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Why the WHO Recommendations for Eosin-Nigrosin Staining Techniques for Human Sperm Vitality Assessment Must Change

Andrology Lab Corner*

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In basic semen analysis and many research-oriented investigations, it is important to distinguish between dead and live spermatozoa. A proportion of live spermatozoa can be identified by their motility, while the ability to distinguish live, immotile spermatozoa from dead spermatozoa requires other techniques. Several dye exclusion techniques have been developed to distinguish live and immotile spermatozoa from dead spermatozoa (Table 1). The underlying principle on which these techniques are based is that spermatozoa with structurally intact cell membranes—supposedly live spermatozoa—are not stained, while dead spermatozoa with disintegrating cell membranes take up the stain. A 2-step staining technique was originally introduced for the examination of bull sperm (Blom, 1950), in which Eosin Opal Blue, bluish eosin, was first added to bull semen and then mixed with a nigrosin solution. This method was also reported to be useful for human sperm (Williams and Pollak, 1950). Further variants have been developed and used in a series of mammals (Campbell et al, 1956; Dott and Foster, 1972, 1975; Beatty, 1957; Dott, 1975). In 1971, Eliasson and Treichl published a technique in which human spermatozoa (in semen) were exposed to eosin Y (yellow) only; negative phase-contrast optics were used to obtain a dark background during assessment. They concluded that concentrations of eosin greater than 7 g/L increased the numbers of stained spermatozoa if an incubation time of more than 15 seconds was used. A variant of Blom's original technique, which consisted of incubating the samples in eosin Y for 15 seconds before nigrosin and then making the smears, was published a few years later (Dougherty et al, 1975). This technique was later compared (Eliasson, 1977) with the eosin Y–only technique (Eliasson and Treichl, 1971) and with a modified version of the protocol of Dougherty et al (lower eosin Y concentration), and it was concluded that nigrosin could be used as a counterstain, provided that the eosin Y concentration was not greater than 10 g/L.

The World Health Organization (WHO) laboratory manual for the examination of human semen (1999) recommends 2 main techniques that use eosin Y as the active stain. The first technique uses eosin Y alone, and negative phase-contrast optics are used to obtain the requisite dark field during evaluation. Two variants of the eosin Y–alone technique are given (WHO, 1999: appendix IV.1.1 and 1.2): the first variant includes 30 seconds of exposure to eosin Y, followed by examination as a wet preparation; the second variant runs with a 60-second exposure to eosin Y, after which smears are prepared and air dried. The second recommended technique, subtitled a modification of Blom's technique, is a 2-step eosin-nigrosin technique that uses nigrosin to obtain the dark background for increased contrast and yields reliable evaluations using ordinary light microscope optics (WHO, 1999: appendix IV.2).

In 1985, Mortimer published a simplified 1-step eosin-nigrosin technique. In a recent study (Björndahl et al, 2003), a slightly modified version of this 1-step technique, in which the dyes were dissolved in saline (9 g/L of sodium chloride) instead of distilled water, was evaluated on sperm in semen samples using data from 1235 consecutive routine samples.

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Table 1. *Evaluated techniques for assessment of sperm vitality, including calculated exposure levels and exposure time of sperm to the different staining solutions; only the first (Blom, 1950) is for bull spermatozoa, and all others are for human spermatozoa**

Publication	Type of Technique, Stain, and Solvent	Procedure	Exposure Time and Concentration of Eosin and Nigrosin
Blom, 1950	2-step eosin-nigrosin (bull) • 50 g/L of eosin (bluish) (Eosin Opal Blue) in distilled water • 100 g/L of nigrosin in distilled water	• Mix 1 volume of semen with 2 volumes of eosin blue. • Add 4 volumes of nigrosin and mix. • Smear after a few seconds. • Mixing and smearing must not exceed about 1 min.	• Eosin OP: 33 g/L (2/3), 15–30 s • Eosin OP: 14 g/L (2/7), 30–60 s • Nigrosin: 57 g/L, 30 s
Williams and Pollak, 1950	2-step eosin-nigrosin • Blom's technique applied to human spermatozoa		
Eliasson and Treichl, 1971	1-step eosin alone • 5 g/L of eosin Y in 0.15 M phosphate buffer	• Mix 1 part (0.1 mL) staining solution with an equal volume (0.1 mL) of semen. • After 1–2 min, a smear is made, air dried, and examined in a negative phase-contrast microscope.	• Eosin Y: 2.5 g/L, 60–120 s
Dougherty *et al*, 1975	2-step eosin-nigrosin • 50 g/L eosin Y in water solution • 100 g/L of nigrosin in water solution	• Mix 1 drop of semen with 2 drops of eosin solution. • Wait about 15 s and add 3 drops of 100/gL of nigrosin solution.	• Eosin Y: 33 g/L, 15 s • Eosin Y: 16.7 g/L, ~15 s • Nigrosin: 50 g/L, ~15 s • Eosin Y: 6.7 g L, 15 s
Eliasson, 1977	2-step eosin-nigrosin • 10 g/L eosin and 100 g/L of nigrosin (or else asx in Dougherty et al, 1975)	• Mix and smear 2 slides; dry on warming plate.	• Eosin Y: 3.3 g/L, ~15 s • Nigrosin: 50 g/L, ~15 s
Eliasson, 1981	1-step eosin-alone 5–10 g/L of eosin Y in 0.15 M (isotonic) phosphate buffer	• Mix 0.1 mL of staining soluiton with 0.1 mL of semen. • After 1–2 min, a smear is made, air dried, and examined in a negative phase-contrast microscope.	• Eosin Y: 2.5–5 g/L, 60–120 s
	2-step eosin-nigrosin 10 g/L of cosin and 100 g/L of nigrosin in distilled water	• Mix 1 drop of semen with 2 drops of eosin solution. • Wait 30 s and add 3 drops of 100 g/L of nigrosin solution. • Mix and make smear; let air dry.	• Eosin Y: 6.7 g/L, 30 s • Eosin Y: 3.3 g/L, ~15 s • Nigrosin: 50 g/L, ~15 s
Mortimer, 1985	1-step eosin-nigrosin 6.7 g/L of eosin Y and 100 g/L of nigrosin in tap water	• Mix 1 drop of semen with 1 drop of eosin-nigrosin mixture. • After 30 s, smears are made, air dried, and examined in a light microscope.	• Eosin Y: 3.3 g/L, 30 s • Nigrosin: 50 g/L, 30 s
WHO, 1999: *appendix IV.1*	1-step eosin alone 5/gL of eosin Y in 9 g/L of sodium chloride solution	• Mix 1 drop of semen with 1 drop of eosin. • Make wet preparation after 30 s and examine in a light microscope (400 ×). • Mix 1 drop of semen with 1 drop of eosin; make smear after 1 min. • Let smear air dry and examine under oil in a negative phase-contrast microscope (1000X).	• Eosin Y: 2.5 g/L, 30 s • Eosin Y: 2.5 g/L, 60 s
WHO, 1999: *appendix IV.2*	2-step eosin-nigrosin 10 g/L of eosin Y in distilled water 100 g/L of nigrosin in distilled water	• Mix 1 drop of semen with 2 drops of 10 g/L of eosin Y. • After 30 s, add 3 drops of 100 g/L of nigrosin. • Make smears within 30 s.	• Eosin Y: 6.7 g/L, 30 s • Eosin Y: 3.3 g/L, 30 s • Nigrosin: 50 g/L, 30 s
Kvist and Björndahl, 2002a	1-step eosin-nigrosin 6.7 g/L of eosin Y and 100 g/L of nigrosin in 9 g/L of sodium chloride	• Mix 50 μL of semen (adjusted for sperm conc) with 50 μL of eosin-nigrosin mixture. • After 30 s, smears are made, air dried, and exmined in an ordinary light microscope.	• Eosin Y: 3.3 g/L, 30 s • Nigrosin: 50 g/L, 30 s

* WHO indicates World Health Organization; conc, concentration.

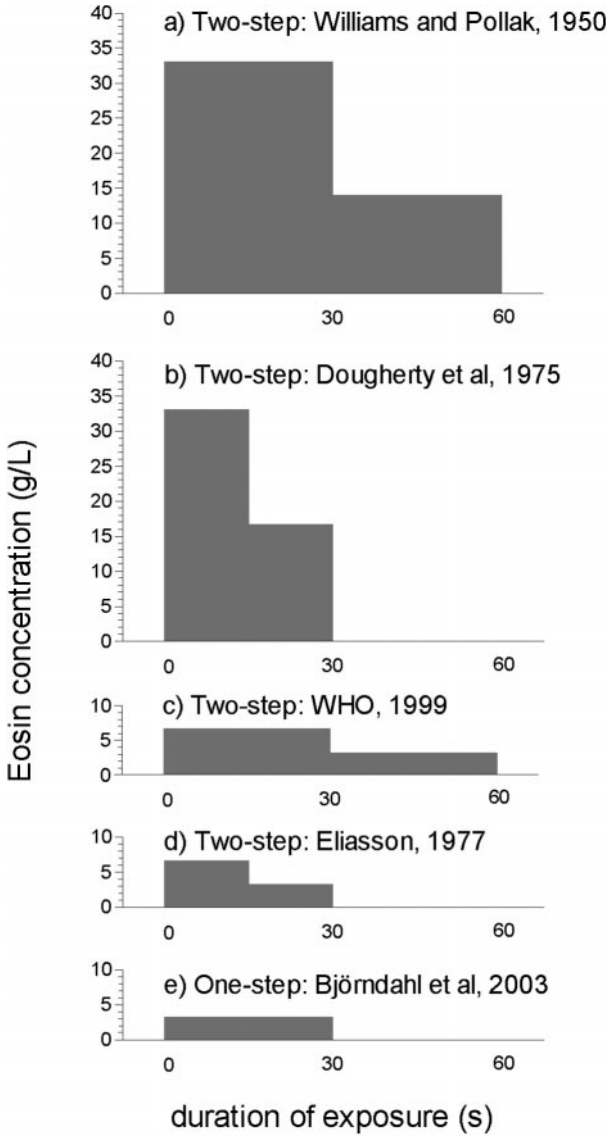

Figure 1. Duration of exposure of sperm in semen samples to eosin Y and dye concentration (grams per liter) according to different protocols.

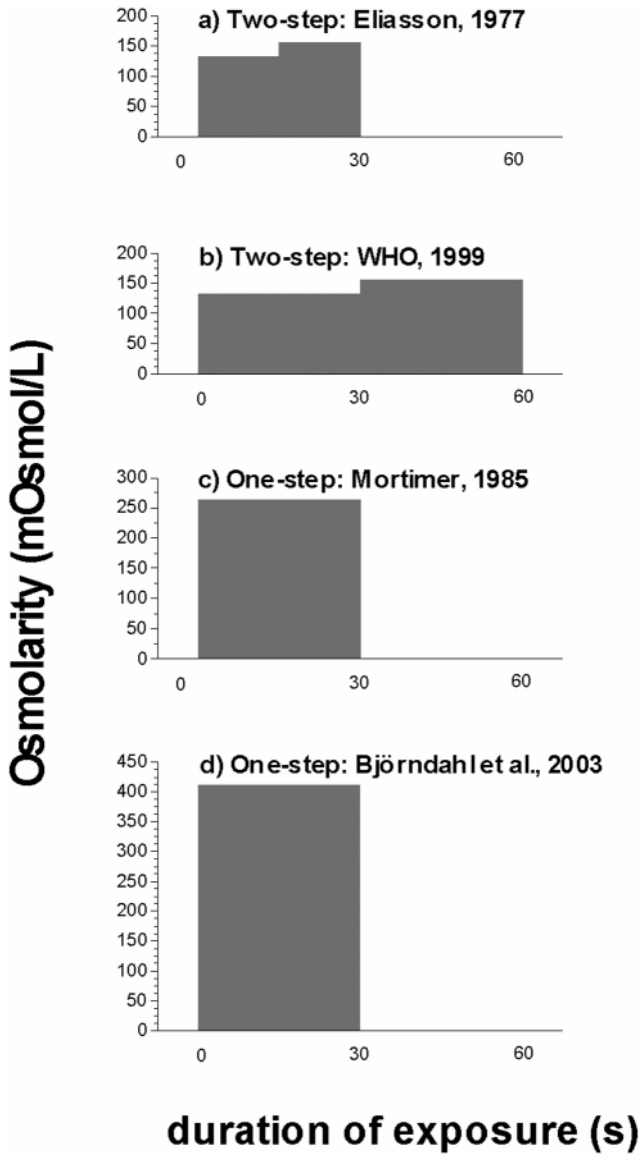

Figure 2. Duration of sperm exposure to different osmolarities according to different protocols. Note the severe low-osmolarity exposure present in the protocols of **(a, b)** and the doubled exposure time in **(b)** compared to the evaluated technique **(a)**.

Discussion—Why Must the WHO Recommendations Be Changed?

For continued progress to be made toward improvement and international standardization in andrology laboratories, it is mandatory that the techniques recommended and used for routine work, training, and external quality control programs be properly evaluated (Kvist and Björndahl, 2002a). It is also important that the recommended protocols be practical, robust, and reliable for routine, daily use in the andrology laboratory.

As is evident from Table 1 and Figure 1 (eosin concentration in relation to duration) and Figure 2 (osmotic conditions in relation to duration), the dye exclusion protocols differ considerably with regard to type and concentration of eosin (in staining solution and after mixing with semen), duration of exposure to eosin, degree of dilution of semen (affecting free concentration of eosin), and osmotic conditions (duration of exposure and type of dilution medium; affecting sperm volume and viability).

The Eosin-Only Protocol—The negative phase-contrast optics required for this technique are not easily obtained, which diminishes its usefulness. However, irrespective of this, there are a number of questions regarding the protocol recommended by the WHO manual in relation to the original protocol. This technique, as introduced by Eliasson and Treichl (1971) for human spermatozoa, used a 5-g/L eosin Y solution in 0.15 M phosphate buffer at pH 7.4. The protocol was later tested on a further 20

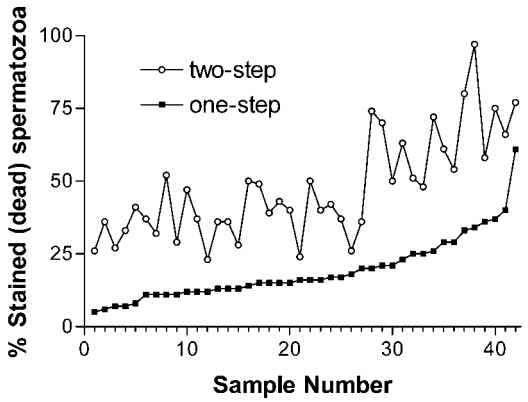

Figure 3. Percentage of stained (dead) spermatozoa in 42 samples, displayed in size-order according to the 1-step protocol, assessed with the 1-step eosin-nigrosin technique (filled squares) and with the 2-step eosin-nigrosin technique recommended by the World Health Organization (WHO) (open circles).

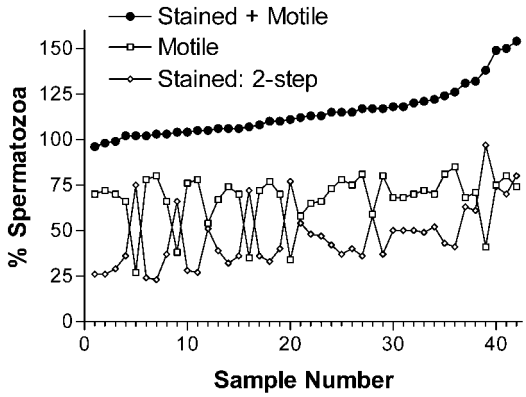

Figure 4. Relation between the percentage of motile spermatozoa (open squares), stained spermatozoa (open diamonds), and sum of stained and motile spermatozoa (filled circles) in 42 samples, displayed in size-order for sum of stained and motile spermatozoa, examined with the 2-step eosin-nigrosin protocol recommended by the World Health Organization (WHO).

semen samples (Eliasson, 1977). However, in the first edition of the WHO manual (see Belsey et al, 1980), the dilution medium for the eosin was given as ''distilled water''; in the second edition (1987), it was given as ''physiological saline''; and in the following editions (1992, 1999), it was given as 0.9% (9 g/L) aqueous sodium chloride solution, indicating a series of small editorial mistakes or changes not supported by experimental data. The validation of the 20 semen samples (Eliasson, 1977) was referenced in all 4 WHO editions. The original protocol included mixing equal parts of the eosin-alone solution and semen; after a wait of 1–2 minutes, a smear was made from a drop of this mixture on a microscope slide. After air drying, the smear was assessed using negative phase-contrast microscopy (Eliasson and Treichl, 1971). This procedure was recommended in the second and third editions of the WHO manual (1987, 1992). However, editorial changes in the fourth edition (1999) provided the option of using 2 variants during assessment: 1) direct assessment using a wet preparation after 30 seconds, or 2) later assessment under oil immersion using an air-dried smear made after 1 minute of incubation.

Thus, neither the recommended solvent (physiological saline) nor the technique to assess sperm vitality for a wet preparation is declared in the stated reference.

The Eosin-Nigrosin Protocols—In our previous validation of the 1-step eosin-nigrosin protocol (Björndahl et al, 2003), we obtained results that indicated this technique was at least as good as the eosin-only protocol (Eliasson and Treichl, 1971) as well as the 2-step eosin-nigrosin protocols (Eliasson, 1977). Moreover, the 1-step protocol appears to yield more reliable results for tests in which low percentages of stained sperm are found (cf Eliasson and Treichl [1971] vs Björndahl et al [2003]). With this background, we were interested in performing a direct comparison of the 2-step eosin-nigrosin protocol (WHO, 1999) with the 1-step protocol (Björndahl et al, 2003). We used 42 semen samples from routine laboratory work. Vitality smears were made simultaneously with both techniques within 30–60 minutes after ejaculation; these were then coded and assessed blindly. The proportions of dead spermatozoa were consistently higher with the WHO 2-step protocol (Figure 3). Evaluating the protocols by using the sum of proportions of dead and motile sperm showed that the WHO 2-step protocol yielded erroneous results by demonstrating that the sum of dead and motile sperm was more than 100% of their combined total (Figure 4), while the 1-step protocol was less than 100% in most samples (Figure 5). As discussed below, a number of factors may have contributed to these errors: the availability of eosin and the dilution of semen, the type of solvent, the osmolarity of the staining solution, and the time in the staining solution.

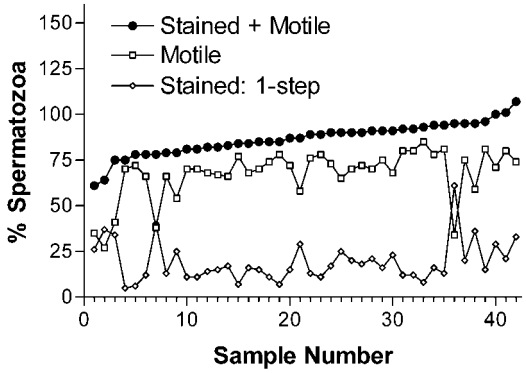

Figure 5. Relation between the percentage of motile spermatozoa (open squares), stained spermatozoa (open diamonds), and sum of stained and motile spermatozoa (filled circles) in 42 samples, displayed in size-order for sum of stained and motile spermatozoa, examined with the 1-step eosin-nigrosin protocol.

Availability of Eosin and Dilution of Semen—The eosin Y dye is toxic to washed human spermatozoa in a salt solution at a concentration of 10 g/L (Eliasson, 1977). Thus, additional seminal plasma components (eg, proteins) that bind eosin appear to reduce the free concentration of eosin to which the spermatozoa are exposed. It could be argued that sample variation in seminal proteins can expose spermatozoa to a critical level of eosin in certain samples when using a standard protocol. Mixing the eosin stain with semen involves dilution of the eosin, which decreases the total eosin concentration, but it also causes a reciprocal dilution of semen, which decreases the concentration of eosin-binding components. Thus, the 2-step 10-g/L eosin staining technique, with a 1:3 dilution of semen with water, will create a total eosin concentration of 6.7 g/L in the first step, with a threefold decrease in eosin-binding components, and a total eosin concentration of 3.3 g/L in the second step, with a sixfold dilution of eosin-binding components. The amount of eosin in relation to the eosin-binding components becomes 20% when steps 1 and 2 are combined (step 1, 6.7 g/L per 33% of eosin binder; step 2, 3.3 g/L per 16.7% of eosin binder). Since the 1-step protocol with 6.7 g/L of eosin includes only a 1:2 dilution, the relative eosin concentration per amount of eosin-binding components is 6.7% (3.3 g/L per 50% of eosin binder). Thus, the 2-step protocol has a threefold higher amount of eosin in relation to the eosin-binding components when compared with the 1-step protocol.

Osmolarity—In Blom's original technique, bull spermatozoa and semen constituents are diluted 1:3 in the first step and then exposed to slightly hypo-osmotic conditions (calculated at 250 mOsm/L; Table 2, 2-step technique; Dougherty et al, 1975) with eosin (33 g/L in water) for 15 seconds.

Dougherty et al (1975) only exchanged Blom's eosin blue for eosin Y, while the final dilution, osmolarity, eosin concentration, and duration remained the same as in the original technique. Eliasson (1977) reduced the concentration of eosin in the stain solution from 50 to 10 g/L. Since distilled water was used as the solvent, the osmolarity of the staining solution was decreased by one fifth (39 mOsm; Table 2, 2-step technique) (Eliasson, 1977). The consequence is that spermatozoa exposed to the eosin solution (Eliasson, 1977) encountered a final osmolarity that decreased from an average of 237 mOsm (range, 223–250) (Table 2) (Dougherty et al, 1975) to an average of 133 mOsm (range, 119–146) (Table 2) (Eliasson, 1977). The 1:3 dilution and the 15-second duration remained unchanged. Thus, by using a more diluted eosin solution to reduce the toxic effect of eosin, spermatozoa were exposed to another severe challenge: an extremely hypo-osmotic environment. The reliability of the modification was based on 20 semen samples in which the sums of the percentages of stained and motile spermatozoa were lower than those found using the protocol of Dougherty et al. One possible reason that the validation (Eliasson, 1977) did not show a tendency for the eosin solution to kill sperm is that the percentages of motile sperm were underestimated (as discussed below).

To investigate whether the dilution of semen to a hypo-osmotic level could increase the proportion of dead spermatozoa, we performed 2 pilot experiments. For practical reasons, we used semen 5–6 hours after ejaculation. Since different aliquots from each semen sample were compared, the observations are valid as proof of principle that hypo-osmotic conditions can increase the proportion of dead spermatozoa. The hypo-osmotic conditions caused a significantly increased proportion of dead sperm (Figure 6; series 2, N = 7; series 3, N = 6). However, not all sperm were killed—a certain proportion of the spermatozoa withstood and survived both the effects of dilution and the effects of a low-osmolarity environment as well as the exposure to eosin. On the other hand, it seems that, for a given sample, there is a certain subpopulation of spermatozoa that is susceptible and that dies because of the effects of dilution and the exposure to hypo-osmotic conditions and eosin. Furthermore, the combined fractions of dead spermatozoa and spermatozoa that were sensitive to (and could be killed by) a low-osmolarity shock remained rather constant, although times from ejaculation to sample testing varied from 1 hour (Figure 6a) and 4–5 hours (series 2, Figure 6) to 6–7 hours (series 3, Figure 6). Obviously, with the 2-step eosin-nigrosin protocol (Belsey et al, 1980; Eliasson, 1981; WHO, 1987, 1992, 1999), both the initially dead sperm and those that have died because of the properties of the solution in which the stains were dissolved will be stained. It could be of interest to quantify the proportion of such susceptible spermatozoa in relation to the outcome of sperm preparation and assisted reproductive treatment. However, it remains unknown how important it is with respect to outcome that a fraction of spermatozoa in a sample has less resistance to dilution and hypo-osmotic conditions.

It is only at ejaculation that the osmotic conditions in semen equal those in other body fluids (290 mOsm). All sperm preparation procedures that use a liquefied semen sample as the starting point expose spermatozoa to a variable hypo-osmotic shock, since the liquefied semen has a higher osmolarity than the preparation media (Björndahl and Kvist, 2003). Nevertheless, for identifying dead spermatozoa, the 1-step eosin-nigrosin protocol appears to be reliable in detecting only the initially dead spermatozoa (Björndahl et al, 2003).

Time of exposure to hypo-osmotic conditions and stains. In the first edition of the WHO manual (Belsey et al, 1980), only the modified protocol (10 g/L of eosin Y in distilled water) (Eliasson, 1977) was recommended;

Table 2. *Osmolarity in staining solutions, semen, and sperm suspensions during human sperm vitality assessment; measured and calculated results (mOsm)**

	Mean mOsm	Range
Semen (n = 50), 25–60 min after ejaculation, stored at 37°C	321	280–361
3 h later	382	292–454
Semen (n = 12), < 1 h after ejaculation	348	299–380
Earle medium	292	286–298
Calculated osmolarity after dilution with equal part of Earle medium	320	299–340
Observed osmolarity after dilution with equal part of Earle medium	316	294–332
Mean difference, $P < .001$	3.8	0.5–6.0
One-step technique (Björndahl et al, 2003)		
6.7 g/L eosin, 100 g/L nigrosin, 9 g/L in saline in distilled water), 30 s	499	498–500
After mixing semen and stain (1 + 1, calc)	410	390–430
One-step technique (Mortimer, 1985)		
6.7 g/L eosin, 100 g/L nigrosin, in distilled water), 30 s (calc)	205	
After mixing semen and stain (1 + 1, calc)	263	243–283
Two-step technique (Dougherty et al. 1975)		
First-step solution: 50 g/L of eosin in distilled water, 30 s (calc as 5 times 5 g/L)	195	
Second-step solution: 100 g/L of nigrosin in distilled water, 30 s	179	
After mixing semen and first-step solution (1 + 2, calc)	237	223–250
After addition of second-step solution (3 + 3, calc)	208	201–215
Two-step technique (Eliasson, 1977; Belsey et al, 1980; WHO, 1987, 1992, 1999)		
First-step solution: 10 g/L eosin in distilled water, 30 s	39	39–39
Second-step solution: 100 g/L nigrosin in distilled water, 30 s	179	178–179
After mixing semen and first-step solution (1 + 2, calc)	133	119–146
After addition of second-step solution (3 + 3, calc)	156	149–163
HOS test (WHO, 1999)		
HOS test solution (cf Buckett, 2003)	150	
Semen (25–60 min as above)	321	280–361
Calculated after mixing 0.1 mL of semen with 1.0 mL of HOS-test solution	166	162–169
Low-osmolarity and semen-osmolarity saline		
1.4 g/L saline	47	46–48
11 g/L saline	399	398–399
Semen, series 2 (n = 6, 4–5 h after ejaculation)	368	348–402
After low-osmolarity dilution (1 + 2, calc)	154	147–165
After ''semen-osmolarity'' dilution (1 + 2, calc)	389	382–400
Semen, series 3 (n = 7, 6–7 h after ejaculation)	431	388–490
After low-osmolarity dilution (1 + 2, calc)	175	161–195
After ''semen-osmolarity'' dilution (1 + 2, calc)	410	395–429

* HOS indicates hypo-osmotic swelling; calc, calculation.

however, all references were given (Blom, 1950; Dougherty et al, 1975; Eliasson, 1977). This modified protocol was further changed in a textbook chapter (Eliasson, 1981) with only 1 reference (Eliasson, 1977): the duration of the first step was doubled from 15 to 30 seconds without any new experimental data to support the change. Thus, with this further modified protocol, spermatozoa encountered a doubled time in the dilution (exposure to eosin and hypo-osmotic conditions of 119–146 mOsm). This editorial mutation became the recommended protocol for the first eosin step in the second (WHO, 1987), third (WHO, 1992), and fourth (WHO, 1999) editions of the WHO manual. In all 3 editions, the same references were given (Eliasson, 1977, 1981).

The second step of the 2-step eosin-nigrosin protocol originally included a 15-second wait after admixture of the nigrosin solution (Blom, 1950; Dougherty et al, 1975). The eosin modification for the first step (Eliasson, 1977) also resulted in a lower osmolarity in step 2 from a calculated range of 201–215 mOsm (Table 2) (Blom, 1950; Dougherty et al, 1975) to 149–163 mOsm (Table 2) (Eliasson, 1977). The duration of incubation in the eosin-nigrosin stain remained 15 seconds (Eliasson, 1977). In the editorial changes of 1981 (Eliasson, 1981), the duration of the second step was changed to a time that was not specified in any other way except that a smear should be prepared after the admixture of the nigrosin solution. This protocol for the second step became the recom-

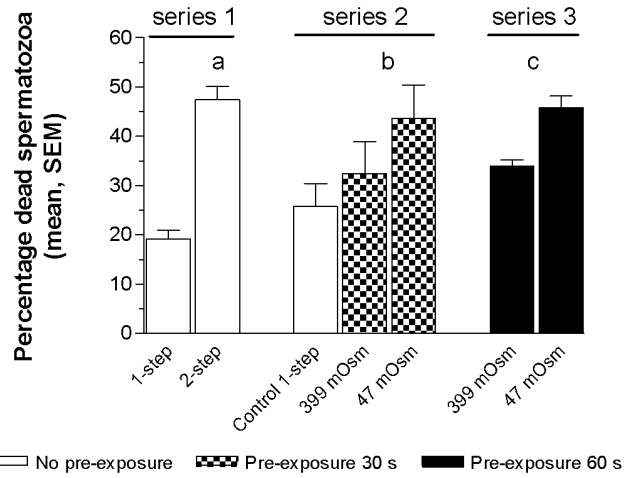

Figure 6. Percentages of dead spermatozoa. **(a)** Without any experimental dilution in low-osmolarity conditions, the World Health Organization (WHO) 2-step protocol (series 1, right bar) yielded more dead spermatozoa than the 1-step protocol (left bar: $P < .001$, paired t test; N = 42). **(b)** After 30 seconds of dilution in 47 mOsm of saline (series 2, right bar [47 mOsm] vs left bar [control], $P = .002$, paired t test; N = 7) or dilution in semenlike osmolarity saline (399 mOsm) (series 2, middle bar [399 mOsm] bar vs left bar [control], $P = .037$, paired t test, 1-tailed; N = 7), more spermatozoa were dead when compared with controls, using the 1-step eosin-nigrosin protocol. **(c)** After 60 seconds of dilution in 47 mOsm of saline, more spermatozoa were dead than after 60 seconds of dilution in saline at semen osmolarity (399 mOsm) (series 3, right bar [47 mOsm] vs left bar [399 mOsm], $P = .002$, paired t test; N = 6), using the 1-step eosin-nigrosin protocol. The percentage of dead spermatozoa did not increase when diluted for 60 seconds compared to when diluted for 30 seconds (''47 mOsm'': series 3, right bar, vs series 2, right bar, not significant [n.s.]; and ''399 mOsm'': series 3, left bar, vs series 2, middle bar, n.s. [Mann-Whitney U test, $N_1 = 7$, $N_2 = 6$]).

mended protocol in the second and third editions of the WHO manual (1987, 1992). In the fourth edition (1999), the only change made to the second step was that the time frame for making the smear was given as ''within 30s.'' Thus, from the initial protocol proposed by Blom, the exposure time increased from 15 seconds plus 15 seconds to 30 seconds plus 30 seconds.

The WHO protocol gave falsely high percentages of dead spermatozoa (Figures 3 and 4). Contributing to this situation could be the general dilution of semen that results in relatively higher amounts of unbound eosin, particularly the hypo-osmotic conditions created during the first 30 seconds of incubation. One question that arises is whether the protocol has given unreliable results since 1977 or 1981. The same dilution and the same low-osmolarity environment were used when the method was validated (Eliasson, 1977), and it seems unlikely that the method became harmful only when step 1 was editorially increased to 30 seconds (Eliasson, 1981). Although only experimental results can answer this question, it can be concluded that an underestimation of the percentage of motile spermatozoa also might have masked cell death due to low-osmolarity conditions in the 20 samples used for validation in 1977. At that time, sperm motility was estimated in 5% intervals, and percentages of high motility were seldom recorded. In support of a technique-based underestimation of the percentage of motile spermatozoa is the original validation of the eosin-alone method (Eliasson, 1971), in which the sums of dead and motile sperm were systematically lower in cases in which the percentages of dead (stained) sperm were 30% or less (Eliasson and Treichl, 1971; data reproduced in Björndahl et al, 2003) compared to cases in which the percentages of dead sperm were greater than 30%. However, since WHO introduced a standardized motility assessment that actually counts motile and immotile cells, along with a protocol that calls for duplicate counting to decrease the occurrence of random errors, this source of errors has essentially been eliminated.

Type of solvent. In a recent textbook chapter (Eliasson, 2002), the author acknowledges that, when water is used as a solvent, the percentage of dead spermatozoa will be artificially high and that the ''stains must be dissolved in 0.15M phosphate buffer,'' but no experimental basis for this conclusion is given. Furthermore, the earlier protocols (Eliasson, 1981; WHO, 1987, 1992, 1999) are acknowledged to be incorrect, but it is stated that the protocol in the original publications was correct. However, in the original publications, all protocols stated that the stains for the 2-step eosin-nigrosin technique were dissolved in water (Blom, 1950; Dougherty et al, 1975; Eliasson, 1977; Belsey et al, 1980).

Robustness. During the assessment of the slides prepared for this study, it became apparent that the dilution factor also had consequences for the time required to complete the analysis: an increased dilution of semen results in decreased numbers of spermatozoa visible in 1 microscopic field. Thus, when semen is more diluted, it is necessary to examine more microscopic fields to assess the same number of spermatozoa. The original Blom technique (1950) and its successors (Eliasson, 1977, 1981; WHO, 1999) create a sixfold dilution (1:6) of the original sample. The eosin-only protocol (Eliasson, 1977, 1981; WHO, 1999) creates a threefold dilution, whereas the 1-step eosin-nigrosin technique gives only a twofold dilution. This is of special importance for semen samples with low sperm concentration—just the type of samples for which the question of sperm viability most often occurs. The number of microscopic fields that must be screened to find at least 200 spermatozoa in a sample with 20 million cells/mL was calculated to be 58 for the 1-step eosin-nigrosin technique, 88 for the eosin-alone technique, and 175 for the WHO (1999) 2-step protocol. Thus, the 1-step eosin-nigrosin protocol is considerably more robust because the assessments are easier when compared with the less dense smears obtained using the other protocols.

Conclusions

The main differences between the protocol we validated earlier (Björndahl et al, 2003) and the protocol recommended by the WHO (1999) appear to be a combination of lower total exposure of spermatozoa to eosin (duration and concentration), avoidance of extremely hypo-osmotic conditions, and lower degree of dilution of the seminal constituents when using a 1-step technique.

The inevitable conclusion is that none of the protocols for human sperm vitality assessment in the most recent WHO manual have been properly validated. The protocols deviate significantly from the published validations on which they are supposed to be based, and it appears as if the reasons for modifications have been editorial rather than scientific. For future editions of the WHO manual, it is mandatory that techniques and protocols be based on scientific evidence and proper laboratory validation rather than editorial judgment.

For continued improvements in andrology standards to occur, it is crucial that the techniques used in routine training and external quality control be reliable. A standardized method for use in all laboratories that produces reliable and comparable results must be robust. The primary criterion for robustness is that the protocol be simple and include as few steps as possible. This makes the protocol easy to learn and perform consistently. Since the 1-step eosin-nigrosin technique uses fewer methodological steps to perform, does not require the assessment of as many fields, and is not dependent on negative phase-contrast optics, it is preferable in terms of standardization and quality management (Kvist and Björndahl, 2002a) and could therefore be recommended for use in basic semen analysis to distinguish between immotile and dead spermatozoa. For these reasons, the 2-step eosin-nigrosin as described in the WHO manual (1999) should be avoided.

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