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The effect of bacteriospermia and leukocytospermia on conventional and nonconventional semen parameters in healthy young normozoospermic males

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ABSTRACT

Bacterial semen inflammation/infection is an important diagnostic and therapeutic problem in contemporary andrology. The molecular mechanism by which inflammatory mediators compromise the fertilizing potential of germ cells is complex and multifactorial, and it remains unclear. To improve the understanding of the pathophysiology of human subfertility/infertility caused or complicated by reproductive tract inflammation/infection, we simultaneously evaluated a set of conventional (standard semen analysis) and nonconventional sperm parameters, including subcellular changes in sperm membranes (phospholipid scrambling, peroxidative damage, and phosphatidylserine (PS) externalization), mitochondria (mitochondrial transmembrane potential, ΔΨm, and oxidoreductive capability), and DNA fragmentation in healthy young normozoospermic males with asymptomatic bacteriospermia and leukocytospermia. Both bacteriospermia and leukocytospermia had a deleterious effect on standard sperm parameters, including sperm concentration, motility and morphology. Bacteriospermia was associated with a simultaneous decrease in mitochondrial transmembrane potential and an increase in PS externalization, and with DNA fragmentation in both live and dead sperm. The highest MDA concentrations in sperm lysates were observed in the presence of leukocytes. This study demonstrates for the first time that bacteriospermia and leukocytospermia compromise sperm quality in healthy young normozoospermic males. Bacteria mainly participate in intrinsic mitochondria-dependent apoptotic cell death mechanisms. Oxidative stress plays a relevant role in decreasing routine sperm parameters during leukocytospermia. The value of these observations may be significant and may support the development of a new diagnostic platform (biomarkers) for infertile males with infections in the reproductive tract.
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1. Introduction

Urogenital tract inflammations/infections represent a significant problem in andrology and are responsible for up to 15% of male infertility cases (Pellati et al., 2008). Despite the increasing number of reports regarding the cause-and-effect relationship between bacterial infections and male infertility, many important aspects remain to be elucidated. There are some pathophysiological concepts to explain how semen bacterial infections might interfere with the functional competence of ejaculated human spermatozoa. They include direct and indirect mechanisms involving both of the main inflammatory mediators: bacteria and infiltrating leukocytes (Fraczek and Kurpisz, 2015).

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According to the kinetics of bacterial urogenital tract infection, in the first stage, shortly after the invasion of the male genital tract by microorganisms, bacteria can be observed in the semen without a significant number of attracted leukocytes. The latter appear in the ejaculate at the second stage, in which they coexist with bacteria, and then they remain in the semen for some length of time following the elimination of the bacteria; the third stage of infection is often represented by isolated leukocytospermia (Sanocka et al., 2004; Fraczek and Kurpisz, 2007). Most probably, the detrimental effects of bacteriospermia and leukocytospermia on ejaculated spermatozoa can vary throughout the course of urogenital tract infection, but can be distinguished by the distinct stages mentioned (Sanocka et al., 2004; Domes et al., 2012).

Many articles have demonstrated the direct and indirect negative effects of individual bacterial agents identified during the routine analysis of the semen of infertile patients. Conventional sperm parameters, including concentration, motility and/or morphology of ejaculated sperm, are markedly affected by both pathogenic and conditionally pathogenic bacteria, including *Escherichia (E.) coli*, *Chlamydia (C.) trachomatis*, *Ureaplasma (U.) urealyticum*, *Mycoplasma (M.) hominis*, *M. genitalium*, *Enterococcus (E.) faecalis*, *Staphylococcus (S.) aureus*, *Pseudomonas (P.) aeruginosa*, *Bacteroides (B.) ureolyticus*, *Streptococcus (Str.) viridans*, *S. haemolyticus*, *S. saprophyticus*, and *Gardnerella (G.) vaginalis* (Fraczek and Kurpisz, 2015). However, most of these findings were obtained from *in vitro* studies and have not yet been confirmed in *in vivo* conditions. As for leukocytes, they were also significantly correlated with standard sperm characteristics (Saleh et al., 2002; Bezold et al., 2007; Domes et al., 2012; Moretti et al., 2014). In contrast, some authors did not see any correlation between leukocyte count and decreased routine sperm parameters (Aitken et al., 1994; Barraud-Lange et al., 2011). In examining the impact of bacteria and leukocytes on conventional sperm parameters, we cannot forget that factors other than standard semen (nonconventional) parameters can also be affected by urogenital tract infections. Moreover, conventional semen parameters do not provide information about the pathophysiological mechanisms through which bacterial infections lead to disturbances in ejaculated human spermatozoa.

Our research group has demonstrated the detrimental effects of different types of bacterial strains and/or leukocytes on human sperm structure and function in a range of papers (Fraczek et al., 2012, 2014, 2015). Here, we attempted to verify the previous results obtained in the experimental *in vitro* model of bacterial semen infection and examined conventional (standard semen analysis) and nonconventional sperm parameters during significant bacteriospermia and/or leukocytospermia *in situ*. In this study, we offer a comprehensive examination of the molecular status of sperm membranes, mitochondria and DNA, which may provide a clearer picture of the subcellular changes occurring in ejaculated spermatozoa over the course of bacterial semen infection. To better understand the pathomechanism of male subfertility/infertility caused or complicated by this pathology, reciprocal relationships between the studied parameters were assessed. To our knowledge, this is the first study in which a set of conventional and nonconventional sperm parameters was simultaneously demonstrated in healthy young normozoospermic men with asymptomatic bacteriospermia and/or leukocytospermia.

2. Materials and methods

2.1. Reagents and chemicals

Phosphate buffered saline (PBS) was purchased from Biomed (Lublin, Poland). The LIVE/DEAD Sperm Viability Kit, JC-1 probe and LIVE/DEAD Fixable Dead Cell Far Red Stain was from Molecular Probes, Inc. (Eugene, OR, USA) and the OxiSelect TBARS Assay Kit for malondialdehyde (MDA) quantitation was from Cell Biolabs, Inc. (San Diego, CA, USA). The Annexin V-FITC Apoptosis Detection Kit was from Beckman Coulter (Fullerton, CA, USA), while the FlowTACS Apoptosis Detection Kit was from Trevigen, Inc. (Gaithersburg, MD, USA). The remaining chemicals were purchased from Sigma Chemical Co (St. Louis, MO, USA).

2.2. Flow cytometry analysis

Cytofluorometric evaluation of spermatozoa was performed using a Beckman Coulter flow cytometer (Cell Lab Quanta SC MPL, Beckman Coulter, Fullerton, CA, USA) equipped with a 488 nm argon-ion laser. Samples were measured at a flow rate of 200–250 cells per second. A minimum 10 000 events were collected for each sample evaluated. The sperm population was gated on the basis of the electronic volume (EV, parameter depends on the cell size) and side scatter (SS, parameter depends on cellular granules) measurements. Green (480–550 nm) and red (590–670 nm) fluorescence was detected using the FL1 and FL3 channels, respectively. The fluorescence data were obtained at a fixed gain setting in logarithmic (FL1, FL3) mode. Fluorescence measurements were repeated two times with distinct samples. Data were analysed using the Cell Lab Quanta SC MPL Analysis software (Beckman Coulter).

2.3. Semen sample collection

Altogether, the study population included healthy young normozoospermic volunteers (n = 101) between 20 and 35 years of age. They were recruited by the Andrology Outpatient Clinic and the Andrology Unit of the University of Medical Sciences in Poznan. Subjects with varicocele were excluded from the study. Freshly ejaculated semen samples were obtained by masturbation after 3–5 days of sexual abstinence and collected into sterile containers. The males were instructed to urinate and to wash their penis, scrotum and hands before ejaculation. The study was approved by the Local Bioethical Committee, Medical University of Poznan, Poland. Written informed consent was obtained from each participant.

2.4. Standard semen analysis

Within 60 min of ejaculation and liquefaction, standard semen analysis was manually performed according to the World Health Organization guidelines (WHO, 2010). In brief, sperm concentration was determined using an improved Neubauer haemocytometer (Paul Marienfeld, Lauda-Königshofen, Germany). Motility characteristics were evaluated using the standard grading system: progressive motility, nonprogressive motility and immotility. Sperm viability was assessed by eosin-fixed smears. Additionally, the hypo-osmotic swelling (HOS) test was performed. Sperm morphology and the teratozoospermia index (TZI) were assessed according to strict Kruger's criteria following Papanicolaou staining of the semen smears. Peroxidase staining was used to count and differentiate peroxidase-positive leukocytes and peroxidase-negative round cells (other round cells) in the ejaculate. Leukocytospermia was indicated by a peroxidase-positive leukocyte concentration $\geq 1 \times 10^6$ per mL (WHO, 2010). All the tested samples were also evaluated for the presence of sperm antibodies using the direct immunobead test (DIBT; Irvine Scientific, Santa Ana, CA, USA).

2.5. Microbiological screening

An aliquot of semen (200 μl) was transported within half an hour to the Microbiology Unit of Poznan Hospital Medical College for comprehensive microbiological screening, including aerobic,

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