

The Acridine Orange test: a clinically relevant screening method for sperm quality during infertility investigation?*

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To determine the clinical usefulness of Acridine Orange (AO) staining of spermatozoa as a screening test for the evaluation of semen quality during basic infertility investigation, semen smears from 103 randomly chosen males of subfertile couples were examined. The median duration of infertility was 4.5 years (range 1–15) and the median age was 33 years (range 21–53). The outcome of AO staining ranged from 5 to 81%, with a median of 24%, green fluorescent spermatozoa. Results were not significantly related to the parameters of semen analysis (sperm count, motility, standard morphology, viability, pH and volume, as well as fructose concentration and number of round cells) or to local sperm antibody testing and semen cultures. Fluorescence after AO staining was also not related to sperm functional capacity (evaluated using sperm–mucus interaction tests *in vitro* and *in vivo*), or the medical history of the patient. No significant differences in the AO test outcome were seen in patients with explained and unexplained infertility, or with regard to subsequent fertility [with a median value of 21% (range 5–46) green fluorescence in the fertile group, compared with a median value of 28% (range 9–81) green fluorescence in the other men]. The results of this prospective study indicate that under the usual conditions of conception, the AO test is not clinically useful as a screening procedure to determine semen quality during basic infertility investigation.

Key words: Acridine Orange test/sperm fertilizing capacity/sperm quality/sperm–mucus interaction/unexplained infertility

Introduction

The routine parameters of sperm analysis, as evaluated by microscopical examination, are usually insufficient to predict

sperm fertilizing capacity (Polansky and Lamb, 1988; Amann, 1989; Bostofte *et al.*, 1992). Acridine orange (AO) staining of spermatozoa may be used to upgrade the information obtained by a semen analysis (Evenson *et al.*, 1980; Ibrahim *et al.*, 1988; Royere *et al.*, 1988; Kosower *et al.*, 1992). As a test for DNA integrity, this cytochemical method allows the differentiation between double- (green fluorescence) and single-strand (red fluorescence) DNA because of the metachromatic properties of AO (Rigler *et al.*, 1969; Darzynkiwicz *et al.*, 1975; Ichimura, 1975). Results of the AO fluorescence staining of semen smears have been reported to be an important indicator of human sperm fertilizing potential, and the AO testing of semen samples has been suggested as a practical and clinically significant procedure to determine sperm quality during infertility investigations (Tejada *et al.*, 1984).

To determine the clinical usefulness of this test as an additional screening method during the evaluation of male fertility, semen samples from unselected subfertile males were examined in a prospective study and the outcome of AO fluorescence staining was related to sperm quality, as determined by a comprehensive semen analysis, sperm–mucus interaction tests *in vitro* and *in vivo*, and the sperm fertilizing capacity under in-vivo conditions of conception.

Materials and methods

Patients

Semen samples of 103 randomly chosen males of subfertile couples were used for AO staining. The median duration of infertility was 4.5 years (range 1–15). Patients' ages ranged from 21 to 53 years, with a median of 33 years. A medical history was obtained and clinical andrological examinations were performed. Males with azoospermia or without motile spermatozoa were excluded from the study.

Semen evaluation

Semen was obtained in the hospital after 5 days of sexual abstinence and evaluated using the criteria of the World Health Organization (WHO, 1987). The ejaculate volume, pH, sperm density, progressive motility after liquefaction, after 2 and 4 h, morphology and viability, as well as the seminal fructose concentration and the number of round cells (undifferentiated in wet preparation), were determined.

Aliquots (10 µl) were inoculated into Port-a-Cul[®] tubes (Becton-Dickinson, Heidelberg, Germany) for the detection of potentially pathogenic aerobic and anaerobic bacteria, and into Shepard's medium for the culture of mycoplasmas. Standard methods were applied for the culture and identification of microorganisms (Department of Microbiology and Microbiological Laboratories, Department of Dermatology, University of Heidelberg, Heidelberg, Germany), as described elsewhere (Eggert-Kruse *et al.*, 1992). Specimens of the

*This research was presented in part at the 15th World Congress on Fertility and Sterility, Montpellier, France, September 17–22, 1995.

Table I. Outcome of Acridine Orange (AO) testing related to sperm analysis^a

Semen parameter	AO test outcome		P ^b
	≤Median (≤24%) Median (range)	>Median (>24%) Median (range)	
Semen volume (ml)	3.5 (1.0–8.0)	3.6 (1.1–6.8)	NS
pH	7.4 (7.0–8.5)	7.5 (7.0–7.2)	NS
Sperm concentration (×10 ⁶ /ml)	40 (3–125)	44 (10–169)	NS
Progressive motility (%)	40 (10–60)	40 (10–70)	NS
Sperm morphology (% normal) ^c	56 (43–70)	60 (40–83)	NS
Sperm viability (%)	60 (45–70)	60 (60–80)	NS
Fructose concentration (μg/ml)	1960 (170–3750)	2020 (460–5800)	NS
No. of round cells (n/HPF)	10 (10–20)	10 (10–25)	NS

HPF = high power field; NS = not significant.

^aPerformed in aliquots of the same ejaculates (n = 103).

^bWilcoxon's rank-sum test.

^cAccording to the World Health Organization classification.

same ejaculates, preferentially from patients with a poor post-coital test (PCT), were screened for local antisperm antibodies using the mixed antiglobulin reaction (MAR; Jager *et al.*, 1978) with either immunoglobulin (Ig) G- or IgA-coated erythrocytes (Eggert-Kruse *et al.*, 1991).

Performance of the AO test

Staining was performed, as described in detail by Tejada *et al.* (1984), in aliquots of ejaculates also used for semen analysis. Briefly, after washing with sterile Tyrode's solution (Difco, Detroit, MI, USA) and adjustment of the sperm count, medium-thick smears on pre-cleaned slides were air dried, fixed overnight in freshly prepared Carnoy's solution (methanol/glacial acetic acid), air dried again and stained with acid AO (Sigma, Deisenhofen, Germany) solution. All slides were prepared in duplicate and read the same day on a fluorescence microscope. Sperm heads were subdivided into those showing a green tinge and those with a red colour or definite red tinge (sometimes orange–yellow), as recommended (Tejada *et al.*, 1984). A total of 200 cells were counted on each slide. All readings were performed in duplicate without knowledge of the other test results by a constant observer (H.K.). The mean value was taken for analysis.

Evaluation of sperm functional capacity

Specimens of the same ejaculates were also used for in-vitro migration testing in the majority of patients. The sperm–cervical mucus penetration test (SCMPT) was performed under standardized conditions, as described previously (Eggert-Kruse *et al.*, 1989a). Briefly, sperm migration in capillary tubes filled with cervical mucus obtained after oestrogen treatment was observed microscopically after 30 min, 2 and 6 h of incubation of the penetration meter (Kremer, 1965). The penetration density, migration distance, quality and duration of motility were examined and summarized in a cumulative score, used to select two groups: SCMPT adequate and SCMPT inadequate. For further analyses, patients were subdivided into four SCMPT groups according to 25% percentiles. Penetration testing was performed with fresh cervical mucus from the female partners of the patients, and in parallel with cervical mucus from fertile donors (crossed SCMPT) and bovine cervical mucus obtained during the oestrous phases of cows.

For post-coital testing, the number of spermatozoa with forward progression in pre-ovulatory cervical mucus 8–12 h after intercourse was counted; the mean of 20 readings was taken. PCT results were subdivided into four groups: PCT negative [no spermatozoa found in cervical mucus/low power field (LPF), but spermatozoa found

in vaginal secretions]; PCT poor [less than two motile spermatozoa/high power field (HPF; ×400)]; PCT good (between two and six motile spermatozoa/HPF); and PCT excellent (seven or more motile spermatozoa/HPF). In patients with an inadequate PCT outcome, a reduced cervical mucus quality or difficulties of timing, the PCT was assessed after hormonal treatment to ensure comparable conditions of the cervical mucus quality (Eggert-Kruse *et al.*, 1989b).

Subsequent fertility

If possible, conception rates were recorded within 6 months after the performance of the AO test. During this time no assisted reproduction techniques such as intrauterine insemination, in-vitro fertilization or gamete intra-Fallopian transfer were applied.

Female partners of the patients (median age 30 years) were submitted to a comprehensive infertility investigation, including multiple cycle-related hormonal analyses and tests for ovarian, pituitary, adrenal and thyroid function (as described previously; Eggert-Kruse *et al.*, 1989a,b, 1991). Based on these findings, treatment was recommended to optimize cycle function. Tubal patency had been proved by hysterosalpingography and/or laparoscopy. Couples were included only when at least one tube offered free patency, no uterine factor was found, ovulatory cycles had been confirmed and no signs or symptoms of genital tract infection were detected following a clinical examination of both partners.

Statistical analysis

Data were processed using the Statistical Analysis System. Wilcoxon's rank-sum test, the Kruskal–Wallis test, the χ^2 test, Fisher's two-tailed exact test and the Spearman rank correlation were used. The level of significance was set at $P < 0.05$.

Results

Relationship of AO testing to semen analysis

The percentage of spermatozoa showing green fluorescence after staining with AO ranged from 5 to 81%, with a median of 24%. Apart from the median, several different cut-off values were used to analyse the relationship of AO testing with other parameters of semen quality, e.g. the 25th percentile (17%), the 75th percentile (41%) and thresholds at AO values of 30 and 50%.

No significant direct correlation of AO testing to sperm

concentration, sperm motility after liquefaction after 2 or 4 h, standard sperm morphology, sperm viability or the other variables of semen analysis was found with correlation coefficients (r) ≤ 0.16 (Spearman rank correlation). The distribution of these parameters in the groups with AO results below or above the median is shown in Table I and did not offer any significant differences. Sperm count was slightly higher in samples with AO results $>24\%$ without reaching significance. When AO staining showed $\geq 50\%$ green-fluorescing sperm heads, none of the samples was oligozoospermic ($<20 \times 10^6/\text{ml}$), severely asthenozoospermic ($<20\%$ progressive motility) or had reduced viability ($<60\%$) (not significant). Sperm morphology was slightly better, with a median of 65%, compared with 58% normal forms in specimens below this cut-off value ($P < 0.03$, Wilcoxon test). No further relevant information was obtained after results were divided into four AO groups, when specimens with AO results $<25\text{th}$ or $>75\text{th}$ percentile were compared with the other samples, or when 30% was taken as the threshold value.

The outcome of the AO test was not influenced significantly by the microbial colonization of semen samples. Potentially pathogenic aerobic bacteria were cultured in 43.1% of ejaculates, mostly enterococci; in addition, mycoplasmas were found in 13.9% of samples, and species of physiological flora were found in the majority of ejaculates (88.0%). The results of AO staining were also not significantly associated with local antisperm antibodies of the IgG or IgA class, as determined with the MAR ($n = 48$); strong positive MAR IgG results ($>60\%$; $n = 4$) were found only in the group with AO results below the median value ($<24\%$; not significant). None of the specimens showed strong positive MAR IgA results.

AO test and the sperm–mucus interaction

The ability of spermatozoa to penetrate cervical mucus *in vitro* (SCMPT) and *in vivo* (PCT) was examined in 76 couples. With regard to SCMPT, the penetration distance and sperm density in cervical mucus (after 30 min, 2 and 6 h) did not correlate significantly with AO test results (Spearman rank correlation), which were also not significantly related to the motility grade in cervical mucus after the three time intervals (χ^2 analysis).

When the cumulative migration score was applied, the SCMPT was adequate in 63.2% and inadequate in 36.8% of couples. No significant differences were found for AO staining results (Table II), either when using other threshold values for AO outcome or when selecting patients according to their SCMPT score in four groups with a poor, moderate, good or excellent ability to penetrate cervical mucus *in vitro*. This lack of a relationship of AO outcome to sperm functional capacity was confirmed when cervical mucus from fertile donors was used in parallel in the crossed SCMPT (regarding all different cut-off values) or when bovine cervical mucus was used as a substitute for human cervical mucus in the sperm migration test.

In addition, post-coital testing did not show any significant relationship with the AO results, as shown in Table II. No further information was obtained when other threshold values were used. In the majority of couples, the PCT was assessed again after oestrogen treatment to consider the hormonal

influence on the quality of the cervical mucus. Again, no significant relationship with AO results in the semen samples was found, although an excellent PCT was more frequently found in the group with an AO test outcome $>24\%$ [in 19.4 (6/31) compared with 8.3% (3/36) of cases (not significant)]. The lack of a significant association of the PCT with staining results was confirmed after the exclusion of patients with a cervical index (according to Insler *et al.*, 1972) <10 .

Relationship of AO testing with general parameters

Several factors of medical history and results of clinical andrological examinations were analysed with regard to the outcome of AO testing without showing a significant relationship, e.g. the duration of infertility, ages of the patient and the female partner, primary/secondary infertility, previous sexually transmitted diseases (3.6%), other genital infections (2.8%), genital injuries (2.4%), varicocele (9.5%), genital surgery (5.6%), mumps (66.7%), known allergies (11.0%), occupational exposure to chemicals, heat or radiation (as indicated by patients on a questionnaire; 25%), smoking (55.2%; also when heavy, moderate and non-smokers were compared), alcohol consumption (74.5%; also when selected in subgroups), andrological medication (mostly kallikrein; 16.9%), general medication (6.1%), stress factors (subjectively caused by working conditions or infertility investigations and treatment; 45.3%), physical activity (regular body exercise; 42.3%) or body weight and length.

AO testing and subsequent fertility

Of the 103 couples, 67 could be followed for subsequent fertility; 20 of them conceived within a period of 6 months after semen evaluation with AO. The outcome of AO staining of the spermatozoa was without significant difference in the patients who did and who did not achieve a pregnancy within this time period, with a median of 21% (range 5–46) in the subsequently fertile group ($n = 20$) compared with 29% (range 9–81) green-fluorescing spermatozoa in the non-fertile group (not significant; Wilcoxon test).

There were also no significant differences when several cut-off values for AO results were used, when results of AO staining were compared in males of the couples with information about subsequent fertility and in those who could not be followed [with a median percentage green fluorescence of 27% (range 12–64) in semen samples of these patients], or when analyses were made in subgroups, e.g. after the additional exclusion of women with minor tubal adhesions on one side (one patent tube had been insured in all patients) and/or those with discrete hormonal disorders before treatment. The AO test outcome also did not differ significantly in males of couples with 'explained' or 'unexplained' infertility, with a median value of 30% (range 10–62) in patients with 'unexplained' infertility compared with 22% (range 5–81) in those with known causes of their infertility.

The 'effective sperm count', as introduced in other reports (Tejada *et al.*, 1984) and obtained by multiplying the percentage of green-fluorescing spermatozoa by the actual sperm count, showed a median of $10.3 \times 10^6/\text{ml}$ ($2.4\text{--}39.4$) $\times 10^6/\text{ml}$ in the non-fertile patients compared with $7.6 \times 10^6/\text{ml}$ (1.8–

Table II. Results of Acridine Orange (AO) testing and sperm–mucus interaction *in vitro* and *in vivo*

Migration test result	Outcome of AO testing						P ^a
	≤Median (≤24%)		>Median (>24%)		Total		
	n	%	n	%	n	%	
<i>In vitro</i>							
Sperm penetration test (SCMPT) with partners' cervical mucus ^b							
Inadequate	15	39.5	13	34.2	28	36.8	NS
Adequate	23	60.5	25	65.8	48	63.2	
Crossed SCMPT with donors' cervical mucus ^b							
Inadequate	15	39.5	15	39.5	30	39.5	NS
Adequate	23	60.5	23	60.5	46	60.5	
<i>In vivo</i>							
Post-coital test							
Negative	13	34.2	11	29.0	24	31.6	NS
Poor	10	26.3	10	26.3	20	26.3	
Good	12	31.6	11	29.0	23	30.3	
Excellent	3	7.9	6	15.8	9	11.8	
Total	38	50.0	38	50.0	76	100.0	

NS = not significant.

^aχ² analysis.^bCervical mucus obtained under hormonally standardized conditions; results of SCMPT after an observation period of 6 h (cumulative score).

24.5)×10⁶/ml in the fertile group of patients (not significant; Wilcoxon test).

Discussion

AO staining of semen smears has been reported to improve the information obtained by semen analysis with respect to sperm fertilizing capacity (Evenson *et al.*, 1980; Tejada *et al.*, 1984; Ibrahim *et al.*, 1988; Roux and Dadoune, 1989; Kosower *et al.*, 1992). AO testing is an established cytochemical method for determining DNA integrity, allowing the differentiation between single- and double-stranded DNA using the metachromatic properties of the dye (Rigler *et al.*, 1969; Darzynkiwicz *et al.*, 1975; Ichimura, 1975). This fluorescence staining method of spermatozoa treated with heat, acid or both to evaluate its resistance to DNA denaturation has been suggested as a valuable test to determine human male fertility, with the simplified procedure described by Tejada *et al.* (1984) making the test suitable as a screening method. These authors reported that a high ratio of green-fluorescing spermatozoa after AO staining indicated a high rate of 'fertile' cells, enabling the AO test to have a large field of potential application in routine infertility investigations. Therefore, in this prospective study, the clinical relevance of the method was evaluated and the outcome of AO fluorescence staining was related to several other parameters of sperm quality and subsequent *in-vivo* fertility.

Semen smears stained with AO exhibited widely ranging proportions of green- and red-fluorescing sperm heads, comparable with the results of other authors (Evenson *et al.*, 1980; Tejada *et al.*, 1984; Royere *et al.*, 1988; Liu and Baker, 1992). Inter-technician variability because of human subjective interpretation of the fluorescence colour shades could be ruled out by using one consistent observer for the whole study. The median AO value of 24% green is in accordance with the findings of Claassens *et al.* (1992), who described this as

the 52nd percentile in a group of subfertile males submitted to *in-vitro* fertilization.

No significant correlation of AO testing with semen analysis results was found. The lack of a relationship with sperm motility confirms other reports (Tejada *et al.*, 1984; Claassens *et al.*, 1992; Liu and Baker, 1992). A potential association with sperm motility after freezing–thawing (Royere *et al.*, 1988), suggesting an influence of the freezing process on the AO test outcome, did not play a role in our study where only fresh semen samples were used. In general, sperm counts were lower in the subfertile population of our investigation than in other studies (Tejada *et al.*, 1984; Liu and Baker, 1992). A weak, but significant, correlation between the proportion of normal forms and the percentage of green-fluorescing spermatozoa (Tejada *et al.*, 1984; Claassens *et al.*, 1992; Liu and Baker, 1992, 1994) could not be confirmed ($r = 0.15$), possibly because of different morphological criteria, although the percentage of normal forms (WHO classification) was higher in the small group of samples with AO values $\geq 50\%$. Studies in animals (e.g. Gledhill, 1966; Wyrobek *et al.*, 1975) and humans (Martin and Rademaker, 1988) did not indicate a significant relationship between the proportion of morphological defects and the proportion of human spermatozoa with chromosomal abnormalities.

The predictive value of microscopical semen analysis for sperm fertilizing capacity is a matter of constant debate (e.g. Barratt *et al.*, 1995; Sukcharoen *et al.*, 1995). There is increasing agreement that groups of tests including functional assays are required to estimate fertility. Valuable clinical information can be obtained when evaluating sperm penetration ability (Kremer, 1965; Ulstein, 1972; Bostofte *et al.*, 1992). While the PCT to evaluate the sperm–mucus interaction *in vivo* might be subject to considerable variability (e.g. Eimers *et al.*, 1994; Oei *et al.*, 1995), based partly on problems of timing, cervical mucus quality or psychosexual function, the *in-vitro* testing of sperm migration can be better standardized. The

SCMPT with cervical mucus from the female partners of patients has been shown to be of clinical significance for the subsequent fertility of couples, and in this respect was superior to the PCT (Eggert-Kruse *et al.*, 1989b). However, our study did not show any significant differences of the AO results in semen samples from patients with a good or poor outcome of the SCMPT performed with specimens of the same ejaculates used for fluorescence staining. As the cervical mucus status is influenced considerably by the endocrine factor, this was controlled. The lack of a relationship of AO testing with the 'intrinsic motility' of the spermatozoa was confirmed when cervical mucus from either fertile female donors or cows was used.

Other potentially influential factors were also considered. As most ejaculates are not sterile and colonization with potentially pathogenic micro-organisms in men without signs or symptoms of genital tract infection is very frequent (Eggert-Kruse *et al.*, 1992, 1995), a possible bacterial interference with fluorescence after AO staining was excluded. Results of AO staining were also not influenced by local sperm antibodies of the IgG or IgA class, determined using the MAR as an established screening method for clinically relevant sperm immunology (Jager *et al.*, 1978; Schumacher, 1988; Eggert-Kruse *et al.*, 1991; Kremer and Jager, 1992). Denaturation of the DNA can be induced by various chemical agents. The frequency of DNA defects might be related to age (Martin and Rademaker, 1987) or the duration of infertility. Therefore these and other potentially associated factors were also analysed in our investigation but no significant relationship with the outcome of AO testing was found. However, it has to be remembered that exposure to, for example, environmental toxins was assessed only as indicated on a questionnaire.

The main point of interest during infertility investigation for the subfertile patients is a pregnancy. Only couples with a reasonable chance of becoming pregnant (ovulatory cycles of female partners, patency of at least one Fallopian tube) were enrolled in this study. No significant relationship of sperm AO testing with subsequent fertility under the usual conditions of conception was found. Indeed the median proportion of green-fluorescing spermatozoa was lower in fertile patients compared with those without a subsequent pregnancy of their partner. However, it has to be considered that AO results were compared in males of subfertile couples with and without subsequent fertility within a certain time period, and not between this group as a whole and, for example, men who had recently fathered a child.

The comparably high conception rate for a population with long-standing subfertility (median duration 4.5 years) in the group of couples who could be followed might be biased by the fact that relatively more pregnant than non-pregnant patients returned to the hospital. As pregnancies were noted within an observation period of 6 months, some fluctuation in the DNA status might be possible, although the frequency and type of chromosomally abnormal spermatozoa has been shown to be stable over time (Bandriff *et al.*, 1985). No significant difference of the percentage of green-fluorescing spermatozoa was found in patients with so-called 'explained' and 'unexplained' infertility. Future studies are necessary to analyse the potential

relationship of AO test results with pregnancy outcome, e.g. the rate and causes of abortion.

With regard to sperm functional quality under in-vitro fertilization conditions (not evaluated here), Claassens *et al.* (1992) found that patients with >24% green-fluorescing spermatozoa had higher oocyte fertilization rates than patients with lower AO values, but described the predictive value of AO testing for subsequent fertility as low and inferior to that obtained with a strict morphological evaluation (Kruger *et al.*, 1986). Others did not find a marked prognostic advantage over zona-binding testing concerning the prediction of fertilization (Liu and Baker, 1992). The relationship with other functional assays (e.g. Brandelli *et al.*, 1995) remains to be determined. Tejada *et al.* (1984), who suggested that the AO test was able to serve as an important indicator of sperm fertilizing capacity, introduced the term 'effective sperm count' and determined an effective sperm count of $50 \times 10^6/\text{ml}$ as a useful cut-off value. This threshold value was beyond the 95th percentile in our study and was therefore not useful. Comparison of the medians and ranges of the effective sperm count in subsequently fertile and infertile patients did not reveal any clinical significance for this parameter.

In summary, the results of our study suggest that the AO testing of semen smears cannot be recommended as a screening procedure for sperm quality and functional capacity, and indicate that the clinical value of this method is low during basic infertility investigation.

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Received on October 30, 1995; accepted on January 16, 1996