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Evaluation of the one-step eosin-nigrosin staining technique for human sperm vitality assessment

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BACKGROUND: The one-step eosin-nigrosin staining technique for assessment of sperm vitality was developed in the 1950s for various mammalian species. Although commonly used on human sperm in semen, a validation for this use has not previously been published. METHODS: The technique was evaluated on 1235 consecutive semen samples. RESULTS: The one-step eosin-nigrosin staining technique gave valid results when evaluated with sperm motility data obtained according to World Health Organization standard (1992, 1999). The mean for the sums of stained (i.e. supposedly dead) and motile sperm using the one-step eosin-nigrosin technique was 91% (SD ± 10%). The distribution of sums for percentage stained and percentage motile sperm was similar, regardless of whether the samples had many or few dead sperm. CONCLUSIONS: Standardization and quality control of basic semen analysis demands robust, reliable and simple techniques that are easy to learn, and easy to continue to perform in the same way. The one-step eosin-nigrosin technique does not need negative phase contrast optics but can be run with ordinary bright-field microscopy. Since it also includes fewer methodological steps to control, it seems preferable in terms of standardization and quality control management. It should therefore be recommended in the basic semen analysis when sperm vitality is to be assessed.

Key words: eosin/nigrosin/semen analysis/sperm viability/sperm vitality

Introduction

Assessment techniques which are robust, reliable and as easy as possible to perform are fundamental to modern basic semen analysis. The assessment of sperm vitality is one of the basic elements of semen analysis, and is especially important in samples where many sperm are immotile, to distinguish between immotile dead sperm and immotile live sperm.

The concept of using eosin to mark dead cells, which take up eosin, and nigrosin as a background stain, to increase the contrast between faintly stained cells and an otherwise bright background, is well known and widely used (Blom, 1950; Williams and Pollak, 1950; Campbell *et al*., 1956; Beatty, 1957; Eliasson and Treichl, 1971; Dott and Foster, 1972; Dougherty *et al*., 1975; Eliasson, 1977; Mortimer, 1985; Mortimer *et al*., 1990; World Health Organization, 1992; 1999). The simplified one-step technique, exposing sperm to a mixture of eosin and nigrosin, was introduced on boar, bull and ram sperm (Campbell *et al*., 1956), on rabbit sperm (Beatty, 1957) and further evaluated for various mammalian sperm (Dott and Foster, 1972). The technique was used on washed human sperm by Mortimer (Mortimer *et al*., 1990). Although Mortimer pioneered its use on human sperm in semen (Mortimer, 1985; 1994), no formal evaluation on its use on sperm in raw semen has been published.

Since the one-step technique is now widely in use for basic semen analysis, it is vital to the ongoing international standardization and quality improvement (Björndahl *et al*., 2002; Kvist and Björndahl, 2002a,b) to evaluate the technique on sperm in semen.

Materials and methods

We studied 1235 consecutive semen samples delivered to our clinical laboratory (mainly men in couples consulting the clinic due to infertility problems, primarily for diagnosis, secondarily for treatment, but also men referred for endocrine disturbances affecting the testicular function). The samples were selected using the following criteria: completely collected sample, sample collected by masturbation and complete vitality and motility assessments performed (excluding samples with severe oligozoospermia).

The one step eosin-nigrosin staining technique

The staining solution for the one-step technique contained 0.67% eosin Y and 10% nigrosin according to Mortimer (Mortimer, 1985; 1994) but dissolved in 0.9% sodium chloride in distilled water. Thus,

Table I. Comparison of four different techniques for vitality assessment with dye exclusion. Techniques from publications1-4 given in Table, experimental data from the present study (technique 1) and from Eliasson (Eliasson, 1977) (Techniques 2–4)

Techniques for vitality assessment with eosin exclusion	No. of samples	% motile + % dead (stained) sperm		
		Mean (SD)	95% CI	Range
One-step eosin-nigrosin (0.67% eosin + 10% nigrosin 30 s)[1]	1235	91 (10)	72–110	45–120
One-step eosin alone 0.5% eosin Y, 60–120 s (dark background, neg. phase contrast)[2]	20	96 (12)	72–120	76–107
Two-step eosin- nigrosin 1% eosin Y 15 s, 10% nigrosin ⩽15 s[3]	20	95 (10)	74–116	68–109
Two step eosin-nigrosin 5% eosin Y 15 s, 10% nigrosin ⩽15 s[4]	20	109 (10)	87–131	97–117

[1]Present study.
[2]Eliasson and Treiche, 1971.
[3]Eliasson, 1977.
[4]Dougherty *et al.*, 1975.

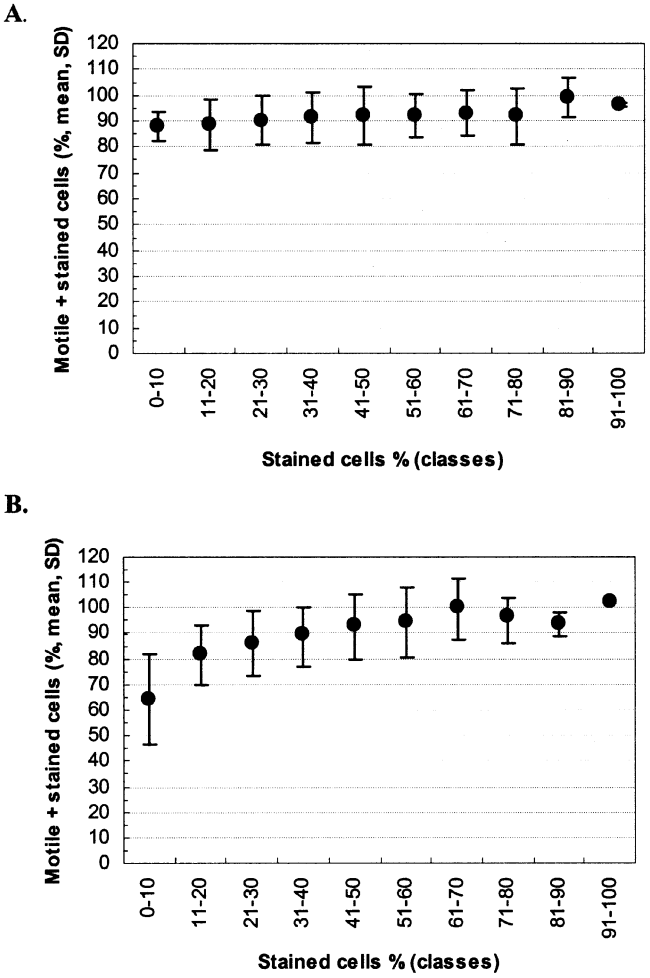

Figure 1. Proportion stained + motile sperm (mean, SD) distributed into classes according to percentage of stained (dead) sperm. (**A**) The present study (n = 1235). (**B**) Graph drawn from data in Eliasson and Treichl (Eliasson and Treichl, 1971), (n = 724).

0.67 g of eosin Y yellow (CI 45380, VWR No 115935) and 0.9 g sodium chloride were dissolved in 100 ml distilled water under gentle heating. Then 10 g of nigrosin (CI 50420, VWR No 115924; VWR™ International, www.vwr.com) was added. The solution was brought to the boil and allowed to cool to room temperature (20°C) after which it was filtered through quality filter paper (Munktell, quality 3: 90 g × m^{-2} retention of coarse and gelatinous precipitates; filtration speed 700 ml × min^{-1} × 100 cm^{-2}; VWR No 108016–9; VWR™ International) and stored in a dark and sealed glass bottle. Before use, the staining solution was brought to room temperature.

Semen samples were liquefied at 37°C for 30 min before analysis. Approximately equal volumes of semen and stain were mixed. One droplet [50 µl taken with an ordinary water-calibrated Finnpipette (5–50 µl) with yellow plastic tips; Treff, Labora 003–2271] of undiluted and well-mixed liquefied semen was mixed in a ceramic well with one droplet of the eosin-nigrosin staining solution (50 µl). The suspension was incubated for 30 s at room temperature (20°C). Then, a 12 µl droplet was transferred with the pipette to a labelled microscope slide where it was smeared by sliding a cover slip in front of it. Two smears were made from each sample.

The smears were air dried and examined directly. At least 200 sperm were assessed at a magnification of 1000× under oil immersion with a high-resolution 100× bright field objective (not phase contrast) and Köhler corrected illumination. Sperm that were white (unstained) were classified as live and those that showed any pink or red colouration were classified as dead, with the sole exception for sperm with a slight pink or red appearance restricted to the neck region ('leaky necks'), which were assessed as live. Reliability and repeatability of assessments were supervised through internal quality control where inter-individual variation was <10% (Mortimer, 1994).

In a pilot experiment, (n = 5), exposure time to eosin-nigrosin was varied from 30–300 s. All smears were assessed blindly, i.e. the person assessing did not know either sample identity or exposure time. All smears were mounted and coded before assessment.

Motility assessment

Sperm motility was determined by assessing at least 400 sperm in each semen sample. Each spermatozoon was categorized as belonging to one of four motility categories (rapid progressive, slow progressive, non-progressive and immotile), using techniques for assessment and quality control according to the World Health Organization (World Health Organization, 1992; 1999). For the comparisons in this study, the percentages of motile sperm (the sum of the three categories of motile sperm) were used.

Calculation and evaluation

The evaluation used here and earlier (Eliasson and Treichl, 1971; Eliasson, 1977) is based on the perception that the subpopulations of (i) motile, (ii) live immotile and (iii) dead immotile sperm include all sperm in a semen sample. From a theoretical point of view the sum of percentages motile and stained (dead) sperm in a sample should be <100% due to the presence of some immotile but live sperm. If assessed without errors the sum of percentages of motile and stained (dead) sperm cannot be >100%. If the sum of dead and motile sperm is >100% this could be due to an overestimation of the percentage of motile sperm, or to an error in the technique for vitality assessment rendering too high a percentage of stained (dead) sperm.

Results

Table I shows the results of this study (Technique 1). The sums of percentages of motile and dead sperm for each of 1235 samples are given as mean (SD), 95% confidence interval and range. For comparison, data from the evaluation of three other techniques (Techniques 2–4, 20 samples each) (Eliasson, 1977) are also given. The mean for sum of stained (dead) and motile sperm using the one-step eosin-nigrosin technique was 91% (SD ± 10%). In Figure 1A (present study), the average sums of the percentage of motile and percentage of stained sperm are plotted for the classes of increasing proportion stained sperm. The distribution of sums for percentage stained and percentage motile sperm was similar regardless as to whether the samples have many or few dead sperm. Figure 1B was drawn from earlier published data of the original evaluation of the eosin-alone method (Eliasson and Treichl, 1971).

The percentage of live sperm did not change with increasing time of incubation (30–300 s) in the eosin-nigrosin staining solution. Mean percentages of live sperm were 81, 80, 81 and 80% for incubation times of 30, 60, 180 and 300 s respectively.

Discussion

In any given semen sample, the sum of percentages for dead and live sperm is 100%. Live sperm are either motile or immotile. Thus the sum of percentages for dead and motile sperm should be somewhat <100%. As shown in Table I, the mean for the sum of percentages for motile and stained (dead) sperm was 91% (95% confidence interval: 72–110) with the present 0.67% eosin-nigrosin technique (Technique 1, Table I).This is in accordance with earlier data (Eliasson, 1977) for the 0.5%-eosin-alone technique [Technique 2, Table I: 96% (72–120); Eliasson and Treichl, 1971] and the two-step 1%-eosin and nigrosin technique [Technique 3, Table I: 95% (74–116); modified (Eliasson, 1977) from the original (Dougherty *et al*., 1975). The present technique gave the narrowest 95% confidence interval. The wider total range in the present results (45–120%)—due to extremes—may be explained by the huge difference in population size (Technique 1: $n = 1235$ versus $n = 20$ for Techniques 2–4).

However, these three techniques clearly differ from the original two-step 5% eosin-nigrosin technique (Technique 4 in Table I) described earlier (Dougherty *et al*., 1975). This technique showed a systematic error giving a sum >100% for stained and motile sperm: on average 109% (87–131). The increased proportion of dead sperm was explained by a toxic effect of the 5% eosin concentration (Eliasson, 1977). Other factors that might contribute to false high percentages of dead sperm include storage of stained and unmounted smears in a humid environment. Once the smears are made and dried, all sperm will be dead. In a humid environment, condensed water vapour could reconstitute the dye solution which then enters the cells. The one-step eosin-nigrosin technique minimizes the exposure of sperm to eosin (concentration of eosin and duration of exposure) when compared to the other techniques (Table I). The total time of exposure includes the 30 s used for mixing and incubation, the time for making the smear and the time for the smear to dry. Since environmental temperature, mixing and incubation, volume used for making the smears and the size of the slides were kept constant, and thus controlled, variations in eosin exposure time was minimized. Furthermore, our data suggest that variations in exposure-time, from 30–300 s in the nigrosin mixture before making the smears, do not affect the final percentage of dead sperm in human semen. The one-step eosin-alone technique (Eliasson and Treichl, 1971) gave, when compared with the one-step eosin-nigrosin method, systematically lower values for the sum of percentages motile and stained sperm in samples with few dead sperm (Figure 1A and B). Whether this was due to an underestimation of the proportion of stained (dead) sperm or an underestimation of the proportion of motile sperm cannot be concluded. Different studies have used different methods for assessment of the percentage of motile sperm, whereas the present study, following the method recommended by the WHO (World Health Organization, 1992; 1999), was run at 37°C and included quality control with comparisons of duplicate counts and systematic goal-oriented training of technicians (Mortimer, 1994).

The modifications of the original one-step technique (Mortimer, 1985) in the present one-step eosin-nigrosin technique are given in detail in the Materials and methods and involve a change in solvent (saline instead of tap water), specification of volumes and the smearing technique used. These modifications conform to the recommendations of the Nordic Association for Andrology and the Special Interest Group in Andrology of the European Society of Human Reproduction and Embryology (Kvist and Björndahl, 2002a).

In conclusion, we found that the one-step eosin-nigrosin staining technique for sperm vitality assessment modified from Mortimer (Mortimer, 1985; 1994; Mortimer *et al*., 1990) gives valid results when evaluated with sperm motility data obtained according to the WHO recommendations (1992, 1999). In contrast to the eosin-alone technique (Eliasson, 1977; Eliasson and Treichl, 1971) the evaluation showed that the one-step eosin-nigrosin technique is reliable in assessing sperm vitality in samples regardless of whether there are few or many dead sperm. Standardization and quality control of basic semen analysis demands robust, reliable and simple techniques that are easy to learn and easy to continue to perform in the same way. The one-step

eosin-nigrosin technique is assessed using standard bright-field microscopy; it does not require the rather uncommon, and difficult to obtain, negative phase contrast optics. Since it includes fewer methodological steps to control than the two-step eosin-nigrosin technique, it seems preferable in terms of standardization and quality control management. It should therefore be recommended in the basic semen analysis when sperm vitality is to be assessed.

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