reported that ciprofloxacin causes a rapid in vitro disintegration of sperm DNA [156], while gentamycin and ofloxacin administered in vivo may affect sperm motility and the extent of testicular apoptosis [157]. In this sense, there is a need to precisely define effective doses and eventual toxicity of antibiotics to male gametes in order to avoid any adverse effects.

Another aspect that must be taken into consideration lies in often-irrational antibiotic overuse in practice, which may endanger antibiotic susceptibility and lead to increased multidrug resistance in a wider range of bacterial species. A recent study has unraveled that more than 56% of all bacterial species found in boar semen were resistant to gentamycin, while only every fifth isolate was susceptible to gentamycin, lincomycin, penicillin, and neomycin [158]. In the meantime, *Pseudomonas aeruginosa* and *Proteus mirabilis* found in boar semen were resistant to spectinomycin, lincomycin, florfenicol, and streptomycin [159]. An alarming resistance of all isolates retrieved from bovine semen to penicillin has been reported, while the majority also presented with resistance patterns to tylosin and lincomycin [160]. With respect to humans, vancomycin and rifampicin were ineffective against G⁺ bacteria detected in Iraqi patients with fertility issues [161]. A similar

study on infertile men unraveled the presence of *Klebsiella pneumoniae*, *Escherichia coli*, and *Staphylococcus epidermidis* with multidrug resistance patterns [162]. Furthermore, 90% of bacterial isolates found in in vitro fertilization (IVF) culture dishes were resistant to at least one of the antibiotics commonly used in ARTs [163], complementing a recent report on increased resistance of *staphylococci* in ART clinics [164].

8.2. Physical Removal of Bacteria

Since different concerns have arisen from an improper use of antibiotics in human or veterinarian andrology, several efforts have been made to develop procedures that would help with the physical removal of bacteria prior to further semen processing. Semen washing with an extra swim-up step has been reported to be more efficient in limiting the occurrence of potentially pathogenic bacteria in semen when compared to antibiotics [165]. Previous studies have also emphasized the advantages of density gradient centrifugation in removing bacteria and viruses from semen. Besides improving the microbial status of ejaculates, density gradient centrifugation also eliminates leukocytes or dead spermatozoa, significantly improving the quality of neat or thawed ejaculates even of suboptimal quality [41,90,165–167]. In addition, sperm filters have been developed for semen processing, causing less damage to the plasma membrane in comparison to centrifugation techniques [168]. Magnetic-activated cell-sorting (MACS), which identifies and eliminates apoptotic cells from semen using annexin V-conjugated superparamagnetic microbeads, could also become a suitable technique to reduce the proportion of damaged or dead spermatozoa prior to ARTs [169].

A new line of antimicrobial defense is represented by nanoparticles. Recent reports on iron oxide nanoparticles [170] or silver-carbon nanoparticles [171] applied to semen reveal that their use did not have any impact on sperm motility, viability, morphology or DNA integrity, while being effective in the elimination of several uropathogens including *Escherichia coli*, *Staphylococcus aureus* or *Pseudomonas aeruginosa*.

8.3. Alternative Antibacterial Supplements

A better prevention or management of bacteriospermia could also be accomplished by complementing conservative prescription medications with traditional remedies. Ethnopharmacological herbs such as copperleaf (*Acalypha wilkesiana*) [172], white weed (*Ageratum conyzoides*) [173], clove basil (*Ocimum gratissimum*) [174], pheasant-berry (*Phyllanthus discoideus*) [175], or Guinea pepper (*Aframomum melegueta*) [176] have been used for centuries now as remedies for male urogenital infections caused by pathogens such as *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Enterococcus faecium* and *Pseudomonas aeruginosa*, all of which may additionally compromise semen quality. Herbal extracts and essential oils prepared from the above-mentioned plants may be used as floral beverages or functional foods with minimal known side effects on health. Furthermore, their use might help in reducing dependence on antibiotics and minimizing antibiotic resistance [177].

Furthermore, oxidative stress resulting from bacterial infestation of semen may be alleviated by products from Ajwain (*Trachyspermum copticum*), juniper (*Juniperus communis*), Gokshur (*Tribulus terrestris*), holy basil (*Ocimum sanctum*) or Ceylon cinnamon (*Cinnamomum verum*), which present with significant antioxidant properties [178]. Preliminary studies have indicated that administration of these remedies in the form of essential oils or extracts improved male steroidogenesis, which was accompanied by increased sperm motility, viability and DNA integrity [179,180].

Finally, the use of plant extracts [181] or pure natural biomolecules [90] as supplements to semen extenders could be yet another strategy to stabilize the oxidative milieu of in vitro processed spermatozoa while at the same time acting as antibacterial agents. A complex assessment of the biological activity of *Schisandra chinensis* revealed that the *Omija* extract was effective against *Streptococcus pneumoniae* and *Enterococcus faecalis*, while acting as a motion-promoting and metabolism-enhancing supplement to bovine sperm in vitro culture [182]. In the meantime, it was observed that marigold (*Calendula officinalis*) extract

exhibited antibacterial activity against *Staphylococcus aureus* and *Enterococcus faecalis* [183] and at the same time provided significant antioxidant protection to extended bull semen [184]. In their experiments, Eini et al. [185] observed that tea tree (*Melaleuca alternifolia*) and rosemary (*Rosmarinus officinalis*) essential oils were capable of exerting similar effects to ampicillin on swine artificial insemination doses deprived of spermatozoa and spiked with *Escherichia coli*. In the meantime, moringa (*Moringa oleifera*) and ginger (*Zingiber officinale*) extracts supplemented with chilled banana shrimp spermatophores were able to maintain a higher percentage of viable sperm while inhibiting growth of pathogenic *Vibrio* and *Pseudomonas* [186]. With respect to pure biomolecules, a pivotal study by Duracka et al. [90] revealed that resveratrol, quercetin and curcumin provided protection to rabbit sperm structural integrity and functional activity during in vitro induced bacteriospermia by *Enterococcus faecalis* while exhibiting partial antibacterial activity against the bacterium.

While the preliminary results on alternative remedies against bacteriospermia collected from currently available literature are undoubtedly promising, precise and detail-oriented toxicological studies of these herbs, extracts and biomolecules are essential to elucidate their suitability for effective in vivo or in vitro management of male reproductive dysfunction caused by bacterial infection.

9. Conclusions

An increasing body of evidence strongly indicates that the presence and/or activity of bacteria in semen may exhibit detrimental effects on the structure and function of male gametes, rendering them to be ineffective in accomplishing their primary role of fertilizing the ovum. While the exact molecular machinery underlying bacteria-inflicted damage to spermatozoa is still not fully understood, it is feasible to state that oxidative mechanisms play a pivotal role in the etiology of bacteriospermia. Further studies unraveling the involvement of reactive oxygen species in bacterial infestation of ejaculates and consequences resulting from this condition will be needed to enable the development of new techniques to diagnose bacteriospermia in a more effective manner, followed by suitable strategies to manage the after-effects of bacterial action and/or oxidative damage to male reproductive cells.

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