

Sperm morphology assessment using strict criteria and male fertility under in-vivo conditions of conception*

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The clinical significance of sperm morphology assessment according to very strict criteria was determined using semen samples of randomly chosen males from couples not submitted to assisted procreation techniques, with a median duration of infertility of 4 years (range 1–17; $n = 89$). The relationships of sperm morphological properties to the results of standard sperm analysis, including the differentiation of round cells in semen by monoclonal antibodies and semen cultures, the testing of sperm functional capacity *in vitro* with the standardized sperm–cervical mucus penetration test (SCMPT) and the subsequent pregnancy rate under in-vivo conditions of conception, were evaluated in a prospective study. The quick staining method (DiffQuick® stain) for sperm morphology proved to be practical and suitable for routine use. The percentage of normal forms according to strict criteria ranged from 1 to 36%, with a median of 12%. Morphological findings were not markedly related to the medical history, but significant relationships between standard parameters of sperm analysis, in particular the sperm count, the progressive motility and standard sperm morphology, were found. The percentage of normal forms was not significantly associated with the microbial colonization of semen samples but was negatively related to high leukocyte rates. Semen samples with a higher percentage of normal spermatozoa (shown, for example, for >4 , >7 or $\geq 14\%$ normal) were significantly more frequent in cases of an adequate SCMPT. The subsequent pregnancy rate within an observation period of 12 months was 20.2%. The pregnancy rate under in-vivo conditions was significantly higher when semen samples had a better sperm morphology, with significant differences for thresholds at 4, 7 and 14% of strictly normal forms. Although sperm morphology is only one among a multiplicity of factors determining fertility, the results suggest that the evaluation of sperm morphology using strict criteria provides valuable information during basic infertility investigations.

*This work was presented in part at the International Meeting on 'Andrology in the Nineties', Genk, Belgium, 21–24 April, 1993.

Key words: male fertility/sperm function/sperm morphology/sperm–mucus interaction/strict criteria

Introduction

Since the early observations of van Leeuwenhoek and Ham (1678) who described the 'animalcules', sperm morphology has been a matter of considerable debate. Although the assessment of the morphological properties of spermatozoa is considered part of the standard sperm analysis of the World Health Organization (WHO), its clinical significance is controversial (e.g. MacLeod and Gold, 1953; Eliasson, 1971; Fredricsson, 1979; Aitken *et al.*, 1982b; Rogers *et al.*, 1983; Mahadevan and Trounson, 1984; Dadoune, 1988; Polanski and Lamb, 1988; Amann, 1989; Dunphy *et al.*, 1989; Bostofte *et al.*, 1990, 1992; Davis and Gravance, 1994). Recently, very strict criteria for selecting morphologically 'normal' spermatozoa have been introduced, referred to by some authors as 'Tygerberg' or 'Norfolk' criteria, and correlated with the success of in-vitro fertilization (IVF; Kruger *et al.*, 1986, 1988a; Menkveld *et al.*, 1990). However, there is no information about the clinical significance of this classification of sperm morphology for male fertility under in-vivo conditions of conception. In this prospective study, these strict criteria were used for a sperm morphology assessment in a subfertile population not submitted to any method of assisted reproduction such as IVF, gamete intra-Fallopian transfer or intrauterine insemination.

In parallel, semen samples were evaluated using the standard procedure of microscopical evaluation, as recommended by WHO. A sperm analysis also included semen cultures covering a broad spectrum of micro-organisms, as well as a round cell differentiation in leukocytes and cells of the early stages of spermatogenesis. Furthermore, aliquots of the same semen samples were used in an in-vitro sperm–cervical mucus penetration test (SCMPT) to determine the sperm functional capacity. The female partners of the patients were submitted to a comprehensive infertility investigation to detect female factors of infertility. The relationship of the sperm morphological assessment with the clinical and laboratory findings and the couples' subsequent fertility under in-vivo conditions of conception within an observation period of 1 year were determined.

Materials and methods

Patients

The study population consisted of 89 randomly chosen couples presenting for infertility investigations at the Infertility Unit of the Women's University Hospital in Heidelberg, Germany. The age of

the male patients varied between 22 and 45 years (median 33.3); the age of the female partners was between 22 and 40 years (median 30.5). The median duration of infertility was 4 years (range 1–17). Primary infertility was found in 70.4% and secondary infertility in 29.6% of patients.

Patients with clinical symptoms or signs of infection of the lower genital tract were excluded from our study, as well as couples with azoospermia of the male partner or a severe female factor of infertility (e.g. women with tubal occlusion, severe adhesions in both Fallopian tubes, severe endometriosis, uterus myomatosus, intrauterine adhesions or uterus duplex).

All female partners were investigated thoroughly for potential infertility factors. Tubal patency was proved by hysterosalpingography and/or laparoscopy. Some pelvic pathologies (mostly discrete adhesions of one Fallopian tube but patency of the other side, or a minor uterine factor such as a slight hypoplasia) were found in 32% of the women, but all had at least one free patent tube and thus a reasonable chance of pregnancy. A comprehensive, cycle-related endocrinal screening was performed on all the women, including several tests for ovarian and pituitary function, as well as adrenal (e.g. adrenocorticotrophic hormone test) and thyroid (e.g. thyrotrophin-releasing hormone test) function, in the early follicular phase, and multiple determinations of progesterone, 17β -oestradiol and prolactin concentration in the luteal phase, which were completed by further hormonal assays if necessary. Luteal insufficiency was defined as a mean progesterone concentration of <10 ng/ml, determined on days 5, 7 and 9 of the hyperthermic phase. In all, 23.0% of the women had regular ovulation without any hormonal disorders and including an adequate luteal phase. Using this differentiated endocrinal examination, minor endocrinal anomalies (mostly discrete luteal insufficiency or preclinical disorders of thyroid or adrenal function) were detected in 64.0% of the women despite spontaneous ovulatory cycles. Oligoamenorrhoea was found in 12.5% of the women. If necessary, specific treatment was given according to the results of the endocrinal examination, which resulted in regular ovulatory cycles with an adequate luteal phase in all women in our study.

A detailed medical history was obtained and an andrological examination was performed in all males. All males were apparently healthy, apart from their complaint of being involuntarily childless. A routine semen analysis gave sperm counts of $<40 \times 10^6$ /ml or a reduced progressive motility ($<40\%$) in nearly half of the men (50.6%). In some patients, andrological treatment had been recommended by colleagues from the Andrology Unit, University of Heidelberg, Heidelberg, Germany, based on the results of standard sperm analyses. The current medication (kallikrein, $n = 16$; pentoxifylline, $n = 2$; testosterone undecanoate, $n = 1$; gonadotrophins, $n = 1$) was not discontinued prior to the study and the strict morphological assessment. During the time of the study, none of the couples were submitted to assisted procreation techniques such as in-vitro fertilization (IVF), gamete intra-Fallopian transfer or intrauterine insemination.

Semen analysis

Ejaculates were obtained by masturbation into sterile glass jars in hospital after a period of sexual abstinence of at least 5 days. Samples were used for all tests directly after liquefaction. A semen analysis, including the determination of sperm volume, pH, sperm count, progressive motility, morphology and fructose concentration, was performed using routine criteria (WHO, 1987).

In parallel, two slides were prepared from each semen sample for an assessment of the sperm morphology using the strict criteria, as described in detail by Kruger *et al.* (1986, 1988a). Liquefied semen (5 μ l) was pipetted onto carefully precleaned standard microscope

slides and a thin smear was prepared. Staining was performed using the DiffQuick[®] stain set (American Hospital Supply dell Caribe Inc., Aguada, Puerto Rico) using the solution for fixation and solutions 1 and 2 for staining, as recommended by the manufacturer. After air drying, slides were rinsed with distilled water, mounted with Glycer-gel[®] (Dako, Hamburg, Germany) and covered with a coverslip. Assessment was carried out on the same day. A total of 100 spermatozoa were evaluated on each slide using the high power magnification ($\times 1000$, in oil) of a light microscope. The percentages of normal and pathological forms, including the morphological index (combination of normal forms and minor abnormalities) according to Kruger *et al.* (1988a), were determined based on the strict criteria. Throughout the whole study, the strict sperm morphology evaluation was performed by the same observer.

When the results of the sperm morphology evaluation using the strict criteria on the two slides prepared from each semen specimen were correlated, the Spearman rank testing offered a correlation coefficient (r) of 0.88 for the percentage of normal forms and 0.89 for the morphological index ($P < 0.0001$). The results of the first slide were used for further comparisons. The percentage of normal forms was chosen for further statistical analyses as the main parameter of sperm morphological quality based on the suggestions of Kruger *et al.* (1986, 1988a).

Examination and differentiation of round cells

Semen smears were prepared and the percentage of leukocytes of round cells was determined as described previously (Eggert-Kruse *et al.*, 1992a). Briefly, the number of round cells was first counted using a Neubauer chamber (LDH, Heidelberg, Germany). For the differentiation of round cells, monoclonal antibodies of high specificity for common leukocyte antigen were used (Dako LC[®]; Dakopatts, Glostrup, Denmark). Staining was performed with a streptavidin-biotin system (HistoStain[®], SP kit; Zymed Laboratories, South San Francisco, CA, USA). Slides were incubated in a blocking solution to eliminate the non-specific background. Primary antibody, biotinylated second antibody, enzyme conjugate and substrate chromogene mixture were applied according to the manufacturer's instructions. Smears were then counterstained with Meyer's haematoxylin and mounted in an aqueous mounting solution (Glycer-gel[®]; Dako). Positive (peripheral leukocytes) and negative controls were included in each of the test series. Using the high power field of a standard light microscope, 100 round cells were examined and the percentage of leukocytes was determined. All readings were performed in duplicate, and the mean value was used in the analysis.

Microbial screening

Aliquots from each ejaculate taken for a sperm morphological assessment were submitted to semen cultures, including mycoplasmas (*Mycoplasma hominis* and *Ureaplasma urealyticum*), potentially pathogenic aerobic and anaerobic bacteria, and species of the physiological flora. In addition, immunoglobulin (Ig) G antibodies to *Chlamydia trachomatis* were determined in serum samples of all patients as a marker for previous infection by these micro-organisms (indirect fluorescence antibody test; Virgo[®]; Schiapparelli Biosystems Inc., Columbia). Standard methods were used for the identification of micro-organisms, as described in detail previously (Eggert-Kruse *et al.*, 1992b; Department of Microbiology and Hygiene and Microbiological Laboratories, Department of Dermatology, University of Heidelberg, Heidelberg, Germany). Simultaneously, a microbial screening was performed in the female partners of patients for the determination of potentially sexually transmitted organisms. Microbial cultures in female patients also included an evaluation of the endocerv-

ical material for *C.trachomatis* (McCoy cell culture) and herpes simplex virus, and of yeasts in the fornix posterior vaginalis.

Sperm function testing

To evaluate sperm functional capacity, the in-vitro SCMPT was performed in parallel with a morphological evaluation of the spermatozoa with aliquots of the same ejaculates. Cervical mucus from the wives of the patients, and additionally cervical mucus from fertile donors in the crossed SCMPT, was used as the penetration medium. The cervical mucus was taken from the endocervix with a special device (Aspiglaire®; IMV, L'Aigle, France). The quality of the cervical mucus was classified according to Insler *et al.* (1972), and the pH was determined with paper strips (pH-Indikatorpapier; Merck, Darmstadt, Germany). To obtain the most reproducible results, freshly obtained cervical mucus from all the women was used, after standardized oral treatment with oestrogens (80 µg/day ethinyl oestradiol) for 1 week preceding the test.

The SCMPT using this standardized hormonal approach has been described in detail elsewhere (Eggert-Kruse *et al.*, 1989a,b). Briefly, capillary tubes were carefully filled with cervical mucus avoiding air bubbles and sealed at one end with modelling clay. With one drop of cervical mucus protruding at the other end, the tubes were placed in the reservoirs of a penetration meter (Kremer, 1968) filled with fresh semen directly after liquefaction. After incubation periods of 0.5, 2 and 6 h in a moist chamber at 37°C, the penetration distance, sperm density and sperm motility grade were determined. Results were summarized in a cumulative SCMPT score. Based on the SCMPT score after an incubation period of 6 h, samples were selected in those offering an adequate or inadequate ability to penetrate the cervical mucus *in vitro*.

Statistical methods

The pregnancy rate was determined after 12 months. Data were processed using the statistical analysis systems SAS and SPSS. χ^2 , Fisher's two-tailed exact tests, Wilcoxon's rank-sum tests, Spearman rank correlation and logistic regression analysis were used. Statistical significance was considered to be achieved at $P < 0.05$.

Results

Microscopical semen analysis

Standard criteria

Semen analyses showed oligozoospermia ($<20 \times 10^6$ spermatozoa/ml) in 15.7%, a progressive motility of $\leq 40\%$ in 49.4% (44/89) and considerable asthenozoospermia ($<20\%$) in 14.6% of patients. Some 27.3% (24/88) of semen samples showed $<60\%$ of normal spermatozoa according to standard WHO criteria, with a median percentage of normal forms of 63% (range 24–77). Median values (ranges) of the other parameters of standard sperm analysis were for ejaculate volume 3.8 ml (0.8–9.4 ml), pH 7.3 (6.7–8.0), viability 65% (45–80) and fructose concentration 1580 µg/ml (520–3450).

Sperm concentration, progressive motility and standard morphology (WHO) were significantly interrelated. In samples offering $\geq 60\%$ normal forms based on WHO criteria, a progressive motility of $>40\%$ was significantly more frequent (found in 67.2% of these samples compared with 8.3% of specimens with $<60\%$ normal forms; $P < 0.0001$), and a sperm count of $\geq 20 \times 10^6$ spermatozoa/ml was found in 92.2% of samples compared with 62.5% in samples with a reduced standard morphology ($P < 0.001$; χ^2 analysis). Spearman rank

testing offered correlation coefficients (r) of 0.73 for the percentage of normal forms based on WHO criteria and sperm motility ($P < 0.0001$), and of 0.49 for sperm count and progressive motility ($P < 0.0001$).

Strict criteria

Markedly less morphologically normal spermatozoa compared with the standard classification were found when the strict criteria (Kruger *et al.*, 1986, 1988a) were applied. The median percentage (range) for normal spermatozoa was 12% (1–36). For statistical analyses, thresholds at 4 and 14% normal forms, based on previous reports (Kruger *et al.*, 1986, 1988a), were used, with an additional cut-off at 7% normal forms.

Sperm morphology according to strict criteria was significantly improved when routine parameters showed adequate progressive motility ($>40\%$), a sperm count of $\geq 20 \times 10^6$ /ml or $\geq 60\%$ normal forms based on the WHO classification, as can be seen in Table I. Spearman rank testing offered a significant correlation of these parameters with strictly determined sperm morphology.

Microbial screening

The majority of ejaculates were colonized with bacteria. Potentially pathogenic aerobic species (*Escherichia coli*, *B. streptococci*, enterococci, *Proteus mirabilis*, *Staphylococcus aureus* and others with a lower prevalence) were found in 53.4% (47/88), potentially pathogenic anaerobic microorganisms in 6.8% and mycoplasmas (*M.hominis* and/or *U. urealyticum*) were cultured in 12.5% of semen samples. Additionally, species of the physiological flora were found in 85.2% of ejaculates. Microbial findings were not significantly related to the morphological properties of the spermatozoa with regard to cut-offs at 4, 7 and 14% strictly normal forms, as well as to medians and ranges.

In addition, no significant relationship was found with respect to IgG antibodies to *C.trachomatis*, which were elevated (titre $\geq 1:256$) in serum samples of 11% of men. Furthermore, the results of the morphological assessment in semen samples did not show a relationship with the microbial findings in the lower genital tract of the female partners.

Differentiation of round cells

The outcome of round cell differentiation using monoclonal antibodies was related to the sperm morphological evaluation using strict criteria in 48 specimens (53.9%). Round cells were found in all specimens and leukocytes in 72.4% of samples. The percentage of leukocytes correlated significantly with the number of leukocytes per ml, as well as the number of leukocytes per ejaculate ($r > 0.8$, $P < 0.0001$).

The median percentage of leukocytes of the round cells was 3% (range 0–53). For a statistical analysis, cut-offs at leukocyte rates of 3 (median), 10 and 15% were used. Ejaculates with $\geq 15\%$ of leukocytes of the round cells were considered 'leukocyte positive', based on previous observations (Eggert-Kruse *et al.*, 1992a).

The number of round cells was not significantly related to strictly determined sperm morphological properties. The association between leukocyte rates and sperm morphology

Table I. Relationship between strict morphological criteria and standard parameters of sperm analysis

Standard parameter	Sperm morphology (strict criteria)								Correlation coefficient (<i>r</i>) ^b		
	% normal forms ^a				% normal forms ^a						Total
	≤7%		>7%		<14%		≥14%		n	%	
	n ^c	% ^d	n	%	n	%	n	%			
Sperm count ≥20 ×10 ⁶ /ml	19	70.4 ^e	56	90.3 ^e	42	77.8 ^e	33	94.3 ^e	75	84.3	0.29 ^e
Progressive motility ≥40%	9	33.3 ^e	36	58.1 ^e	21	38.9 ^e	24	68.6 ^e	45	50.6	0.31 ^f
Standard morphology ≥60% normal	12	44.4 ^f	52 ^g	85.3 ^f	32 ^h	60.4 ^e	32	91.4 ^e	64	72.7	0.26 ^e
Total	27	30.3	62	69.7	54	60.7	35	39.3	89	100.0	

^aχ² analysis, compared with corresponding group below the threshold indicated.

^bSpearman rank correlation.

^cNumber of patients per group.

^dPercentage of patients per group.

^e*P* < 0.05.

^f*P* < 0.005.

^gTotal number per group with >7% normal forms = 61.

^hTotal number per group with <14% normal forms = 53.

Table II. Relationship of leukocyte rates after round cell differentiation and sperm morphology using strict criteria

Normal forms (strict criteria)	Leukocyte rate ^a											
	<3%		≥3%		<i>P</i> value ^b	<15%		≥15% ^c		<i>P</i> value ^b	Total	
	n ^c	% ^d	n	%		n	%	n	%		n	%
>4%	20	90.9	24	92.3	NS	40	95.2	4	66.7	<0.05	44	91.7
>7%	19	86.4	16	61.5	<0.05	33	78.6	2	33.3	<0.05	35	72.9
≥14%	13	59.1	14	53.9	NS	27	64.3	0	0.0	<0.05	27	56.3
Total ^f	22	45.8	26	54.2		42	87.5	6	12.5		48	100.0

NS = not significant.

^aImmunologically determined percentage of leukocytes of the round cells in semen.

^bFisher's two-tailed exact test, compared with corresponding groups below the threshold indicated.

^cNumber of patients per group.

^dPercentage of patients per group.

^eConsidered 'leukocyte positive', based on previous reports.

^fTotal number and percentage of patients.

based on the strict criteria is shown in Table II. In 'leukocyte positive' samples, normal sperm morphology was significantly less frequent (*P* < 0.05). None of these samples showed >14% normal forms evaluated with strict criteria. Significant differences were also seen when a threshold at 10% leukocytes was used (*P* < 0.05 for cut-offs at 4, 7 and 14% normal forms, Fisher's two-tailed exact tests), e.g. ≥14% strictly normal sperm forms were found in 64.1% (25/39) of samples when the leukocyte rate was <10% compared with 22.2% (2/9) in cases where round cell differentiation offered a higher leukocyte rate (≥10%).

Sperm function testing

The sperm–mucus interaction *in vitro* was evaluated using the *in-vitro* SCMPT with cervical mucus from the female partners in 82 couples (92.1%). The outcome of this migration test, based on the cumulative SCMPT score after 6 h observation, was inadequate in 39% and adequate in 61% of cases. The relationship between SCMPT results and sperm morphology assessment is shown in Table III. Sperm morphology based on strict criteria was significantly better in the group with

adequate SCMPT outcome. This could be demonstrated for thresholds at 4, 7 and 14% normal forms.

The significance of sperm morphological properties for mucus migration ability *in vitro* was confirmed when the cervical mucus of fertile donors, also obtained under standardized conditions, was used for the crossed SCMPT (*n* = 81; see Table III).

Medical history

The medical histories revealed general illnesses in 11.2%, mumps in 69.0% and previous genital infections in 14.0% of the patients. Of the men, 20.9% reported genital surgery and 8.1% varicocelectomy. None of the men remembered any genital injury.

In all, 22.5% of the patients (*n* = 20) received andrological medication, predominantly kallikrein (16/20). In 4.5% of the men the duration of andrological treatment was ≥2 years. These variables, as well as the age of the patients, smoking (>10 cigarettes/day in 33.3%), alcohol consumption (55.7%), stress due to working conditions (28.4%) or infertility investigation and treatment (11.3%), and infertility factors in their

Table III. Relationship of sperm morphology using strict criteria and sperm functional capacity, evaluated with the standardized in-vitro sperm-cervical mucus penetration test (SCMPT)^a

Normal forms (strict criteria)	Adequate ^b		Inadequate ^b		Total		P value ^c
	n	%	n	%	n	%	
SCMPT I (with partners' cervical mucus) ^d							
>4%	48	96.0	22	68.8	70	85.4	<0.04
>7%	39	78.0	18	56.3	57	70.2	<0.04
≥14%	27	54.0	4	12.5	31	37.8	<0.03
Total ^f	50	61.0	32	39.0	82	100.0	
Crossed SCMPT (SCMPT II) (with donors' cervical mucus) ^e							
>4%	48	96.0	21	67.7	69	85.2	<0.04
>7%	39	78.0	17	54.8	56	69.1	<0.04
≥14%	27	54.0	3	9.7	30	37.0	<0.03
Total ^f	50	61.7	31	38.3	81	100.0	

^aSperm-mucus interaction testing performed *in vitro* under hormonally standardized conditions.

^bBased on the total SCMPT score after 6 h of incubation.

^c χ^2 analysis or Fisher's two-tailed exact test, compared with the corresponding group below the threshold value indicated.

^dSCMPT performed with fresh cervical mucus from the female partners of the patients.

^eCervical mucus used for crossed SCMPT obtained from fertile female donors.

^fTotal number and percentage of patients.

Table IV. Sperm morphology using strict criteria and subsequent fertility under in-vivo conditions of conception^a

Normal forms (strict criteria)	Pregnancy rate (%)	No. pregnant/total no. of couples per group	P value ^b
≤4%	10.0	1/10	NS
>4%	21.5	17/79	
≤7%	3.7	1/27	<0.02
>7%	27.4	17/62	
<14%	11.1	6/54	<0.01
≥14%	34.3	12/35	
Total	20.2	18/89	

NS = not significant.

^aDetermined after 12 months.

^bFisher's two-tailed exact test.

female partners, did not offer a significant relationship with the outcome of the sperm morphological assessment using strict criteria.

Pregnancy rate

The pregnancy rate after 12 months in this prospective study was 20.2% (18/89). The relationship of sperm morphology using strict criteria and subsequent fertility is shown in Table IV. Statistical analysis revealed marked differences for the percentage of normal forms based on strict criteria when 4, 7 or 14% normal forms were taken as cut-off values.

The pregnancy rate was more than three times higher when semen samples had ≥14% normal forms compared with those having <14% normal spermatozoa (34.3 versus 11.1%; $P < 0.01$). For a threshold value of 7% normal forms, pregnancy rates were 27.4% when semen samples had ≥7% normal forms compared with 3.7% for those having <7% normal spermatozoa. Significant differences were also found when 20% strictly normal was taken as an additional cut-off value. The subsequent pregnancy rate was 14.1% (10/71) when morphological assessment based on strict criteria offered ≤20% normal forms, compared with 44.4% (8/18) when samples showed >20% normal spermatozoa ($P < 0.01$,

Fisher's two-tailed exact test). However, poor sperm morphology (≤4% normal) did not exclude a subsequent pregnancy.

Significant differences in the sperm morphological properties of those patients who did and those who did not achieve a pregnancy within the observation period were also found when median and range values were compared with a median percentage of strictly normal spermatozoa of 16.5% (range 3–33) in the fertile group compared with 10.0% (range 1–36) in the non-fertile group of men ($P < 0.01$, Wilcoxon's rank-sum test).

A logistic regression analysis including, in addition to strictly determined sperm morphology, the basic parameters of routine sperm analysis (sperm count, motility and standard morphology) offered a P value of 0.02 for the percentage of normal forms according to strict criteria; the other parameters did not achieve statistical significance. When standard sperm morphology was excluded from the logistic regression model, sperm motility also achieved significance ($P < 0.02$), whereas the impact of the other parameters remained unchanged (significant with $P < 0.02$ for strictly determined sperm morphology, not significant for sperm count).

The clinical relevance of the morphological properties for subsequent sperm fertilizing capacity was confirmed in further statistical analyses, after exclusion of, for example, those couples offering potentially influencing female factors of infertility (e.g. minor tubal pathology or hormonal disorders such as oligoamenorrhoea prior to treatment). The pregnancy rate in this subgroup was slightly higher (14/52, 26.9%). Again, the percentage of strictly normal forms in semen samples (with regard to the different threshold values and the medians and ranges) was significantly related to the subsequent fertility of the couples under in-vivo conditions of conception, e.g. the pregnancy rate was 47.6% in cases when ≥14% normal forms were found compared with 12.9% when a morphological semen evaluation using the strict criteria proved <14% normal spermatozoa ($P < 0.01$), as seen in Table V.

Table V. Sperm morphology using strict criteria and subsequent fertility under in-vivo conditions of conception (evaluated after the exclusion of couples with minor factors of female infertility)^a

Normal forms (strict criteria)	Pregnancy rate (%)	No. pregnant/total no. of couples per group	P value ^b
≤4%	14.3	1/7	NS
>4%	28.9	13/45	
≤7%	6.3	1/16	<0.05
>7%	36.1	13/36	
<14%	12.9	4/31	<0.01
≥14%	47.6	10/21	
Total	26.9	14/52	

NS = not significant.

^aDetermined after 12 months.^bFisher's two-tailed exact test.

Discussion

The role of the traditional semen parameters, including sperm morphology as a prognostic indicator of sperm fertilizing capacity, is a matter of constant debate (e.g. Falk and Kaufman, 1950; Page and Houlding, 1951; Hinting *et al.*, 1980; Aitken *et al.*, 1982b; Hargreave and Elton, 1983; Bostofte *et al.*, 1985, 1992; Jeulin *et al.*, 1986; Liu and Baker, 1988, 1992; Polanski and Lamb, 1988; Amann, 1989). Some authors have indicated that the morphological evaluation of spermatozoa using a very strict classification is of value in IVF protocols (Kruger *et al.*, 1986, 1987a, 1988a,b), but there is a lack of information for these criteria to be useful under in-vivo conditions.

Using this strict classification, markedly less 'normal' spermatozoa are found than with the standard procedure employed in most laboratories. The quick staining method used in our study proved to be easy and practical, and allowed the clear differentiation of spermatozoa. Advantages of the DiffQuick® stain technique over the classic Papanicolaou stain are a complete staining to reading time of <7 min, commercially prepared reagents and an easy staining procedure. Care has to be taken that slides are cleaned thoroughly and that a very thin slide is prepared to avoid background stains that could negatively influence the results.

A sperm morphological evaluation is considered to be a highly subjective procedure. Unlike the haematopoietic cells for example, the difficulty in classifying human sperm morphology is compounded by the large variety of abnormal forms encountered in the semen of infertile men. Only certain types of abnormality can be quantitated objectively. Comparative studies carried out >20 years ago concluded that the assessment of sperm morphology was personality orientated, qualitative, non-repeatable and difficult to teach to students and technicians (Freund, 1966). On the other hand, a low inter- and intratechnician variability using strict criteria has been reported (Kruger *et al.*, 1987b). In our study, the laboratory error in determining the sperm morphological properties (by strict criteria) was minimized by using one observer for all assessments.

The percentage of normal forms was taken as a parameter for morphological quality, and offered a median percentage of normal forms of 12% (range 1–36%). This corresponds well with the 12% (range 0–29) reported by Kruger *et al.* (1987b) but is lower than in other studies of the Tygerberg group

(Kruger *et al.*, 1988a,b). Unlike the selected study population of these authors (Kruger *et al.*, 1986, 1987a,b, 1988a,b), samples with <30% motility or <20×10⁶ spermatozoa/ml were not excluded. Our findings confirm previous reports, some of which were published many years ago, which demonstrated that the different characteristics of semen quality are significantly interrelated (Falk and Kaufman, 1950; Page and Houlding, 1951; MacLeod and Gold, 1951, 1953; Overstreet *et al.*, 1981; Bostofte *et al.*, 1984). In the present study, this correlation could be shown particularly for standard morphology and other semen parameters, but also with regard to strictly determined sperm morphological properties and, for example, sperm count and motility. These factors must be taken into consideration when interpreting the morphological findings.

Sperm morphology is regarded as a relatively stable parameter. Abnormal sperm morphology may be a reflection of poor testicular physiology. No significant relationship was found between the medical history and the results from the clinical examination. As the impairment of testicular function might be caused by inflammatory processes, all of the ejaculates were screened for microbial colonization and leukocytospermia, although all patients were asymptomatic in terms of genital tract infection. The majority of ejaculates were colonized by bacteria, in accordance with the findings of a much larger subfertile population (Eggert-Kruse *et al.*, 1992b). However, this did not interfere with the outcome of the strict morphological assessment. Nevertheless, these observations do not exclude the fact that the results might be quite different in men with current infections of the lower genital tract.

Elevated rates of leukocytes in semen are a potential marker for a subclinical infection, which may have an effect on sperm functional capacity and subsequent fertility. The relationship between leukocyte rates and sperm morphology using strict criteria has not been reported previously. It was shown that round cells mainly represented early spermatogenic cells, with a median percentage of leukocytes of only 3%. Because the relevance and upper limit of normality of white blood cells in semen is controversial (Wolff, 1995), different cut-off values were used to relate the leukocyte rates to the morphological findings. In 'leukocyte positive' samples, defined according to previous reports (Eggert-Kruse *et al.*, 1992a), a poor sperm morphology according to strict criteria was significantly more frequent, possibly reflecting impaired spermatogenesis.

Our study indicates that sperm morphological properties according to strict criteria are related to sperm functional capacity. A poor sperm morphology based on strict criteria was significantly more frequent when the results of the SCMPT with cervical mucus from the female partners were inadequate. The significance of morphological properties for the ability of spermatozoa to migrate could be confirmed when donor cervical mucus was used in the crossed SCMPT. This highlights the marked filtering capacity of human cervical mucus for abnormally configured spermatozoa (Fredricsson and Björk, 1977; Perry *et al.*, 1977; Jeulin *et al.*, 1985), particularly for spermatozoa with abnormal heads (Kremer, 1968; Hanson and Overstreet, 1981). On the other hand, the significant correlation of sperm morphology with progressive motility (Overstreet

et al., 1981; Katz *et al.*, 1982), also known to be important for cervical mucus penetration (Kremer, 1968; Aitken *et al.*, 1985; Eggert-Kruse *et al.*, 1989b), has to be considered. The relevance of a more differentiated sperm pathology (with regard to the different types of anomaly of the sperm head, neck and tail according to strict criteria) for the sperm–mucus interaction *in vitro* and *in vivo* is evaluated in another study (Eggert-Kruse *et al.*, 1995).

The association of semen quality and male infertility has been recognized for >40 years (MacLeod and Gold, 1953). However, there is no agreement as to what characteristics are unique to a fertile spermatozoon. The definition of morphological 'normality' is a matter of debate, as are the clinically relevant limits for the rate of pathological forms. The barrier function of the cervix is an important factor for fertility under *in-vivo* conditions of conception. Unlike reports on morphology and IVF outcome (Hinting *et al.*, 1980; Mahadevan and Trounson, 1984; Jeulin *et al.*, 1986; Kruger *et al.*, 1986, 1987a, 1988a,b; Liu and Baker, 1992), this factor has not been overcome with assisted reproductive technologies in our study. Under *in-vivo* conditions of conception, it can also be demonstrated that the pregnancy rate was significantly higher in cases of better sperm morphology based on strict criteria when threshold values for the rate of normal forms, defined in IVF studies (e.g. Kruger *et al.*, 1988a,b), were used; this was also the case when the median rate of normal spermatozoa was used. For example, the pregnancy rate within 12 months of a sperm morphological assessment was 34.3% when $\geq 14\%$ normal forms were found in the semen samples, and 11.1% when $< 14\%$ were found ($P < 0.01$); with regard to a cut-off of 7%, cumulative pregnancy rates were 27.4 versus 3.7% ($P < 0.02$) when $\geq 7\%$ versus $< 7\%$ normal forms were found in the semen samples. Significant differences were also found when 20% normal forms was taken as an additional limit ($P < 0.01$). However, it could also be demonstrated that very poor sperm morphology ($< 4\%$ normal forms) did not exclude subsequent fertility under *in-vivo* conditions.

Particularly under the usual *in-vivo* conditions of conception, pregnancy rates are influenced by a multiplicity of different parameters. Subfertility has to be considered as a problem of the couple. Therefore, both partners were submitted to a comprehensive infertility investigation in our study. Couples with a clinically considerable female factor of infertility, e.g. severe tubal pathology, were excluded. With regard to their female partners, all patients had a reasonable chance of achieving a pregnancy. The clinical relevance of discrete hormonal disorders, e.g. some luteal insufficiency or borderline results of function tests, in cases of spontaneous ovulatory cycles is controversial. However, because minor disorders in female partners could also potentially influence the pregnancy rate, additional statistical analyses were performed in this study paying particular attention to these variables, which confirmed the significant relationship of strictly determined sperm morphology and subsequent fertility.

On the other hand, with regard to the male factor, strictly assessed sperm morphology must be considered as only one of many clinically relevant variables. The medical history and the results of a clinical andrological examination of the patient

have to be carefully analysed, as well as psychosexual factors and potential environmental influences. The interrelationships of the different parameters of microscopical semen analysis should also be considered, as well as biochemical factors and the ability of the spermatozoon to penetrate the cervical mucus and reach the site of fertilization [no pregnancy was achieved in our study when SCMPT offered an inadequate result ($P < 0.01$), confirming previous findings (Eggert-Kruse *et al.*, 1989a,b). In addition, the duration of the patients' infertility is also important (e.g. MacLeod and Gold, 1953; Aafjes *et al.*, 1978), as well as many other andrological factors. Therefore, during infertility follow-up, it is necessary to base the estimation of fertility prognosis and therapeutic decisions not only on one but on several different determinants. Although the evaluation of strict morphology alone is not sufficient for an exact definition of sperm fertilizing capacity, a sperm morphological assessment according to strict criteria provides valuable information about sperm quality and is therefore useful for basic infertility investigations.

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Received on August 8, 1994; accepted on September 22, 1995