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Gram Staining Applied to Human Spermatozoa: a Simple Method for Studying Chromatin Condensation Status

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ABSTRACT. Gram staining applied to human spermatozoa from fertile donors is described. The stain revealed populations of Gram-positive and Gram-negative spermatozoa. Data showed a significant and progressive decrease in the percentage of Gram-positive spermatozoa at different times during the chromatin decondensation procedure (SDS-BSA and SDS-EDTA). No significant correlation could be found between Gram staining and other functional tests used for spermatozoa; only the aniline blue staining test showed a poor correlation. Our study demonstrates that normal spermatozoa with regular chromatin condensation appear Gram-positive, while spermatozoa with altered chromatin condensation appear Gram-negative.

Key words: Gram staining, spermatozoa, chromatin, nuclear condensation

The Gram stain has been used almost exclusively for bacteriology and there are few reports regarding the histological application of this technique. Pearse (1960), in an exhaustive review, reported data of McLetchie who observed strongly Gram-positive β-granules in formalin fixed sections of human pituitary. Foster and Wilson (1973) demonstrated that α-acidophile granules of the pituitary gland were Gram-negative. Panijel (1950) studied Gram staining of *Ascaris* spermatozoa and suggested that Gram positivity is related to the presence of an envelope similar to that found in amphibian eggs (Brachet 1946). Monnè (1955) examined Gram-positive coat fibrous proteins in Nematode eggs and concluded that the positive reaction was associated with carbonyl groups, acid polysaccharides, and phenolic substances.

In our earlier studies investigating the adhesion mechanism of bacteria to spermatozoa and the bacteriospermia/infertility ratio, Gram staining was applied to smears of fresh human semen (unpublished data). We found it possible to detect clearly two sperm subpopulations: one Gram-positive (blue) and the other Gram-negative (pink).

The aim of the present investigation was to clarify the reasons for these different staining affinities in human sperm and to evaluate the possible use of Gram staining for clinical diagnosis.

MATERIALS AND METHODS

Donors collected semen samples in a sterile container by masturbation after 3–5 days of sexual abstinence. Following collection, semen samples were placed in an incubator for 30 min at 36 C to ensure complete liquefaction. Two aliquots of each sample were used to evaluate standard seminal parameters (sperm concentration, motility, viability, morphology) according to the WHO manual (1992) and to carry out Gram stain experiments using neat semen samples incubated at 36 C in 5% CO_2 from 1 to 48 hr, according to the following protocol.

Semen samples from 10 healthy fertile subjects were used for each experiment. One drop of semen was smeared on each microscope slide at room temperature, air dried and fixed for 15 min in ethanol.

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The classic Gram staining technique was applied using crystal violet as primary stain (Hucker's solution: 2% crystal violet, C. I. 42555, 20% ethanol and 0.8% ammonium oxalate), followed by Lugol's solution (1.3% iodine, 2% potassium iodide and 10% polyvinylpyrrolidone) and finally, 0.25% safranin (C. I. 50240) solution as a counterstain (bioMérieux Laboratory, 69280 Marcy-l'Etoile, France). The preparations were examined under an optical microscope using a 100× immersion lens. Three hundred cells were counted to calculate percentages.

In an attempt to explain the different staining of different sperm populations, the results obtained with Gram staining and those obtained using common methods for the functional study of the spermatozoa were compared. In particular, a morphological-functional study of the cell membrane was performed using the swelling test (Jeyendran et al. 1984), sperm viability was evaluated by the eosin-nigrosin (eosin, C. I. 45380, nigrosin, C. I. 50420) staining technique (Eliasson and Treichl 1971), nuclear protein was stained using aniline blue (C. I. 707) (Dadoune et al. 1988), and of nuclear sperm decondensation was evaluated using either 1% sodium dodecyl sulfate and 5% bovine serum albumin (SDS/BSA) or 1% of sodium dodecyl sulfate and 6 mM ethylenediamine tetracetic acid (SDS/EDTA) (Huret 1986, Gopalkrishnan et al. 1991). Briefly, sperm pellets were washed twice in 0.05 M borate buffer (pH 9) from collected semen samples, exposed to SDS/BSA or SDS/EDTA in the same buffer at 37 C. The reaction was stopped and the percent of decondensation calculated after an incubation of 15, 30, 45, 60, 90 and 120 min.

Results were analyzed by linear correlation of Bravais-Pearson, linear regression, one-way variance analysis and variance analysis for repeated measures (ANOVA) (Armitage 1971) using the computer program BMDP/386 (Statistical Software 1991, Los Angeles, CA).

RESULTS

Table 1 compares Gram staining with the swelling test (SW) at 1, 24, and 48 hr. The ANOVA test for repeated measurements applied to these data revealed significant variation in the percentage of swollen cells ($p < 0.001$) and stability in the percentage of Gram-positive sperm ($p = 0.623$). Linear regression analysis of the results obtained at different time points from swelling test vs. Gram staining showed no correlation (1 hr, $r = 0.488$, $p = 0.194$; 24 hr, $r = 0.292$, $p = 0.413$; 48 hr, $r = 0.261$, $p = 0.466$).

Table 1 also shows data concerning viability variations among sperm as determined by eosin-nigrosin staining. We observed a significant variation in vital sperm percentage at the different time points considered using eosin-nigrosin ($p < 0.001$), but no change was observed over time with Gram staining. Linear regression analysis of these results revealed no correlation (1 hr, $r = 0.590$, $p = 0.073$; 24 hr, $r = 0.481$, $p = 0.160$; 48 hr, $r = 0.618$, $p = 0.057$). A linear regression analysis of aniline blue and Gram staining 1 hr after semen collection show poor correlation ($r = 0.640$, $p = 0.046$) (Table 1 and Fig. 2).

To evaluate further the involvement of the nuclear structure in differential sperm stain we Gram stained spermatozoa before and at different time points after chromatin decondensation by SDS/BSA and SDS/EDTA (Figs. 1C and D, and 3). In particular, a significant and rapid decrease in the number of Gram-positive cells were evident after 45 and 60 min of incubation, with additional, slower decrease until 120 min with both SDS/BSA and SDS/EDTA curves. Variance analysis for repeated measures demonstrated a significant time-related variation among sperm in both experiments ($p < 0.001$).

DISCUSSION

We were unable to demonstrate any relation between Gram staining and either membrane integrity as shown by the swelling test or viabil-

Table 1. The mean percentage and standard deviation of swollen sperm (swelling test) vs. Gram-positive sperm; vital sperm (eosin-nigrosin staining) vs. Gram-positive sperm at 1, 24 and 48 hr after semen collection in 10 fertile subjects; and unstained sperm (aniline blue staining) vs. Gram-positive sperm at 1 hr after semen collection in 10 fertile subjects.

	1 hr	24 hr	48 hr
Swollen vs. Gram-positive sperm	66.0 ± 9.2 (64.7 ± 9.3)	35.5 ± 8.9 (63.9 ± 11.5)	17.8 ± 7.3 (65.1 ± 11.0)
Vital vs. Gram-positive sperm	63.6 ± 6.3 (65.6 ± 8.4)	34.5 ± 8.1 (65.9 ± 9.3)	16.2 ± 3.6 (64.9 ± 8.1)
Unstained vs. Gram-positive sperm	62.1 ± 11.4 (63.8 ± 12.0)		

Intra- and inter-assay variation coefficients calculated for the Gram-positive cells for 10 samples were 2.86% and 4.74%, respectively.

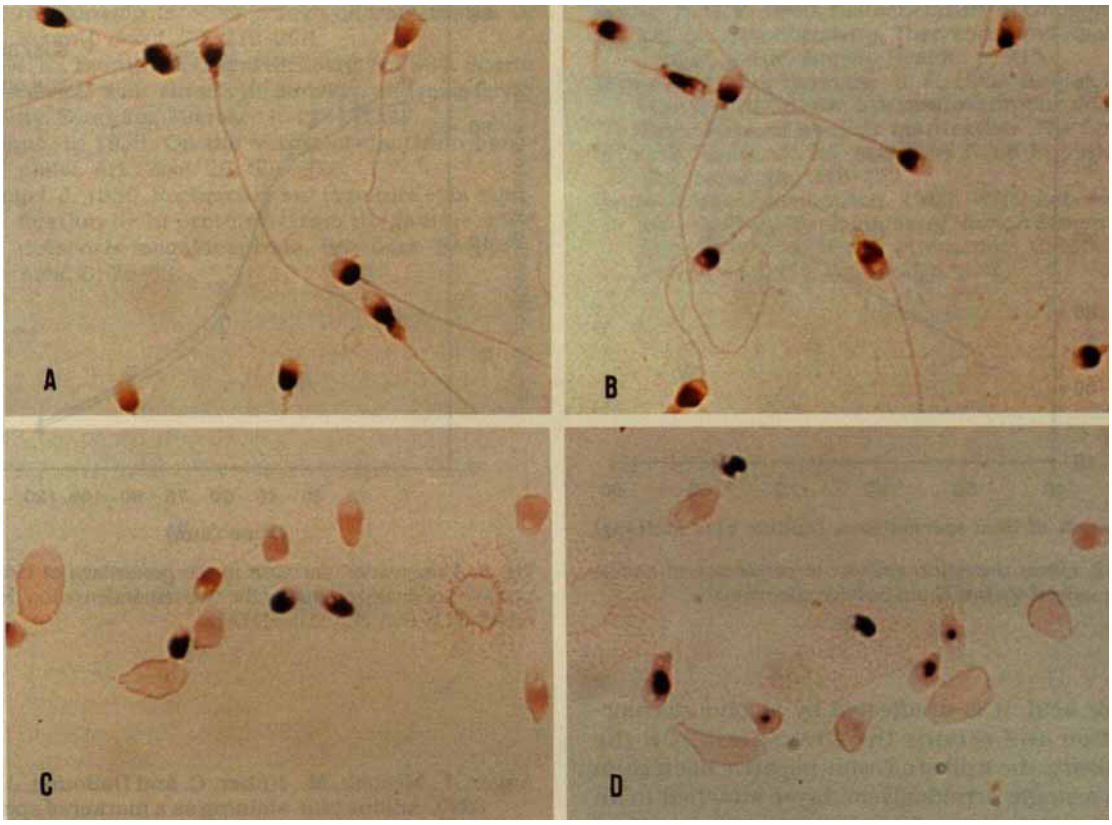

Fig. 1. Gram stained human spermatozoa from fertile, healthy donors. Two populations of sperm are distinguishable. A and B) After 30 min of decondensation (SDS-BSA), most of the heads are pink. C) After 90 min decondensation (SDS-EDTA), all the heads are pink and swollen. D) A blue central spot in some cells is still recognizable and it is probably due to residual condensed chromatin because decondensation proceeds from the periphery to central region of the nucleus. All panels 100×.

ity as assessed by the eosin-nigrosine. On the other hand, the significant time-related variation obtained in the experiments using SDS-BSA and SDS-EDTA led us to hypothesize that nuclear condensation plays an important role in differential Gram staining.

After ejaculation, zinc from prostatic fluid reaches sperm chromatin and binds to free thiol groups, thus stabilizing the quaternary structure and assuring a balance between disulfide bonds and free thiols (Kvist et al. 1987). During spermiogenesis, changes in nuclear morphology are preceded by changes in the biochemical composition of DNA-associated basic proteins (Gusse et al. 1986).

The equilibrium among disulfide bridges, free SH groups and zinc-bound SH groups is important for both sperm chromatin condensation and sperm nuclear decondensation during egg penetration. Impairment of the condensation process could produce precocious decondensation or incomplete condensation of sperm nuclei, both of which are incompatible with normal fertilization potential. The study of sperm chromatin in infertile patients with various andrological diseases showed a greater degree of decondensation (Auger et al. 1990).

The poor correlation between aniline blue and Gram staining obtained in our study could be explained by differences in intrasperm concentration of zinc; it is generally assumed that zinc ions play an important role during chromatin condensation. Moreover, Terquem and Dadoune (1983) demonstrated that aniline blue staining is influenced only by changes in basic nucleoprotein composition of human sperm, whereas our system may be affected also by ion concentration.

The Gram-positive bacterial wall is a thick peptidoglycan layer with large amounts of tei-

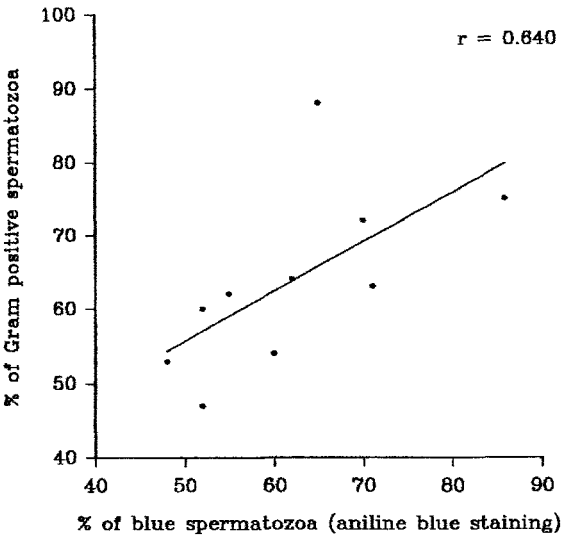

Fig. 2. Linear regression analysis of percentage of aniline blue stained sperms Gram-positive spermatozoa.

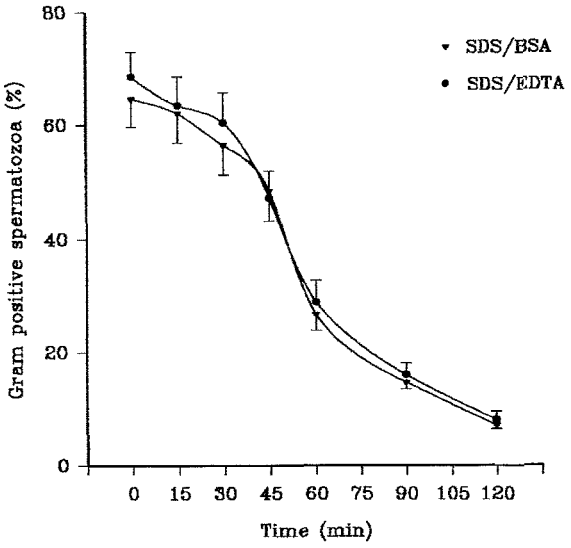

Fig. 3. Time-related decrease in the percentage of Gram-positive spermatozoa during the two decondensation treatments (SDS-BSA and SDS-EDTA).

choic acid. It is unaffected by alcohol decolorization and retains the Gram stain. On the contrary, the wall of a Gram-negative bacterium has a single peptidoglycan layer attached to an asymmetric lipopolysaccharide-phospholipid bilayer, which may be damaged by alcohol decolorization, allowing the crystal violet-iodine complex to leak out.

We have demonstrated with the swelling test experiments that differential Gram staining of human sperm is not dependent on the state of the membrane. We hypothesize, therefore, that extensive condensation of the nuclear chromatin prevents loss of the crystal violet-Lugol complex from the nucleus by decolorization. We believe that Gram staining may distinguish spermatozoa with normal chromatin condensation (blue heads) from spermatozoa with altered chromatin condensation (pink heads). Studies are underway to establish the clinical importance of this procedure in pathological conditions.

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