REVIEW

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Mechanisms of the harmful effects of bacterial semen infection on ejaculated human spermatozoa: potential inflammatory markers in semen

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Abstract

The invasion of the male reproductive tract by microorganisms, and its subsequent consequences for sperm fertilizing potential, has been intensely discussed. The role of the bacteria that are responsible for the colonization and contamination of the male urogenital tract, rather than its infection, in diminished sperm parameters raises the most controversy. There are numerous premises suggesting that bacterial semen infection is associated with male infertility. However, the molecular mechanism by which the fertility is affected is complex and multifactorial, and still presents a puzzle. Some authors have suggested that direct interactions between bacteria and human spermatozoa facilitate sperm immobilization, affect sperm morphology, and thus weaken the ability of sperm to fertilize. On the other hand, the massive infiltration of activated leukocytes into the inflammatory site may be associated with impairment of sperm fertilizing potential, due to oxidative, apoptotic, and immune processes. This review presents current research trends and aims to summarize the present knowledge of semen inflammation and causative bacterial agents in the male urogenital tract, with its consequence on seminological parameters, and male fertility status. *(Folia Histochemica et Cytobiologica 2015, Vol. 53, No. 3, 201–217)*

Key words: bacterial semen infection; sperm quality; oxidative stress; sperm apoptosis

Introduction

Urogenital tract inflammations and infections are thought to be responsible for up to 15% of cases of male infertility. According to clinical data, as many as 60% of patients treated with assisted reproductive technology (ART) had suffered local inflammation or infection. The direct relationship between acute or chronic inflammations and infections of the male urogenital tract and the subsequent development of infertility is actively debated, and constitutes an important problem in contemporary andrology. Due to the anatomical topography, local inflammations and infections include chronic urethritis, orchitis, epididymitis, prostatitis, vesiculitis, and obstruction of the seminal tract. However, these rarely occur in only one gland, which complicates their diagnosis and proper treatment. The course of the inflammatory process and its detrimental effect on sperm depends on the type of the initiating factor, the type and activation of infiltrating leukocytes, the duration of contact of spermatozoa with inflammatory mediators, and the genetic predisposition to the inflammatory response [1]. The majority of inflammatory disorders within the male genital tract are of infectious origin. The bacteria, viruses, *Chlamydiae*, and fungi responsible for semen infection may originate from the urinary tract or may be sexually transmitted; their role in causing male infertility has been discussed in recent years. However, the role of different types of bacteria that colonize and contaminate the male urogenital tract in diminishing sperm parameters is the issue that raises the most controversy. The clinical and experimental data suggest that bacteriospermia and leukocytospermia may inhibit male fertility by affecting the

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sperm characteristics directly or indirectly by acting on the regulatory systems. The purpose of this review is an attempt to summarize the current knowledge of the main pathophysiological concepts describing the harmful effects of bacterial semen infection on sperm cells.

Direct effect of bacteria and leukocytes on sperm quality

There are many reports demonstrating that bacterial invasion could contribute to sperm quality deterioration that is visible in routine semen analysis, especially in infertile men (Table 1) [2–31]. Decreasing sperm concentration, loss of motility, sperm morphological alterations, and impairment of acrosome reactions are the most frequent alterations revealed in spermatozoa attributable to bacteria in both *in vivo* and *in vitro* conditions. Most data concern well-known causative agents of urogenital tract infections, such as *Escherichia (E.) coli*, *Staphylococcus (S.) aureus*, *Enterococcus (E.) faecalis*, *Ureaplasma (U.) urealyticum*, *Mycoplasma (M.) hominis*, and *Chlamydia (C.) trachomatis*. However, some authors have suggested that other bacteria, responsible for the colonization and contamination of the male urogenital tract, rather than infection, could also contribute to the decrease in sperm quality [32, 33]. During bacterial semen infection, sperm motility and normal morphology loss may be consequences of adhesion phenomena and sperm agglutination. The sperm surface is rich in glycoproteins and is thus susceptible to bacteria–spermatozoa interactions at the receptor–ligand level [34, 35]. The tight adhesion between bacteria and male gametes was found for *E. coli*, *C. trachomatis*, T-mycoplasma, and *U. urealyticum* [10, 24, 36]. Interestingly, in our morphological *in vitro* study, the adhesion of *Staphylococcus (S.) haemolyticus* and *Bacteroides (B.) ureolyticus* (bacteria of questionable clinical significance) to the apical part of the acrosome and to the flagellum of spermatozoa have also been revealed (Figure 1) [10]. In particular, *E. coli* strains are known for their ability to immobilize and damage the morphology of spermatozoa by direct contact, mediated by attachment organelles such as pili or type-1 fimbriae (projections) and mannose receptor-dependent interactions. Another type of adhesins, namely P fimbriae, are important and widely studied mannose-resistant adhesion molecules present in 40–60% of uropathogenic (UPEC) isolates [37]. High incidences of P-fimbriated *E. coli* strains have been found in acute prostatitis [38]. Our experience with P-fimbriated *E. coli* serotype O75:HNT has confirmed direct contact adhesion to the surface

of sperm midpiece, principal piece, and acrosome, mediated by projections and sperm agglutination, and resulting in ultrastructural alterations in regions with attached bacteria (Figure 1) [39]. It should be emphasized that despite the ultrastructural evidences observed in both *in vitro* and *in vivo* conditions, there is no general consensus on a negative relevance of the bacteriospermia on sperm morphology parameters, assessed as a part of the routine sperm analysis. On the one hand, some authors demonstrated poor sperm morphology characteristics in the presence of several types of bacteria (Table 1). Also, the improvement in sperm parameters including sperm morphology abnormalities in men with urogenital tract infections after long-term antibiotic treatment has been observed [40]. On the other hand, sperm morphology alterations associated with elongation and reduced acrosomal inducibility have been found in men with inflammatory chronic prostatitis/chronic pelvic pain syndrome, and these changes were attributed rather to leukocytes [41]. There is an ongoing discussion whether chronic epididymitis additionally induces inflammatory semen alterations, especially sperm morphology abnormalities such as 'tapering' of sperm heads and differences in tail colouring [42]. Taking into consideration the fact that the increased sperm head length is usually accompanied by the sperm nuclear anomalies, factors other than conventional semen parameters can be a major issue to diagnose urogenital tract infections/inflammations. In this context, the assessment of DNA integrity can provide more information about the underlying pathophysiological mechanism of infertility [43]. This will be discussed in details later in this review.

The harmful effects of numerous microbial pathogens on spermatozoa not only result from the tight adhesion of interacting cells, but also from the expression of other surface virulent factors, such as lipopolysaccharides (LPS), cytotoxic necrotizing factor, α-haemolysins, β-haemolysins, and from the release of soluble spermatotoxic factors such as sperm immobilization factor (SIF) [37, 44]. For example, *E. coli* haemolysins might be involved in the molecular mechanism that ultimately alters the membrane integrity [45]. In turn, SIF can inhibit sperm motility and viability by decreasing mitochondrial ATPase activity [35]. However, a loss of sperm motility concomitant with the integrity of the sperm mitochondrial membrane potential ($\Delta\psi$m) during experimental *in vitro* semen infection has also been reported in the presence of bacteria that do not produce SIF, such as known pathogenic *C. trachomatis* [15] and the conditionally pathogenic *S. haemolyticus* and *B. ureolyticus* [9, 10]. Most probably, a complex balance between

Table 1. Effects of individual bacterial strains on sperm quality including conventional as well as nonconventional seminal parameters demonstrated in both *in vivo* and *in vitro* conditions

Bacterial strain	Consequences on semen quality	References
Escherichia coli	↓ sperm count	[2]
	↓ sperm motility	[2, 3]
	↓ sperm morphology	[3]
	↓ sperm motility (*in vitro*)	[4–9]
	↓ sperm viability (*in vitro*)	[7, 9]
	↑ MDA (*in vitro*)	[8, 9]
	↓ ΔΨm	[7, 8, 10]
	↑ mitochondrial ROS	[8]
	↑ PS externalization (*in vitro*)	[11]
	↓ acrosome reaction (*in vitro*)	[5, 12]
Chlamydia trachomatis	↓ sperm count	[13–15]
	↓ sperm progressive motility	[15, 16]
	↓ sperm motility	[13]
	↓ sperm morphology	[13]
	↑ MDA	[17]
	↓ ΔΨm	[15]
	↑ PS externalization (*in vitro*)	[18]
	↑ DNA fragmentation	[15, 19]
	↑ DNA fragmentation (*in vitro*)	[18]
Ureaplasma urealyticum	↓ sperm count	[2, 20–22]
	↓ sperm motility	[2, 21, 23]
	↓ sperm morphology	[21]
	↓ sperm viability	[2, 22, 23]
	↓ sperm motility (*in vitro*)	[24, 25]
	↓ sperm morphology (*in vitro*)	[24]
	↓ viability (*in vitro*)	[24, 25]
	↓ pH value	[20]
	↑ viscosity	[20]
	↑ ROS	[26]
Mycoplasma hominis	↓ sperm count	[27]
	↓ sperm motility	[23]
	↓ sperm morphology	[27]
	↓ sperm motility (*in vitro*)	[5]
	↓ acrosome reaction (*in vitro*)	[5]
Mycoplasma genitalium	↓ sperm count	[27]
Enterococcus faecalis	↓ sperm count	[28]
	↓ sperm morphology	[28, 29]
Staphylococcus aureus	↓ sperm count	[2]
	↓ sperm motility	[2, 3]
	↓ sperm morphology	[2, 3]
	↑ PS externalization (*in vitro*)	[11]
Pseudomonas aeruginosa	↓ sperm progressive motility (*in vitro*)	[4]
Bacteroides ureolyticus	↓ morphology	[30]
	↓ sperm motility (*in vitro*)	[9]
	↓ sperm viability (*in vitro*)	[9, 10]
	↑ MDA (*in vitro*)	[9]
	↓ ΔΨm (*in vitro*)	[10]
	↑ PS externalization (*in vitro*)	[31]
	↑ DNA fragmentation (*in vitro*)	[31]

→

Table 1 (cont.). Effects of individual bacterial strains and sperm quality including conventional as well as nonconventional seminal parameters demonstrated in both *in vivo* and *in vitro* conditions

Streptococcus viridans	↓ sperm count	[29]
	↓ sperm morphology	[29]
Staphylococcus haemolyticus	↓ sperm motility (*in vitro*)	[9]
	↓ sperm viability (*in vitro*)	[9, 10]
	↑ MDA (*in vitro*)	[9]
	↓ ΔΨm (*in vitro*)	[10]
	↑ PS externalization (*in vitro*)	[31]
	↑ DNA fragmentation (*in vitro*)	[31]
Staphylococcus saprophyticus	↓ motility	[3]
	↓ sperm morphology	[3]
Gardnerella vaginalis	↓ concentration	[21]
	↓ motility	[21]
	↓ morphology	[21]

the expression of virulent factors in different types of bacteria and the host defence status marks a thin borderline between semen contamination/colonization and infection, especially in the context of bacteria that have so far been considered 'nonpathogenic' to sperm cells. The establishment of a minimal threshold for particular bacterial strains seems to be essential in evaluating their negative influence on sperm quality with real consequences for fertilization potential and the decision to take targeted therapy.

Infectious factors trigger the infiltration of leukocytes to the inflammatory site. Peroxidase-positive leukocyte concentration in excess of 1×10^6 mL^{-1} ejaculate, a condition called leukocytospermia, is generally attributed to the inflammation or infection of semen [46]. However, some authors have postulated the necessity to reevaluate this threshold [47–50]. According to the kinetics of the inflammatory process in the urogenital tract previously proposed by our group, leukocytes appear in semen as the addition to bacteriospermia at the second stage of the urogenital tract infection, and remain present in semen for some length of time following the elimination of the bacteria in the third stage (isolated leukocytospermia) [51]. There is an ongoing controversy concerning the biological role of the leukocytes attracted into the semen. Some authors have indicated a lack of any connection between leukocytospermia and diminished semen quality [52–54]. However, the vast majority of clinical and experimental *in vitro* reports have shown a direct association between leukocytospermia and deterioration in semen parameters, in terms of total sperm count [55, 56], sperm motility [9, 55, 57–60], sperm morphology [56, 59, 60], and sperm viability [60]. Moreover, according to some authors, leukocytospermia represents an essential or additional

risk factor that needs to be treated if sperm quality is to be improved [61, 62]. On the other hand, the observed alterations of standard sperm parameters accompanying leukocytospermia have not always been associated with a decreased fertilizing ability of the sperm, especially in assisted conception procedures [63, 64]. These data are indirectly in agreement with the results that we obtained in an *in vitro* model of semen bacterial infection, indicating the detrimental effect of leukocytes alone on sperm motility and the hypoosmotic swelling (HOS) test. However, the decrease in sperm function reflected in the sperm penetration assay (SPA) was visible when leukocytes were used together with bacteria [9].

Almost 90% of the leukocytes attracted into semen during bacterial semen infection are phagocytic cells such as polymorphonuclear granulocytes (PMN) and macrophages. To date, little is known about direct associations between the presence of macrophages in semen and male subfertility or infertility, mainly due to the fact that the establishment of this second abundant leukocyte subpopulation within semen is not a part of the routine peroxidative test recommended by the World Health Organization [46]. However, some authors have suggested that activated macrophages are frequently observed in nonleukocytospermic infertile men and that they may be associated with altered sperm parameters [49, 65, 66]. The tight adhesion of both leukocyte types, neutrophils, and macrophages to the surface of the sperm results in phagocytic process (Figure 1). Interestingly, our morphological ultrastructural findings have revealed, for the first time, that the immobilization of human spermatozoa by activated leukocytes may be mediated by three different mechanisms: by direct cell-to-cell attachment, by leukocytic processes at the early stage
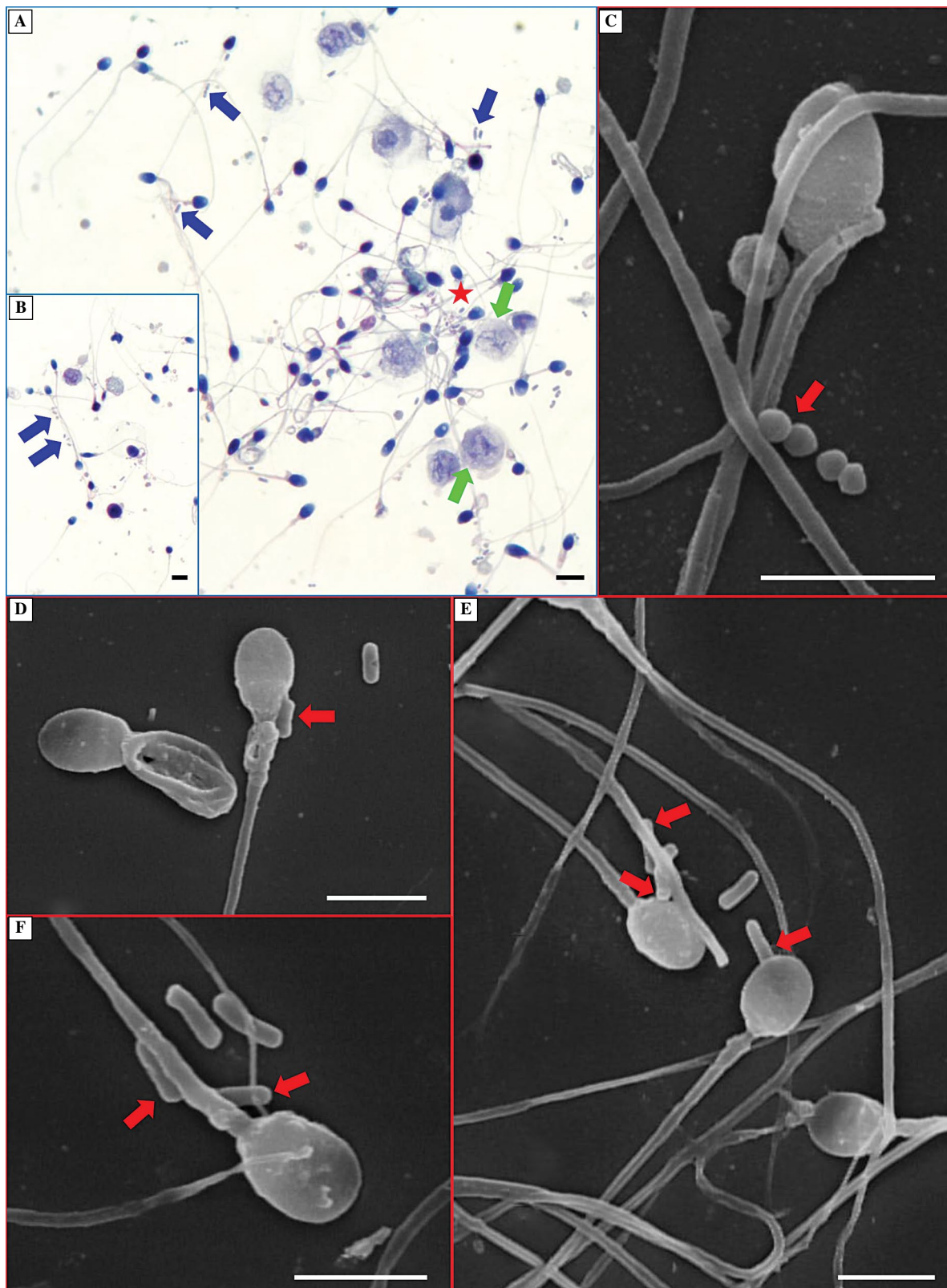
Figure 1. Interactions among bacteria, leukocytes and human spermatozoa. Papanicolaou-stained smear of human semen with bacteriospermia and leukocytospermia *in situ*. Sperm agglutination in the presence of bacteria (asterisk); adhesion of bacteria (blue arrows) and leukocytes (green arrows) to the sperm structures (**A, B**). Scanning electron micrographs of ejaculated human spermatozoa incubated with *Bacteroides ureolyticus* (**C**) or with *Escherichia coli*, serotype O75:HNT (**D–F**) in *in vitro* conditions. Adhesion of bacteria to sperm structures (red arrows). Scale bar: 5 μm. Photographs by Malgorzata Piasecka, Department of Histology and Developmental Biology, Pomeranian Medical University, Szczecin, Poland

of sperm phagocytosis, and by extracellular structures related to extracellular traps that help to eliminate male gametes [39]. Furthermore, these processes of sperm elimination can occur in human semen in isolated leukocytospermia, as well as in leukocytospermia coexisting with bacteriospermia. In the latter condition, sperm phagocytosis and entrapment appear to be more aggressive, and they both may lead to a disturbance of the natural system controlling semen quality [39]. During semen bacterial infection, the engulfment of spermatozoa due to phagocytosis is accompanied by the formation of reactive oxygen and nitrogen species, proteases, and immune factors. In bacterial semen infection, the induction all of the inflammatory reactions in the seminal tract through the activation of neutrophils and macrophages may indirectly exert a deleterious effect on male fertility.

Indirect effect of bacteria and leukocytes on sperm cells

Role of oxidative stress

The role of reactive oxygen species (ROS) in sperm cell biology is beyond doubt. At low levels, ROS play a physiologically important role in sperm hyperactivation, capacitation, and acrosome reaction; at higher levels, they cause oxidative stress that limits the fertilizing potential of the male gametes as a result of peroxidative damage to cellular macromolecules [67]. Growing evidence indicates that the imbalance between ROS levels and total antioxidant capacity (TAC) in semen leads to disorders of male gametes (also visible in routine semen analysis), and may be associated with distinct pathologies in the male reproductive tract, including varicocele, leukocytospermia, cryptorchidism, idiopathic infertility, testicular cancer, and urogenital tract inflammation and infection [68]. The dominant external source of ROS (usually generated as part of the host response) in seminal plasma are neutrophils and macrophages. Indeed, leukocytospermic patients are characterized by oxidative stress. However, patients presenting peroxidase-positive leukocytes at concentrations below 1×10^6 mL^{-1} ejaculate also revealed seminal oxidative stress, reflected as high ROS levels or low fertility index (ROS-TAC score) [69, 70]. Moreover, seminal oxidative stress during bacterial prostatitis has also been found, regardless of leukocytospermia status [71]. Male accessory gland infections (MAGI) are closely related to oxidative stress as a result of leukocyte-derived ROS overproduction or reduced antioxidant potential [72–75]. Interestingly, some authors have postulated the existence of a relation-

ship between the degree of oxidative stress and the extension of MAGI. Vicari et al. [76] found higher levels of seminal ROS in prostatovesiculitis than in prostatitis alone. However, the highest ROS production in semen was observed in prostatovesiculoepididymitis. Furthermore, the oxidative stress level in inflammatory semen can be related to the type of infecting, contaminating, or colonizing bacterial strain. It is well documented that bacteria themselves, due to their toxic metabolites and virulence factors, stimulate ROS production in leukocytes; in this context, they can also be a source of external ROS in bacterial semen infections [77]. For example, molecular mechanisms underlying LPS-induced ROS generation in macrophages are well recognized [78]. Both superoxide and hydrogen peroxide are metabolic products of *U. urealyticum* [79]. Indeed, abnormally elevated ROS levels in semen samples among prostatitis patients with positive *U. urealyticum* cultures have been shown [26]. It is known that *B. urealyticus* produces superoxide dismutase (SOD) that supplies additional amounts of hydrogen peroxide, which is the most toxic to sperm [80].

The detrimental effects of ROS on sperm function include alterations in both conventional and nonconventional sperm parameters. Patients with MAGI, especially when associated with *C. trachomatis* or *U. urealyticum* infection, often present significantly reduced sperm concentration, total sperm number, motile spermatozoa, or sperm with normal morphology [13, 24]. However, the degree of impaired semen quality seems to be directly related to the extension of MAGI [72]. Semen hyperviscosity turns out to be associated with increased oxidative stress in infertile patients with bacterial prostatitis, especially when the infection extends to the seminal vesicles [81]. Apart from the changes in sperm characteristics, seminal oxidative stress may be a cause of interaction with all sperm components, especially with polyunsaturated fatty acids, which are present in large amounts in sperm membranes. Sperm membrane lipid peroxidation status, measured by malondialdehyde (MDA) concentration, is the main nonconventional measurable marker of oxidative stress in semen. Malondialdehyde levels have often been correlated with deterioration of sperm quality [82–84]. An association between the presence of some known pathogens, such as *U. urealyticum* or *C. trachomatis*, and the induction of sperm lipid peroxidation, as judged by the MDA levels, has also been suggested [17, 26]. Moreover, there are suggestions that, in the presence of bacterial urogenital infections and varicocele, MDA might be considered a marker of altered sperm quality, and especially reduced sperm motility [85]. A recent

study by our group supports evidence that, under *in vitro* conditions, some types of bacteria (both known pathogens and conditionally pathogenic types) may be important inducers of oxidative stress responsible for the destruction of sperm membranes, which in turn may lead to subfertility expressed as a reduction in penetrated oocytes in SPA. Additionally, our measurements have demonstrated that the harmful activity of bacterial agents towards spermatozoa do not require the mediation of leukocytes. However, these latter were additional mediators that worsened sperm fertilizing potential through peroxidative damage of sperm membranes [9]. Regardless of whether MDA may be a marker of semen infection or not, the process of lipid sperm membrane peroxidation is a crucial factor in the aetiology of defective sperm function during bacteriospermia and leukocytospermia.

Although seminal leukocytes have the potential to cause oxidative stress, the intrinsic ROS production of spermatozoa themselves also leads to oxidative damage in male gametes, once it exceeds the ability of intrinsic sperm enzymatic and nonenzymatic systems to its neutralization [86, 87]. Some investigators have reported stronger relationships between intrinsic than extrinsic ROS production in semen and sperm DNA integrity [49]. The factors responsible for excessive ROS generation by spermatozoa are not entirely clear. Among them, age, environmental, and lifestyle factors are most frequently mentioned in the literature [88–91]. Interestingly, leukocytospermia has been shown to play a role in stimulating ROS production by the sperm themselves [58, 92]. The interactions between spermatozoa and leukocytes in the context of the oxidoreductive potential of ejaculated spermatozoa have also been studied by our group. Previously, we have demonstrated that ejaculates contain sperm populations that differ in oxidoreductive potential. This may influence their function and response to the surrounding environment [93]. In another study, the presence of bacteria was found to decrease the effective neutralization of leukocyte-derived ROS by sperm, depending on the type of bacterial strain and the sperm subpopulation used [80]. These are strong suggestions that inflammatory mediators, such as bacteria and leukocytes, are responsible for quantitative and qualitative changes in the oxygen metabolism of spermatozoa; this, in turn, determines the magnitude of the interaction between toxic oxygen metabolites and cell macromolecules with subsequent consequences for sperm fertilizing potential.

There is now increasing evidence that mitochondria are a major source of intracellular ROS in spermatozoa [94, 95]. One novel concept suggests that mitochondrial ROS generation may decide whether

spermatozoon remains alive or enters an apoptotic state; its role in the aetiology of male infertility has most recently been postulated in the literature [96].

Apoptosis/necrosis

Apoptosis is a natural process that regulates sperm cell numbers through the stages of spermatogenesis, and is one of the principal mechanisms by which abnormal or dead spermatozoa are eliminated. However, the induction of an apoptotic process in ejaculated human sperm is still controversial. Several authors have suggested the involvement of sperm apoptosis in impaired men's fertility with consequences for reducing sperm fertilizing potential [97–99]. Mature spermatozoa have been reported to express distinct markers of early and late apoptosis-related cell damage, including the externalization of phosphatidylserine (PS) from the inner to the outer membrane, caspase activation, the loss of mitochondrial membrane potential ($\Delta\psi$m), and DNA fragmentation [96]. According to a novel theory regarding apoptotic process in mature spermatozoa, the increase in the production of ROS by the mitochondria is one of the first signs of intrinsic apoptosis. Mitochondrial ROS generation is closely associated with the lipid sperm membrane peroxidation cascade that generates electrophilic lipid aldehydes. The latter form adducts with sperm proteins, and also with proteins of the mitochondrial electron transport chain, and triggers mitochondrial ROS production with a subsequent detrimental effect on sperm motility [96, 100]. At the same time, there occur other features of the apoptotic process, such as the externalization of PS and the activation of caspases. Finally, oxidative stress, as a result of the self-perpetuating cascade of mitochondrial ROS production and lipid peroxidation, culminates in DNA fragmentation and sperm death caused by hydrogen peroxide, which is known to readily diffuse through the membranes [96, 101].

The increase in the percentage of sperm with apoptotic phenotype has often been reported to be higher in ejaculates from subfertile and infertile men with clinical conditions associated with oxidative stress, including varicocele [102], idiopathic infertility [103], and urogenital tract inflammation and infection [104, 105]. During bacterial semen infection, changes in sperm apoptotic markers can be attributed to both leukocytes and bacteria. The direct relationship between seminal leukocytes and apoptotic sperm characteristics has been postulated in both leukocytospermic [59] and nonleukocytospermic patients [106]; and in both cases, the mediating role of phagocyte-generated ROS was strongly suggested [72, 107]. The involvement of bacteria in the induction of apoptosis of ejaculated

human sperm is the subject of intense research. There are strong premises that the direct contact of bacteria and their toxins with spermatozoa is an initial signal for germ cell death. This is evidenced by experimental reports in which different types of bacterial strains directly increased apoptotic features in ejaculated human spermatozoa, without the mediation of external ROS generated by leukocytes [11, 31]. The proapoptotic effect of bacteria on sperm may mostly or at least partially be the consequence of bacterial endotoxins, including LPS, porins, and peptidoglycans *via* Toll-like receptors (TLR) 2 and 4, of which expression in the membranes of human spermatozoa has been demonstrated [108]. To date, there have been relatively few studies of the direct influence of individual bacterial strains infecting male urogenital tract on sperm apoptosis. In this context, the apoptosis-inducing mechanism of the well-known pathogenic species *C. trachomatis* and *E. coli* is best documented. The exposure of sperm to *C. trachomatis* LPS has been reported to cause an increase in the production of ROS and caspase-mediated sperm apoptosis, as a result of interaction of LPS with CD14 on the sperm surface [109]. Satta et al. [18] observed sperm PS externalization and DNA fragmentation during experimental *C. trachomatis* semen infection. Lastly, *C. trachomatis* infection of semen *in vivo* has been found to increase mitochondrial depolarization, as well as caspase-3 activation [15]. A significant reduction in $\Delta\psi$m, viability, and motility was also observed after the incubation of sperm with *E. coli* and its soluble factors [7]. In another experimental study, the loss of sperm motility induced by the soluble products of *E. coli* was accompanied by the increased intrinsic mitochondrial generation of ROS and membrane lipid peroxidation [8]. In line with these findings, our original experimental data have demonstrated a direct role for conditionally pathogenic bacterial strains, *S. haemolyticus* and *B. ureolyticus*, in the induction of sperm apoptosis, as reflected by reductions in sperm motility, mitochondrial depolarization, lipid peroxidation, loss of sperm membrane asymmetry, PS externalization, and DNA fragmentation with subsequently reduced competence of sperm–oocyte fusion [9, 31]. All the above data have provided molecular evidence for the induction of sperm death by distinct bacterial agents (differing in pathogenicity and metabolism), partially due to intrinsic apoptotic cascade.

There are strong suggestions that apoptosis is induced by different types of bacteria. However, the origin of sperm DNA damage during bacteriospermia may be more complex. Our data have demonstrated that, during experimental semen infection *in vitro*, ejaculated human spermatozoa exhibited reduced viability visible in HOS tests and increased proportions

of dead sperm [9]. However, DNA fragmentation was observed in both apoptotic and necrotic sperm, and the most part of the latter [31]. This is in agreement with the ultrastructural findings of Collodel et al. [110], who observed a high percentage of sperm with anomalies typical of apoptosis, as well as of necrosis, from individuals affected by urogenital bacterial infection, although the percentage of necrotic sperm was predominant in the majority of infected semen samples. Moreover, sperm apoptosis and necrosis were also induced under experimental high-ROS conditions [111]. These observations are supported by a recent report that showed a lack of correlation between ROS and DNA fragmentation in semen, as well as a correlation between sperm-damaged DNA and mitochondrial depolarization, suggesting that intrinsic mitochondrial-dependent apoptotic pathways might not have a major impact on sperm DNA fragmentation [112]. It should be mentioned, however, that low co-presence of DNA fragmentation, oxidative damage and apoptotic markers in live sperm have also been demonstrated recently [113]. There is no doubt that the bacterial infection of semen causes severe damage to ejaculated sperm that results in ROS-generated cell death, due to both apoptosis and necrosis. However, the mechanism underlying ROS-induced necrosis under inflammatory conditions is questionable. Future studies could explain whether bacterial semen infection causes sperm death due to the necrosis itself or by necrosis as the final step of the apoptotic process, as suggested by some authors [110].

With respect to the relationship between sperm apoptotic markers and the fertility potential of spermatozoa, the published data are still controversial. However, the vast majority of authors agree that the determination of apoptotic markers in spermatozoa, usually measured by flow cytometry, has better potential to predict fertility in clinical practice than conventional semen parameters. Some authors have shown that, among the apoptotic markers, DNA status is the best predictor of both natural [99] and assisted [114, 115] conception. In contrast to these studies, Zhang et al. [116] have suggested that two early apoptotic changes, such as PS externalization and decreased $\Delta\psi$m, might be the best markers for diagnosing male infertility. Sperm with normal phospholipid asymmetry, characterized by a lack of PS externalization, has been demonstrated to have a superior ability to capacitate and a high capability of achieving full fertile potential estimated in a SPA assay [97, 117]. These findings correspond with our recent study demonstrating numerous correlations between reduced penetration of hamster zona-free oocytes by

human spermatozoa and an increase in the scrambling of the plasma membrane phospholipids, as measured by merocyanine 540 (M540) dye during experimental semen bacterial infection [9]. It should be emphasized that, in human spermatozoa, a loss of lipid asymmetry in the M540 test also reflects degenerative membrane modifications that occur during apoptotic events [118, 119]. In turn, other investigators have found a positive correlation between peroxidative lipid sperm membrane damage, as reflected by MDA levels, and sperm fertility index, calculated on the basis of the number of sperm free of structural defects, sperm necrosis, and apoptosis (visible in transmission electron microscopy) in a group of infertile men with urogenital tract infections [85]. Taking into consideration all these results, we can state that, although human sperm DNA is susceptible to damage as a result of bacterial semen infection, changes in lipid sperm membrane structures are critical for sperm fertilizing potential in this pathological condition. Moreover, this statement supports the general opinion postulated by some authors regarding the role of sperm DNA integrity as a predictor of the genetic quality of the embryo rather than of fertility [120, 121]. However, further research is required in order to recommend the marker with the best prognostic power in clinical practice reflecting lipid sperm membrane damage during bacterial semen infection.

Role of the immune/autoimmune reactions

An interplay between the immune and reproductive systems is involved in the mechanism of sperm damage as a consequence of local inflammation or infection; the participation of immune factors including cytokines, chemokines, and growth factors must also be taken into account. The main source of immune factors in the male reproductive tract is the testes, where they are involved in the regulation of spermatogenesis and the maintenance of the privileged immunosuppressive status of gonads [122]. The local production of cytokines in the secondary sex glands, irrespective of spermatogenesis, has also been postulated [123–126]. The proinflammatory cytokines produced and released in large amounts by infiltrating leukocytes (such as macrophages, lymphocytes, monocytes, and dendritic cells) support host defence mechanisms in cases of local infection, but also participate in various pathophysiological reactions. Studies using experimental models of autoimmune orchitis have demonstrated that the complex network of proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, interferon (IFN)-γ, and interleukins (IL)-6, IL-12, IL-17, and IL-23 play critical roles in inducing permeability of the blood–testis barrier and

immature germ cell apoptosis, due to their direct effects on adherens and tight junction restructuring events [127–129]. Finally, these factors can be involved in the disruption of tolerance and the production of autoantibodies against germ cell antigens (ASA). Opinions about the relevance of ASA in semen, as measured by the mixed antiglobulin reaction (MAR) and immunobead tests in urogenital tract infections are divided. There are reports demonstrating a significantly higher ASA incidence in seminal plasma from patients with prostatitis and epididymitis, as compared with noninflammatory group [130, 131]. In contrast to these studies, others suggested the lack of correlation between the presence of ASA and inflammation/infection in semen [132]. Probably, it may be connected with molecular similarities between different strains of bacteria and sperm antigens, as previously demonstrated [133]. Regardless of a clinical relevance of seminal ASA in the urogenital tract inflammations/infections, the mediating role of upregulated cytokines during orchitis and epididymo-orchitis, leading to the impairment of spermatogenesis and loss of tolerance, remains to be fully appreciated.

The modulating role of cytokines in inflammatory reactions due to the direct effect on pro-oxidative and antioxidative systems (to the advantage of the ROS) has been postulated by our group and other investigators [134–137]. In this context, cytokines may play a crucial role in the perpetuation of semen inflammation and infection, and for this reason these bioactive substances may constitute an important link between the inflammation or infection of the urogenital tract and infertility status [138]. The adverse effect of cytokines on sperm membrane properties might constitute one of the several mechanisms by which these immune factors interfere with sperm quality during semen bacterial infection. An increase in lipid sperm membrane peroxidation, as judged by MDA levels, has been demonstrated in the presence of some proinflammatory cytokines under both *in situ* [139, 140] and *in vitro* conditions following the incubation of sperm with human recombinant proinflammatory cytokines [141, 142]. However, the harmful effect of cytokines on sperm membranes appears to be closely associated with the accompanying leukocytospermia. This has been confirmed by numerous reports that have documented correlations between seminal leukocyte count and proinflammatory cytokine concentrations [140, 143–148]. Also in our own *in vitro* observations, the peroxidative damage of sperm membrane lipids was markedly enhanced in the simultaneous presence of cytokines and leukocytes [149]. Most probably, oxidative stress during semen inflammation and infection is modulated by the levels of certain cytokines;

even more, during leukocytospermia, the ROS and cytokines produced by the leukocytes cooperate with each other, provoking a destructive effect on sperm membranes.

The participation of cytokines in the induction of human ejaculated sperm apoptosis has also been suggested. Among the various inflammatory cytokines, TNF-α, one of the major cytokines produced during inflammation and infection, is most often presented as the inducer of human sperm apoptosis. In a few experimental *in vitro* studies, this cytokine has been found to increase the percentage of ejaculated spermatozoa with PS externalization or DNA fragmentation [150, 151]. These observations are supported by the findings of Allam et al. [104], who detected a correlation between seminal TNF-α level and apoptotic sperm in ejaculates from men with chronic urogenital tract inflammations. The proapoptotic effect of TNF-α on mature sperm cells can be mediated through ROS and nitric oxide production [152–154]. Little is known about the pro-apoptotic properties of IL-6, a proinflammatory cytokine produced in large amounts during semen infection. In our experimental study, IL-6 concomitantly applied with IL-8 was able to increase the DNA fragmentation of ejaculated human spermatozoa [155]. The effect of IL-6 most likely occurred through binding to its receptor, IL-6R alpha, of which presence has been reported in ejaculated human spermatozoa [156]. However, the hypothesis of IL-6 or IL-8 contribution to sperm apoptosis has not so far been confirmed in clinical studies. The influence of other proinflammatory cytokines, such as IL-1β and IL-18 on apoptosis *via* induction of the Fas/Fas ligand (FasL) system has been already documented in somatic cells [157, 158]. Some authors have described the harmful effects of IL-18 on sperm concentration and motility in infertile men with urogenital tract infections [159, 160]. Additionally, a combination of IL-18 with IL-12 was linked with a high sperm fragmentation index in an *in vitro* model of semen bacterial infection [155]. However, the mechanism by which these cytokines induce apoptosis in mature spermatozoa is not yet understood, and is currently under investigation.

In the case of bacterial urogenital tract infections, many cytokines have been demonstrated in elevated concentrations in semen [135, 161]. The list of published articles indicating direct connections between various cytokine levels in seminal fluid and sperm quality is long [81, 162]. Most investigators have suggested that seminal cytokines are mostly associated with the presence of leukocytes [163–165]. Elevated cytokine presence in the semen has been also attributed to pathogens [135, 162, 166]. However, there are studies in which seminal pathogens have been

found to have no influence on cytokine levels [167, 168]. Among the various inflammatory cytokines, seminal IL-1β, IL-6, and IL-18 have been proposed for the role of specific biomarkers discriminating between patients with or without MAGI [165, 169, 170]. Moreover, according to some clinicians, some cytokines can be a part of the evaluation of the host inflammatory response in patients with complicated MAGI, in order to identify subjects who require additional antioxidant administration after antimicrobial treatment [76]. In turn, the seminal chemokine IL-8 has been also suggested as one of the most reliable and predictive surrogate markers of prostatitis [171]. Undoubtedly, cytokines, chemokines, and growth factors are an essential part of the inflammatory effect caused by bacteria and leukocytes. The number of clinical studies on the significance of the detection of cytokines in seminal plasma, especially in the context of bacterial urogenital tract infection, continues to increase. Assuming that cytokines do not act in isolation, but rather as part of a network, we cannot exclude the possibility that the toxicity of one cytokine to spermatozoa can be increased in the presence of the other cytokines. Most probably, interactions among numerous immune factors can create specific micropatterns of cytokines in semen that could delineate the infectious entity responsible for male infertility. It appears that controlled prospective studies that include a large number of patients and analyse a wide range of cytokines are urgently required to answer the question of the applicability of these factors as potential biomarkers.

Conclusions

Bacterial infection of the male urogenital tract has been associated with subfertility and infertility. However, the mechanism by which it affects fertility is complex and multifactorial (Figure 2). Distinct inflammatory reactions within the male urogenital tract are inevitably connected to oxidative stress, being the sum of microenvironment and sperm intrinsic damage. The induction of sperm apoptosis contributes as an important pathogenic mechanism by which both bacteria and leukocytes can alter human spermatozoa. However, understanding of the nature of sperm–leukocyte interactions during male urogenital tract bacterial infection is not yet complete. Most probably, the monitoring of a complex network of molecular, biochemical, immunological, oxidative, and inflammatory parameters in semen may offer a chance to better understand the mechanisms leading to subfertility and infertility caused or complicated by microbial infection of the semen. The value of such observations would be significant,
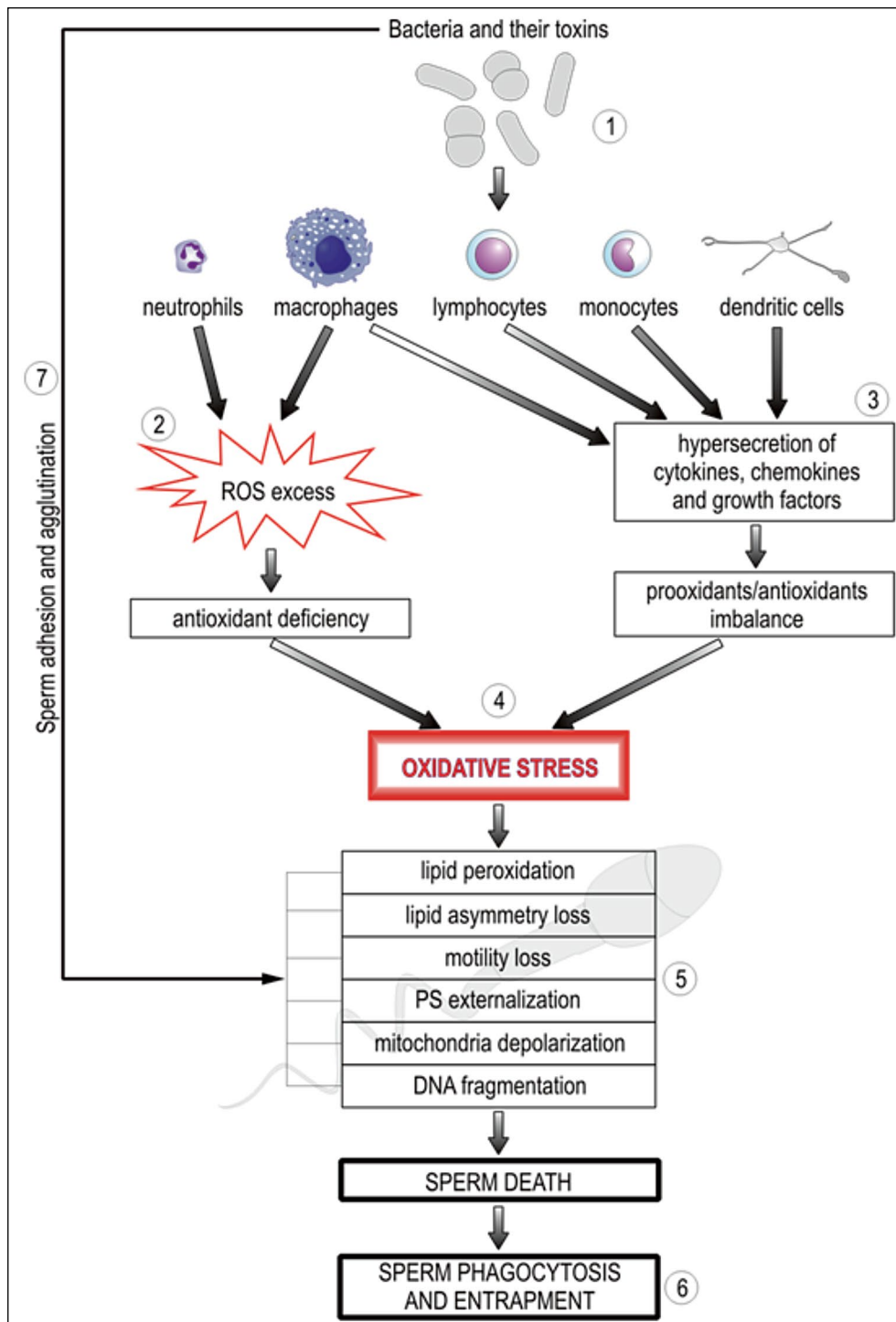
Figure 2. Proposed mechanisms of the harmful effect of bacterial semen infection on human spermatozoa. The diagram has been developed on the basis of our previously published results [9, 10, 31, 39, 51]. **(1)** Bacteria and their toxins trigger the infiltration of immune cells connected with **(2)** the production and release of large amounts of ROS (neutrophils, macrophages) as well as **(3)** immune regulatory factors (from macrophages, lymphocytes, monocytes, and dendritic cells); the cytokines may modulate the activities of the prooxidative and antioxidative systems, which may also result in the enhanced secretion of ROS; **(4)** When the amounts of ROS exceed the potential of the antioxidative defence, oxidative stress occurs; **(5)** Oxidative stress induces lipid sperm membrane peroxidation and leads to a series of detrimental defects in the spermatozoa, including lipid asymmetry loss, motility loss, PS externalization, and loss of ΔΨm. The changes culminate in DNA fragmentation and sperm death (apoptosis and necrosis); **(6)** Damaged and dead spermatozoa are eliminated by traditional phagocytosis and trapping mechanisms; **(7)** Bacteria kill sperm without the mediation of external ROS generated by leukocytes. Bacteria immobilize sperm and damage their morphology and function by tightly adhering to spermatozoa as well as by expressing surface virulence factors, and releasing soluble spermatotoxic factors. Modified from [162]. Abbreviations: ROS — reactive oxygen species; PS — phosphatidylserine

and would support the development of new diagnostic platforms (biomarkers) for infertile males with semen infections, which are seen in a considerable number of men attempting both natural and assisted procreation. Key points:

— Sperm motility and phospholipid sperm membrane status are the most sensitive indicators of sperm damage during bacterial semen infection, which can be attributed to both bacteria and leukocytes.

— Bacteria and their toxins may be harmful to ejaculated spermatozoa independently of leukocyte contamination, although the concomitant presence of bacteria clearly enhances the harmful effects of leukocyte-generated oxidative stress by inducing the peroxidative damage of sperm membranes to a level above which their ability to penetrate and fuse with an oocyte may be significantly reduced.

— Induction of intrinsic apoptotic pathway could be an important pathomechanism by which both pathogenic and conditionally pathogenic bacteria can kill sperm cells. Extended semen microbiological diagnostics are recommended, especially in patients consulted for infertility and qualified for assisted reproduction techniques.

— Future research should be focused on the search for new seminal biomarkers for the noninvasive early diagnosis of asymptomatic urogenital tract infection.

Acknowledgements

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