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Evaluation of phagocytosis in human neutrophils using enhanced green fluorescent protein (EGFP) expressing E. coli

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Abstract

Introduction: Phagocytosis plays a very important role in innate immunity and helps the body against bacterial infections. Patients who have defect in phagocytosis suffer from recurrent bacterial infections that may be life threatening. It is important to detect the defect in phagocytosis as early as possible in life. Patients who have received immunosuppressive medication may also have suppressed phagocytosis. There are different laboratory tests for evaluation of phagocytosis such as NBT (Nitroblue Tetrazolium) and DHR (Dihydrorhodamine) which use chemical compounds not real bacteria. Nitroblue Tetrazolium is yellow chemical substance, in NBT test neutrophils are isolated first and then for Stimulation of respiratory burst in neutrophils add PMA (Phorbol Myristate Acetate). PMA and NBT are exposed to neutrophils, if a respiratory burst occurs in neutrophils, the color of NBT changes from yellow to purple, that purple neutrophils can be seen under microscope. In DHR test Dihydrorhodamine 1,2,3 was exposed to neutrophils that were stimulated with PMA. Normal neutrophils oxidize DHR after ingestion; finally neutrophils will be fluorescent that can be detected by flow cytometry. The behavior of neutrophils when exposed to chemicals compounds such as NBT and DHR may be different and abnormal, so when real bacteria such as *E. coli* are exposed to neutrophils the behavior of neutrophils phagocytosis will be normal. The aim of this study was to evaluate a simple and quick method for testing phagocytosis using real bacteria instead of chemical compounds.

Materials and Methods: An EGFP (Enhanced green fluorescent protein) sequence was cloned into a pColdI expression vector. *E. coli* (Bl21 strain) was transformed by EGFP containing vector. EGFP expression in bacteria was detected by a fluorescent microscope and flow cytometry. EGFP expressing bacteria were added to the heparinized blood of healthy volunteers. Phagocytosis and digestion of fluorescent bacteria by neutrophils were detected using flow cytometry at different time points.

Results: Neutrophils that engulfed the fluorescent bacteria showed high fluorescent activity and, were identified by flow cytometry. Bacterial digestion over time led to a decrease in fluorescent of neutrophils.

Conclusion: EGFP expressing bacteria and flow cytometry technique can be used to evaluate phagocytosis. It can be optimized for clinical as well as research uses.

Keywords: Phagocytosis, EGFP, *E. coli*, Fluorescent, Flow cytometry

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Introduction

All blood cells, such as red blood cells and platelets, as well as white blood cells, are derived from hematopoietic stem cells in the bone marrow (1). Hematopoietic stem cells divide into lymphoid and myeloid cells. Myeloid cells also differentiate into two cell types including monocytes and granulocytes. Granulocytes are differentiated into three types of cells neutrophils, eosinophils and basophils. Neutrophils have phagocytic properties and are among the first cells to respond to infection (1, 2, 3). In adults, about 10^{11} neutrophils are produced daily in the bone marrow. Neutrophils cannot proliferate and their lifespan does not exceed a few days. During infection, Neutrophils are activated by bacterial cell wall products such as lipopolysaccharide, (LPS) and muramic dipeptide (MDP) and inflammatory mediators such as interferon-gamma (IFNγ) (4,5). Neutrophils with respiratory burst produce such oxygen radicals, Hydroxyls and their antibacterial peptides (defensins) are able to destroy pathogens (6). Neutrophils have Azerophilic cytoplasmic granules (primary) and have specific granules (secondary). Activation of neutrophils leads to phagocytosis of foreign antigens. Phagocytosis is a type of endocytosis. Endocytosis is a process of removal of environmental substances by the cell. In phagocytosis, the plasma membrane of the cell surrounds a particle. These particles may contain the entire body of a pathogen. Phagocytosis is an active process that uses energy to swallow particles larger than 0.5μm. Phagocytosis has four basic stages, which include chemotaxis, opsonization, swallowing and digestion (7). Neutrophils in the bloodstream circulate naturally, but stimulated by the adhesion of endothelial cells, the blood vessels allow neutrophils to leave. Stimulated endothelial cells produce adhesive proteins that include selectins and integrins. These proteins bind to neutrophils and cause migration of neutrophils into the tissues.

When neutrophils are drawn to the source of foreign objects, First the pseudopod and then the bulk of the cell are pushed forward. The neutrophil pseudopod cytoplasm consists of a filamentous network containing actin and myosin proteins. The status of this network determines the fluidity of the cytoplasm. When the neutrophil encounters the bacteria, the pseudopod neutrophil is drawn to the bacteria and encloses it and the opsonins on the organism and the neutrophil surface receptors bind to each other. Eventually, the bacteria are drawn into the cell and then the bacteria are trapped in a vacuole called a phagosome. Neutrophils include or produce deadly molecules, including a number of active oxygen proteases and mediators that are able to destroy pathogens in the body (8). Destruction of the swallowed bacteria is accomplished through two distinct processes: first, the production of oxygen radicals, (process of respiratory burst) and second, the release of decomposing enzymes and antimicrobial peptides from inside the cell. One of the most important steps in responding to inflammation by neutrophils is respiratory burst, which is characterized by high oxygen consumption and the production of active oxygen species (9). There are different laboratory tests for evaluation of phagocytosis such as NBT (Nitroblue tetrazolium) and DHR (Dihydrorhodamin) which use chemical compounds, not real bacteria. To study the behavior of phagocytic cells during phagocytosis, it is more appropriate to use real bacteria such as *E. coli*. These bacteria can be transformed to expressed Enhanced Green Fluorescent Protein (EGFP). The phagocytosis of fluorescent bacteria can be detected by flow cytometry.

NBT test is used to measure the production of active oxygen mediators in a respiratory burst, in the short time that neutrophils attach to the bacterium, oxygen consumption increases by about 100 times; this, increase in consumption is the result of the activation of a cellular enzyme called NADPH oxidase. This enzyme causes the transfer of electrons to oxygen in the cell wall. By the action of the enzyme myeloperoxidase, hydrogen peroxide is converted to its bactericidal compound (10). In this study, the *E. coli* was transformed using an EGFP containing plasmid. Expression of EGFP by *E. coli* was detected using fluorescent microscopy and flow cytometry. Phagocytosis of EGFP expression bacteria by neutrophils then was assessed using flow cytometry at different time points.

Materials and Methods

The steps of material and method for this study are divided into two parts. The first step was to prepare *E. coli* strain BL21 that can express a bright green fluorescent protein (EGFP). The second step was to use

fluorescent bacteria to evaluate phagocytosis and obtained the optimal time for the stages of ingestion and digestion of phagocytosis. Preparation of bacterial competent cells from *E. coli* strain BL21were done with the Calcium chloride chemical method. For this purpose inoculated bacteria to 10ml LB (Luria Bertani) media incubated at 37 ºC for 16 hours. Take some colony and inoculated to 20ml LB media and use shaker incubator at 37 ºC to OD600=0.4-0.6. Culture media was transferred to 1.5ml microtubules and placed on ice for 10 minutes. Centrifuge microtube in refrigerated centrifuge at 4 ºC for 20 minutes at 4000 rpm and transferred to ice. Supernatant out and added 0.75 ml of CaCl2 100Mm to each microtube. Microtubes were placed on ice for an hour and then continue like centrifugation step. Finally supernatant out and added 200 µl glycerol stock solution and store -70 ºC.

PEGFP Transformed to Pcold Expression vector with Electroporation Method. For this propose, 5µl of recombinant vector was added to 100ml competent bacterial cell and placed on ice for 30 minutes. The thermal shock was caused by plasmid passing through the bacterial membrane so that microtubes containing competent bacteria were put in Bain Marie at 42 ºC for 90-120 seconds and then microtubes were placed on ice for 10 min. 400µl sterile LB culture medium was added to the contents of the microtube and pipette and was placed at 37 ºC in shaker incubator for bacterial reproduction for an hour. After an hour, the microtubes were centrifuged for 10 minutes at 5000 rpm. The supernatant was out and 100µl of LB medium was added to the bacterial sediment and pipetted. The contents of the microtube were transferred to a plate containing LB agar culture medium and incubated overnight at 37 ºC. Reproduction and plasmid extraction for evaluating the attendance of EGFP was done by GenetBio kit. For plasmid extraction from the bacteria that contain the EGFP plasmid, cultured the bacteria overnight then the following steps are performed.

LB medium containing bacteria from overnight culture was transferred to 1.5 ml microtubes and centrifuged for 3 minutes at 8000 rpm. Supernatant out and added 250µl suspension solution and pipetted. Added 250µl of lysis buffer to each microtube and each microtube was slowly turned upside down about 4 to 6 times for 5 minutes. After that add 350µl neutralizing solution to each microtube was slowly turned upside down about 4 to 6 times. The sample was centrifuged at 12500 rpm for 10 minutes; supernatant was carefully transferred to the purification column, purification column was centrifuged at 12500 rpm for 1 minute and was out micro tube sediment. After that added 700µl washing solution to column and centrifuged at 12500 rpm for 30 seconds and was out supernatant. In order to remove the residual wash buffer, which contains ethanol, the column was centrifuged again for 2 minutes and placed at room temperature for 2 minutes to completely remove the alcohol. The column was transferred to a new microtube and 50 µl of the dissolution buffer was added to the center of the purification column to separate the plasmid DNA extracted from the column and incubated for 2 minutes at room temperature. In the last step, the column was centrifuged at 12500 rpm for 2 minutes. The collected solution was stored in the microtube in a freezer at -20 °C. After plasmid extraction to check the accuracy of plasmid extraction, 2µl of the plasmid extraction product was electrophoresed on 1% agarose gel. In order to quantitatively and qualitatively verify the expression of EGFP in transform bacteria with pEGFP, samples were first prepared for observation by fluorescent microscope and then read by flow cytometry. Evaluation of protein expression by bacteria done with different concentrations of IPTG (Isopropyl β-D-1-thiogalactopyranoside). IPTG with a concentration of 0.3mmol was selected to express high volume EGFP protein. Evaluation of phagocytosis of neutrophils, Performed by DHR(Dihydrorhodamine) test. Select 3 FACS tubes and add 100µl heparinized blood and added 0.15ngr/ml DHR just to the second and third tube and incubate at 37ºC for 20min. After incubation added 10ngr/ml PMA (Phorbol Myristate Acetate) just to third tube and incubate at 37ºC for 20min. Added to tree tubes 2ml aluminum chloride lysis buffer 1x. In this study flow cytometry techniques were performed on a BD FACSCalibur flow cytometry (Partec PAS, Germany). The number of bacteria selected for each sample was 10µl from the medium with OD600 = 0.4-0.6, which contains about 6×106 bacteria. To determine the optimal time for phagocyte bacteria by neutrophils, samples with different duration of time (from 20 minutes to 90 minutes) were incubated in the presence

of EGFP bacteria, and used flow cytometry techniques for evaluating optimal time of phagocytosis. Preparation of samples for reading by flow cytometry similar to the steps performed with DHR but difference is that instead of DHR in the tubes, was used the *E. coli* bacteria strain Bl21, which contains EGFP plasmid, was stimulated expression by IPTG. Selected 2.5hours to determine the optimal time to digest phagocyte bacteria by neutrophils.

Results

PEGFP plasmid extraction was done with Kit Company and the result of gel electrophoresis shows band PEGFP plasmid in figure 1.

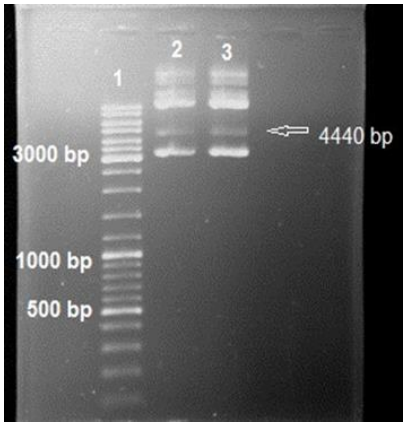
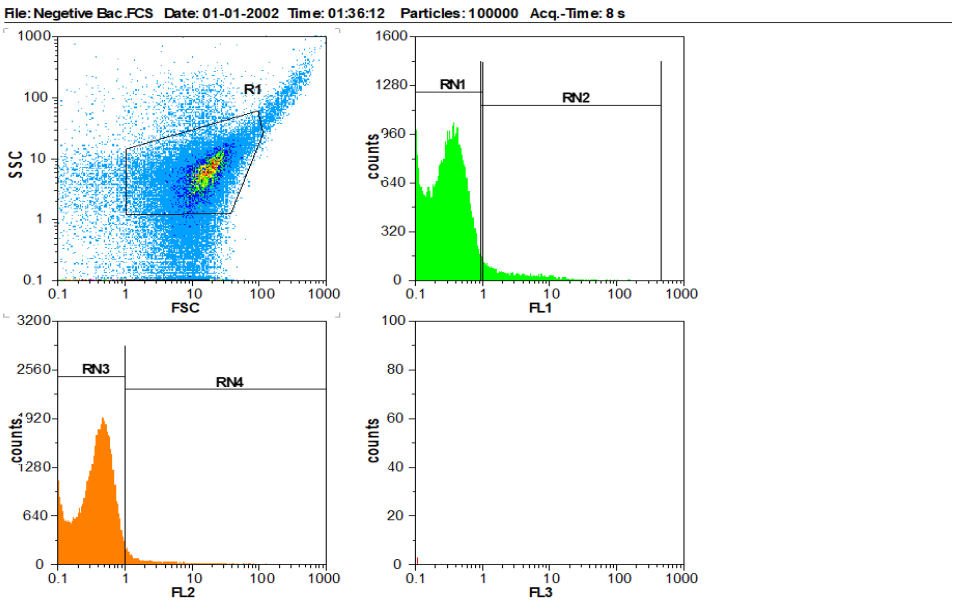


Figure 1. Plasmid extraction of EGFP. Column 1: 1Kb DNA Ladder. Column 2: 2µl of plasmid extraction product. Column 3: 5µl of plasmid extraction product. The pcoldI vector is 4407 bp, which contains the EGFP = 32.7 bp.

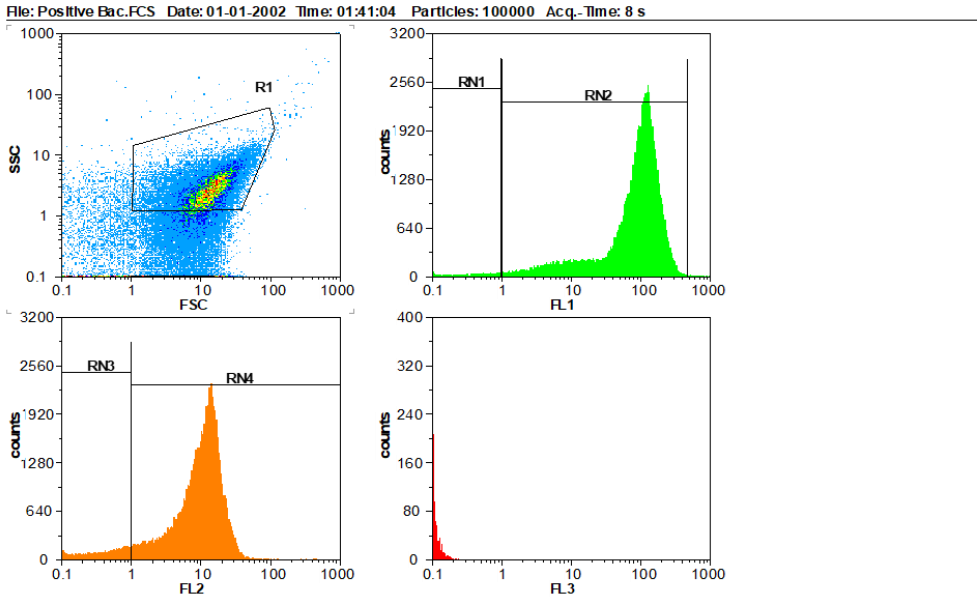
Expression Confirmation of EGFP done with Flow cytometry and Fluorescent Microscope. Figure 2 shows negative control of bacteria that includes dot diagram (Dot Plot) of the FSC and SSC bacterial characteristics. The FSC shows the size of the EGFP bacteria and the SSC shows their granulocyte characteristics. As can be seen in the figure, most of the bacteria are present inside the gate. The majority of them are in the same range in terms of size and granulocyte. The upper and left sides of the histogram show auto Fluorescent. These bacteria don't have the EGFP gene and don't have fluorescents, and their auto Fluorescent is shown on their histograms. The autofluorescent shown in their histogram is the result of the device absorbing the laser beam and emitting it in the same range. The RN1 gate is the negative fluorescent range and the RN2 gate is the positive fluorescent range. Numerical data for RN1 gate calculated by FlowMax software. Mean x for RN1 represents the evaluation index, the value of which is 0.21 and doesn't have any fluorescent.



Region	Gate	Ungated	Count	Count/ml	%Gated	GMn-x	Mean-x	CV-x%	GMn-y	Mean-y	CV-y%
R1	<None>	65077	65077	-	65.08	14.71	18.25	69.55	5.52	6.88	73.14
RN1	<None>	96414	96414	-	96.41	0.16	0.21	86.12	-	-	-
RN2	<None>	3775	3775	-	3.77	2.85	4.59	153.54	-	-	-
RN3	<None>	95814	95814	-	95.81	0.24	0.32	70.56	-	-	-
RN4	<None>	4208	4208	-	4.21	2.42	3.70	153.34	-	-	-

Figure 2. Flow cytometer analysis, dot blot and histogram of negative bacteria.

Figure 3 shows Bacterial positive control, at the top left of the dot plot diagram for EGFP-transformed bacteria is plotted by the software showing the FSC and SSC characteristics of the bacteria. At the top right of the figure, a histogram diagram is plotted for fluorescent bacteria. Bacterial fluorescent peak is in the positive range of fluorescent and its average fluorescent intensity is 103.44, Which indicates successful transformation and high expression of EGFP protein (0.21 in negative control compared to 103 in transformed bacteria).



Region	Gate	Ungated	Count	Count/ml	%Gated	GMn-x	Mean-x	CV-x%	GMn-y	Mean-y	CV-y%
R1	<None>	56342	56342	-	56.34	13.38	16.05	61.89	2.83	3.26	63.43
RN1	<None>	8879	8879	-	8.88	0.14	0.19	110.48	-	-	-
RN2	<None>	91136	91136	-	91.14	70.10	103.44	66.02	-	-	-
RN3	<None>	20057	20057	-	20.06	0.16	0.22	104.21	-	-	-
RN4	<None>	79947	79947	-	79.95	9.51	11.94	65.96	-	-	-

Figure 3. Flow cytometer analysis, dot blot and histogram of positive EGFP bacteria.

For confirmation of the transformation and expression of EGFP protein Bacteria were evaluated with a fluorescent microscope. Untransformed bacteria were used as negative control.

As shown in Figure 4, *E. coli* bacteria strain of BL21 transformed by EGFP (A) is shown to be completely bright green under the fluorescent microscope that compared to untransformed Bacteria of the same strain (B), but don't have the EGFP gene. To determine the optimal time for engulfment and digestion of bacteria by neutrophil, fresh blood was exposed with EGFP-expressing bacteria at times 15, 30, 45, And 60 minutes.

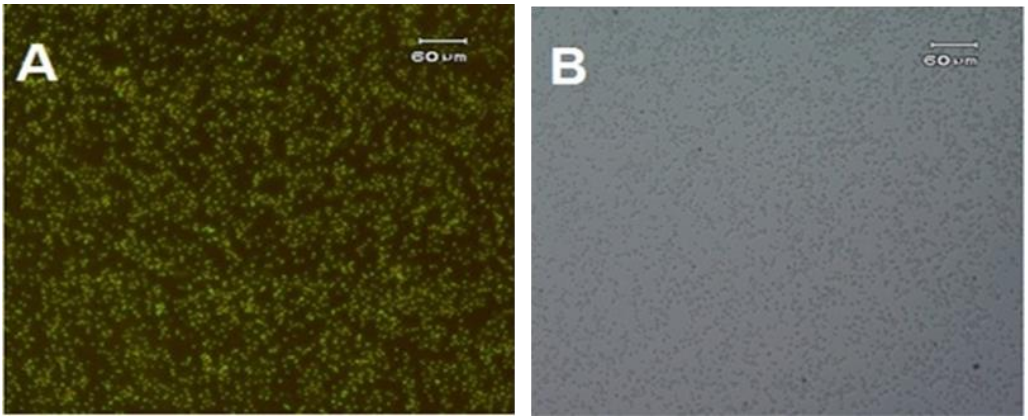


Figure 4. Image of bacterial fluorescent microscopy, before and after transformation.

Figure 5 shows respectively from top to down proximity time of neutrophils with EGFP- expressing bacteria at times 15, 30, 45, And 60 minutes. Due to decreased fluorescent intensity from top to down, after 45 minutes, the optimal time for phagocytosis was selected 45 minutes. Comparison between fluorescent intensity at different times showed best time for engulfment is 45 minutes.

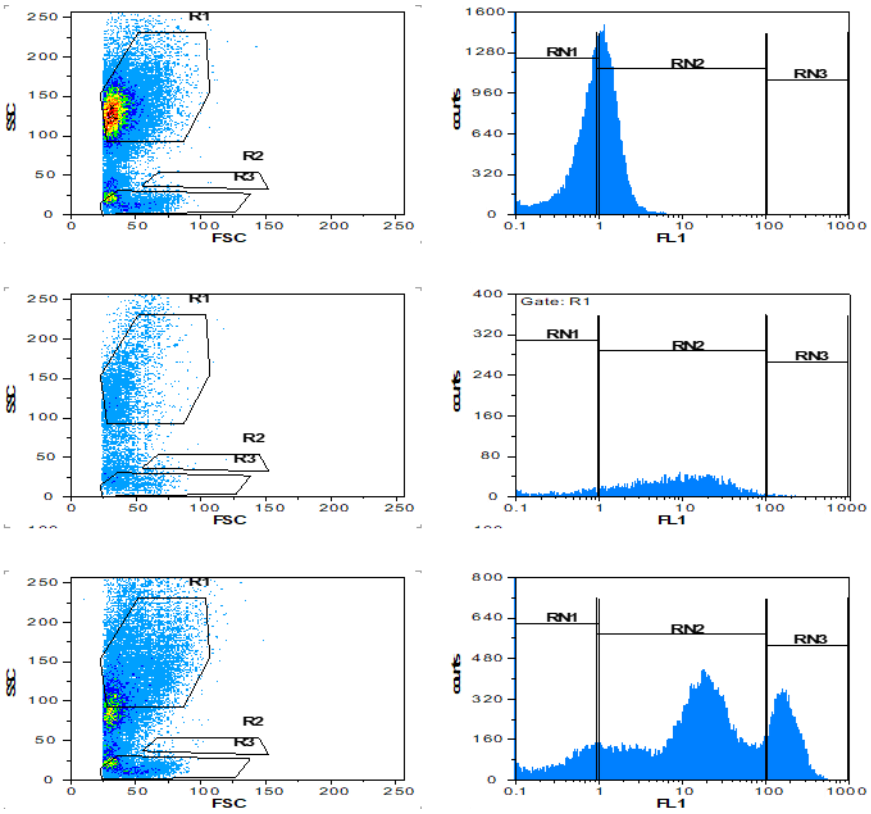


Figure 5. Flow cytometer analysis for determination of phagocytosis of bacteria with neutrophils.

Figure 6 demonstrates that fluorescent intensity significantly reduced after 45 minutes.

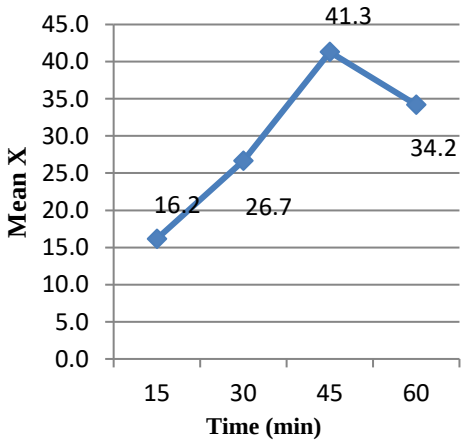


Figure 6. Comparison between fluorescent intensity at different times for determination best time of engulfment.

Determination of optimal time for digestion of EGFP-expressing bacteria by neutrophils showed that the results after about 45 minutes, the intensity of fluorescent neutrophils gradually decreased and after 2.5hr of incubation, there were no significant changes in fluorescent intensity. Therefore, the results of 2.5hr were considered as the optimal time for incubation to digest bacteria by neutrophils (Figure 7).

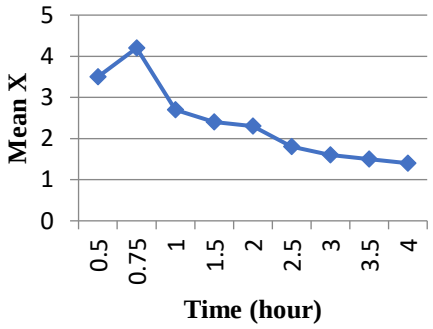


Figure 7. Comparison between fluorescent intensity in different times for determination best time of digestion.

Discussion

This study tried to suggest a suitable method for evaluating phagocytosis in which real bacteria instead of chemicals compounds like DHR and NBT. Phagocytosis is one of the vital and essential activities of the immune system. Defect in phagocytosis leads to severe infections. Neutrophils, which are a group of phagocytic cells, play a very important role in the initial response to annoy pathogens such as bacteria and fungi. People with neutrophil dysfunction, confront recurrent and severely treatable infections (11, 3). The microscopic method is a basic method for evaluating phagocytosis based is on counting bacteria or particles phagocytes by phagocytic cell (12, 7). Another method for evaluating phagocytosis is NBT test used by Nitro Blue Tetrazolium, which is yellow normally and after phagocytosis by phagocytic cell changes to purple seen by microscope. Flow cytometry techniques are including methods that are utilized for evaluation of phagocytosis (13, 9, 4). Use DHR in most methods of Flow cytometer techniques that is a chemical compound and it doesn't indicate the response of neutrophils to live bacteria. In cases where the bacteria are used in flow cytometer, dead bacteria that have been labeled with fluorescent chemical compound such as FITC is used. In this method, the bacteria may be destroyed by the dye, which is considered a defect and it shows the conditions of the study far from the natural conditions governing the process of phagocytosis. Also, other flow cytometry methods which depend on fluorescent dyes that use live bacteria have surface staining defects (14, 15). GFP protein doesn't alter the behavior of bacteria with host cells and doesn't have a detrimental effect on the pathogenicity of some bacteria expressed in them such as *S.typhimurium*, *Y.pseudotuberculosis*, *Mycobacteria sp* (16, 17, 18). Since 2000, GFP use in the study of bacterial phagocytosis by fluorescence methods has increased (19, 20, 21).

In this study, an EGFP sequence was cloned into a pColdI expression vector, *E. coli* (Bl21 strain) was transformed by EGFP containing vector. EGFP expression in bacteria was detected by a fluorescent microscope and flow cytometry. Gating analysis revealed that 99% of EGFP-*E. coli* cells expressed EGFP. The number of bacteria for determining the optimal time for neutrophils to swallow bacteria was selected in the amount of 10 µl of medium with OD_{600} = 0.4-0.6 which contains about 6×10^6 bacteria. Determining the optimal time for bacteria to be swallowed by neutrophils (Time Response) by flow cytometry was founded 45 minutes, and determining the optimal time for digestion of bacteria swallowed by neutrophils was founded 2.5 hours.

Conclusion

GFP as fluorescent protein has always been used extensively in biological studies, which can be used to study the interactions between pathogenic bacteria and mammalian hosts. This protein can also be detected in infected laboratory animals' tissue that by use flow cytometry methods can also detect the type of infectious cells. Thus, it is useful for studying the pathogenicity of bacteria in acute and chronic infections (22, 23). GFP after expression accumulates in the cytoplasm of the bacteria and remains bacteria to stable and doesn't affect the survival of the bacteria. Bacterial labeling with this protein eliminates the disadvantages of surface painting with fluorescent dyes and can traverse microbial killings (24, 25, 26). Due to the High efficiency of EGFP in biology, EGFP was used for evaluating phagocytosis. EGFP expressing bacteria and flow cytometry technique can be used to evaluate phagocytosis. It can be optimized for clinical as well as research uses.

Author contributions

HAH done this research and write manuscript, **SHZE** and **ZE** Guidance and assistance in laboratory work and analysis of the results, **EM** helps and guidance in statistical analysis.

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Conflict of interest

The authors declare that they have no conflict of interest.

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