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In vitro reconstruction of inflammatory reaction in human semen: effect on sperm DNA fragmentation

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ABSTRACT

The study was aimed at evaluating an *in vitro* induction of DNA damage in three sperm subpopulations exposed to selected inflammatory mediators, such as leukocytes, two combinations of pro-inflammatory cytokines (interleukin [IL]-6 + IL-8 and IL-12 + IL-18) and two bacterial strains (*Escherichia coli* and *Bacteroides ureolyticus*). Semen samples from normozoospermic volunteers were differentiated by swim-up (swim-up fraction) and Percoll gradient procedures (90% and 47% Percoll fractions). Leukocytes were isolated from the whole heparinized blood using the density gradient centrifugation technique. DNA fragmentation in sperm fractions was evaluated using flow cytometry with TUNEL labeling and Comet assay. Out of the inflammatory factors tested, bacteria were found to have a greatest toxic effect on sperm DNA, especially in fractions isolated by Percoll gradient, compared with untreated cells ($P < 0.05$). The results indicate that inflammatory mediators can be a direct cause of DNA fragmentation in ejaculated spermatozoa, which can ultimately lead to limited fertilizing abilities of the germ cells. In contrast to the swim-up technique, the selection of spermatozoa by gradient procedures increases the vulnerability of mature spermatozoa to the harmful effects of infectious agents on DNA integrity. This observation may have some meaning for recommendations concerning laboratory techniques used in assisted reproductive therapy.

1. Introduction

The 'poor' sperm DNA quality appears to be one of the important factors affecting male reproductive ability, both in natural and assisted procreation (Simon et al., 2011; Aitken et al., 2009; Carrell et al., 2006; Comhaire et al., 1999; Sergerie et al., 2005). This has been confirmed by numerous reports in which a higher percentage of spermatozoa with fragmented DNA has been found in infertile men compared with fertile individuals (Baccetti et al., 1996; Hughes et al., 1996; Lopes et al., 1998; Smit et al., 2010; La Vignera et al., 2012). Sperm DNA fragmentation can be attributed to various pathological conditions including: local and systemic diseases, environmental factors, sperm preparation protocols and infection/inflammation in the male reproductive tract (Erenpreiss et al., 2006; Muratori et al., 2006). Three mechanisms described in the literature can disrupt sperm DNA integrity, such as defective chromatin packaging, apoptosis and oxidative stress (Aitken and De Iuliis,

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2007; Schulte et al., 2010; Tamburrino et al., 2012). In the case of inflammation of the genitourinary tract, the redox imbalance is probably one of the etiological factors responsible for the destructive effects of the inflammatory process on male gametes, which is mainly associated with peroxidation of sperm macromolecules (Comhaire et al., 1999; Aitken and Baker, 2006; Fraczek and Kurpisz, 2007).

Patients with semen urogenital infection/inflammation have more frequently shown a higher number of spermatozoa with DNA fragmentation than fertile controls (Allam et al., 2008; La Vignera et al., 2012). Moreover, many authors claim that the percentage of DNA-fragmented spermatozoa in semen is connected to semen contamination with bacterial species (Moskovtsev et al., 2009; Domes et al., 2012), although others have not found any relationship between bacteriospermia and sperm DNA integrity (Rybar et al., 2012). Published studies also reported conflicting results on the harmful impact of leukocytospermia on sperm DNA integrity as measured by DNA fragmentation assays (Ochsendorf, 1999; Henkel et al., 2003; Moskovtsev et al., 2007; Fariello et al., 2009; Domes et al., 2012). These contradictory opinions may be connected to the specific site of infection/inflammation within the reproductive tract, with a different diagnostic profile for semen microbial culture, and colonization of the male genital tract by specific bacterial strains. Furthermore, bacteriospermia and/or leukocytospermia do not necessarily mean an infection/inflammation with negative consequences for fertility (Merino et al., 1995; Köhn et al., 1998; Rodin et al., 2003; Lackner et al., 2006; Gdoura et al., 2008).

The direct association between the presence of infectious factors in semen and sperm fertilizing potential has been intensely discussed and constitutes a significant problem in contemporary andrology. It is impossible to avoid the issue of sperm DNA integrity when analyzing the influence of male genitourinary tract inflammation on oxygen metabolism, and its effect on sperm structure and function. Thus, we decided to reconstruct semen inflammation *in vitro* and to analyze the effect of selected inflammatory mediators on an *in vitro* DNA fragmentation of different sperm subpopulations. Out of the many factors participating in the inflammatory process, previously studied regarding lipid sperm membrane peroxidation vulnerability, we chose peripheral blood mononuclear cells (PBMC), two combinations of human recombinant pro-inflammatory cytokines (interleukin (IL)-6 + IL-8 and IL-12 + IL-18) and two pathogenic bacterial strains isolated from semen samples (*Escherichia coli* and *Bacteroides ureolyticus*) for the present study.

2. Materials and methods

2.1. Sample collection and preparation

Semen specimens were obtained from ten healthy volunteers attending the Outpatient Andrology Clinic, Poznan, Poland after four days of sexual abstinence. Following 30 min of sample liquefaction at room temperature, sperm parameters were assessed according to the World Health Organization criteria (WHO, 1999) and Kruger's strict criteria for morphology (Kruger et al., 1986). Each semen specimen was also checked for the presence of peroxidase-positive cells by Endtz test (Endtz, 1974). All samples tested were subjected to extended microbiological examination, including aerobic, anaerobic, and atypic bacteria (BioMerieux, Marcy-L'Etoile, France). Only normozoospermic semen samples with leukocytes $<0.2 \times 10^6$/mL and negative bacterial culture were utilized for further experiments (Table 1). Semen samples selected for the study were fractionated by the swim-up technique and the Percoll gradient procedure as previously reported (Fraczek et al., 2004, 2007). The cells from swim-up, 90% and 47% Percoll sperm fractions, were finally washed in phosphate-buffered saline (PBS) and adjusted to a density of 2×10^7 spermatozoa/mL.

Heparinized venous blood samples were collected from ten healthy adults donating to the Regional Blood Centre, Poznan, Poland. Leukocytes were isolated using the density gradient centrifugation technique (Histopaque-1.077 (Sigma, St. Louis, MO, USA)) as described elsewhere (Fraczek et al., 2004, 2007). The peripheral blood mononuclear cells (PBMC) suspensions were diluted to a concentration of 1×10^7 cells/mL for further use.

The bacterial isolates used in this study were obtained from the Outpatient Clinic of Poznan Hospital Medical University, using the following biochemical test kits(BioMerieux, Marcy-L'Etoile, France): ID 32 E for Gram-negative rods and API 20 A for anaerobic bacteria. The bacterial strains were isolated from semen samples, with significant bacteriospermia ($>3 \times 10^5$ cells/mL and $>1 \times 10^6$ cells/mL of semen for *E. coli* and *B. ureolyticus*, respectively) and leukocytospermia, of our infertile patients. Suspensions of all isolates containing 3000 bacteria per mL were prepared in a sterile 0.85% saline no more than 3 h before the experiment in which they were to be used. A specific anaerobic atmosphere generator system (GenBag Anaer, BioMerieux) was used for the transport of anaerobic strain.

2.2. Incubation of sperm fractions with inflammatory mediators

One million spermatozoa of all the three sperm fractions resuspended in PBS were then incubated with PBMC (1×10^6 per mL of sperm suspension), human recombinant proinflammatory cytokines (200 pg, 500 pg, 50 pg, and 500 pg per mL of sperm suspension respectively for IL-6, IL-8, IL-12, and IL-18) or bacteria (1×10^3 cells per mL of sperm cells, for both *E. coli* and *B. ureolyticus*) for 1 h at 37 °C. Leukocytes were then removed from the co-incubated mixtures using a Dynal MPC-1 immunomagnetic cell isolation system (Fraczek et al., 2004, 2007, 2008).

2.3. Tunel labeling

Once induced in spermatozoa, DNA fragmentation was evaluated using the TUNEL (Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling) assay (FlowTACS Apoptosis Detection Kit, R&D Systems, Minneapolis, MN, USA) following the manufacturer's instruction. Sperm samples were fixed with 3.7% formaldehyde solution and permeabilized with Cytonin. Next, biotinylated nucleotides