Ability of *Escherichia coli* to produce hemolysis leads to a greater pathogenic effect on human sperm

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Objective: To determine the effect on human sperm of *Escherichia coli* strains separated on the basis of their ability to produce hemolysis.
Design: Experimental study.
Setting: University-based laboratory.
Patient(s): Semen samples from healthy donors.
Intervention(s): Five million sperm, selected via the swim-up method, were incubated with 3 *E. coli* concentrations to obtain ratios of sperm to *E. coli* of 1:2, 1:16, and 1:128. The *E. coli* strains were: a hemolytic isolated strain (H), a nonhemolytic American Type Culture Collection strain (NH-ATCC), and a nonhemolytic isolated strain (NH-I).
Main Outcome Measure(s): Aliquots of human sperm were used to measure progressive motility using computer-aided sperm analysis, mitochondrial membrane potential ($\Delta\Psi$m) with a JC-1 (5,5′,6,6′ tetrachloro-1,1′,3,3′-tetraethylbenzamidazolocarbocyanin iodide) and propidium iodide stain, and intracellular reactive oxygen species (iROS) with a dihydroethidium (DHE) stain. Sperm $\Delta\Psi$m and iROS were measured by flow cytometry. Sperm vitality was considered the mean of propidium iodide–negative and DHE-negative cells.
Result(s): Sperm incubated with the H strain in a 1:2 sperm to bacteria ratio demonstrated a significant decrease in motility and $\Delta\Psi$m, and an increase of iROS. The NH-ATCC strain decreased sperm motility and $\Delta\Psi$m, but in a ratio of sperm to bacteria of 1:128; it increased iROS at a ratio of 1:16. The NH-I strain did not affect the analyzed sperm functions, even at a 1:128 sperm to bacteria ratio.
Conclusion(s): Results show a greater pathogenic effect on human sperm of *E. coli* strains with, versus without, hemolytic capacity. (Fertil Steril® 2015;103:1155–61. ©2015 by American Society for Reproductive Medicine.)
Key Words: Human spermatozoa, uropathogenic *Escherichia coli*, mitochondrial membrane potential, motility, reactive oxygen species

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The presence of bacteria in semen, in contrast to the semen in men without infection, has been associated with male infertility (1). Additionally, males with urinary tract infection have impaired sperm motility and vitality (2). Gram-negative bacteria have been associated with male accessory gland infection and included in this group; *Escherichia coli* represents 60%–85% of isolates from prostatic secretion (3). Other authors have reported that *E. coli* represents 69% of the microorganisms isolated from semen in patients with bacterial prostatitis (4), and it has been associated with non–sexually transmitted epididymitis (5).

Escherichia coli is a broad species that colonizes environmental and animal niches (6). There are many intestinal and extraintestinal pathogenic *E. coli* strains, of which the most important is the uropathogenic (UPEC; reviewed in [7]). The O serogroups that are frequent in UPEC are: O1, O2, O4, O6, O7, O8, O16, O18, O25, and O75 (8). These are the same *E. coli* O serogroups that have been reported to cause prostatitis (9). The most-frequent O serogroups of *E. coli* found in the semen of infertile patients are O1, O2, O4, and O6 (10). The UPEC strains have virulence factors such as an adherence system, siderophores, and toxins, highlighting the alpha hemolysin (11), which produces erythrocyte lysis (12).

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Uropathogenic *E. coli* impairs sperm quality because it induces sperm motility loss (13), which has been linked to an adherence effect of *E. coli* on sperm (14), accompanied by sperm agglutination, a mannose-dependent effect (15). Another hypothesis is that *E. coli* affects the sperm membrane architecture (16). In addition, sperm motility has been shown to be impaired by reactive oxygen species (ROS), which are in turn induced by leukocytes that respond to *E. coli* (17). In addition to sperm motility, UPEC has been reported to impair sperm mitochondrial membrane potential ($\Delta\Psi$m) (18). Uropathogenic *E. coli* has been reported to decrease sperm $\Delta\Psi$m and induce lipid membrane scrambling (19). *E. coli* toxins, such as lipopolysaccharide and porins, have been found to induce cell death in sperm (20). In addition, lipopolysaccharide induces DNA (deoxyribonucleic acid) fragmentation in human sperm (21).

We have reported recently that serogroups are not relevant to predicting the pathogenicity of *E. coli* on human sperm in vitro (22). To date, the identity of the toxins that determine higher pathogenicity in human sperm is unknown; therefore, the objective of this work was to determine the in vitro effect on human sperm of *E. coli* strains that have been separated on the basis of their ability to produce hemolysis.

MATERIALS AND METHODS

Sperm Collection and Selection

Human semen was obtained from apparently healthy men, via masturbation, according to recommendations from the World Health Organization (23). Semen donors were interviewed and signed the informed consent form, which was reviewed and approved (file 45/012) by the Ethics Committee of the Faculty of Medicine at the Universidad de La Frontera, Temuco, Chile.

Sperm selection was conducted by the direct swim-up method. Briefly, 150 μL of semen were deposited in the bottom of a sterile conical polystyrene tube containing 500 μL of human tubal fluid (HTF) (24), without antibiotics, and incubated for 1 hour at 37°C at a 45° angle. After swim-up incubation, 400 μL of the top phase was collected and transferred to another sterile tube. Next, a cell count of the collected sperm was performed in a Neubauer chamber.

Escherichia coli Collection, Identification, Typification, and Growth

Strains of UPEC were isolated from the urine of patients who had a urinary tract infection. The *E. coli* were identified according to Bergey's Manual (25), and the hemolytic condition of *E. coli* was established according to its growth in blood agar. Typification of *E. coli* was performed with O antigen–specific antisera (SSI Diagnostica), according to manufacturer instructions.

The experiments were conducted with 3 UPEC strains: an isolated hemolytic *E. coli* strain (H), an isolated nonhemolytic *E. coli* strain (NH-I), and nonhemolytic American Type Culture Collection (NH-ATCC) strain 25922. *Escherichia coli* O-antigen typification was: O25 to the H strain, O75 to the NH-I strain, and O6 to the NH-ATCC strain. The UPEC and sperm were incubated in HTF without antibiotics, because streptomycin could inactivate the metabolism of *E. coli* strains and would interfere with the pathogenic activity of the UPEC strains. The 3 UPEC strains were incubated with HTF, and with HTF without antibiotics, to test growth capacity. The growth of the UPEC strains at 1 hour of incubation with both media was observed to be negligible (data not shown); therefore the initial number of bacteria added to each was kept constant during the incubation time.

Experimental Procedure

Five million sperm selected via the swim-up method were incubated with each of the 3 *E. coli* strains, separately: H, NH-I, and NH-ATCC. Each UPEC strain was used at 10 x 10^6 colony-forming units (CFU), 80 x 10^6 CFU, and 640 x 10^6 CFU, to obtain a ratio of sperm to *E. coli* of 1:2, 1:16, and 1:128. As a baseline control, sperm were incubated without *E. coli* (a sperm to *E. coli* ratio of 1:0).

The 3 experimental groups, and the baseline control without bacteria, were adjusted to 1 mL with HTF without antibiotics and were incubated for 1 hour at 37°C. Later, aliquots of sperm suspension were removed from each experimental group and the baseline control to assess progressive motility, vitality, $\Delta\Psi$m, and intracellular ROS (iROS). A total of 4 independent replicates were performed on different days for each *E. coli* strain.

Sperm motility. Ten microliters of each sperm suspension were used to measure sperm progressive motility by conducting computer-aided sperm analysis with Integrated Sperm Analysis System (ISAS) software, version 1 (Proiser). Each measurement was taken twice.

Sperm mitochondrial membrane potential. Changes in sperm $\Delta\Psi$m were measured with 5,5′,6,6′ tetrachloro-1,1′,3,3′-tetraethylbenzamidazolocarbocyanin iodide (JC-1; Mît-E-Ψ mitochondrial permeability detection kit; Enzo Life Sciences Inc). The JC-1 stain enters all the cell compartments in a monomeric form, fluorescing green. When JC-1 enters mitochondria, it fluoresces orange, because it aggregates and cannot exit the mitochondrial matrix, owing to differences in charges between the matrix and the mitochondrial intermembrane space (26). Thus, changes in sperm $\Delta\Psi$m can be measured as changes in the fluorescence of JC-1 aggregates.

The JC-1 stain was combined with propidium iodide (Sigma Aldrich) as the vital stain, which enters cells that have membrane disruptions, binds DNA, and fluoresces red. Briefly, 1 x 10^6 sperm suspended in 1 mL of HTF were stained with 1 μL of JC-1 100X. After 15 minutes, 1 μL of 1 mmol/L propidium iodide was added to each test and incubated for 2 minutes at 37°C. After that, sperm were centrifuged at 500 g for 5 minutes. The supernatant was discarded and the cells were suspended with 300 μL of phosphate buffered saline 1X, to be measured by flow cytometry (see later section,