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Original Article

Improving precision in the assessment of round cell numbers in human semen

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Abstract

The aim of this study was to increase the precision of assessment of the number of round cells observed in the peroxidase test for detection of seminal leukocytes (granulocytes). The dilution of semen was reduced and the volume of suspension examined was increased for semen samples containing between 0.6 and 6 million round cells per mL. A 1 + 5 (1:6) dilution was compatible with measurable peroxidase activity and a sufficiently clear background for cell assessment. At this dilution, and with assessment of all 18 grids on both sides of the Neubauer-improved counting chamber, only three of the 10 samples (nominal cell concentrations of 1.9×10^6–3.3×10^6 mL^{-1}) presented 400 round cells or more. As lower seminal dilutions were incompatible with easy detection of round cells or their peroxidase reaction product, it was not possible to provide precise measurements (sampling error 5%) of the suggested lower reference limit of 1×10^6 cells per mL. The results indicate that this poor precision of measuring 1×10^6 round cells per mL could explain the discrepant reports on the acceptability of the cut-off values for leukocytospermia. Such reference limits need to be established with statistically sound methods.

1   Introduction

There is debate over whether the World Health Organization (WHO)-suggested concentration of 10^6 leukocytes per mL in semen is a valid threshold value for leukocytospermia. Some have found this value too low [1], others too high [2, 3], depending on the end-point examined (semen quality, *In Vitro* Fertilization [IVF] results, bacterial presence, sperm response to reactive oxygen species). Other values from 0.5×10^6–1.0×10^6 polymorphs per mL or 1×10^6–2×10^6 leukocytes per mL have been suggested [1]. One aspect of this problem that has not re-

ceived attention is the precision of the methods by which these low concentrations have been generated. For any concentration, how certain one can be that the estimate provided is close to the true, but unknown, value depends solely on the number of cells counted (Poisson distribution). The precision of published methods for a semen sample containing 10^6 round cells per mL is given below.

Sharma *et al.* [2] examined one-in-four diluted semen in a Makler chamber. The number of cells counted was not given but the volume of the Makler chamber grid is 10 nL so theoretically only 2.5 cells should be present for a suspension of 250 000 cells per mL. The sampling error (%SE) for 3 cells is 58% with a rounded 95% confidence interval (CI) ± 116%, so that a true value of 1×10^6 per mL could be assessed as high as 2 160 000 per mL, with an equal chance of finding no cells in that volume. Politch *et al.* [4] diluted semen 1 + 1 with reagent and further diluted this 1 + 1 with PBS before counting in a Neubauer chamber. Assuming that only the central grid were examined, for the expected 25 cells visible (%SE 20%), estimates would range

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between 1 400 000 and 600 000 cells per mL. Wolff *et al.* [5] diluted semen 1 + 1 with reagent, which should present 50 cells (%SE 14%) in the central Neubauer grid, providing estimates of 1 280 000 to 720 000 cells per mL. Punab *et al.* [3] assessed the leukocyte concentration by comparing their number per microscope field relative to those of spermatozoa and multiplying by the sperm concentration. As a total of 200 round cells was counted, which gives a %SE of 7%, a true value of 10^6 mL^{-1} could be assessed as 1 140 000 or 860 000 cells per mL, although the accuracy of the method of assessing sperm concentration was not given.

Thus, none of the above reports provides the minimum precision recommended for assessing sperm concentrations accurately (400 cells, %SE 5%; WHO 1999 [6]), that provides a confidence interval spanning 1 100 000 to 900 000 cell/mL. The WHO 1999 manual [6] suggests that peroxidase-positive cells are counted in a haemocytometer chamber. At the recommended dilution of 1 + 9 for the cut-off concentration, the resulting 100 000/mL provides only 10 cells per central Neubauer grid, or 20 in both chambers (%SE 22%) so that values between 1 440 000 and 560 000 per mL would be reported. If solely the two central grids are examined, only samples containing 20 × 10^6 round cells per mL would achieve a counting error of 5% after a 1 + 9 dilution.

As the whole Neubauer chamber comprises nine 1 mm × 1 mm grids, assessing them all should increase the number of cells encountered and increase the precision of any value obtained. However, even in this case of a 1 + 9 dilution, a total of only 90 cells (or 180 in both chambers; %SE 7.5%) would be observed, providing an estimate between 1 150 000 and 850 000 cells per mL; still an unacceptably large confidence range. As the precision of assessment is number-dependent, it could be increased further by reducing the extent of semen dilution. However, the high background found at lower dilutions could interfere with recognition of cells or peroxidase activity. With Endz's reagent, low (1 + 1) semen dilutions can be used successfully [2, 5] but the use of *o*-toluidine makes reading easier, as less H_2O_2 is required and fewer bubbles interfere with measurement [7] and is the method recommended by WHO [6].

In this study, the dilution of semen in the standard peroxidase test [6] was reduced in order to determine which dilution was the best compromise between the anticipated improved counting precision and the reduced visibility of cells or the inhibition of peroxidase activity due to the higher background.

2 Materials and methods

Semen from ten patients attending the Centre of Reproductive Medicine and Andrology who had between

0.6×10^6 and 5.5×10^6 round cells per mL, as quantified by the standard (imprecise) method for peroxidase detection [6], was examined. Each patient signed a University Ethics Committee-prepared consent form that their semen may be used for research purposes. For the standard procedure, duplicate aliquots of 20 μL semen were each added to 180 μL ortho-toluidine reagent providing a 1 + 9 (1:10) dilution. In variants of the standard protocol, lower dilutions were prepared by mixing 20 μL semen with 140, 100, 60, and 20 μL reagent to provide 1 + 7 (1:8), 1 + 5 (1:6), 1 + 3 (1:4) and 1 + 1 (1:2) dilutions, respectively. Samples were vortexed for 10 s and incubated at 37°C for 20 min. Thereafter aliquots from each duplicate dilution were transferred to one side of a Neubauer improved counting chamber, allowed to settle for 2 min in a humid chamber, and the numbers of round and peroxidase-positive cells were assessed in the central grid of both chambers in an Olympus BX40 phase contrast microscope (Olympus, Hamburg, Germany). Values were accepted if the difference between the duplicates was within acceptable limits [6]. Round cells were also assessed in the additional 8 grids in each chamber so that all cells within the entire grid area of each side (900 nL; total 1.8 μL) were assessed.

The improved Neubauer haemocytometer has two separate counting chambers, each of which has a microscopic 3 mm × 3 mm pattern of gridlines etched on the glass surface. It is used with a special, thick coverslip (0.44 mm, thickness No. 4), which lies over the grids and is supported by glass pillars 0.1 mm above the chamber floor. Each counting area is divided into nine 1 mm × 1 mm grids each with a volume of 100 nL. The concentration of peroxidase-positive cells in semen is calculated from their number (N) divided by the volume of the total number (n) of grids examined for the replicates (where the volume of a grid is 100 nL), multiplied by the dilution factor. For a 1 + 9 dilution, the concentration (C) = (N/n) × (1/100) × 10 cells per nL: thus (N/n) was divided by 10 to obtain peroxidase-positive cells per nL (or million cells per mL). When all nine grids in each chamber of the haemocytometer were assessed, the total number of peroxidase-positive cells was divided by the total volume of both chambers (1.8 μL), and multiplied by the dilution factors (10, 8, 6, 4, 2), to obtain the concentration in cells per μL (thousand cells per mL).

3 Results

As expected, the number of round cells observed was greater when nine grids rather than only the central grid were assessed and as the dilution was reduced (Figure 1). At a 1 + 9 dilution, the maximum number of round cells observed in these samples in the two central grids was 109, whereas in the total 18 grids it was 599 (Table 1). In the 18

grids, 400 cells or more were only found in two samples, both of which had nominal round cell concentrations of 3.3 $\times 10^6$ per mL. At a 1 + 1 dilution a maximum of 351 cells was observed in 2 grids, still short of 400, yet up to 3 354 round cells were observed in 18 grids (Table 1) and seven of the ten samples (with nominally > 0.7 $\times 10^6$ cells per mL) contained more than 400 cells (Figure 1). Between these extremes, dilutions of 1 + 3, 1 + 5 and 1 + 7 provided sampling errors < 5% for three to five of the samples when the entire chamber was evaluated.

Theoretically a 4.5-fold dilution of semen containing 1 $\times 10^6$ cells mL^{-1} provides the precision needed (400 cells) if all grids are scored; however, peroxidase activity may not be measurable at such a low dilution. For three semen samples in this study, the background found in dilutions of 1 + 1 and 1 + 3 made round cells and the peroxidase activity difficult to discern and a dilution of 1 + 5 was the optimum for these samples and adequate for the others. At this dilution for the 10 samples studied, up to 147 cells were found in the 2 central grids and up to 1 271 in all 18 (Table 1). However, only three of the present samples had the required 400 cells and these had more than 2.9 $\times 10^6$ cells per mL (Figure 1).

4   Discussion

The WHO Laboratory Handbook for the examination of human semen [6] recommends that a simple peroxidase test be performed in 1 + 9-diluted semen to assess leukocoytes (granulocytes); it also suggests 1 $\times 10^6$ cell per mL as consensus reference limit. Which portion of the haemocytometer grid area to be assessed is not explained, but for sperm concentration only the central grid is mentioned. The recommended 1 + 9 (1:10) dilution of semen, if containing the consensus limit of 1 $\times 10^6$ round cells per mL, would provide only 10 cells in the central grid of the Neubauer chamber, 20 in duplicates, so that the sampling error (%SE 22%) is high and the precision of the estimated value is low (95% CI: 1 220 000 to 780 000 cells mL^{-1}). This was borne out in practice as examination only of the central

grid did not permit 400 cells to be counted in any of the samples assessed.

Precision should be increased by assessing the whole Neubauer chamber (nine grids per side, 18 in total) in which 90 (%SE 11%) and 180 (%SE 7%) cells, respectively, would be found, and for the latter, an estimate of 1 070 000 to 930 000 cells per mL provided. Although assessing all 18 grids in both Neubauer chambers for round cells in semen diluted 1 + 9 should provide a 5% counting error only for samples containing at least 2.22 $\times 10^6$ mL^{-1}, only two of the present samples (3.3 $\times 10^6$ mL^{-1}) presented more than 400 cells.

Thus with the current WHO-recommended procedure, neither in theory nor practice, was it possible to assess a round cell concentration of 1 $\times 10^6$ per mL with a sampling error of 5%. As this provides a 95% CI (± 10%) considered adequate for assessment of sperm concentration [6], it is hardly surprising that the "cut-off" value for leukocy-
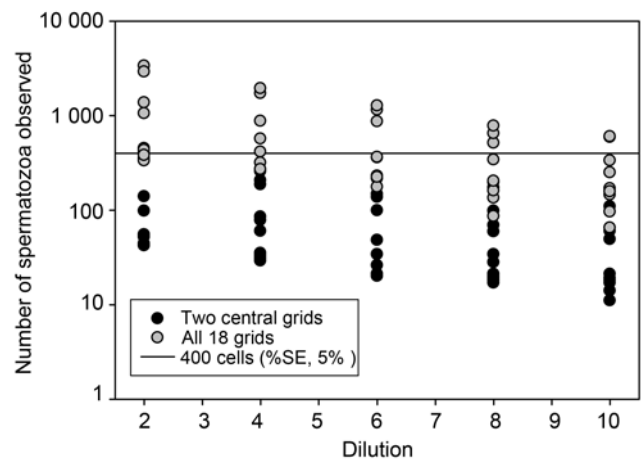

Figure 1. Plot of the number of spermatozoa observed (ordinate, log scale) at various semen dilutions (abscissa) in the two central grids of each chamber (●) and in all 18 grids of both chambers (◉). The horizontal line represents 400 cells that provide a 5% sampling error (%SE).

Table 1. Minimum (Min) and maximum (Max) numbers of round cells, and the calculated sampling error (%SE) and rounded 95% confidence intervals (CI) for the mean numbers, assessed in both central grids and in all 18 grids of the Neubauer improved counting chamber for different dilutions of 10 semen samples containing between 0.6 $\times 10^6$ and 5.5 $\times 10^6$ round cells per mL.

Dilution	Both central grids					All 18 grids				
	Min	Max	Mean	%SE	95% CI (± %)	Min	Max	Mean	%SE	95% CI (± %)
1 + 9	11	109	33	17.4	34.2	65	599	255	6.6	12.3
1 + 7	17	98	38	16.3	31.7	86	777	322	5.6	10.9
1 + 5	20	147	58	13.1	25.6	176	1 271	507	4.4	8.7
1 + 3	29	206	77	11.4	24.3	256	1 939	688	3.8	7.5
1 + 1	42	351	130	8.8	17.2	332	3 354	1 187	2.9	5.7

tospermia is disputed: none of the estimates provided so far with this method is statistically sound. With the modifications introduced here of reducing semen dilution to 1 + 5 (1:6), and analyzing the entire volume of the Neubauer haemocytometer (a total volume of 1.8 µL), a 5% counting error is theoretically achievable with a concentration of 1.33×10^6 round cells per mL semen and three samples, nominal concentration $2.9 \times 10^6 \, mL^{-1}$, did fulfil this criterion.

If the total number of leukocytes per ejaculate is required to describe patients with immunological infertility, such values need to be obtained by statistically robust methods on a sufficient numbers of cells. The new WHO manual [8] provides a method for assessing even lower sperm concentrations than 1×10^6 per mL precisely, and even larger volumes (by using more or larger chambers) could be assessed if 1×10^6 round cells per mL really needs to be assessed precisely.

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