Original Article

Zahedan Journal of Research in Medical Sciences

Journal homepage: www.zjrms.ir

Comparison of Neutrophil Apoptosis by the Pseudomonas Aeruginosa Exotoxins between Healthy Individuals and Term Infants

Soheila Khazaei,[1]* Abdollah Karimi,[2] Alireza Fahimzad,[2] Abolfazl Afjei,[2] Fatemeh Fallah,[3] Soodabeh Taheree,[3] Ahmadreza Shamshiri[4]

1. Department of Pediatric, Iran Ministry of Health and Medical Education, Tehran, Iran
2. Department of Pediatric, Shaheed Beheshtee University, Tehran, Iran
3. Department of Microbiology , Shaheed Beheshtee University, Tehran, Iran
4. Department of Epidemiology, Shaheed Beheshtee University, Tehran, Iran

Article information

Article history:
Received: 5 Sep 2011
Accepted: 19 Nov 2011
Available online: 5 Nov 2012
ZJRMS 2013; 15(4): 6-11

Keywords:
Pseudomonas
Exotoxin
Apoptosis
Neutrophil

*Corresponding author at:
Iran Ministry of Health and Medical Education
E-mail:
khazaei@health.gov.ir

Abstract

***Background*:** *Pseudomonas aeruginosa* may be colonized in different human tissues and result in some infections potentially. Thus, considering that these bacteria are resistance to most of the current antibiotics, an examination on pathogenesis mechanisms of such bacteria can be effective in controlling the infections developed by it.

***Materials and Methods*:** In this project, among 40 blood samples (20 healthy persons, 20 infants), an amount of 5 ml (2 ml in the infants) heparinized blood was collected form each and then neutrophils were isolated by a standard method and were counted by neubauer lam. After culturing Pseudomonas bacteria in broth medium, some tubes with densities of 1, 2, 3 and 4 McFarland were prepared and the bacteria were isolated by centrifuge method with 3000rpm for 10 minutes and then its exotoxin were exposed to neutrophils of the groups under study. The effect of time and the bacteria count on the amount of the secreted toxin and in adjacency to neutrophils was measured.

***Results*:** There were 11 men and 9 women in the health group and the infants group consisted of 12 boys and 8 girls. Death cell percentage of neutrophils was 100% in the health group and 8.90% in the infants group. Percentage of bacterial growth in the medium 1 and 2 McFarland was zero; in the medium 3 McFarland, it was 12.5% in the healthy group and 1% in the infants group (p<0.10). The average rate of cell death in the minute 15th was different in two groups (68.5% in health group vs. 92.5% in the infants) (p<0.0005).

Conclusion: This study showed the effect of Pseudomonas bacteria on the development of early cell death in the infants very well. As it was shown, this effect is time-dependent and this cell death (apoptosis) is occurred in the infants earlier than health people.

Introduction

The neutrophil cells as a main component of acute inflammatory response, after the infection overcomes the defensive mechanisms are evoked from peripheral blood [1]. The neutrophils' longevity in peripheral blood is 24 hours and their death is regulated through cell death. Cytokines of the body control the cell death process; though such control will lose its balance in some cases of infection and will not have their physiological system. Body cells movement process is not only controlled by meiosis speed control, but the programmed cell death or apoptosis is also a component of this process. The recent process is called programmed cell death or apoptosis derived from its Greek word (which means as leaves falling from trees).

Body cells will be destroyed of necrosis type in response to any type of damage. Necrosis may cause to stimulate inflammatory process, while the cell death is contrary and does not cause to activate inflammatory process. In fact, this process does not only cause immune stimulation, but activation process of proteolytic cascades in the cell death will cause to prepare intracellular components for the construction and subdivision of other cells. In this process before the cell components are released, macrophage cells swallow the cells with programmed death and cause preventing to release intracellular components [4].

Some bacteria such as pseudomonas may cause early cell death through reducing the number and the function of neutrophils. Pseudomonas bacteria secrete toxins which may affect the cell death. A question which is addressed in the present study is: "Is there any difference between the pseudomonas exotoxins on the development of cell death in healthy people and in term infants". It is essential to provide appropriate therapeutic strategies considering the time necessary for the interaction between pseudomonas aeruginosa and neutrophils (treatment before suffering) before these special bacteria is colonized in high-risk individuals. Finding some drugs with adverse effect, i.e. preventing early cell death in neutrophils being exposed to pseudomonas, may be one of those effective therapeutic strategies.

In this investigation, cellular relationship between neutrophil, bacteria and cell death has been examined. To do this, blood samples were collected from healthy people and the infants (40 samples: 20 healthy individual. 20 term infants). Considering the results of this study, new medical solutions may be designed and suggested for high-risk people.

Materials and Methods

The present investigation is an observational and applied study in which 40 blood samples from 20 healthy person and 20 infants admitted to Mofid Children Hospital were examined and evaluated; the results achieved were compared. Sampling was carried out by simple random approach. In this work, a comparison between the effect of pseudomonas aeruginosa exotoxin on the cell death of neutrophils in healthy people and term infants was studied.

How to isolate neutrophils from peripheral blood: Firstly 5 ml blood was drawn from the person under study (2 ml from the infants) by anticoagulant heparin coated syringes and then we added 3 ml of dextran 3% solution with molecular weight of 500,000 dalton to it. The blood sample in syringe or plastic tubes were placed inside incubator for 30-45 minutes at room temperature or 37ºC; after incubation period is finished, red and other blood cells are deposited at the bottom of syringe or tube, but the neutrophils were collected in the supernatants. The supernatant was transferred into a plastic tube such as a falcon tube and is centrifuged by 1500 rpm for 10-15 minutes; the neutrophils were deposited at the bottom of the tube and the supernatant will be cell-free. The supernatant was taken away and 1 ml of distilled water was added to the deposit at the bottom of the tube in order to collapse remaining red blood cells, it was shaken for 30 minutes and then adding 5ml of BPS buffer, it was centrifuged for 10-15 minutes by 1500 rpm. The supernatant was discarded and then BPS was added to the deposit increasing its volume to 1 ml. Afterwards, we took some cells and counted it by neubauer lam.

How to prepare counted microbial strains: we cultured in broth medium the pseudomonas aeruginosa bacteria prepared by department of microbiology; after 24 hours, we prepared tubes with densities of 1, 2, 3 and 4 McFarland by comparing McFarland tube unit. After calculation of bacteria count, pseudomonas microbe was isolated from the medium by centrifuge method with 3000 rpm for 10 minutes and just a fluid containing secreted exotoxins will remain in the medium.

Placing neutrophils exposed to microbial exotoxins: for this purpose, 50 µl of ready-made neutrophils were poured into a sterile test tube and added to it 50 µl of the above bacteria medium separately from 1, 2, 3 and 4 McFarland densities (PRMT1640) and put the tube during a certain period into the water bath.

NBT test conduction: We added an amount of 50 µl of NBT ready-made solution and then put the tube for 30 minutes inside the water bath at 37ºC temperature. Then, we brought it out and centrifuged for 3 minutes y 1500 rpm. From the deposit at the bottom of tube, we prepared a blood smear on the lam and fixed it by methanol and then staining with Gimsa, the lam was washed for 15 minutes and the neutrophils were counted by lens 100X light microscopy. Reduction rate of NBT material was stated as 100 percent. We determined the death cell in neutrophils based on their capability to revive the NBT material so that cell-deceased neutrophils will be unable to revive NBT and consequently to be converted to formazan crystals. In this stage, the rates of neutrophils' programmed cell death were determined separately based on NBT at different times including 15, 30, 45 and 60 minutes and with different microbial densities.

The collected data were analyzed and evaluated using SPSS-15 software as well as Kruskal Wallis test, non-parametric tests, post-hoc test and a confidence level of 95%.

Internal and external research obligations: Healthy people under the test should have no disease for at least one recent month and receive no medicine.

All tests were conducted in pediatric infections research center under the supervision of a senior microbiology and immunology expert. Ethical and humanitarian issues of the project were observed and a testimonial was taken from the infants' parents and the health individuals after a full justification.

Results

There were 11 men (55%) and 9 women (45%) in the health group. The infants group consisted of 12 boys (60%) and 8 girls (40%). Due to the lack of variance analysis prerequisites, non-parametric test Kraskal-Wallis test was used that showed significant difference between two groups at zero time and 30th minute (p was respectively less than 0.01 and 0.002).

Reduction percentage on 30th and 60th minutes as well as at zero time is shown in table 2 and its analysis is seen in Fig. 1. There is significant difference on 30th minute in the infants group (zero vs. 5.2 respectively in healthy and infants groups, $p<0.04$).

As it was argued, neutrophils with programmed cell death have no power for NBT reduction. According to the examinations, cell death has been occurred more in the infants. In table 2, NBT reduction rate has been expressed by percentage at the times zero, 30 and 60 for different groups under study with confidence level of 95%.

Taking the above diagram into consideration, NBT reduction rate in primary control sample in the infants were less than that in the healthy group. In 30th minute, NBT reduction has been decreased at a considerable rate in both groups. In 60 minute, NBT reduction rate was nearly zero. As it was already said, rate of cell death in neutrophils has an inverse relationship with NBT reduction rate. In the groups under study, this percentage had been 12.5 for healthy people and 1.00 in the infants; such difference is significant. About the bacterial growth rate in McFarland media with different densities, this

study showed that the bacteria count in media 1 and 2 McFarland has been insufficient for production of necessary level of toxin. The percentage of neutrophil reduction in medium McFarland 3 has been stated in the following table. In medium 4 McFarland in both groups, cell death rate was 100%; toxin rate was in an amount that caused all the neutrophils be destroyed. These results indicate that the infants are more susceptible to pseudomonas toxin than healthy people. Furthermore, studies showed that NBT reduction percentage at 30th and 60th min after combination of toxin with neutrophil is dependent on the time as obviously apparent in figure 1; as the time is increased, reduction rate is decreased.

NBT reduction rate in term infants is less than healthy people. It was decreased rather in the infants in 30th min. In 60th min, neutrophil reduction has been close to zero. Using Kruskal Wallis test, the difference at zero and 30th min was significant (p=0.0001 & p=0.002).

Table 1. Percentage of neutrophils reduction in the groups under study

Group		Neut % could reduce NBT	Neut% reduced NBT after 30 min	Neut% reduced NBT after 60 min
Healthy	Median	100,0000	0.0000	0.0000
	Minimum	100,00	0.00	0.00
	Maximum	100,00	70.00	0.00
Neonate	Median	95.0000	2.5000	0.0000
	Minimum	60.00	0.00	0.00
	Maximum	98.00	50.00	10.00

Table 2. NBT reduction percentage and standard deviation at the times zero, 30 & 60 min in the groups under study

NBT Time	Group	Mean(%)	SD
	Healthy	100.0000	0.00000
	Neonate	90.8000	10.07655
	Total	81.1481	30.37808
0.001	Healthy	6.0000	18.75044
	Neonate	7.5000	12.72172
	Total	4.6914	12.30715
	Healthy	0.0000	0.00000
	Neonate	0.5000	2.23607
	Total	0.3086	1.64804
60.00	Healthy	35.3333	47.38829
	Neonate	32.9333	42.39558
	Total	28.7160	41.70723

Table 3. Percentage of pseudomonas growth in medium Mcfarland 3 in different groups under study

Group		McFarland 3
Healthy	Number	20
	Mean	12.50%
	Median	0.00
	SD	22.21
	Minimum	0.00%
	Maximum	50.00%
Neonate	Number	20.00
	Mean	1.00%
	Median	0.00
	SD	4.47
	Minimum	0.00%
	Maximum	20.00%

Table 4. Comparison of cell death percentage in Mcfarland 3 & 4

		MacFarland 3 Mean	McFarland 4 Mean
Group	Healthy	12.50	100.00
	Neonate	1.00	100.00

Table 5. The amount of cell death between the groups at different times

APPO-Time	Group	Mean±SD	N
15.00	Healthy	68.5±27.19	20
	Neonate	92.5±12.72	20
	Total	88.7±19.43	81
30.00	Healthy	94.0±18.75	20
	Neonate	92.5±12.72	20
	Total	95.3±12.30	81
45.00	Healthy	97.5±11.18	20
	Neonate	99.5±2.23	20
	Total	99.1±5.76	81
60.00	Healthy	100.0±0.00	20
	Neonate	99.5±2.23	20
	Total	99.7±1.64	81
Total	Healthy	90.0±21.28	80
	Neonate	96.0±9.62	80
	Total	95.7±12.62	324

As it is seen in Fig. 5, average cell death rate in 15th min is different between groups (68.5% in healthy group vs. 94.7% in the infants group). Statistical analysis of Kruskal Wallis test results is indicative of statistically significant difference between the numbers listed in 15th min (p<0.002). In all other times, there have been some differences which were little with no statistical value (respectively p<0.596 and p<0.314) (Table 5). Considering the difference between cell death rate in two groups under our study including infants and control samples in this investigation, it was concluded that the effect of pseudomonas bacteria on the programmed cell death (apoptosis) is time-dependent and it is occurred in the infants earlier than the healthy people, before and after being exposed to pseudomonas toxin. Inhibition of early cell death and later examinations on the causes of early cell death in the infants as well as inhibitory factors of cell death will help considerably to treat such patients.

Discussion

The key role of neutrophils' cell death in the improvement of inflammation is accepted by all. Neutrophils cell death prevents the loss of bacteria and causes the infection development [5, 6]. This procedure may be inhibited by some pathogens through developing early cell death and the organism may escape from the host immune and defense [7]. In our study, 40 patients including 20 healthy persons and 20 term infants were examined. This investigation showed very well the impact of pseudomonas bacteria toxins on the development of early cell death in the infants. As already mentioned, this effect is time-dependent and the cell death is occurred in the infants earlier than the healthy people.

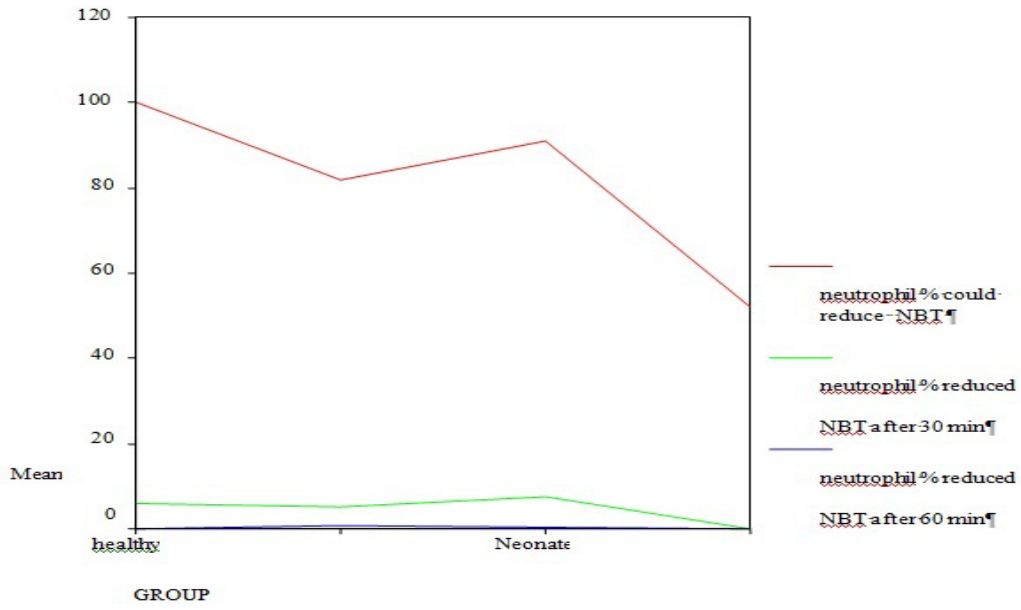

Figure 1. Comparison of neutrophil reduction rate at different times in two groups under study

All previous studies conducted by other researchers suggested merely to an investigation on the difference between various strains of bacteria with or without special exotoxins including pyocyanin which differentiate our study from other ones.

In an investigation carried out by Hanna et al, the difference of cell death in the infants and adults were evaluated. The results suggested that phosphatidyl serine of neutrophils' cell membrane and caspase 3 as well as the occurrence of apoptotic proteins including Bak, Bad and Bax has been decreased in the infants more than adults and there is a delayed cell death in the infants compared to that in the adults [8]. Our study indicated an accelerated cell death in term infants compared to those adults that is contrary to the above results.

Molly et al examined neutrophils' cell death in 30 adult persons and 30 infants and indicated that there has been a serious delayed cell death of neutrophils in the normal term infants as compared to the adults. Such delay in the infants born by natural childbirth has been more than those born by cesarean delivery [9 10]. In this study, a reduced caspase3 was found in funiculus neutrophils as a possible cause for delayed cell death [11, 12]; Cortisol causes delayed cell death. And increased serum cortisol and IL-6 after natural childbirth may cause such delay. There was a considerable delayed cell death in the infants compared to that in the adults. According the investigation carried out by Mozinbo (1994) and Peng (1999), immature neutrophils are increased in preterm infants [13, 14]. And in the studies on HL-60 cells, neutrophils have shown a delayed cell death [15]; this is contrary to the results of our study. Preterm infants have not been examined in this study and pseudomonas toxin has not been used to induce cell death.

In an investigation conducted by Joei et al in NICU ward of Royal Hospital as a prospective study on 100 on-chip sample of ventilated infants, it was concluded that in the preterm infants with low level of IL-10 in on-chip fluid, there is a reduced neutrophil cell death and such infants are disposed to impairment of lung repair and chronic pulmonary disease [16]. In this investigation unlike our study, neutrophils of peripheral blood have not been evaluated. In addition to pseudomonas, the impacts of other organisms on the cell death have been also investigated.

Van Zandbergen et al showed in their study that Chlamydia infection decreases neutrophils' cell death. This study was conducted outside the body. As per this investigation, procaspase3, Lipopolysaccharide 1 and IL-8 has caused to inhibit the cell death [17]. It was also shown that *Leishmania major* and mycobacteria has had such inhibitatory effect too [18, 19].

Some organisms including *E. coli* [20, 21] and candida albicans [22, 23] induce neutrophils' cell death. Knowing how these organisms affect will help us to develop new methods of treatment including vaccines for the treatment of the patients with some immune deficiencies [24-26].

Patricu et al. exanimated the effects of granulocyte-colony stimulating factor (G-CFS) on the neutrophils of healthy people. This investigation was carried out on volunteer individuals and cyclohexamide was used to induce the cell death. According to this study, G-CFS has affected the cell death considerably [27]. The study conducted by Kobota in 1994 has verified this effect too [28]. Squier concluded in his study in 2002 that outside the body, cytokines including IL-6, TNF, IL-1B, LPS, GM-CFS and INF Gama cause to decrease neutrophils' cell death [29-31]. These studies argued on of the approached to reduce cell death that may be used like pseudomonas for the treatment of the infections [32]. In our investigation, we have not examined the factors effective on the cell death.

In this study, considering the difference of cell death rate in two groups under study including infants and

control samples, it was determined that the effect of pseudomonas bacteria is time-dependent and this cell death is occurred in the infants earlier than the healthy people. Inhibition of early cell death and later examinations on the causes of early cell death in the infants as well as inhibitory factors of cell death will help considerably to treat such patients [32, 33]. Furthermore, in the patients with chronic diseases such as chronic granulomatous, it might be possible to change the disease process by making use of the delayed cell death [34]. It is hopefully to provide a new treatment for such patients in the near future by conducting more comprehensive studies on the biochemical differences of cell death between the healthy and immunodeficiency people.

Acknowledgements

The author of this paper is expressing his deep sense of gratitude to the colleagues of Mofid Hospital research center for their valuable assistance for the project implementation and the related laboratory studies under the code 1156.

Authors' Contributions

All authors had equal role in design, work, statistical analysis and manuscript writing.

Conflict of Interest

No conflict.

Funding/Support

Shahid Sadoughi University of Medical Sciences.

References

1. Usher LR, Lawson RA, Geary I, et al. Induction of neutrophil apoptosis by the Pseudomonas aeruginosa exotoxin pyocyanin: A potential mechanism of persistent infection. J Immunol 2002; 168(4): 1861-1868.
2. Dacheuax D, Attree I, Schneider C and Toussaint B. Cell death of human Pseudomuna aureginosa cystic fibrosis isolate requires a function type III secretion system. Infect Immun 1999; 67(11): 6164-67.
3. Terada LS, Johansen KA, Nowbar S, et al. Pseudomonas aeruginosa hemoloytic phospholipase c suppress neutrophil respiratory burst activity. Infect Immun 1999; 67(5): 2371-2376.
4. Stephan F, Yang K, Tankovic J, et al. Impairment of polymorphonuclear neutrophil functions precedes nosocomial infections in critically ill patients. Crit Care Med 2002; 30(2): 315-22.
5. Rocha CL, Coburn J, Rucks EA and Olson JC. Characterization of Pseudomonas aeruginosa exoenzyme S as a bifunctional enzyme in J774A.1 macrophages. Infect Immun 2003; 71(9): 5296-305.
6. Maresso AW, Riese MJ, Barbieri JT. Molecular heterogeneity of a type III cytotoxin, Pseudomonas aeruginosa exoenzyme S. Biochemistry 2003; 42(48): 14249-57.
7. Allen L, Dockrell DH, Pattery T, et al. Pyocyanin production by pseudomonas aeruginosa induces neutrophil apoptosis and impairs neutrophil-mediated host defenses In Vivo. J Immunol 2005, 174(6): 3643-3649.
8. Hanna N, Vasquez P, Pham P, et al. Mechanisms underlying reduced apoptosis in neonatal neutrophilsmechanisms underlying reduced apoptosis in neonatal neutrophils. Pediatr Res 2005; 57(1): 56-62.
9. Molloy EJ, O'Neill AJ, Grantham J, et al. Gender-specific alterations in neutrophil apoptosis: The role of estradiol and progesterone. Blood 2003; 102(7): 2653-2659.
10. Ojielo CI, Cooke K, Mancuso P, et al. Defective phagocytosis and clearance of Pseudomonas aeruginosa in the lung following bone marrow transplantation. J Immunol 2003; 171(8): 4416-24.
11. Haslett C. Granulocyte apoptosis and inflammatory disease. Br Med Bull 1997; 53(3): 669-83.
12. Whyte M, Renshaw S, Lawson R, and Bingle C. Apoptosis and the regulation of neutrophil life span. Biochem Soc Trans 1999; 27:802.
13. Peng CT, Lin HC, Lin YJ, et al. Early dexamethasone therapy and blood cell count in preterm infants. Pediatrics 1999; 104(3 Pt 1): 476-81.
14. Mouzinho A, Rosenfeld CR, Sanchez PJ and Risser R. Revised reference ranges for circulating neutrophils in very-low-birth-weight neonates. Pediatrics 1994; 94(1): 76-82.
15. Alaoui-el-Azher M, Jia J, Lian W and Jin S. ExoS of Pseudomonas aeruginosa induces apoptosis through a Fas receptor/caspase 8-independent pathway in HeLa cells. Cell Microbiol 2006; 8(2): 326-38.
16. Oei J, Lui K, Wang H and Henry R. Decreased neutrophil apoptosis in tracheal fluids of preterm infants at risk of chronic lung disease. Arch Dis Child Fetal Neonatal Ed 2003; 88(3): F245-9.
17. van Zandbergen G, Gieffers J, Kothe H, et al. Chlamydia pneumoniae multiply in neutrophil granulocytes and delay their spontaneous apoptosis. J Immunol 2004; 172(3): 1768-1776.
18. Jansson AL, Yasmin L, Warne P, et al. Exoenzyme S of Pseudomonas aeruginosa is not able to induce apoptosis when cells express activated proteins, such as Ras or protein kinase B/Akt. Cell Microbiol 2006; 8(5): 815-22.
19. Kirschnek S, Gulbins E. Phospholipase A2 functions in Pseudomonas aeruginosa-induced apoptosis. Infect Immun 2006; 74(2): 850-60.
20. Uguz A, Coskun M, Yuzbey S, et al. Apoptosis of cord blood neutrophils and their response to colony-stimulating factors. Am J Perinatol 2002; 19(8): 427-434.
21. Leavey PJ, Sellins KS, Thurman G, et al. In vivo treatment with granulocyte colony-stimulating factor results in divergent effects on neutrophil functions measured in vitro .Blood 1998; 92(11): 4366-74
22. Adachi S, Kubota M, Lin YW, et al. In vivo administration of granulocyte colony-stimulating factor promotes neutrophil survival in vitro. Eur J Haematol 1994; 53(3): 129-34
23. Albertine KH, Plopper CG. DNA oxidation or apoptosis: Will the real culprit of DNA damage in hyperoxic lung injury please stand up? Am J Respir Cell Mol Biol 2002; 26(4): 381-383
24. Kotecha S, Mildner RJ, Prince LR, et al. The role of neutrophil apoptosis in the resolution of acute lung injury in newborn infants. Thorax 2003; 58(11): 961-967.
25. Doyle BT, O'Neill AJ, Fitzpatrick JM and Watson RW. Differentiation- induced HL-60 cell apoptosis: A mechanism independent of mitochondrial disruption? Apoptosis 2004; 9(3): 345-52.
26. Jimenez MF, Watson RW, Parodo J, et al. Dysregulated expression of neutrophil apoptosis in the systemic

inflammatory response syndrome (SIRS). Arch Surg 1997; 132(12): 1263-9.
27. Leavey PJ, Sellins KS, Thurman G, et al. In-vivo treatment with granulocyte colony-stimulating factor results in divergent effects on neutrophil functions measured In-vitro. Blood 1998; 92(11): 4366-74.
28. Adachi S, Kubota M, Lin YW, et al. In vivo administration of granulocyte colony stimulating factor promotes neutrophil survival in vitro. Eur J Haematol 1994; 53(3): 129-134.
29. Squier MK, Sehnert AJ, Cohen JJ. Apoptosis in leukocytes. J Leukoc Biol 1995; 57(1): 2-10.
30. Rotstein D, Parodo J, Taneja R and Marshall JC. Phagocytosis of Candida albicans induces apoptosis of human neutrophils. Shock 2000; 14(3): 278-83.
31. Aga E, Katschinski DM, van Zandbergen G, et al. Inhibition of the spontaneous apoptosis of neutrophil granulocytes by the intracellular parasite Leishmania major. J Immunol 2002; 169(2): 898-905.
32. Suttmann H, Lehan N, Bohle A and Brandau S. Stimulation of neutrophil granulocytes with Mycobacterium bovis bacillus Calmette-Guérin induces changes in phenotype and gene expression and inhibits spontaneous apoptosis. Infect Immun 2003; 71(8): 4647-4656.

Please cite this article as: Khazaei S, Karimi A, Fahimzad A, Afjei A, Fallah F, Taheree S, Shamshiri A. Comparison of neutrophil apoptosis by the pseudomonas aeruginosa exotoxins between healthy individuals and term infants. Zahedan J Res Med Sci (ZJRMS) 2013; 15(4): 6-11.