Leukocytospermia Quantitation (ENDTZ) Test

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1 Introduction

Peroxidase positive granulocytes (neutrophils and macrophages) are identified by histochemical staining. This test is also referred to as Myeloperoxidase or Endtz test. It is performed when routine semen analysis shows that the number of round cells is $\geq 0.20 \times 10^6$/mL for male infertility patients [1–3]. In the case of no female factor infertility samples, the cutoff for performing the Endtz test is $\geq 1.0 \times 10^6$/mL. In this case, it is necessary to differentiate granulocytes such as neutrophils, polymorphonuclear leukocytes, and macrophages (primary sources of reactive oxygen species (ROS), which can lead to male infertility) from germinal cells, all of which are seen as round cells. The procedure is done using suspended cells in a liquefied semen specimen and quantitated by counting stained cells using a Makler counting chamber [4].

2 Equipment and Materials

A. Preparation of Stock Solution (stable for 6 months):

Mix these chemicals in a clean 100-mL bottle. The solution should be clear and yellow. Cover the bottle with aluminum foil and store in the dark. Fresh stock solution needs to be prepared if the solution gets dark in color or forms a cloudy precipitate.

CAUTION: Benzidine is carcinogenic and should be handled carefully. Wear gloves and a face mask to avoid accidental inhalation when handling. Prepare the solution in a biological safety cabinet. The expired Endtz test solution should be discarded in concentrated Clorox solution.

1. Ethanol—50 mL of 96 %
2. Benzidine—0.125 g reactive oxygen species (ROS)
3. Sterile water—50 mL

B. Preparation of Working Solution:

1. Mix 2.0 mL of stock solution and 25 µL of 3 % H_2O_2 in a 6 mL polystyrene tube (use 3 % H_2O_2 or dilute 30 % stock H_2O_2 1:10).
2. Cover the tube with aluminum foil (Fig. 7.1).
3. Prepare fresh working solution from stock every week and discard old solutions.

C. Phosphate-buffered saline

D. Makler counting chamber (Fig. 7.2)

E. Microcentrifuge tubes

F. Pipette (5 µL, 20 µL, 40 µL) tips

3 Quality Control

A. A positive control should be run weekly to check reagents.

B. Endtz test results should be positive with the new and old reagent.

C. If the results are negative, mix new reagents and retest the control. If still negative, try a new control specimen.

Note: If a semen specimen is not available, an EDTA anti-coagulated blood specimen may be used. Centrifuge the blood specimen to obtain the buffy coat. Remove supernatant by using a transfer pipette. Remove the buffy coat using a transfer pipette, dilute it into 2 mL of PBS buffer, and aliquot (0.1 mL). These aliquots may be used for 1 month.

4 Procedure

A. Take 20 µL of liquefied semen specimen in a dark-colored microcentrifuge tube (Fig. 7.3); add 20 µL of PBS solution and 40 µL of working Endtz solution. Vortex and incubate at room temperature for 5 min.

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Fig. 7.1 Endtz working solution. [Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2015. All Rights Reserved.]

Fig. 7.2 Makler counting chamber. [Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2015. All Rights Reserved.]

Fig. 7.3 Dark-colored microcentrifuge tube. [Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2015. All Rights Reserved.]

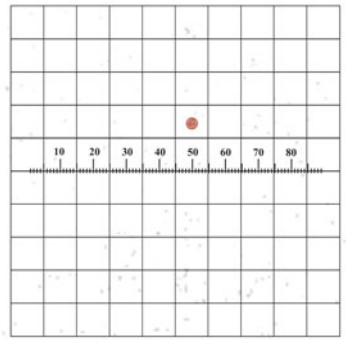

Fig. 7.4 Stained leukocyte as seen on Makler chamber grid under 10× objective

B. Load a Makler counting chamber with 5 μL of the above solution and observe under a 10× bright-field objective lens.
C. All granulocytes will stain dark brown in color and retain their round shape (Fig. 7.4).
D. Count the cells in all 100 squares of the Makler grid (Fig. 7.4).
E. The number of WBCs can be calculated by multiplying the total number of cells by four to correct for the dilution factor. The total WBC number will be 10^5/mL semen. This number should be corrected to 10^6/mL by dividing by 10.

Endtz Calculation:
WBC × 4 (dilution factor) = 10^5/mL semen
10^5/mL semen divided by 10 to give result in 10^6/mL semen (million/mL)

Example:
1. 5 WBCs are counted in 100 squares on the Makler grid
2. Endtz positive cells = WBC × 4/10 = 5 × 0.40 = 2.0 M/mL
3. Report results as million/mL.
4. A normal concentration of white blood cells in semen is < 0.20×10^6/mL), and therefore anything greater or equal to 0.20×10^6/mL will be considered as significant.

5 Reference Range

< 0.20×10^6/mL (normal): Routine semen analysis and semen profile
< 1.0×10^6/mL (normal): IUI samples and basic semen analysis

Panic value: Any Endtz positive test should be reported to the ordering physician or nurse immediately (e-mail, pager, or phone). Any Endtz positive test should be communicated to the nurse or the inseminator before the insemination.

References

1. Athayede KS, Cocuzza M, Agarwal A, Krajcir N, Lucon AM, Srougi M, Hallak J. Development of normal reference values for seminal reactive oxygen species and their correlation with leukocytes and semen parameters in a fertile population. J Androl. 2007;28:613–20.
2. Henkel R, Kierspel E, Stalf T, Mehnert C, Menkveld R, Tinneberg HR, Schill WB, Kruger TF. Effect of reactive oxygen species produced by spermatozoa and leukocytes on sperm functions in non-leukocytospermic patients. Fertil Steril. 2005;83:635–42.
3. Sharma RK, Pasqualotto AE, Nelson DR, Thomas Jr AJ, Agarwal A. Relationship between seminal white blood cell counts and oxidative stress in men treated at an infertility clinic. J Androl. 2001;22:575–83.
4. Endtz AW. A rapid staining method for differentiating granulocytes from "germinal cells" in papanicolaou-stained semen. Acta Cytol. 1974;18:2–7.

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Leukocytospermia Test or Endtz Test

Procedure

Peroxidase positive granulocytes (neutrophils and macrophages) are identified by histochemical staining. This test is also referred to as Myeloperoxidase or Endtz Test.

Performed when routine semen analysis shows that the number of round cells is ≥0.20 million/mL for male infertility patients

For IUI or basic semen analysis samples, the cut off for performing the Endtz test is >1.0 million/mL

Reagents

I. Preparation of Stock Solution (stable for 6 months):

1. Ethanol - 50 mL of 96%
2. Benzidine - 0.125 g
3. Sterile water - 50 mL

Mix these chemicals in a clean 100-mL bottle.

II. Preparation of Working Solution:

1. Mix 2.0 mL of stock solution and 25μL of 3% H_2O_2 in a 6 mL polystyrene tube (use 3% H_2O_2 or dilute 30% stock H_2O_2 1:10). Cover the tube with aluminum foil

Figure 1. Tube covered with aluminum foil containing Working Endtz solution

A. Take 20 μL of liquefied semen specimen in a dark-colored microcentrifuge tube (**Figure 3**); add 20 μL of PBS solution and 40 μL of working Endtz solution.Vortex and incubate at room temperature for 5 minutes.

Figure 3. Brown microcentrifuge tube.

B. Load a cell counting chamber with 5 μL of the above solution and observe under a 10X bright-field objective lens.

Figure 2. Makler chamber with a coverslip

C. All granulocytes will stain dark brown in color and retain their round shape (**Figure 4**).

Count the cells in all 100 squares of the Makler grid

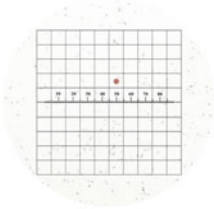

Figure 4. grid of a Makler chamber showing one Endtz positve granulocyte

Endtz Calculation:

WBC X 4 (dilution factor) = 10^5/mL semen
10^5/mL semen divided by 10 to give result in 10^6/mL semen (million/mL)

Results: >1x10^6 WBC/mL of semen condition seemed as leukocytospermia