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Phagocytosis: A Practical Approach for Evaluating Neutrophil Function in Health and Disease

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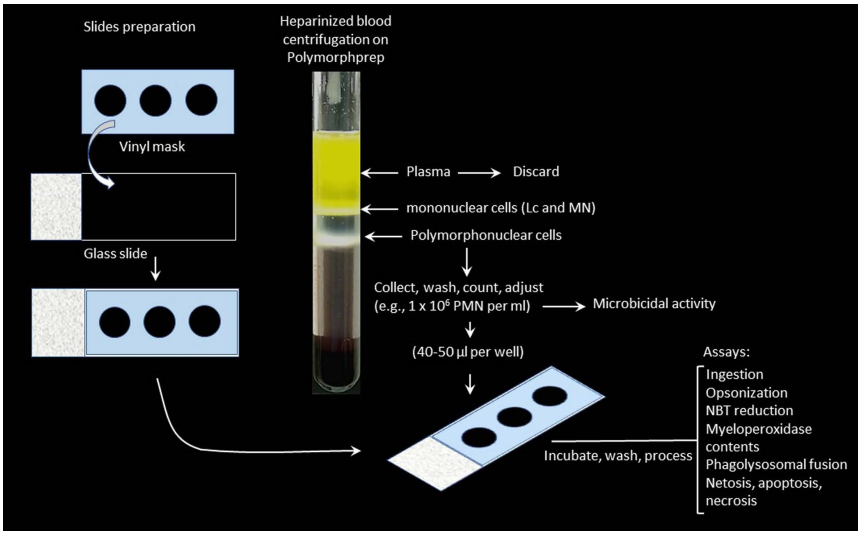
Abstract

Neutrophils, crucial players in the effector phase of the immune response, are recognized as important mediators of both innate and adaptive immune responses. Through the production of pro- and anti-inflammatory cytokines, they modulate the function of T and other lymphoid cells. Countless reports have highlighted the importance of these cells as efficient antimicrobial agents and annotated their involvement in the pathology of infectious and noninfectious diseases. The development of modern, sophisticated technologies has allowed the study of the functions of these cells in clinical settings. These advanced technologies include fluorescence-activated cell sorters, confocal microscopy, automated cell image analyzers, and live cell analysis instruments. Unfortunately, the cost of these modern instruments, maintenance, reagents, and the need for qualified technicians prohibit their use in low-income laboratories and universities in developing countries. With this in mind, we propose a series of basic tests that can be used in low-input clinical laboratories and universities to evaluate the function of neutrophils in health and disease. Our methodology allows us to assess in a practical and low-cost manner the functions of neutrophils in the phagocytic process, including opsonization, ingestion, ROI production (NBT reduction), myeloperoxidase content, phagosome-lysosome fusion, microbicidal activity, and NET production. Thus, under a disadvantageous ambiance, this may guide physicians in deciding whether a patient's illness involves phagocytic defects without imposing a heavy financial burden.

Graphical Abstract



Keywords

Phagocytosis, Neutrophils, Microbicidal Activity, Netosis

1. Introduction

Phagocytosis is a crucial mechanism of the innate immune system, and neutrophils play a vital role in this process [1] [2]. They are the most abundant cells in circulation, making up 40% - 75% of blood leukocytes. Neutrophils have 2 to 5 nuclear lobes and measure between 8 - 9 µm (in flow cytometry) and 12 - 15 µm (in blood smears). They have an average lifespan of 5 days in circulation and are known for their high microbicidal capacity. Neutrophils contain various granules filled with microbicidal and proinflammatory substances [3] [4], and they are equipped with multiple surface and intracellular receptors that enable them to interact with microorganism-associated molecules [5]-[7]. Additionally, neutrophils have an active cytoskeleton that allows them to migrate from blood vessels to tissues and participate in intracellular organelle transport, chemotaxis, endocytosis, and exocytosis [8]-[10]. They also possess adhesive surface molecules that enable interaction with inflamed endothelial vessels for diapedesis [11]. Furthermore, neutrophils have opsonic receptors that facilitate interaction with antibody-, complement-, and collectin-coated microorganisms, thereby improving their endocytic function.

Neutrophils utilize a variety of oxygen-independent and oxygen-dependent microbicidal mechanisms [12]. Most oxygen-independent microbicidal mechanisms rely on the activity of hydrolytic enzymes and antibiotic peptides found in cytoplasmic granules or lysosomes. Lysosomal enzymes, which include proteases, nucleases, lipases, and glycosidases, are acid hydrolases. Some lysosomal enzymes, like myeloperoxidase (MPO), contribute to the oxygen-dependent kill-

ing of microorganisms. This enzyme acts on hydrogen peroxide, a member of reactive oxygen intermediates (ROIs), to generate an unstable intermediary ($H3O^+$), which binds to Cl^- to produce hypochlorite, a highly toxic oxygen derivative [13] [14].

The phagocytic process initiates when the neutrophil encounters the microorganism through pattern recognition receptors (PRRs) or opsonic receptors that bind C3b/C3d moieties, collectin tails, or Fcγ antibody fragments [15] [16]. These ligand-receptor interactions trigger multistep signaling cascades, leading to various but related events. The signals transduced into cells activate the mechanisms responsible for endocytosis and simultaneously trigger the mechanisms that produce reactive oxygen intermediaries or oxygen species (ROIs or ROS). Endocytosed microorganisms remain confined in a vacuole known as the phagosome, exposing them to the toxic action of ROIs. Many microbes are killed at this stage, while others survive with varying degrees of damage.

Ingested microorganisms are wholly killed and destroyed when phagosomes merge with lysosomes and other endosomal vesicles, forming phagolysosomes (PLs). In the PL, damaged microorganisms mix with lysosomal hydrolases, which become active when the acidity drops to ≤5.0 due to the action of a proton-ATPase pump. This pump captures hydrogen ions in the cytoplasm and transports them to the interior of the PL [17] [18]. The success or failure of NEUs' microbicidal activity can lead to different outcomes: NEUs may undergo apoptosis, necrosis, or NETosis, depending on the phagocytic stimulus [19]. Cell corpses are removed when ingested by macrophages, dendritic cells, and fresh granulocytes. Furthermore, netosis extends the microbicidal capability of neutrophils, as NETs (neutrophil extracellular traps) contain various lysosomal hydrolases and antimicrobial molecules that exterminate trapped microorganisms in an extracellular manner.

The phagocytic steps, such as adherence, chemotaxis, endocytosis, respiratory burst, chemiluminescence, and microbicidal ability, can be assessed in vitro. Chemiluminescence, which represents the overall oxidative response of neutrophils, is typically measured using a chemiluminometer, while endocytosis, acidification, and ROI production are measured using a flow cytometer. Despite the practicality of automated assessment of phagocytic function, the high cost of equipment, maintenance, and reagents means this modern technology is only occasionally available in many laboratories in developing countries. To address these challenges, we have enhanced the NBT test in this study, transforming it into a reproducible, reliable, and practical test for qualitatively evaluating endocytosis and ROI production (NBT reduction). Our study also includes a methodology for examining the importance of opsonization in the phagocytic process and a means to observe the fusion of phagosomes with lysosomes to form the phagolysosome, where most microorganisms are ultimately destroyed. Additionally, we have implemented a test to measure the microbicidal activity of neutrophils and a practical test for studying NET release and other cell changes.

Aside from their application in clinical settings, these techniques also serve as valuable teaching aids, providing students with the opportunity to witness and understand phenomena in real time rather than merely from dot distributions or histograms on a computer screen of a flow cytometer or other machines (based on our teaching experience).

2. Materials and Methods

The techniques outlined in this article have been developed in our laboratory, with some adapted from existing strategies to enhance results and practicality. All steps were carried out using sterile materials.

Chemicals: Unless stated otherwise, chemicals were purchased from Sigma–Aldrich Co., Toluca, México.

Yeast preparation: A uniform yeast suspension was prepared following the protocol below:

1) Weigh and disperse 1.0 g of baking yeast in 100 ml of distilled water in a 250 ml Erlenmeyer flask, then autoclave at 121˚C for 10 min. Allow the suspension to cool.

2) Divide the suspension into two 50-ml Nalgene tubes and centrifuge at 1000 rpm (112 × g) for 5 min.

3) Collect the supernatant and discard the larger yeast pellet.

4) Repeat steps 2 and 3 three times.

5) Collect the fine yeast-containing supernatant and count the particles in a Neubauer hemocytometer. Adjust the suspension to 40×10^6 particles/ml with a physiologic saline solution (PSS, 0.85 g NaCl/100 ml DW) or distilled water. This process ensures the creation of a stable suspension of small, uniform particles for visual assessment of the phagocytic process.

6) Prepare 1.0 ml aliquots of the adjusted yeast suspension and store them at 4˚C. This sterile suspension remains stable for over a year.

NBT dye

Dissolve 10 mg of NBT in 10 ml of PSS; shake the suspension thoroughly until the salt completely dissolves. Filter the solution through 0.2 - 0.5 μm membranes and store it at 4˚C. The sterile solution will remain stable for months (discard when a yellow precipitate appears).

Gelatin (3%) in Alsever's solution

In a 100 ml screw-capped flask, sequentially dissolve 1.025 g of dextrose (glucose), 0.44 g of sodium citrate (dehydrate), 0.028 g of citric acid (monohydrate), and 0.210 g of sodium chloride in 50.0 ml of distilled water (this forms Alsever's solution). Next, add 1.5 g of gelatin, stir the mixture, and autoclave it at 121˚C for 15 minutes. This process will fully dissolve the gelatin and produce a sterile solution. Allow the solution to cool and store it at 4˚C. Melt the gel in a boiling water bath or microwave for 10 - 15 seconds to use the solution. Ensure that the solution remains sterile for repeated use.

Phosphate-buffered saline glucose solution (PBSG)

Sequentially dissolve 8.0 g of sodium chloride (NaCl), 0.2 g of potassium chlo-

ride (KCl), 1.44 g of dibasic sodium phosphate (Na_2HPO_4), and 0.24 g of monobasic potassium phosphate (KH_2PO_4) in 800 ml of pyrogen-free (injectable) water. Adjust the pH to 7.4 with 0.1 - 1.0 N HCl, add 1.0 g of glucose, adjust the volume to 1 liter, and autoclave at 121˚C for 15 minutes. For storage, it is recommended to autoclave the solution into 50 to 100-ml working aliquots in glass, screw-capped flasks, or bottles to minimize the risk of the whole lot contamination.

Türk's solution

Dilute 3 ml of glacial acetic acid and 1.0 ml of gentian violet (a drugstore product) in 100 ml of distilled water. This solution remains stable for months at room temperature.

Yeast opsonization

1) Take two 1-ml aliquots of yeasts in 1.5 ml microcentrifuge tubes and centrifuge them at 10,000 rpm (11,178 × g) for 5 minutes in a microcentrifuge.

2) Discard the supernatant from one aliquot and re-suspend the sediment in 1.0 ml fresh human serum.

3) Discard the supernatant from the second aliquot and re-suspend the sediment in 1.0 ml of PSS or PBSG.

4) Incubate both aliquots at 37˚C for 30 minutes.

5) Centrifuge the aliquots at 10,000 rpm (11,178 × g) for 5 minutes, discard the supernatants, and re-suspend the serum-opsonized and non-opsonized yeasts in 1.0 ml of 0.1% NBT.

Glass slide preparation

1) Rather than purchasing Teflon-coated glass slides (Tekdon Inc., Myakka City, Fl. 34251, USA), we opted to save costs by creating our own using homemade vinyl self-adhesive masks with circular areas punched in a local shop (see the graphical abstract). The number of circular areas (wells) can be customized based on the experiment's requirements. We used 3-well/10 mm or 4-well/8 mm vinyl masks.

2) After preparing the masked slides, place them into 150-mm Petri dishes, wrap them with kraft (brown) paper, and sterilize them at 121˚C for 10 minutes.

3) Once sterilized, recover the humid-wrapped dishes and allow them to dry at room temperature. Your slides are now ready to be used.

Cell preparation

1) Use a 1.00 cc insulin syringe preloaded with 0.2 ml of 3% gelatin in Alsever's solution to collect 0.8 ml of venous blood. To expedite the blood collection process, use a 21-22-gauge needle.

2) After collecting the blood, invert the syringe 3 to 5 times to mix the contents. Expel any air and then incubate the syringe upright at 37˚C until the erythrocyte's sedimentation is complete (approximately 15 - 20 minutes).

3) Once the sedimentation is complete, keep the syringe upright, angle the needle to about 45˚, and push down the syringe barrel to dispose of the first two drops of plasma. Then, collect the leukocyte-enriched plasma into a 15-ml conical Nalgene screw-capped centrifuge tube.

4) Dilute the collected plasma to 12 ml with PBSG. Mix the contents by inversion three times and then centrifuge at 1500 rpm (252 × g) for 5 minutes at 4˚C.

5) After centrifugation, discard the supernatant, gently loosen the cell sediment, and suspend it in 2.0 ml of PBSG.

6) To count the neutrophils, use Turk's solution (1:10 or 1:20 dilution) and adjust the cell number to 1×10^6 per ml.

Cell monolayers, endocytosis, and NBT-reduction

1) Prepare a masked slide in a sterile Petri dish and add 40 µl of the cell suspension (4×10^4 or 40,000 neutrophils) on each well. This volume will cover the entire delimited area. Add 10 µl of PBSG to the cell suspension in well 1 (control cells), 10 µl of opsonized yeasts to well 2, and 10 µl of nonopsonized yeasts to well 3, which will give a multiplicity of infection (MOI) of approximately 10:1. Note: For endocytosis and NBT reduction, yeasts are suspended in the NBT solution, for only endocytosis yeasts are suspended in plain PBSG.

2) Incubate the slide for 30 min at 37˚C under a humid atmosphere produced by placing a piece of damp tissue paper on the inner surface of the cover plate.

3) After incubation, recover the glass slide and wash the cell monolayers by carefully dropping approximately 1.0 ml of PBSG on each well, not on the monolayer itself but at its external edge, using a 1 to 3 ml transfer pipette to perform the washing. Gently oscillate the slide and tilt it to drop the washing. Wipe any liquid around the cell monolayers.

4) Cover the cell monolayers with 50 µl of 0.5% safranin and stain for 10 min.

5) Dip the slide thrice in a Copling jar or a beaker to eliminate excess dye, wipe off any liquid around the cell monolayers, and let the slides dry.

6) remove the adherent vinyl mask and mount the slides with synthetic resin. We use Entellan-New, Merck KGaA, 64271 Darmstadt, Germany.

MPO contents

1) Prepare a vinyl-masked slide and add 40 µl of the cell suspension to each well.

2) Incubate the slide at 37˚C for 30 minutes to promote cell adhesion (CO_2 is recommended but not compulsory).

3) Gently wash the cell monolayers with PBSG three times (1.0 ml each) to eliminate nonadherent cells. Drop the PBSG from the transfer pipette at the edge of the cell monolayer, not on it.

4) Remove the liquid from each cell monolayer and add 40 µl of a solution containing 3.0 mg of ortho-dianisidine dissolved in 1.0 ml of isopropanol or methanol, 9 ml of PBSG, and 10 µl of 30% H_2O_2 (or 100 µl of 3% - 4% H_2O_2).

5) Incubate the slide for 10 - 15 min at room temperature and wash it with distilled water to clean up the cell preparation.

6) Air-dry the cell monolayer, peel off the vinyl mask and mount it under Entellan-Neu resin. No counterstain is necessary because the final insoluble product is orange-brown and is easy to visualize.

Phago-lysosomal fusion

1) Prepare a vinyl-masked slide as described previously and add 40 µl of the cell suspension to each well.

2) Add 10 µl of PBSG to the cells in well 1 (control cells) and 10 µl of opsonized yeast to the cells in wells 2 and 3 (duplicate). For this assay, opsonized yeast must be suspended in plain PBSG, not in NBT solution, as this will mask the result.

3) Incubate the slide for 60 minutes at 37˚C/5% CO_2 in a humid chamber or incubator.

4) Gently wash the cell monolayers by carefully dropping 1.0 ml of PBSG three times from a transfer pipette onto a horizontally tilted slide to remove excess yeast.

5) Cover the cell monolayers with 40 µl of the chromogenic substrate solution described for the MPO assay for 10 to 15 minutes at room temperature.

6) Carefully rinse the cell preparation by dipping the slide twice in distilled water, air-dry it, and then peel off the vinyl mask. Finally, mount the cell preparation with Entellan-New resin. Analyze the sample under immersion oil (100×). No counterstain is recommended because this may mask the brown color in the phagolysosomes resulting from the discharge of MPO into the phagosomes.

Bactericidal activity

1) Prepare two 5-ml snap-cap polystyrene tubes (9219F24 Falcon or similar tubes) and label one as the control (C) and the other as the test (T) tube.

2) Place 1×10^6 neutrophils in 0.5 ml of PBSG in each tube.

3) Add 10 µl of PBSG containing 5×10^6 *Staphylococcus aureus* to tube T and nothing to tube C.

4) Incubate both tubes for 10 minutes at 37˚C/5% CO_2 (ingestion time).

5) Add 4.0 ml of PBSG to each tube, tighten the cap, and mix by inversion three times.

6) Centrifuge the tubes at 1300 rpm (189 × g) at 4˚C for 5 minutes.

7) Pour off the supernatants by carefully inverting the tubes, ensuring the cell sediments are undisturbed.

8) Gently tap the bottom of the tubes to loosen the cell sediment, then add 0.5 ml of PBSG.

9) Incubate the tubes for 1 hour at 37˚C 5% CO_2.

10) Centrifuge the tubes at 1300 rpm (189 × g) for 5 minutes, and carefully pour off the supernatants.

11) Gently tap the bottom of the tubes to loosen the cell sediment and add 0.5 ml of 0.125% Triton-X100 in PBSG. At this time, add 10 µl of the *S.aureus* suspension to the control (C) tube.

12) Swirl the tubes to promote cell dissolution, checking visually for dissolution.

13) Prepare three serial decimal dilutions (1:10, 1:100, and 1:1000) of each cell lysate. (Ten µl of the original lysate in 90 µl of PBSG is 1:10; 10 µl of the 1:10 dilution in 90 µl of PBSG is 1:100, and 10 µl of the 1:100 dilution in 90 µl of PBSG is 1:1000).

14) Deposit 10 µl of each dilution in triplicate on a standard Petri dish containing 1% nutritious agar (any brand) and incubate the dishes at 37˚C for 18 - 24 hours.

15) Count the number of surviving bacterial colonies from each dilution and calculate the bactericidal index.

Neutrophils isolation

1) Layer 3.0 ml of heparinized blood on 3.0 ml of PolymorphPrep in a 15 ml conical tube or a 13 × 100 mm glass tube for better cell separation.

2) Centrifuge the tubes at 1500 rpm for 60 minutes at 25˚C.

3) Remove the upper plasma and mononuclear cell layers, then collect the polymorphonuclear (neutrophil) layer.

4) Dilute the collected neutrophil suspension to 12.0 ml with PBSG and centrifuge at 1500 rpm for 3 minutes.

5) Remove the supernatant, loosen the cell sediment, and suspend it in 1.0 ml of PBSG for cell counting. Adjust the cells to 1×10^6 per ml in PBSG.

Netosis

1) Prepare a clean glass slide with a 3-well vinyl adherent mask.

2) Place the slide in a 100-mm Petri dish with moist tissue paper.

3) Deposit 50 µl of cell suspension (50×10^3 cells) in each well.

4) Incubate the cells for 30 minutes at 37˚C/5% CO_2 to allow cell adherence.

5) Add 10 µl of PBSG to the cells in well 1 (negative control), 10 µl of PMA (0.1 µg/10^6 cells) to the cells in well 2 (positive control), and 10 µl of the test particles (for a MOI 10:1) to well 3.

6) Incubate for four hours at 37˚C/5% CO_2 with humidity.

7) Remove the supernatant from each well and add 50 µl of 2% paraformaldehyde in PBS (pH 7.4) without washing. Let the fixation proceed for 10 minutes.

8) Wash the slide by dipping it twice in distilled water.

9) Wipe off the liquid around the wells and add 50 µl/well of Hoechst solution to stain the cells' nuclei and extruded DNA for 5 minutes.

10) Wash the slide by dipping it twice in distilled water and let it dry.

11) Peel off the vinyl mask and mount the slide with an anti-fade reagent.

12) Examine under UV light; nuclei and extruded DNA stain blue.

3. Results

Adherent cells: Figure 1 shows the cellularity of the cell suspension obtained by blood sedimentation in a gelatin-Alsever's solution after 30 min of adherence. More than 90% are polymorphonuclear cells (primarily neutrophils); less than 10% are monocytes, with a few lymphocytes present. This highly reproducible result shows the method's validity for studying diverse neutrophil functions.

Endocytosis (phagocytosis): Figure 2 shows the results obtained when opsonized and nonopsonized yeast were used in the assay. A higher degree (percent) of phagocytosis can be observed when opsonized yeasts are used. The re-

sults also revealed more particles ingested per cell when opsonized yeasts were used.

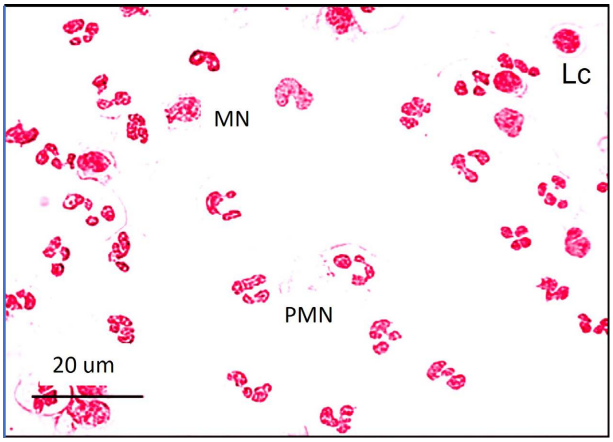


Figure 1. The image displays blood leukocytes separated by sedimentation in a gelatin-Alsever solution. The separated cells were then permitted to adhere to glass slides for 30 minutes at 37˚C. The adherent cells primarily consisted of polymorphonuclears (PMN) and monocytes (MN), with some lymphocytes (Lc) also observed. Subsequently, the cells were stained using a Safranin stain and examined at a magnification of 100×.

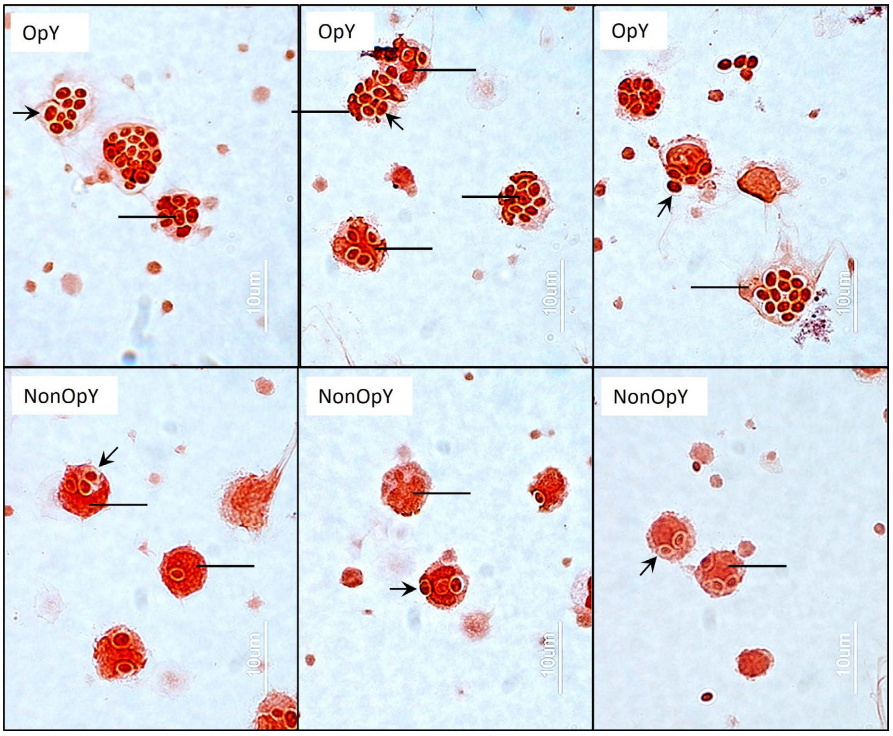


Figure 2. This figure depicts the phagocytosis process in neutrophils of healthy individuals. The neutrophils were observed to engulf opsonized (OpY) and non-opsonized (NonOpY) yeasts. Short arrows indicate some ingested yeasts, while the long lines point to the nucleus in some cells. The study revealed that opsonized yeasts were ingested at a higher rate (average of 8) compared to non-opsonized yeasts (average of 2 - 3), underscoring the significance of opsonization during phagocytosis. The cells were stained with safranin and magnified at 100×.

Reactive oxygen species (ROS) production: Figure 3 illustrates the ability of neutrophils to produce ROS while ingesting microorganisms (yeasts in this case). The NADPH oxidase system is activated in cells that recognize a variety of PAMPS/MAMPS to produce superoxide (SO) anion. Superoxide reduces the oxidized nitroblue tetrazolium (NBT) and transforms it into an insoluble, blue formazan, which precipitates where NBT is reduced. Most NBT reduction occurs within the yeast-containing phagosomes, producing an image of "blue-stained" yeasts that can be visually quantified.

Myeloperoxidase content: Figure 4 illustrates the presence and distribution of myeloperoxidase in resting, normal polymorphonuclear cells. The enzyme's homogeneous and fine granular distribution in the cytosol is evident (MPO activity was detected with H_2O_2 as the substrate in the presence of ortho-dianisidine, which, when oxidized, produces an insoluble brown compound).

Phagosome-lysosome fusion: Figure 5 is a representative image of phagolysosomes in polymorphonuclear cells that have ingested opsonized yeasts. Because the MPO content of lysosomes is discharged into the phagosomes, the oxidized chromogen ortho-dianisidine is deposited at this site, leading to a brown residue on the ingested yeasts.

Neutrophil separation: Figure 6(A) shows the effective separation of neutrophils by centrifugation on PolymorphPrep. Three cell regions are observed from top to bottom: mononuclear cells, polymorphonuclear cells (primarily neutrophils), and erythrocytes. Figure 6(B) illustrates the homogeneous and

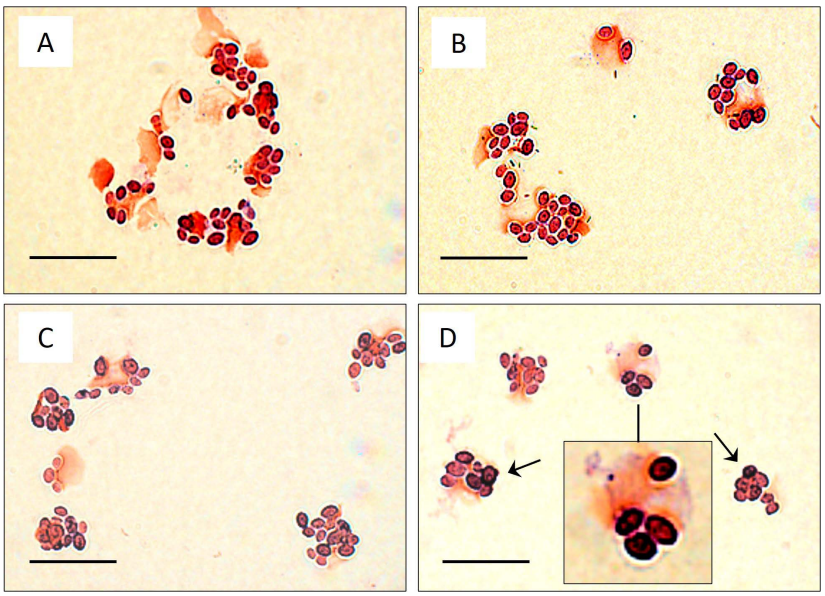


Figure 3. This figure shows neutrophils that were exposed to opsonized yeasts for 30 minutes in the presence of NBT. The dark purple color of the yeasts indicates that the cells reduced the NBT to insoluble formazan, which accumulated on the ingested particles. In image D, the ingested yeasts with reduced NBT are highlighted, although this feature is present in all images. The inset in image D is an amplified image that reveals the intraphagosomal location of the yeasts, as shown by the Safranin stain. The scale bars in the photos represent 20 μm, and the magnification is 100×.

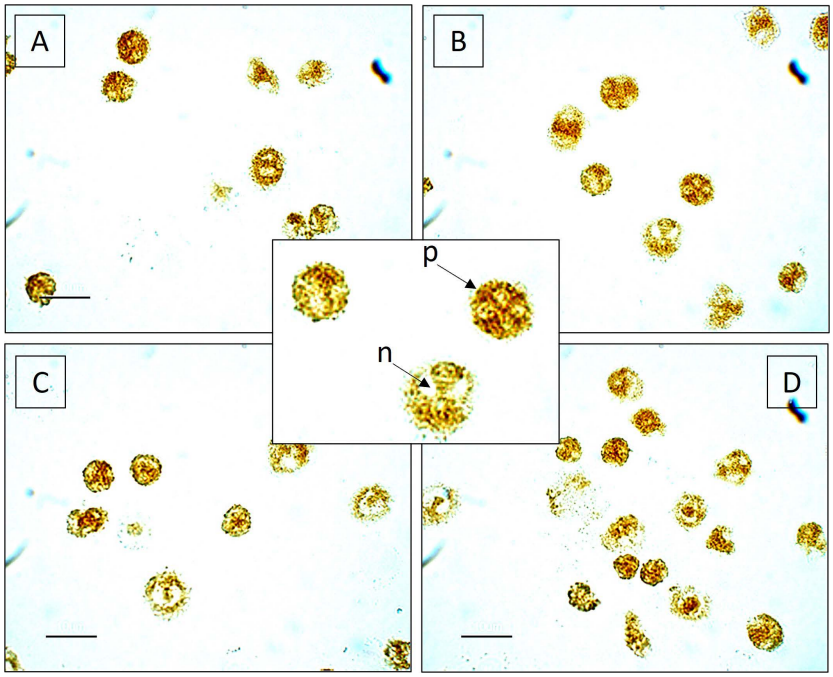


Figure 4. This figure displays images from an experiment conducted in quadruplicate (A-D) to show the presence of neutrophils MPO. The images highlight the cytoplasmic localization of the enzyme (brown granules) and the unstained nuclei within the cells. The inset illustrates the localization of peroxidase (p) and the nucleus (n) in two cells. The horizontal lines in the images represent a length of 10 μm. The magnification is 100×.

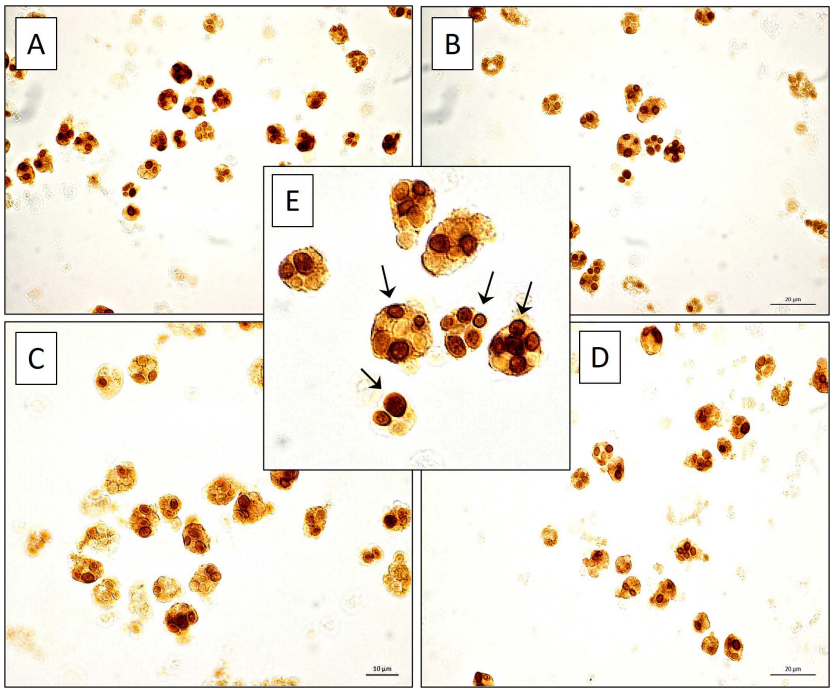


Figure 5. This figure shows images of four experiments (labeled A-D) where neutrophil monolayers were fed with opsonized yeasts for one hour and then stained for myeloperoxidase. MPO-stained yeasts are visible in all four experiments. The inset in E provides an amplified image of cells displaying yeast-containing phagolysosomes (indicated by arrows). No counterstain was used, and the magnification is 100×.

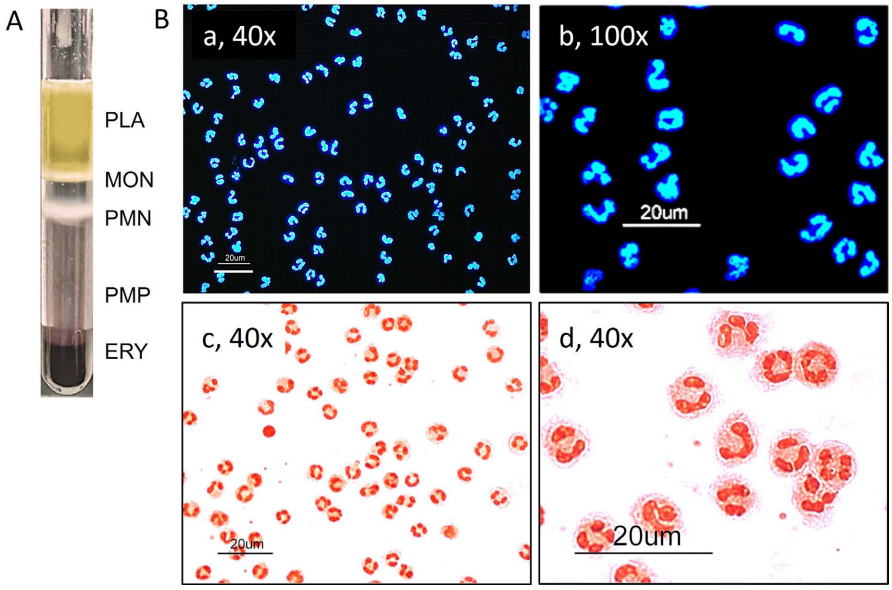


Figure 6. (A) PMN can be effectively separated using PolymorphPrep at 1500 rpm and 25˚C for 60 minutes. Here, PLA represents plasma, MON mononuclear cells, PMN polymorphonuclear cells, PMP PolymorphPrep, and ERY erythrocytes. In (B), all cells are identified as polymorphonuclear (neutrophil) leukocytes. The images display cell monolayers stained with Hoechst stain ((a), (b)) or safranin ((c), (d)). Notably, the cells exhibit high purity and a healthy appearance.

healthy appearance of neutrophils isolated by this method. Careful collection produces highly purified (100%) neutrophil populations. Cells were stained with the Hoechst reagent or safranin.

NET production: Opsonized yeast is a good inducer of NETs and can be used as a positive control when examining NETs for other microorganisms. Pre-staining the yeast with a fluorescent dye, such as iris fuchsia, allows us to visualize the yeast's location within phagocytic cells or trapped within the NETs, as shown in Figure 7.

Two popular reagents for DNA and chromatin staining in NETs are the fluorescent DAPI and Hoechst reagents. However, if a fluorescence microscope is unavailable, NETs can still be detected using non-fluorescent nuclear dyes such as hematoxylin or safranin, as shown in Figure 8.

Figure 9 displays a typical image of neutrophils treated with various substances that induce NET formation, including PMA, yeasts, *Staphylococcus aureus*, *Mycobacterium tuberculosis*, and *Trypanosoma cruzi*. Other stimulants, such as viruses, liposomes, and chemicals, can also activate NETs. Aside from NETs, this method can also help identify other changes in cell structure, such as autophagy, apoptosis, or necrosis, depending on the specific stimulus used [20].

Bactericidal activity: Figure 10 shows a representative result of an experiment where 1×10^6 PMNs from a healthy donor were exposed to 5×10^6 *S. aureus* for 60 min and then processed as described in the Materials and Methods. Notice that in the illustrated experiment, the number of colonies was smaller in the T tube than in the C tube at all dilutions tested. Calculations should be performed

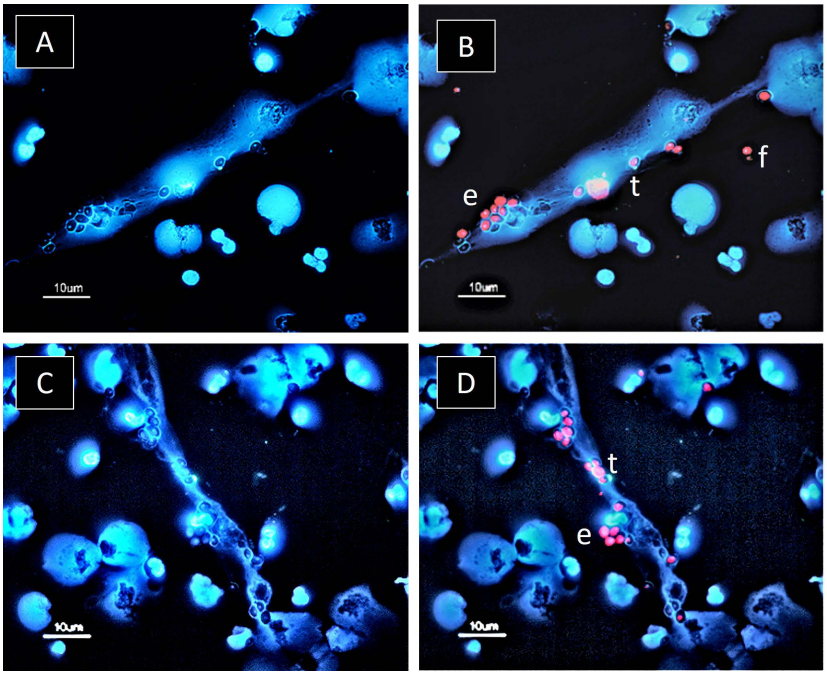


Figure 7. This figure shows neutrophil extracellular traps produced by PMN cells that were incubated with iris fuchsia-stained yeasts for four hours at a ratio of 5:1. Images (A) and (C) were captured at 461 nm (blue channel), whereas images (B) and (D) were taken at 588 nm (red channel) and then superimposed on the blue images. Some yeasts appear endocytosed (e), while others seem trapped in the nets (t). The cell monolayers were stained with the Hoechst reagent and photographed at a magnification of 100×. If a confocal microscope is not available, composite images can be generated by overlaying images using a standard fluorescence microscope.

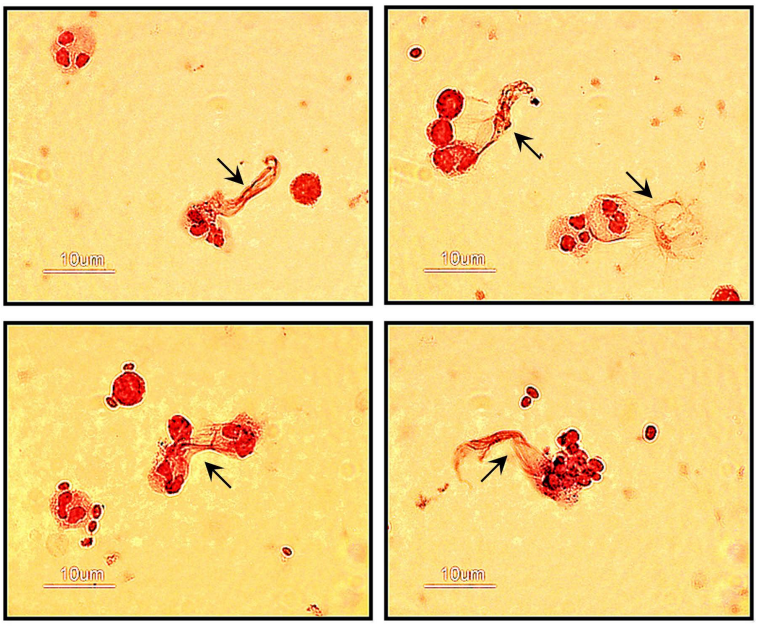


Figure 8. In the absence of a fluorescence microscope, NETs can still be identified using a standard microscope with safranin staining. The arrows in the image highlight the NETs produced by neutrophils that were incubated with opsonized yeasts for three hours at 37˚C and 5% CO_2. These images were captured at 100× magnification.

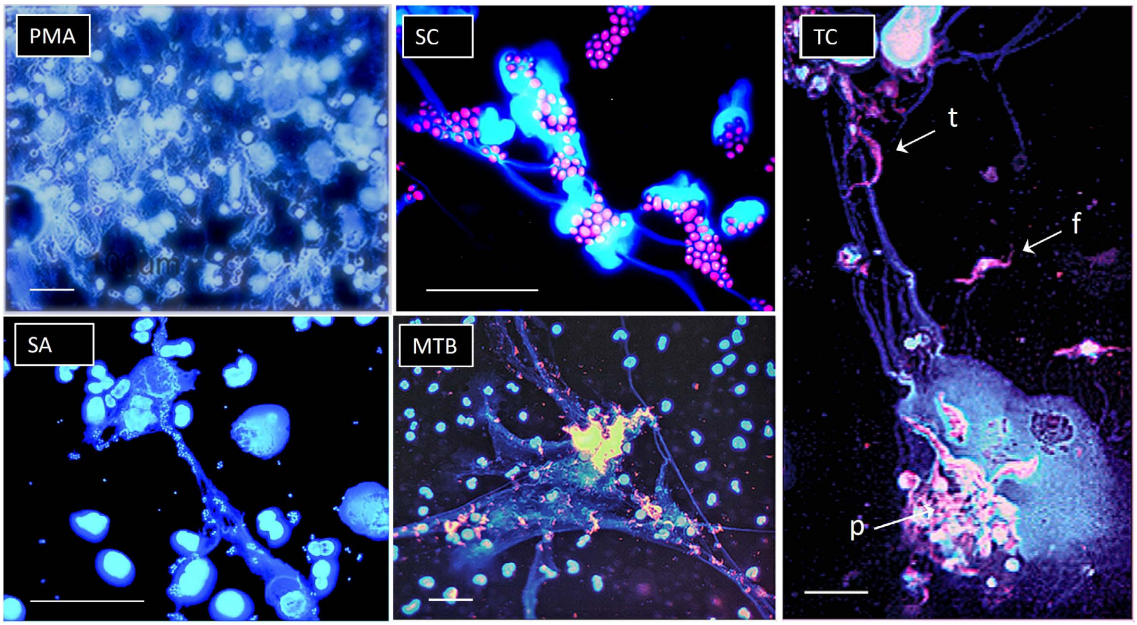


Figure 9. Shows Neutrophil Extracellular Traps (NETs) that were produced by neutrophils after a three-hour incubation with phorbol myristate acetate (PMA), *Saccharomyces cerevisiae* (SC), *Staphylococcus aureus* (SA), *Mycobacterium tuberculosis* (MTB), and *Trypanosoma cruzi* (TC). SC, MTB, and TC were pre-stained with iris fuchsia. In the TC image, arrows indicate trapped (t), free (f), and phagocytosed (p) parasites. Scale lines are 20 μm. Hoechst stain was used. Images of PMA and MTB were captured at 40× magnification, while images of SC, SA, and TC were captured at 100× magnification.

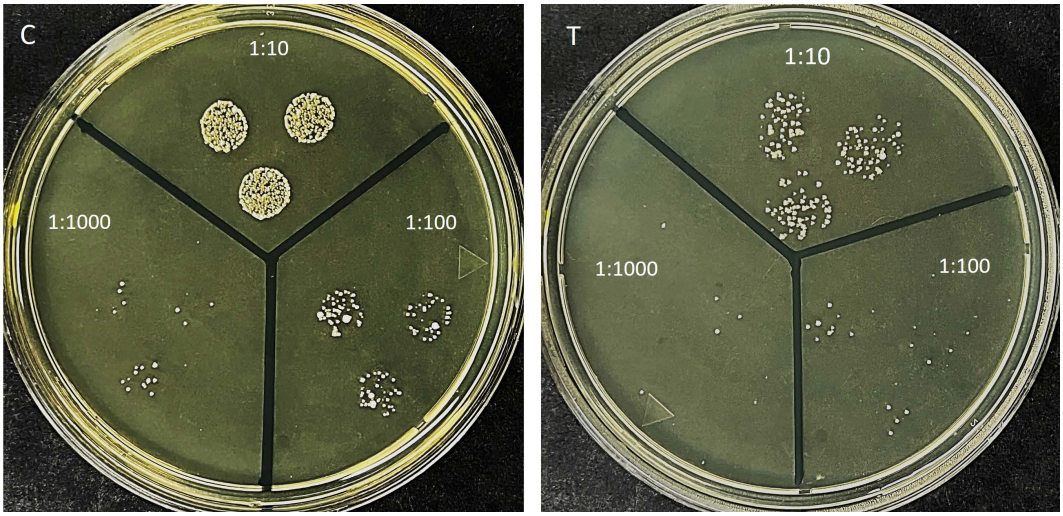


Figure 10. This experiment illustrates the bactericidal activity of neutrophils on *S. aureus* (plate T) compared to the control experiment (plate C). The results show that the number of colony-forming units on the test plate (T) is consistently lower than that on the control plate (C) at all dilutions tested. The average number of CFUs was calculated from the 1:100 dilution, revealing an average of 30 CFUs on the control plate and 7 CFUs on the test plate, indicating an approximate 80% bactericidal efficiency.

on plates with easily countable colonies, possibly at dilutions of 1:100 or 1:1000. The percent of bactericidal activity in the assay can be calculated by dividing the number of colonies in the T tube at the same dilution by that in the C tube × 100.

4. Discussion

The importance of evaluating neutrophils' phagocytic capacity cannot be overstated. These cells are crucial for protecting the body from invading microorganisms through phagocytosis. Advancements in technology, such as flow cytometry and microscopy with automatic image analysis, have made it possible to assess various phases of the phagocytic process rapidly and accurately. However, many laboratories, especially those in developing countries, may need more funds, equipment, and reagents to conduct these assessments. As a result, clinical specimens often have to be sent to national or foreign reference laboratories. Despite these challenges, it's important to note that the basic steps of phagocytosis can still be evaluated practically and cost-effectively using low-input techniques as described in this article.

This methodological platform focuses on preparing neutrophil monolayers to study various aspects of phagocytosis, including endocytosis, opsonization, production of reactive oxygen species, and bactericidal activity. It's important to understand that endocytosis is an energy-consuming process that involves cell surface receptors, a healthy cytoskeleton, and complex intracellular signal transduction proteins [21]. Endocytosis occurs more avidly in the presence of opsonins [22], and a series of diseases have been associated with deficiencies in C3b, MBL, and IgG. Microbial killing requires the production of reactive oxygen and reactive nitrogen species (ROS and RNS), and the lack of ROS is the cause of severe clinical anomalies, such as in chronic granulomatous disease [23]-[25]. Most microorganisms are contained within phagocytic vacuoles or phagosomes after ingestion. Inside these vacuoles, they are damaged by reactive oxygen species (ROS). Effective killing occurs when the phagosome merges with lysosomes and other vesicles to form the phagolysosome. In the phagolysosome, the ingested microorganisms are exposed to the full microbicidal arsenal of neutrophils [26].

The final step of the phagocytic process in neutrophils is cellular death, which may occur in diverse modes depending on microorganisms and external factors [27]. One of these forms of cell death is netosis, a process in which neutrophils extrude nuclear chromatin that carries microbicidal elements, including hydrolases, antibiotic peptides, ROS, and other toxic components. Netosis is also an efficient mechanism for trapping and killing extracellular microorganisms [28] [29]. All these microbial-killing mechanisms are also responsible for tissue damage without regulation.

The assessment of phagosome-lysosome fusion may not have direct clinical implications, but it is relevant to research. This is because some intracellular disease-causing microorganisms, such as *Mycobacterium tuberculosis* and *Mycobacterium leprae,* can disrupt this process, avoiding the bactericidal actions of macrophages [30]-[32]. On the other hand, other disease-causing mycobacteria, like *M. lepraemurium,* actually encourage fusion. This allows lysosomal enzymes to weaken their cell wall, making them permeable and permitting the entry of

nutrients or metabolites that promote their growth [33] [34].

Myeloperoxidase (MPO) is a heme-containing protein mainly found in human neutrophils and monocytes' lysosomal granules. When neutrophils are activated during phagocytosis, they undergo a process known as a respiratory burst. This burst produces superoxide, hydrogen peroxide, and other reactive oxygen derivatives, all toxic to microbes. During the respiratory burst, granule contents are released into phagolysosomes, allowing the released cargo to contact the enclosed microorganisms. MPO plays a crucial role in this process by catalyzing the conversion of hydrogen peroxide and chloride ions (Cl) into hypochlorous acid (HOCl-), which is a potent microbicidal agent [35]. Neutrophils deficient in MPO can still ingest microbes, but their ability to kill microorganisms may be impaired, depending on the specific microorganism.

Neutrophils and other cells, such as eosinophils, mast cells, macrophages, and basophils, release extracellular traps (NETs) in response to infectious or noninfectious stimuli. In suicidal NETosis, this process relies on ROI production, and neutrophils from patients with chronic granulomatous disease (CGD) do not produce H_2O_2 or release NETs. These NETs comprise DNA and nuclear proteins like histones and lysosomal constituents such as elastase, myeloperoxidase, cathepsins, and antibiotic peptides. NETs are effective in trapping and killing microorganisms [36], and they can also aid in their capture and destruction by other cells, such as fresh neutrophils, macrophages, and potentially other phagocytic cells. While initially identified as microbicidal structures in 2004 [37], NETs are currently the subject of much research due to their involvement in cancer, chronic degenerative diseases, and autoimmune disorders, and therapies for their elimination have been initiated [38].

In summary, while advanced techniques and equipment are valuable for studying phagocytosis, practical and low-cost methods can still provide useful insights, especially in resource-limited settings. Understanding the intricacies of phagocytosis is essential for improving patient management and advancing our knowledge of infectious diseases.

5. Conclusion

The long-time neglected role of neutrophils as more than just phagocytic cells has rapidly evolved into a critical mechanism of immunity. Their participation in the effector phase of the adaptive immune response, including pathology, is now well recognized. Assessing their function using techniques such as those described in this paper is usually sufficient for detecting diseases related to anomalies in phagocytosis.

Ethical Considerations

This project was reviewed and approved by the Committee of Ethics in Investigation of the National School of Biological Sciences, National Polytechnic Insti-

tute, Mexico City, under the code SH-007-2020. The research adhered to the principles of the Declaration of Helsinki on medical research (updated 2013) and included obtaining informed consent from all participants.

Limitations and Deficiencies

This descriptive article follows a methodological approach, and its clinical applicability will be assessed for usefulness.

Authors' Contributions

O.R-E: Design, supervision, writing, formatting, and editing;
P.A-P: Methodology, documentation, and experimentation;
S.I-T: Technical assistance;
E.B-V: Experimentation;
M.D.P-R: Blood sampling.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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