Fertilizing potential of ejaculated human spermatozoa during in vitro semen bacterial infection

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Objective: To assess the in vitro effect of three bacterial isolates (*Escherichia coli*, serotype O75:HNT, *Staphylococcus haemolyticus*, and *Bacteroides ureolyticus*) and/or leukocytes on sperm motility, subcellular changes in sperm plasma membranes, and sperm fertilizing potential.
Design: An in vitro model of semen bacterial infection.
Setting: Basic research laboratory.
Patient(s): Healthy normozoospermic volunteers and healthy blood donors.
Intervention(s): None.
Main Outcome Measure(s): Sperm plasma membrane stability was evaluated with a LIVE/DEAD Sperm Viability Kit and with the merocyanine 540 (M540) test both performed using flow cytometry. An oxiSelect TBARS Assay Kit was used for quantitative measurement of malondialdehyde content. Functional ability of spermatozoa was assessed by hypo-osmotic swelling (HOS) test and sperm penetration assay (SPA).
Result(s): The incubation of sperm with bacteria and/or leukocytes was associated with the reduction of their fertilizing potential demonstrated in both the HOS test and SPA, and this effect can be considered as a natural consequence of diminished motility and sperm membrane injury of lipid bilayers. *Bacteroides ureolyticus* demonstrated the most significant detrimental effect on sperm structure and function.
Conclusion(s): Sperm motility and lipid sperm membrane status might be the earliest and the most sensitive indicators of sperm damage with negative consequences for male factor fertility, which can be attributed to both bacteria and leukocytes action. (Fertil Steril® 2014;102:711–9. ©2014 by American Society for Reproductive Medicine.)
Key Words: Semen bacterial infection, sperm plasma membranes, sperm fertilizing potential

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The potential negative influence of genitourinary tract inflammation/infection on sperm fertilizing ability has been long debated (1). Recently, some investigators have discussed the long-term effects of the local inflammatory process and have postulated its role as the one of possible reasons for impaired reproductive quality in middle-aged men (2). In the context of these reports, the appropriate diagnostic algorithm of urogenital inflammation/infection and rapid initiation of anti-inflammatory treatment (before the reproductive potential of the sperm is severely affected) became important issues in contemporary andrology. Attention has been focused on the search for new biomarkers of semen inflammation/infection, which are often beyond the scope of routine seminological

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analysis. The direction of research has been determined by the kinetics of the inflammatory process showing the relationship among the infectious factor, leukocytes, and proinflammatory cytokines (3).

Data concerning a significantly increased number of leukocytes and/or bacteria in semen are inconsistent. The presence of seminal peroxidase-positive leukocytes at an amount $\geq 1 \times 10^6$/mL of ejaculate, defined by the World Health Organization (WHO) (4) as leukocytospermia, is still considered to be pathological for sperm, although this threshold value has been repeatedly questioned in the literature (lower concentrations have been proposed), particularly with regard to monitoring the intensity of inflammatory reaction, which differs in time (5–7). Regardless of the number of leukocytes in the semen, most investigators agree that the final effects of the cells of the immune system on spermatozoa may depend on their activity (1, 3, 8). As for bacteriospermia, it may represent semen contamination, colonization, or infection (9, 10). The problem is further complicated by the fact that the same common bacterial strains are often isolated from the semen of both fertile and infertile patients. Moreover, there is a lack of uniformity in defining the critical number of bacteria, above which a decrease in sperm fertilizing potential occurs. On the one hand, according to WHO recommendations (11), a concentration $\geq 1 \times 10^3$ colony-forming unit (CFU)/mL of common uropathogenic bacteria per milliliter of ejaculate is regarded as bacteriospermia. On the other hand, a concentration $\geq 1 \times 10^4$ CFU of pathogenic and nonpathogenic bacterial strain per milliliter of ejaculate has been suggested by most investigators as significant bacteriospermia requiring diagnostics, although practically during active in situ bacterial infection elevated concentrations of bacteria have been often observed (5, 10, 12). It seems that the location of infection in the male genitourinary tract and the diagnostic profile of routine semen microbial culture are of great importance in the assessment of the clinical significance of bacteria in semen.

Among the accepted pathogenic microbial strains causing genitourinary infections associated with suspected male factor infertility, *Escherichia coli*, *Enterococcus faecalis*, *Chlamydia trachomatis*, *Ureaplasma urealyticum*, and *Mycoplasma hominis* are the most often mentioned (13). However, there are also reports of harmful effects on sperm induced by conditionally pathological bacterial strains belonging to *Staphylococci*, *Streptococci*, and anaerobic Gram-negative and Gram-positive rods and other groups (14–16). It seems that an unequal effect of individual species and types of bacteria on germ cells could be significant in the personal assessment of inflammation and the monitoring of anti-inflammatory therapy.

Experimental approaches have shown a possible mechanism of interaction between sperm and single inflammatory mediator, which obviously cannot be observed in situ. There are many experimental data that have revealed the induction of structural changes in purified sperm suspensions by a single bacterial strain, as compared with uninfected sperm, with respect to sperm viability and motility (17–20), mitochondrial membrane potential (20), and phosphatidylserine translocation (20–23); however, none of them have been exclusively focused on sperm fertilizing potential. In the present study, we have attempted to compare the changes in sperm plasma membrane integrity observed during classic experimental in vitro bacterial semen infection with results from functional sperm tests, such as the hypo-osmotic swelling (HOS) test and the sperm penetration assay (SPA). Moreover, these functional results would complement the findings of our earlier molecular, morphological, and cytochemical studies regarding an experimental model of semen infection (16).

MATERIALS AND METHODS

Semen and blood sample collection was approved by the Local Bioethical Committee, Medical University of Poznan. Experiments with animals were approved by the Local Ethical Committee for the Animal Experiments, Poznan University of Life Sciences.

Reagents and Chemicals

Phosphate buffered saline (PBS) was purchased from Biomed. The LIVE/DEAD Sperm Viability Kit (SYBR-14 and propidium iodide [PI]) was from Molecular Probes, and the OxiSelect TBARS Assay Kit for malondialdehyde (MDA) quantitation was from Cell Biolabs. Pregnant mare serum gonadotropin (Folligon, 200 IU) was purchased from Intervet International B.V., and hCG (Pregnyl, 1,500 IU) was from Organon. The remaining chemicals used were purchased from Sigma Chemical Co.

Flow Cytometry Measurements

Flow cytometry analysis of sperm samples was performed using a Beckman Coulter flow cytometer (Cell LabQuanta SC MPL) equipped with a 488-nm argon-ion laser. For each sample, 10,000 events, at a rate of 150–250 events per second, were recorded within the characteristic flame-shaped region in the electronic volume (parameter depends on the cell size) and side scatter (parameter depends on cellular granules) dot plot corresponding to the sperm population. The green (480–550) and red (590–670) fluorescence were detected using the FL1 and FL3 channels, respectively. The fluorescence data were obtained at a fixed gain setting in logarithmic (FL1, FL3) mode. Data were analyzed using Cell LabQuanta SC MPL Analysis software (Beckman Coulter). Fluorescence reading was repeated 2 times from distinct samples.

Semen Sample Collection and Preparation

The study population included healthy volunteers (n = 15), between 20 and 35 years of age, recruited at an andrology outpatient clinic in Poznan, Poland. Selected donors were asymptomatic for genitourinary inflammations and varicocoele. Freshly ejaculated semen samples were collected in sterile containers after 3–5 days of sexual abstinence. Within 60 minutes after ejaculation and liquefaction, the conventional semen analyses were performed according to the WHO 2010 criteria (4). The peroxidative test as originally