ISSN NO. 2320-5407

Journal homepage:http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

EFFECTS OF SILVER NANOPARTICLES ON PATHOGENIC E.COLI & SOME HEMATOLOGICAL PARAMETERS

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Manuscript Info

Manuscript History:

Received: 14 January 2016
Final Accepted: 29 February 2016
Published Online: March 2016

Key words:
Silver nanoparticles, White blood cells, Red blood cell, cytotoxicity, pathogenic E.coli.

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Abstract

Background:-Silver nanoparticles (AgNPs) have become one of the commercialized nanoparticles because of its properties. It is used in biomedical applications as antimicrobial agent, drug delivery systems and targeting agent.

Objective:-to study the antibacterial effects of silver nanoparticles on *E.coli* isolated from blood and the effect of these particles on hematological parameters: total WBCs count, hemoglobin concentration and the morphology of WBCs and RBCs.

Methods:- Silver nanoparticles of 20 nm size and 99.9 % purity were used. The effect of different concentrations of silver nanoparticles on *Escherichia coli* (*E.coli*) isolated from blood was studied. The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of these AgNPs against pathogenic strainsof *E. coli* were determined by micro dilution technique and measured by spectrophotometer. The impact of silver nanoparticles on white blood cells count and hemoglobin was investigated using different concentrations of silver nanoparticles and different incubation times. The effects on WBC and RBC morphology were studied by incubating equal volumes of silver nanoparticles and blood.

Results:- The MIC and MBC values of silver nanoparticles on *E.coli* were 10µg/mL and 50 µg/mL respectively. The total white blood cells (WBCs) counts decreased depending on the concentrations and incubation time. The lowest WBC count was observed at concentration of 100 µg/mL of silver nanoparticles and after 4 hours of incubation. Hemolysis was observed at all concentrations of silver used in the study. WBCs morphology was not affected and they showed no evidence of phagocytosis.

Conclusions:-

1. Silver nanoparticles inhibit *E.coli* at the concentration 10 µg/mL while 50 µg/mL kills this bacteria.
2. Silver nanoparticles when mixed with blood lead to the decrease in the total WBCs count, the decease ratio depend on the concentration of the silver nanoparticles and on the time of the incubation.
3. Silver nanoparticles cause complete hemolysis of the RBCs.
4. There is no evidence of silver particles phagocytosis by WBCs while there are moderate and severe degree of RBC damage according to the concentration of silver nanoparticles in blood film.

Introduction:-

Medical applications of silver nanoparticles has been extremely active. More and more innovative applications are being proposed and evaluated.[1] Because of their large surface area-to-volume ratios, silver nanoparticles (Ag NPs) offer a large antimicrobial spectrum and greater efficacy against bacteria than common antibiotics.[2-3]

The Action of this metal on microbes is not fully known. It has been hypothesized that silver nanoparticles can cause cell lysis or inhibit cell transduction.[4]

Resistance of bacteria to bactericides and antibiotics has increased in recent years due to the development of resistant strains. The preparation of uniform nano sized drug particles with specific requirements in terms of size, shape, and physical and chemical properties is of great interest in the formulation of new pharmaceutical products.[5]

In some biomedical applications, AgNPs are directly in contact with blood. Because of their reduced particle size and increased surface area AgNPs may also enter the body and translocate into systemic blood flow after inhalation, ingestion, dermal contact or systemic administration,[6] therefore the interaction between AgNPs and blood parameters should be investigated.

Aims of the study:-

This study was focused on the effects of AgNPs on *E.coli* isolated from blood & the effects of these particles on total white blood cells count and on the hemoglobin and on WBC and RBC morphology.

Materials and methods:-

The study was approved by the ethical committees of both pathology and microbiology departments in conformity with Helsinki declaration. Oral informed consent was obtained from all volunteers.

Silver nanoparticles were obtained from the HongWu nanometer as a powder of 20 nm in size and 99.9% purity. They were suspended in sterilized D.W at a stock concentration of 100 µg/mL and dispersed by ultrasonic vibration for 5 min.

Test of the antibacterial effects of AgNP on *E.coli* by dilution method:-

The pathogenic *E.coli* was isolated from blood sample and taken from Al-Yarmouk Teaching Hospital. Various concentrations of 100, 50, 25, 10, 0.1, 0.01, and 0.001 of silver nanoparticles were used, negative and positive controls were prepared with nutrient broth and cultured nutrient broth respectively for comparison. Sterile tubes were incubated aerobically in shaker incubator at 37°C for 24 hr ,which contained 10 mL of nutrient broth with approximate 10^8 CFU bacterial cells and 0 µg/mL (the control group),100,50,25,10,1 ,0.1,0.01,0.001µg/mL silver nanoparticles. The tube without visible growth of the bacterial cells was the MIC. Then these tubes were cultured on nutrient agar and incubated for 24 hr, the plate without bacterial growth was the minimal bactericidal concentration MBC. [7] Thereafter, tubes were tested with spectrophotometer at wavelength of 600 nm, and optical density (OD) was adjusted to 0.1, corresponding to 10^8 CFU/mL at 600 nm.

Test of the effect of AgNPs on total WBC count:-

Blood samples were obtained from 10 healthy human donors who were free from any medication for at least two weeks. Their age range was (20-30) years. One mL of Blood was collected by venipuncture into EDTA tubes. Twentyµlof blood samples were taken and mixed with 400 µl of white blood cells solution (Glacial acetic acid) and mixed for 10 min .Total WBC count was performed using Improved Neubauer Chamber by holding the pipette at angle 45 degree and touching the space between the cover slip and the chamber by the point of the pipette, an appropriate drop of the mixture is allowed to run under the cover slip by capillary action. Two minutes were allowed for cells to settle before counting. The effect of AgNPs on WBCs was tested by adding 40 µl of different concentrations (100,50,25,10) µg/mL of AgNPs to blood samples then adding the white blood cells solution as previous section. Thereafter, the tubes were placed in shaker incubator at 37°C for 1 h and 4 hours respectively. Total WBC count was performed usinghemocytometer after 1hr and 4hr and results recorded. The negative control without the addition of silver nanoparticles are used for 1h and for 4 hours. The count was adjusted for the dilutional effect of AgNP solution.

Microhematocrit:-
Hematocrit was determined using capillary tubes and microcentrifuge according to ICSH reference method. [8]

Effect of silver nanoparticles on WBC and RBC morphology in blood film:-
A mixture of equal amount (400 µl) of AgNP solution 10 µg/mL and 100 µg/mL and EDTA blood sample was prepared. The mixture was left for incubation at room temperature for 15 minutes. Afterwards,blood smear was prepared using Leishman staining technique. The morphological changes in WBCs and RBCs were then studied by clinical microscopy technique (conventional light microscopy) under high power field. Control blood film without the addition of silver NP was used for comparison. [9]

Statistical data analysis:-

Analysis of data was carried out using the available statistical package of SPSS-22 (Statistical Packages for Social Sciences- version 22). Data were presented in simple measures of frequency, percentage, mean, standard deviation, and range (minimum-maximum values). The significance of difference of different means (quantitative data) were tested using Students-t-test for difference between two independent means or Paired-t-test for difference of paired observations (or two dependent means), or ANOVA test for difference among more than two independent means.Statistical significance was considered whenever the P value was equal or less than 0.05.

Results:-

The results showed that concentration (50) µg/mL was lethal to the *E.coli* while the other concentrations were inhibitors .The minimal inhibitory concentration was 10 µg/mL. Results are shown in figure 1.

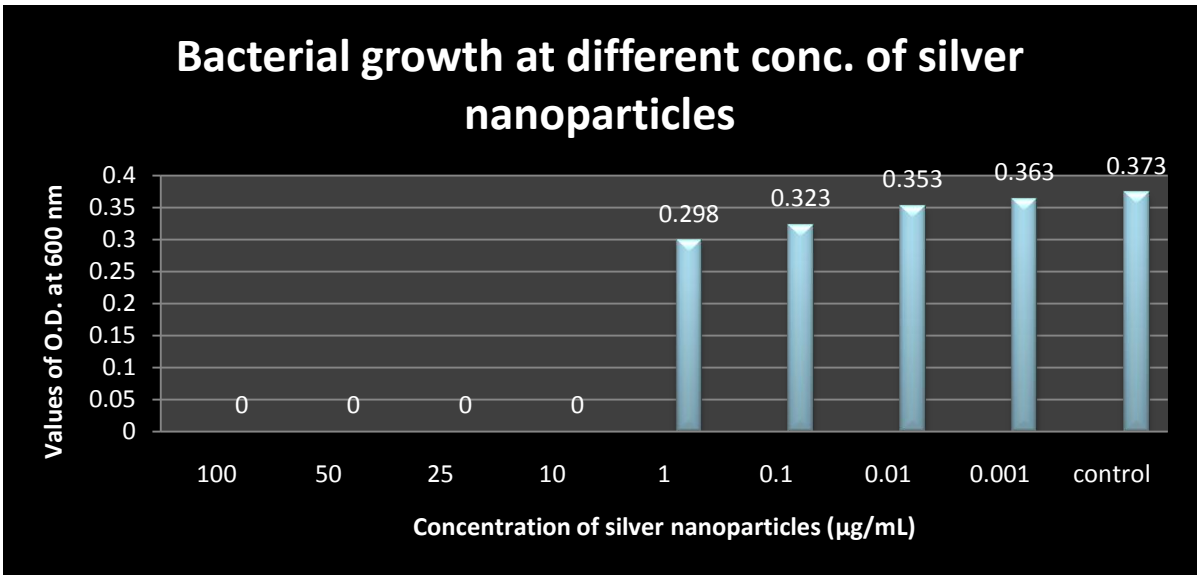

Figure 1.Inhibitoryeffects of silver nanoparticles on *E.coli* growth on values of OD at 600 nm.

Effects on total WBC count is shown in figure 2 and table 1. Figures 4 and 5 show the effect on WBC and RBC morphology.

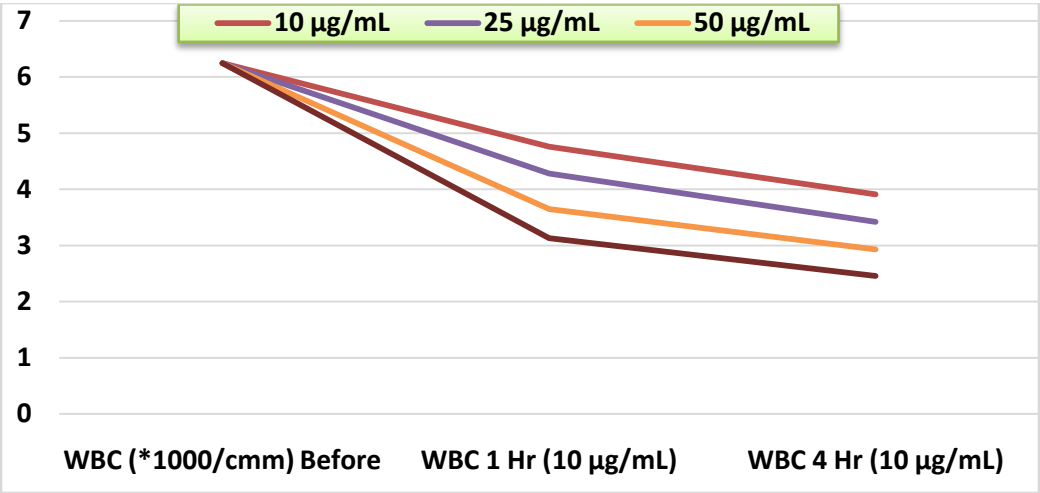

Figure 2. Graph showing the effects of different concentration of AgNPs on total WBC count after 1 hr and 4 hr incubation.

Table 1**:-**The effect of silver nano on total WBC count at different concentrations after 1 hour and 4 hour. The effect of time was studied by counting one sample total WBC count at zero, 1 hr and 4 hr without any addition.

	Control	10µg/mL	25µg/mL	50 µg/mL	100 µg/mL	P value
WBC (*1000/cmm) Before	5.50±	6.25±1.71 (4.0-9.7)	6.25±1.71 (4.0-9.7)	6.25±1.71 (4.0-9.7)	6.25±1.71 (4.0-9.7)	0.999
WBC 1Hr (10 µg/mL)	5.40±	4.76±1.01 (3.7-6.3)	4.28±0.85 (3.4-5.8)	3.65±0.70 (2.6-4.6)	3.13±0.60 (2.1-4.0)	0.0001#
WBC 4Hr (10 µg/mL)	5.20±	3.91±0.83 (3.0-5.4)	3.42±0.63 (2.75-4.8)	2.93±0.64 (2.0-4.2)	2.46±0.62 (1.8-3.8)	0.0001#
P value 1Hr xBefore	-	0.002*	0.001*	0.0001*	0.0001*	
P value 4Hr xBefore	-	0.0001*	0.0001*	0.0001*	0.0001*	
P value 1Hr x4Hr	-	0.011*	0.008*	0.003*	0.003*	
*Significant difference using Paired t-test for two dependent means at 0.05 level						
#Significant using ANOVA test at 0.05 level						

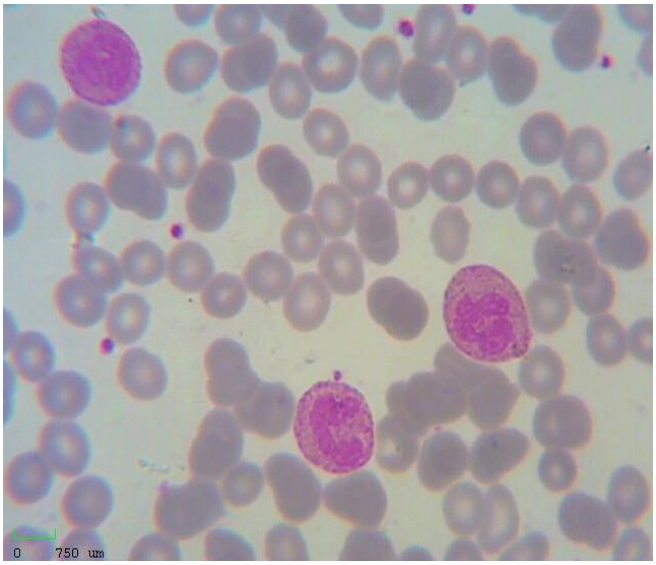

Figure 3. Photomicrograph of control blood film before the addition of silver nano. Normal WBCs and RBCs. (Leishman stain. X1000).

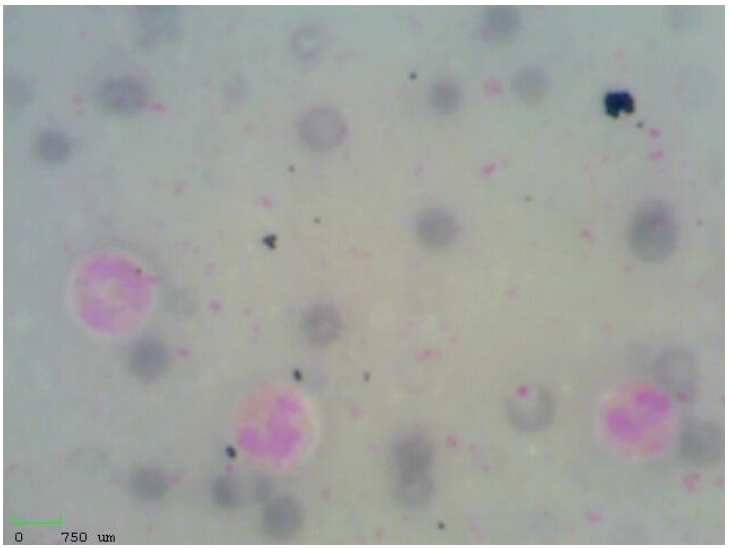

Figure 4. Photomicrograph of blood film after addition of silver nanoparticles at concentration 10 µg/mL. There is no evidence of silver particles phagocytosis by WBCs. However, there is moderate degree of RBC damage and hemolysis. (Leishman stain. X1000)

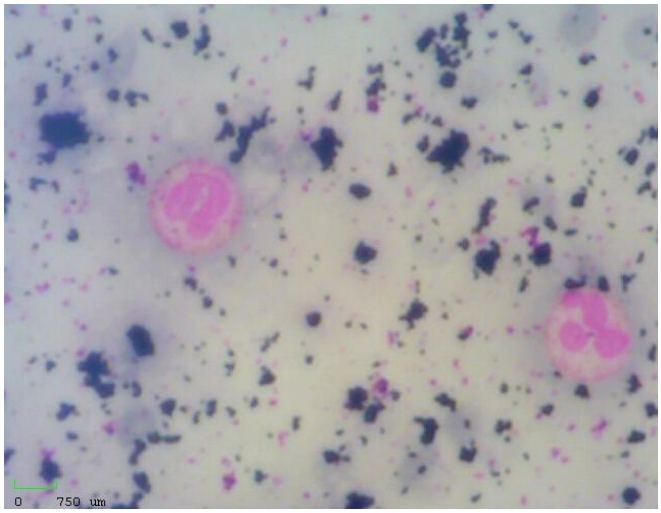

Figure 5. Photomicrograph of blood film after addition of silver nanoparticles at concentration 100 µg/mL. There is no evidence of silver particles phagocytosis by WBCs. However, there is severe degree of RBC damage and hemolysis. Notice the marked extracellular aggregates of silver nanoparticles (Leishman stain. X1000)

Discussion:-

There are various theories on the action of silver nanoparticles on microbes to cause the microbicidal effect. Silver nanoparticles have the ability to anchor to the bacterial cell wall and subsequently penetrate it, thereby causing structural changes in the cell membrane like the permeability of the cell membrane and death of the cell. There is formation of 'pits' on the cell surface, and there is accumulation of the nanoparticles on the cell surface. [10] The formation of free radicals by the silver nanoparticles may be considered to be another mechanism by which cells die. There have been electron spin resonance spectroscopy studies that suggested that there is formation of free radicals by the silver nanoparticles when in contact with the bacteria, and these free radicals have the ability to damage the cell membrane and make it porous which can ultimately lead to cell death.[11,12] The efficacy of silver also depends on particle size:the smaller the diameter (the bigger surface),the better the antibacterial efficacy.[13] The MIC value in the current study is higher than that reported by Radzig et al [1] who found that the MIC was 1 µg/mL while they reported a similar MBC value that was 50 µg/mL. These differences could be attributed to differences in AgNP surface charge and size. Generally, NPs with smaller sizes have a large surface area available for interaction with the cell membrane, which could alter some primary functions of bacteria, such as permeability and cell respiration.

Nevertheless, other authors have proposed that the antimicrobial activity of AgNPs is also dependent on the initial concentration of bacteria. [14]

The effect of different concentrations of AgNPs (10, 25, and 50,100) µg/mL and different times on the total WBC are shown in figure 2 and table 1. All concentrations used caused decrease in the total WBC count depending on the concentration and time. The decrease was more pronounced with higher concentration of AgNPs and longer time. The lowest WBC counts produced by the concentration of 100 µg/mL of silver nanoparticles after 4 hours of exposure. Most of the future applications of silver nanoparticles are based on systemic administration, experiments on their interaction with human blood parameters are very important. A similar in vitro experiment by Barkhordari et al found that cytotoxic effect of AgNps on suspensions of blood mononuclear cells increased with higher concentrations and longer incubation times. The maximum cytotoxicity was observed at the concentration of 500 µg/mL and markedly increased when exposure time increased to 24 hours.[15]

Silver nanoparticles caused hemolysis at the lowest concentration used in this experiment as shown in figure 4. A similar result is found by Laloy et al.[6] who measured the degree of hemolysis by spectrophotometry.

In the current study, leukocytes did not show evidence of AgNps phagocytosis even at 100 µg/mL concentration as shown in figure 5. This is in contrast to Wiwanitkit et al who reported phagocytosis of gold nanoparticles by leukocytes.[16, 17] This might be explained by different physical and chemical properties between gold and silver nanoparticles. There is paucity of literature on this effect. Further studies are needed in this regard.

Conclusion:-

1. Silver nanoparticles inhibit *E.coli* at the concentration 10 µg/mL while 50 µg/mL kills this bacteria.
2. Silver nanoparticles when mixed with WBC leading to the decrease in the total WBCs count, the decease ratio depend on the concentration of the silver nanoparticles and on the time of the incubation.
3. Silver nanoparticles cause complete hemolysis of the RBCs.
4. There is no evidence of silver particles phagocytosis by WBCs while there are moderate and severe degree of RBC damage according to the concentration of silver nanoparticles in blood film.

References:-

1. Radzig MA, Nadtochenko VA, Koksharova OA, Kiwi J, Lipasova VA, Khmel IA. Antibacterial effects of silver nanoparticles on gram-negative bacteria: influence on the growth and biofilms formation, mechanisms of actions. Colloids and Surfaces B: Biointerfaces.2013 Feb 1; 102:300-6.
2. Ahamed M, Alsalhi MS, Siddiqui MK. Silver nanoparticle applications and human health. ClinChimActa. 2010 Dec 14; 411(23-24):1841-8.
3. Shahverdi AR, Fakhimi A, Shahverdi HR, Minaian S. Synthesis and effect of silver nanoparticles on the antibacterial activity of different antibiotics against Staphylococcus aureus and Escherichia coli. Nanomedicine. 2007 Jun;3(2):168-71.
4. Sondi, I, Salopek-Sondi, B. Silver nanoparticles as antimicrobial agent: a case study on E. coli as a model for Gram-negative bacteria. J. Colloid Interface Sci. 2004 Jul 1;275(1):177-82.
5. Ivan Sondi and BrankaSalopek-Sondi. Silver nanoparticles as antimicrobial agent: a case study on *E. coli* as a model for Gram-negative bacteria. J Colloid Interface Sci. 2004 Jul 1;275(1):177-82.
6. Laloy J, Minet V, Alpan L, Mullier F, Beken S, Toussaint O, et al.(2014) Impact of Silver Nanoparticles on Haemolysis, Platelet Function and Coagulation. Nanobiomedicine. 2014 September 29; **1**:4 .doi: 10.5772/59346.http://www.intechopen.com/journals/nanobiomedicine/impact-of-silver-nanoparticles-on-haemolysis-platelet-function-and-coagulation
7. Chao Wang, Lian-Long Liu, Ai-Ting Zhang, PengXie, Jian-Jun Lu and Xiao-Ting Zou. Antibacterial effects of zinc oxide nanoparticles on Escherichia coli K88. Afr. J. Biotechnol. 2012 May; 11(44):10248-10254.
8. Briggs C. and Bain BJ. Chapter 3; Basic Haematological Techniques. In: Bain BJ, Bates I, Laffn MA and Lewis SM, editors. Dacie and Lewis Practical Haematology. 11th edition. Churchill Livingstone. P61
9. Wiwanitkit V, Sereemaspun A, Rojanathanes R. Identification of gold nanoparticle in lymphocytes: a confirmation of direct intracellular penetration effect. Turk J Hematol. 2009 March; 26(1): 29-30.
10. Sondi I, Salopek-Sondi B. Silver nanoparticles as antimicrobial agent: a case study on E. coli as a model for Gram-negative bacteria. J. Colloid Interface Sci. 2004 Jul 1; 275(1):177-82.

11. Danilczuk M1, Lund A, Sadlo J, Yamada H, Michalik J. Conduction electron spin resonance of small silver particles. SpectrochimActa A MolBiomolSpectrosc. 2006 Jan; 63(1):189-91.
12. Kim JS, Kuk E, Yu K, Kim JH, Park SJ, Lee HJ,et al. Antimicrobial effects of silver nanoparticles. Nanomedicine. 2007; 3: 95–101.
13. Guzman M, Dille J, GodetS.Synthesis and antibacterial activity of silver nanoparticles against gram-positive and gram-negative bacteria. Nanomedicine. 2012 Jan;8(1):37-45.
14. Paredes D, Ortiz C and Torres R.Synthesis, characterization, and evaluation of antibacterial effect of Ag nanoparticles against Escherichia coli O157:H7 and methicillin-resistant Staphylococcus aureus. Int J Nanomedicine. 2014 Apr 3;9:1717-29.
15. Barkhordari A, Barzegar S, Hekmatimoghaddam H, Jebali A, RahimiMoghadam S, N Khanjani N. The Toxic Effects of Silver Nanoparticles on Blood Mononuclear Cells. Int J Occup Environ Med. 2014; July 5(3): 164-68.
16. Wiwanitkit V, Sereemaspun A, Rojanathanes R. Effect of gold nanoparticle on the microscopic morphology of white blood cell. Cytopathology. 2009 Apr;20(2):109-10.
17. Wiwanitkit V, Sereemaspun A, Rojanathanes R. Identification of gold nanoparticle in lymphocytes: a confirmation of direct intracellular penetration effect. Turk J Hematol 2009; 26: 29-30.