SHORT PAPER

Stable Down-Regulation of HLA Class-I by Serum Starvation in Human PBMCs

Mahsa Rahmani[1], Hamid Reza Khorasani[2], Monireh Golpour[2], Ali Shabestani Monfared[3], Hosein Nattaj[4], Saeeid Abedian[4], Amrollah Mostafazadeh[2*]

[1]Students Research Committee, [2]Cellular and Molecular Biology Research Center, Health Research Institute, [3]Department of Medical Physics, Babol University of Medical Sciences, Babol, [4]Immunology Department, Mazandaran University of Medical Sciences, Sari, Iran

ABSTRACT

Background: The human leukocyte antigen (HLA) matching between organ donor and recipient is an acceptable strategy in clinical transplantation since 1964. However, in bone marrow transplantation, finding matched donors is often problematic. Thus new method for down regulation of HLA can be an alternative strategy to solve this problem. **Objective:** To examine the effect of serum starvation on HLA class I expression in human peripheral blood mononuclear cells (PBMCs). **Methods:** PBMCs were cultured in RPMI-1640 supplemented with 10% FBS (non-starved cells) as well as in medium only (starved cells) for 16, 24, 48, 72, 96h under standard cell culture conditions. The pattern of cell death and HLA class I expression was determined by flowcytometry. Antigenicity of the starved PBMCs was evaluated in a one-way mixed lymphocyte culture by MTT assay. **Results:** Mean fluorescence intensity (MFI) of different indicated starved PBMCs gradually decreased and this reduction was stable after 96h of re-feeding with medium containing FBS. Under serum starvation condition, PBMCs showed apoptotic cell death pattern. There was a linear correlation between percentages of cells, which exhibited the late apoptosis death pattern and serum starvation period ($r=0.88$, $p<0.01$). Surprisingly, the starved PBMCs lost their stimulatory property in mixed culture with allo-reactive lymphocyte. **Conclusions:** Membrane HLA class I expression could be stably reduced in 96h starved human PBMCs culture condition, decreasing their allo-reactivity while their viability rate is enough for possible clinical application.

Rahmani M, et al. Iran J Immunol. 2016; 13(1):54-63.

Keywords: Expression, HLA class I, PBMC, Transplantation

---

*Corresponding author: Dr. Amrollah Mostafazadeh, Cellular and Molecular Biology Research Center, Health Research Institute, Tel: (+) 98 11 32197667, Fax: (+) 98 11 32234650, e-mail: amrolah65@yahoo.com

INTRODUCTION

Lymphocytes may encounter some harsh pathological condition like wound (1,2), pus, atheroma (3) and tumors (4,5), where due to blood circulation shortage which is prevailed in such conditions, the occurrence of hypoxia and growth factors insufficiency are plausible (6). Thus the study of lymphocyte behavior in a growth factor deprived condition can be considered as an experimental model to speculate the behavior of lymphocytes in the above-mentioned pathological situations. The biometrical data originated from such studies would form a reliable basis to design some new drugs to regulate the function of immune cells, which have pivotal roles in harsh conditions. Moreover, there are adequate evidence indicating that some intracellular nucleic acid sensors, such as nucleotide-binding oligomerization domain receptor C5(NLRC5), sense cellular stress which in turn are able to regulate the human leukocyte antigen class I (HLA class I) expression in lymphocytes (7-11). Additionally, Interferones (IFNs), the ubiquitously expressed proteins, are also able to up-regulate these antigens (12,13). We recently showed that under serum free cell culture conditions the human dermal fibroblasts up-regulated the IFN-β and HLA class I molecules at a significant level. HLA class I molecules play some important roles in the immune response against different viruses as well as a variety of cancerous cells (14-16). These molecules also are the major obstacles in different tissue and organs transplantations due to their potent immunogenicity (17). Therefore, the exploration of strategies that can modify HLA class I expression based on the different clinical needs would be helpful in the treatment of autoimmune diseases, cancer and virus derived infectious disease or overcoming the mismatched HLA barrier in clinical transplantation.

Fetal calf/bovine serum (FCS or FBS) as a supplier of different growth factors is a vital component in different mammalian cell culture systems (18). It is well known that in absence of serum (serum starvation) the cells execute apoptotic cell death (19). The lesson we have already learned from starved human fibroblasts. However, our preliminary data on the effect of serum starvation on human peripheral blood mononuclear cells showed that a remarkable proportion of lymphocytes resisted to the serum starvation induced cell death while the expression of HLA class I molecule was stably decreased at a significant level.

MATERIALS AND METHODS

Isolation of Human Peripheral Blood Mononuclear Cells (PBMCs). Ten mL of whole blood was collected in a heparinized tube under sterile condition from each of the six healthy volunteers (three women and three men, age: 20-27). We obtained the written informed consent from blood donor individuals. Ethics committee of Babol University of Medical Sciences approved this research proposal. The blood specimens were diluted with the same volume of Hanks balance salt solution (HBSS) [GE. healthcare, PAA]. PBMCs were isolated by Ficoll-Paque gradient centrifugation method [Amersham, UK] and viability of the cells was determined by trypan blue dye exclusion staining.

Cell Culture and Serum Starvation/re-Feeding Protocols. 8×10^5 of isolated PBMCs were cultured in duplicates in RPMI-1640 [GE. healthcare, PAA] supplemented by 10% FBS [Gibco, Carlsbad, CA] and penicillin (100 units/mL), streptomycin (100 µg/mL),

as non-starved control. The same number of the cells was cultured as duplicates in medium only (starved cells) for 16, 24, 48, 72, 96h at 37°C in humidified, 5% CO2, 95% air atmosphere. The cultivation was performed in 12-wel1 flat-bottomed cell culture plates. After indicated incubation time, cells were counted by a neobar slide and HLA class I expression was measured by flowcytometry. Another series of the above-mentioned starved cells were re-fed with complete medium for four days under standard cell culture conditions in parallel with non-starved cells, then the HLA class I expression was measured by flowcytometric analysis [Partec, Germany].

Annexin V/Propidium Iodide Staining. According to the instruction of Annexin V-FITC Apoptosis Detection Kit [Biovision USA], 2×10^5 of cultured cells were collected and then washed with cold PBS. The cells were re-suspended in 1X binding buffer and subsequently the same amount of Annexin V-FITC and propidium iodide dye were added and the cell contained tubes were incubated for 5 minutes at room temperature. After this time, the viability of the cells was analyzed by flowcytometry [Partec, Germany].

Analysis of HLA Class I Expression. Almost 3×10^5 cells were fixed with 4% paraformaldehyde and were incubated for 15 minute at room temperature. After washing the cells with cold phosphate buffered saline (PBS) containing 10% FBS, the blocking buffer which was included in cold PBS, 10% bovine serum albumin, and 0.3 M Glycine was added and tubes were incubated for 30 minutes at room temperature. Then the cells were stained with mouse anti-HLA class I- FITC conjugated monoclonal antibody [Abcam, ab20313]. After 30 minutes, the expression of these molecules was analyzed by flowcytometry [Partec Germany].

Allogenic Mixed Lymphocyte Culture. To determine the response of allogeneic lymphocyte to starved PBMCs, one way mixed lymphocyte reaction was performed by mixing 10^5 responder cells prepared from two individuals (average age: 24-26) with 5×10^4 starved PBMCs (triplicate wells)as stimulator cells which have already been inactivated by Cobalt-60 Gama radiation (Theratone780, Canada) at a dose rate of 1.54 Gy/min with a source surface distance (SSD) of 80 cm and fixed field size of 10×10 cm^2 at room temperature (3000 rad) according to the method described, previously (21). The same number of lymphocytes (triplicate wells) were cultured in medium only and stimulated with phytohemagglutinin-A (PHA) as background and positive controls respectively, also the irradiated starved and non-starved lymphocytes were stimulated with PHA to check the effect of irradiation on proliferation rate of stimulator cells. All cells were cultured in 200µl RPMI-1640 containing 10% FBS, penicillin (100 units/mL), streptomycin (100 µg/mL) in 96-wel1 flat-bottomed plates for 4 days and then the stimulation indices were measured by MTT assay as already described (Mosmann, 1983). SI (Stimulation Index) was calculated by the following formula:

$$SI = \text{Stimulator OD}/\text{Background OD}$$

Statistical Analysis. Statistical analysis was performed by repeated measures ANOVA, Independent-sample *t*-test and Pearson Bivariate Correlation test using SPSS version 16.0 software. Graphs were drawn by Microsoft Excel and SPSS software. P values less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

We recently observed that serum starvation up-regulates *in-vitro* HLA class I expression in human newborn skin fibroblasts [unpublished data].

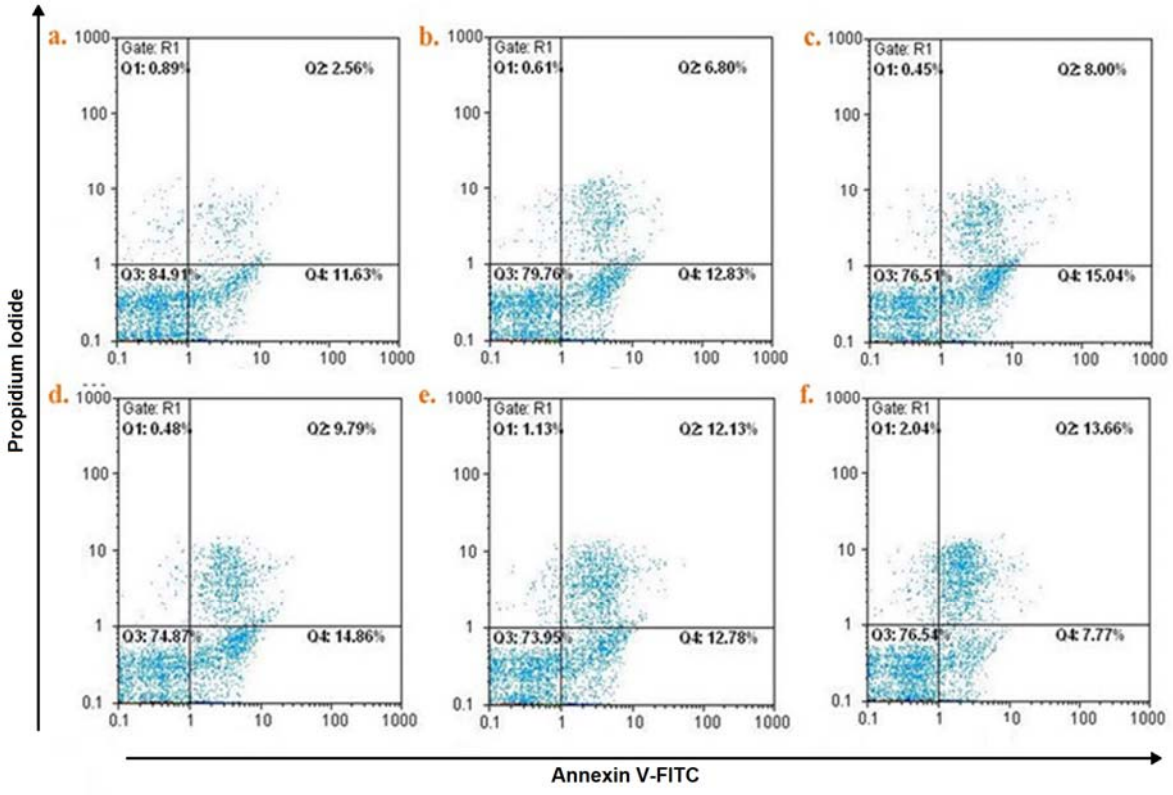

Figure 1. Scatter plots and quadrants of Annexin V/Propidium iodide staining as representative of three independent samples. PBMCs were cultured in RPMI-1640 + FBS 10% for 96h as non-starved control (a) as well as in medium only for 16, 24, 48, 72, 96h (respectively b-f), then cells were stained with Annexin/PI and viability and death pattern were determined with flowcytometry.

Because of a common derived embryonic mesodermal layer and also a close cross-talk between these cells with lymphocyte we decided to investigate the serum starvation effect on *in-vitro* expression of this antigen in lymphocyte.

Surprisingly we obtained an inverse result in comparison with our fibroblast study, i.e. the expression of HLA class-I was down-regulated by serum starvation while a remarkable percentage of lymphocytes were alive (Figure 1). As it can be seen in Figures 2 and 3, the percentage of live cells gradually decreased while the percentage of apoptotic cell increased during serum starvation.

As a representative of the six independent PBMCs specimens, Figure 4 illustrates the MFI histogram of non-starved and 96h starved PBMCs. Mean fluorescence intensity (MFI), as well as the number of HLA class I positive cells of different indicated starved PBMCs gradually decreased (Figure 5).

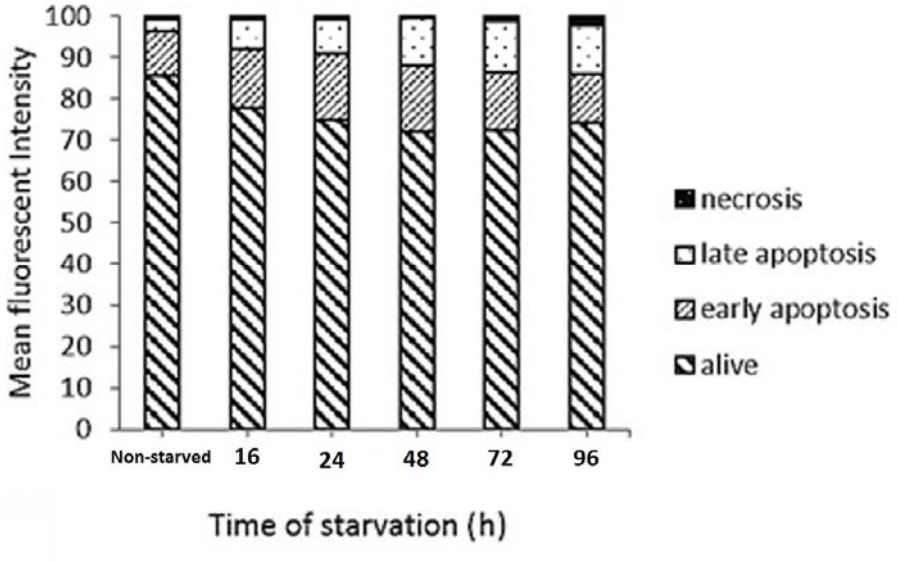

Figure 2. The percentage of Live/dead cells with different death pattern after culturing in indicated period of serum starvation condition. The live cell number significantly decreased after 24h ($p<0.05$). The percentage of the cells with early pattern of apoptosis cell death, reached its maximum level after 48h of culturing under serum starvation condition. However this increasing was not statistically significant when compared with non-starved control. The percentage of the cells which showed the late pattern of apoptosis cell death, gradually increased following the starvation periods and reach its maximum level after 72h which had a significant difference when we compared it with non-starved control ($p<0.05$). The low number of starved cells (1-2%) showed a necrosis pattern of cell death with no significant difference with non-starved control.

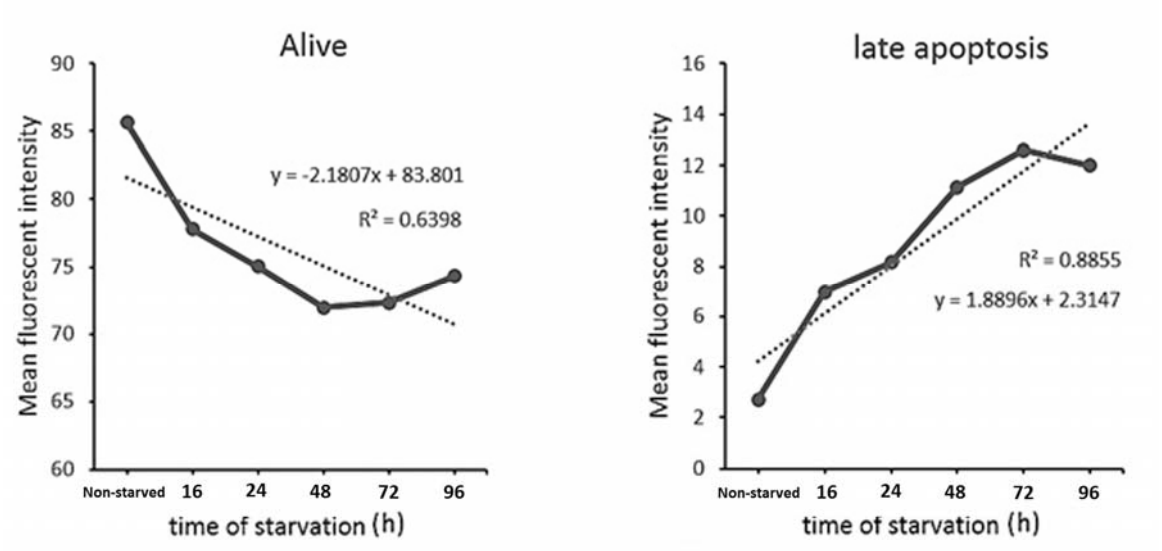

Figure 3. The linear correlation between time of serum starvation and live or late apoptotic cell percentages. There was an inverse correlation between time of serum starvation and live cells percentage and strong positive correlation between time of serum starvation and percentage of late apoptosis cell death ($r=-0.64$, $p<0.05$ and $r=0.88$, $p<0.01$, respectively).

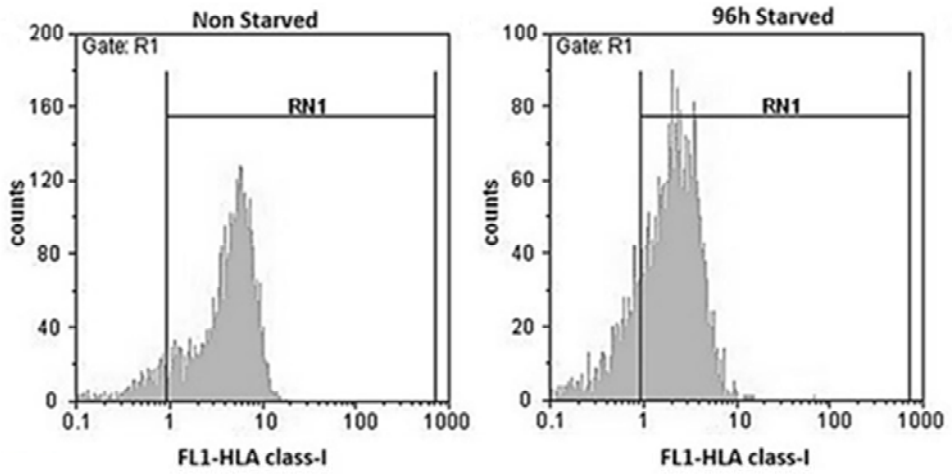

Figure 4. Flowcytometric representative histogram of six samples (two independent experiment, n=3). It shows that serum starvation down regulated the HLA class-I expression in PBMCs.

Even in comparison with non-starved control cells, almost 17% of the 96h starved cells failed to express a detectable level of HLA class 1 molecule on their surface and more than 20% of the PBMCs were negative for this antigen in comparison with fresh blood isolated PBMCs (data not shown). Interestingly, there was a strong inverse correlation (r=-0.91, p=0.009) between time of starvation and HLA class I expression on PBMCs (Figure 5B).

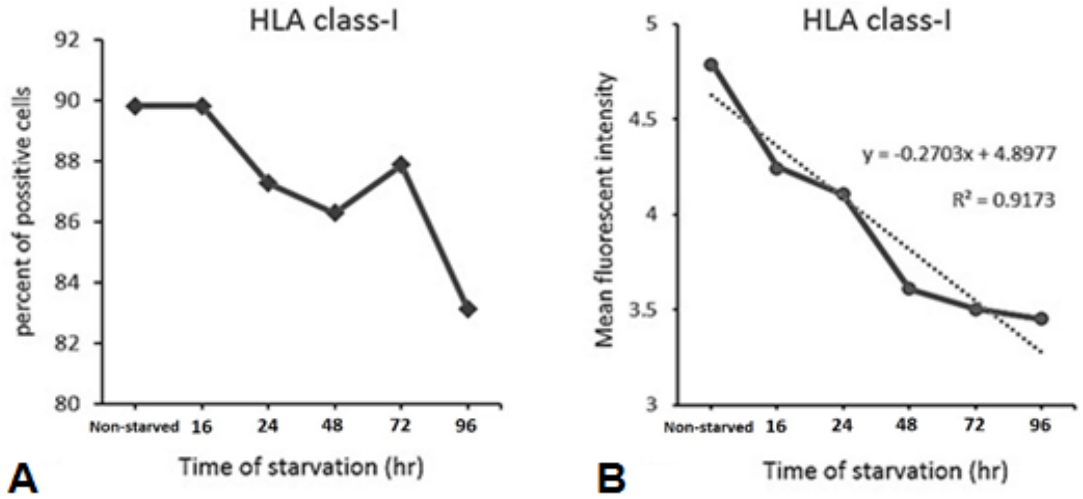

Figure 5. Flowcytometric analysis of HLA class-I surface expression. (A) The percentage of HLA class-I positive cells tended to decrease with serum starvation period and after 96h it reached a significant level statistically in comparison to non-starved control. *p<0.05. (B) There was a strong negative correlation (r=-0.91, p=0.009) between time of starvation and HLA class-I expression on PBMCs surface.

To evaluate the possibility of return to normal expression of HLA class I molecule after growth factors became available again, we continued the starved PBMCs culturing in RPMI-1640 supplemented with FBS 10% for 96h (re-feeding) and analyzed the HLA class I molecule expression by flowcytometry. Interestingly, as it is shown in Figure 6, down-regulation of HLA class I was stable after 96h of re-feeding with FBS and even the expression of this antigen underwent further decrease with the most significant difference in 48h starved PBMCs ($p<0.001$).

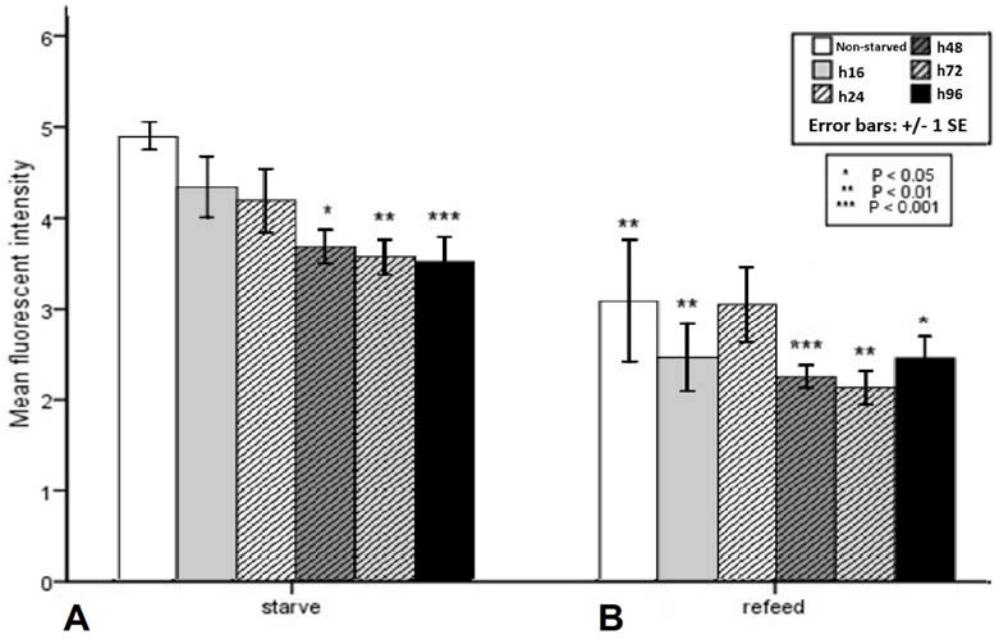

Figure 6. Flowcytometric analysis of HLA class-I surface expression on PBMCs after starvation and re-feeding. (A) This antigen expression significantly diminished in 48h, 72h and 96h starved PBMCs in comparison to non-starved control. (B) 96h after re-feeding, the starved cells were not able to return to normal HLA class-I expression even the trend of decrement in HLA class-I expression continues to further lower levels significantly when we compared them with their related starved cells

To examine the applicability of the culture of immune cells in serum free condition for bone marrow transplantation we did the one way mixed lymphocyte culture method in which different indicated time points of serum starved PBMCs were used as stimulator cells. As it can be seen in Figure 7, all stimulator cells exhibited a stimulation index (SI) lower than 2 while for non-starved PBMCs and PHA (positive control) this value was 2.06 and 2.43, respectively. Interestingly, the SI value for 96h starved PBMCs was 0.98.

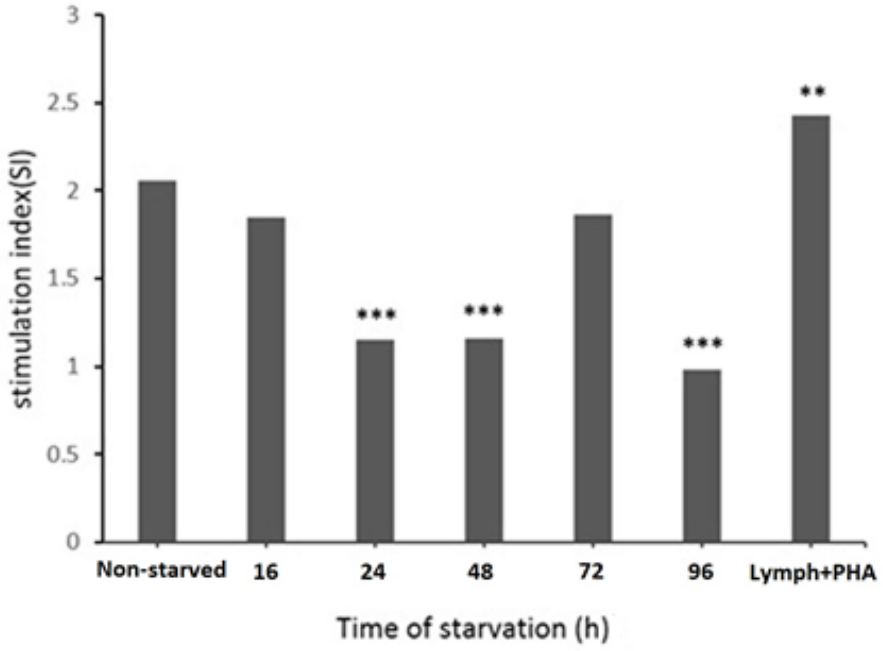

Figure 7. One way mixed lymphocyte culture with starved peripheral blood mononuclear cells (PBMCs). PBMCs were cultured under normal as non-starved control and serum starvation condition for different time points and then these cells were inactivated by gamma radiation with cobalt 60 source for 32 min. The fresh isolated lymphocyte from two volunteers were co-cultured with these cells as well as with phytohemagglutinin-A (PHA) as positive control and without stimulation as background in 96-well cell culture plate for 96h under standard condition for animal cell culture. The stimulation indicia were obtained for each time point and positive control by dividing of OD (optical density) of stimulated samples to their background and compared to non-starved control. The allo-lymphocyte stimulatory activity of PBMCs was significantly diminished in 24h, 48 and 96h starved cells, but not in 16h and 72h starved PBMCs. PHA was able to stimulate the non-starved PBMCs.
** $p<0.01$ and *** $p<0.001$

It means that these cells were not able to stimulate the allo-reactive lymphocytes at all. To our knowledge there are a few previous studies that investigated the effect of serum starvation on lymphocyte viability or on HLA molecule expression. In attempting to optimize the lymphocyte cell culture for karyotyping test, Wolff *et al.* reported that cultured lymphocytes in a medium without fetal calf serum and antibiotic show faster proliferation rate (22). Although it is well known that the HLA class I gene is a housekeeping gene and does not need any inducer for its expression at normal level, some tumor cells as well as some virus infected cells are able to decrease HLA class I expression on their surface. Here we can add a new condition; the serum starvation to do so but we need perform further studies to reveal that this process being regulated at the level of transcription, translation or translocation onto the cell surface.

Apart from the underlying mechanisms, which resulted in down-regulation of HLA class I molecules, we assumed that these results could be applied to HLA-tailoring in bone marrow transplantation. Therefore, we conducted the mixed lymphocyte culture in which starved allo-lymphocytes were used as stimulator cells. It is well known that 1-10% of our lymphocytes are allo-reactive cells (23). It means that they respond to other individuals HLA molecule directly. Thus MLC can be considered as an *in vitro* model

of allo-transplantation. Since we observed that the starved lymphocytes lost their stimulatory property during starvation period while a remarkable proportion of them remained alive this idea seems to be a legitimate theme. However, appropriate animal model is needed to examine this idea *in vivo*. This strategy can be effective to solve the problem, which exists in finding the HLA-matched people for bone marrow transplantation in clinical practice.

Taken together this preliminary study showed that serum starvation circumstances might mimic some real condition in which lymphocyte must exert their effects in harsh environment such as wound, tumor, atheroma and necrotic tissues. In these conditions lymphocytes maintain to be alive and down regulate the expression of HLA class I molecule on their surface. These finding can be considered as a base for further studies in HLA class I tailoring for lymphoid tissue transplantation in clinical practice as well as a new window in fueling immunity (24).

ACKNOWLEDGEMENTS

We would like to thank the Students Research Center, Babol University of Medical Sciences for financial support to conduct this research project and Narges Amirjamshidi for grammatically and linguistically support.

REFERENCES

1. Eming SA, Krieg T, Davidson JM. Inflammation in wound repair: molecular and cellular mechanisms. J Invest Dermatol. 2007; 127:514-25.
2. Schaffer M, Barbul A. Lymphocyte function in wound healing and following injury. Br J Surg. 1998; 85:444-60.
3. Mach F, Sauty A, Iarossi AS et al. Differential expression of three T lymphocyte-activating CXC chemokines by human atheroma-associated cells. J Clin Invest. 1999; 104:1041-50.
4. Harris AL. Hypoxia--a key regulatory factor in tumour growth. Nat Rev Cancer. 2002; 2:38-47.
5. Holmes EC. Immunology of tumor infiltrating lymphocytes. Ann Surg. 1985; 201:158-63.
6. Caldwell CC, Kojima H, Lukashev D et al. Differential effects of physiologically relevant hypoxic conditions on T lymphocyte development and effector functions. J Immunol. 2001; 167:6140-9.
7. Kobayashi KS. NLRC5/CITA: a novel regulator of class I major histocompatibility complex genes. J immunodefic Disord. 2012; 1:1000e102.
8. Kobayashi KS, van den Elsen PJ. NLRC5: a key regulator of MHC class I-dependent immune responses. Nat Rev Immunol. 2012; 12:813-20.
9. Meissner TB, Li A, Biswas A et al.NLR family member NLRC5 is a transcriptional regulator of MHC class I genes. Proc Natl Acad Sci U S A. 2010; 107:13794-9.
10. Neerincx A, Castro W, Guarda G, Kufer TA. NLRC5, at the Heart of Antigen Presentation. Front Immunol. 2013; 4:397.
11. Neerincx A, Rodriguez GM, Steimle V, Kufer TA. NLRC5 controls basal MHC class I gene expression in an MHC enhanceosome-dependent manner. J Immunol. 2012; 188:4940-50.
12. Zanon RG, Cartarozzi LP, Victorio SC et al. Interferon (IFN) beta treatment induces major histocompatibility complex (MHC) class I expression in the spinal cord and enhances axonal growth and motor function recovery following sciatic nerve crush in mice. Neuropathol Appl Neurobiol. 2010; 36:515-34.
13. Zhou F. Molecular mechanisms of IFN-gamma to up-regulate MHC class I antigen processing and presentation. Int Rev Immunol. 2009; 28:239-60.
14. Bukur J, Jasinski S, Seliger B. The role of classical and non-classical HLA class I antigens in human tumors. Semin Cancer Biol. 2012; 22:350-8.

15. Seliger B. Novel insights into the molecular mechanisms of HLA class I abnormalities. Cancer Immunol Immunother. 2012; 61:249-54.
16. Seliger B, Ritz U, Ferrone S. Molecular mechanisms of HLA class I antigen abnormalities following viral infection and transformation. Int J Cancer. 2006; 118:129-38.
17. Thorsby E. A short history of HLA. Tissue Antigens. 2009; 74:101-16.
18. Brunner D, Frank J, Appl H, Schoffl H, Pfaller W, Gstraunthaler G. Serum-free cell culture: the serum-free media interactive online database. ALTEX. 2010; 27:53-62.
19. Schamberger CJ, Gerner C, Cerni C. Caspase-9 plays a marginal role in serum starvation-induced apoptosis. Exp Cell Res. 2005; 302:115-28.
20. Golpour M, Fattahi S, Niaki HA et al. Starved human fibroblasts secrete acidic proteins inducing post re-feeding proliferation and in vitro cell migration: a potential tool for wound healing. Biol Cell. 2014; 106:139-50.
21. Shirazi A, Khadem Shariat H, Mahdavi SR, Samiei F, Hajati J, Alimoghadam K. Evaluation and Measurrment of Cobalt-60 Gamma-ray for the Blood Lymphocytes Inactivation. Journal of Iran University of Medical Science. 2003; 10:397-406.
22. Wolff S, Afzal V, Lindquist PB. Cultured human lymphocytes proliferate faster in medium lacking fetal calf serum and antibiotics. Mutat Res. 1984; 129:207-13.
23. Smith C, Miles JJ, Khanna R. Advances in direct T-cell alloreactivity: function, avidity, biophysics and structure. Am J Transplant. 2012; 12:15-26.
24. Pearce EL1, Poffenberger MC, Chang CH, Jones RG. Fueling immunity: insights into metabolism and lymphocyte function. Science. 2013; 342:1242454.