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# Analysis of Hyperthermia Effect on Peripheral Blood Natural Killer Cell Cytotoxicity against SW-872 Cell Line

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## Abstract

**Background:** Natural killer (NK) cells are a type of cytotoxic lymphocyte. It is revealed that hyperthermia which is used in cancer treatment, increases natural killer cell activity. In this study, our aim was to analyze effects of in-vitro hyperthermia on human Natural Killer cytotoxicity against SW-872 cell line.**Materials and Methods:** In this study, we used SW-872 liposarcoma cell line as a target cell. Peripheral blood NK cells were isolated from normal individuals by MACS. NK cells were treated at 39°C for 1hr .The expression of CD69 on the surface of NK cells was examined by flow cytometry at different time points including 0, 6 and 12 hrs. NK cell cytotoxicity was measured by LDH assay 12hrs after co-culture with SW-872. The results were compared to the conditions that both NK cells and SW-872 cells were heat treated at 39°C for 12hrs. **Results:** Our results revealed that cell killing activity of NK cells treated with heating alone was increased 6hrs after heat treatment at 39°C compared with heat-treated NK-target cells co-culture.While in heat-treated NK-target cells co-culture the maximum cytotoxicity was observed 12hrs after heat treatment at 39°C. **Conclusion:** These results showed that thermotherapy could be a feasible method to stimulate immune response against tumor cells. **[GMJ. 2015;4(3):75-81]**

**Keywords:** ; NK Cell; Immunologic Cytotoxicity; Liposarcoma; SW-872

## Introduction

Hyperthermia is a type of cancer treatment in which body tissue is exposed to high temperatures (up to 113°F) to damage and kill cancer cells. Reports have shown the positive effect of hyperthermia in combination with other cancer treatments such as radiation therapy, surgery and chemotherapy. Many studies have been performed on the effect of local and whole body hyperthermia on immune cells activity such as natural killer cells (NK cells)[1]. NK cells are a group of lymphocytes and constitute about 5 to 15 percent of peripheral blood lymphocytes [2, 3] . These cells are rapid respondent to kill virus-infected cells [4, 5]. The function of NK cells is regulated by a balance between inhibitory and stimulatory receptor signal transmission. In normal mode, MHC class I molecules are expressed on the surface of body cells but in tumoral or viral conditions is reduced on the cell surface. Instead, most of the molecules including ULBP, MIC-B and MIC-A are expressed. MHC class I molecules (HLA- A, B, C and G) are as a ligand for inhibitory receptors on

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NK cells. MIC or ULBP are known as a ligand for stimulatory receptors of NK cells. Tumor cells have small number of MHC class I. When NK cells recognize tumor cells, inhibitory receptors are not activated. Because increasing MIC and ULBP molecules on the surface of tumor cells lead to binding activating receptors to these molecules and NK cells are activated. NK cells apoptosis of target cell through the release of perforin, granzyme and FasL expression [4, 6, 7] .

Liposarcoma is one of the malignant soft tissue cancers. It grows in the soft tissue gently and cancer occurs at elder ages specifically more than 40. Nowadays, the only treatment is surgery. Due to problems after surgery, other methods including radiotherapy and thermotherapy are required along with surgery [8,9].

The aim of this study is to investigate the killing ability of NK cells after hyperthermia .NK cells are heated for one hour at temperatures 39°C and 42 °C. The cytotoxicity of these cells in the co-culture with liposarcoma cells line (SW-872) after 0, 6 and 12 hours was evaluated with LHD-release assay kit. LHD is a stable cytoplasmic enzyme that is present in all cells and is released from cytoplasmic membrane damaged in cell culture [10]. CD69 expression on NK cells was determined by flow cytometry as a marker of activation.

## Materials and Methods

*Tumor Cell Lines*

Human liposarcoma cell line SW-872 was purchased from cell bank Pasteur Institute of Iran and was cultured in RPMI 1640 medium (Gibco, Karlsruhe, Germany) supplemented with 10% FBS (Biosera, Brighton, UK), penicillin (100 units/ml) and streptomycin (100µg/mL) in a humidified incubator with 5% CO2 at 37°C and 2mM L-glutamine. After 5 passages, cells reached logarithmic phase and harvested by trypsin.

*Health Donor Selection and Bleeding*

NK cells were isolated from healthy donors and the history was taken from donors because NK cells are activated in infectious viral diseases. 130ml of blood sample was taken in Heparinized syringes and diluted with 80ml RPMI 1640.

*NK Cell Isolation*

Diluted blood was added to 6 tubes slowly. Each of them contained 15ml ficoll. To reach a volume of 50ml, centrifuge 25min, 2000 RPM and after several time centrifuge are separated from PBMC. NK cells are selected through negative selection in MACS. In this case, the isolation of CD56+CD16+ NK cells is performed in a two-step procedure. First, non-NK cells and CD56+CD16+ NK cells are labeled with a cocktail of biotin-conjugated antibodies against lineage-specific antigens and a cocktail of MicroBeads. Upon subsequent magnetic separation of the cells over a MACS column which is placed in a magnetic field of a MACS Separator, the magnetically labeled cells are retained within the column, while the unlabeled cells run through. In the second step, the target cells are labeled with CD56 MicroBeads and isolated by positive selection from pre-enriched cell fraction using a second column. After determination of cell number, the centrifuges of cell suspension at 300x g for 10 minutes, suspended cell pellet in 40µl of buffer per 107 cells. Add 10µl of NK cell biotin-antibody cocktail per 107 cells. Mix well and keep cold for 10 minutes then add 30 µl of buffer and 20 µl of NK cell microbead cocktail per 107 cells. Mix well and keep cold for 15 minutes. (MACS system, Miltenyi Biotech, Bergisch Gladbach, Germany).

*Determination of Appropriate Concentration and Ratio of Cells for LDH Cytotoxicity Assay*

Cell ranges from 5000 to 50,000 per chamber were chosen. The maximum amount of LDH released was considered as a high control in the presence of Triton and as low control in the presence of medium alone. The maximum difference between OD high control and low control was considered as the optimal cell for determination of the appropriate ratio in target cells. Rates of 10:1, 5:1, 2.5:1 and 1.25:1 were considered for determination of the appropriate concentration in target cell i.e. NK cells.

*Heat Treatment*

After target cells reach logarithmic phase, the

cells attached to the bottom chamber after 8hr incubation at 37°C plates were incubated for one hour at a temperature of CO2 between 39 and 42°C with 95% humidity for times 0, 6 and 12 hours after heat .We considered four plate tests for each of temperatures of 39°C and 42°C. We heat NK cells at 39 and 42°C for one hour. Plates were transferred to the incubator at 37°C and co-culture.

*LDH Cytotoxicity Assay*

Natural killer cell cytotoxicity activity against cell lines is measured by lactate dehydrogenase (LDH) cytotoxicity detection kit (Roche Applied Science Bavaria, Germany). This method is based on a colorimetric assay to quantify cell death based on LDH released from damaged cells. Target cell lines were cultured at 104 per well for 8 hrs. Then, plates were subject to heat treatment and 105 heat-treated NK cells in 100µL of complete medium containing 5% fetal bovine serum was added to plates and incubated at 37°C for 12hrs. Also effector: target mix was subject to heat treatment. Cell culture plates were centrifuged at 250g 10 minutes. Cell-free culture supernatant was collected. The amount of LDH released from cells was measured by enzymatic reaction. Tests were performed in triplicate. The percentage of cytotoxicity was calculated based on the formula in kit.

*Flowcytometry*

The purity of isolated NK cells followed by PE-Conjugated anti-human CD56/CD16 by flow cytometry was achieved at two separated times immediately. To determine the surface expression of CD69 on NK cells, the following monoclonal antibodiy (mAb) was used: FITC Conjugated anti human CD69 (BD Pharmingen™). Cells were incubated with anti-human CD69 and Isotype Control 30min. Data analysis was performed with software Flowjo.

*Statistical Analysis*

To compare NK cell cytotoxicity results, GraphPad Prism software and ANOVA test were performed. $P<0.05$ was considered significant in all experiments.

**Results**

*Flow Cytometry for the Purity of Isolated NK Cells*

NK cells were isolated and purity was followed by PE-Conjugated anti-human CD56/CD16 by means of flow cytometry (Figure1).

*The Effect of Hyperthermia on Expression of CD69 Surface Molecules on NK Cells*

Heat-treated NK cells at 39°C for 1hr and incubated at 37°C at time points 0, 6 and 12 hrs. After heat treatment, expression of CD69 molecule was analyzed by flow cytometry on the surface of these cells. Expression of CD69 increased along with heat treatment at 39°C. After 0, 6 and 12 hours, the rates of expression were 5/46 percent, 13/2 percent and 22/0 percent, respectively (Figures 2, 3).

*Determination of Appropriate Concentration of Test Cells for LDH Cytotoxicity Assay*

For determination of optimal concentration of cells in each chamber and determination of the greatest amount of released LDH from target cells, the range of cells from 5000 to 50,000 was chosen per chamber. The maximum amount of LDH was considered as a high control in the presence of triton and as a low control in the presence of medium alone. The maximum difference between OD high

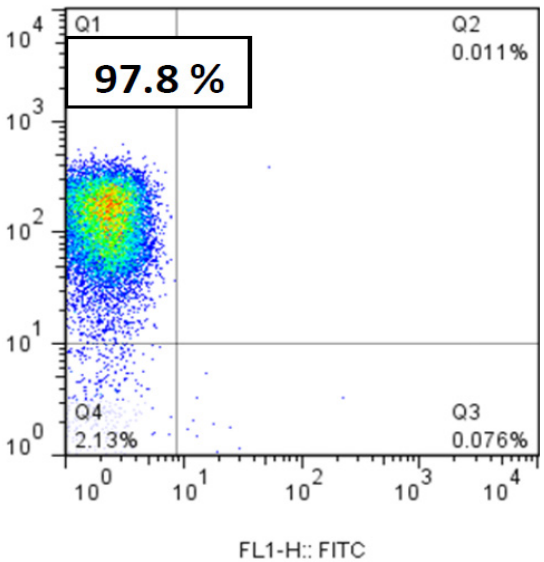


**Figure 1.** Flow cytometric analysis of human NK cell purity after isolation by MACS from fresh peripheral blood lymphocytes

control and low control is considered as the optimal cell for determination of the appropriate ratio target cells. 10000 SW-872 cells were chosen for cytotoxicity assay (Table 1).

*Determination of Appropriate Ratio of NK Cells to Target Cells*

For determination of optimal rate of NK cells to target cells, we co-cultured various rates of

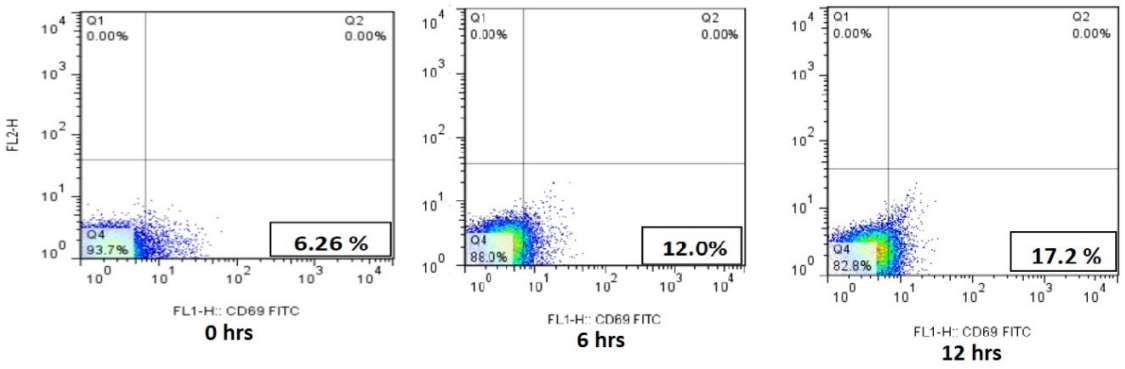


**Figure 2.** Analysis of Expression of CD69 on the surface of NK cells in 37 ° C after 0,6 and 12 hours by flow cytometry

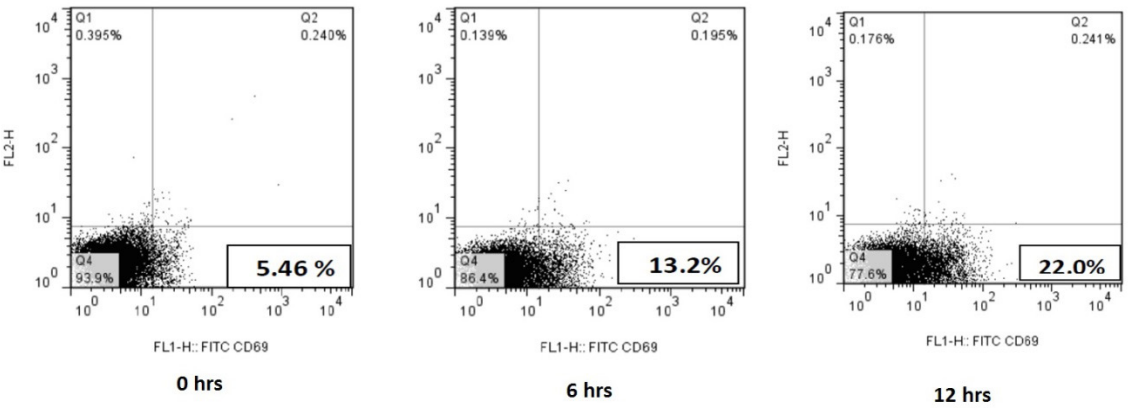


**Figure 3.** Analysis of Expression of CD69 on the surface of NK cells after temperature 39 ° C after 0,6 and 12 hours by flow cytometry

**Table 1.** Determination of the optimum concentration SW-872 cell line for LDH cytotoxicity assay

| Sw-872 cell/well | Low control | High control | High-low |
|---|---|---|---|
| 50000 | 0.842 | over | - |
| 20000 | 0.585 | over | - |
| 10000 | 0.257 | 2.136 | 1.871 |
| 8000 | 0.177 | 1.96 | 1.783 |
| 7000 | 0.145 | 1.739 | 1.594 |
| 5000 | 0.912 | 1.118 | 0.206 |

NK cells: target cells which were assessed by LDH assay. Ratio of 10:1 was chosen as the optimum rate (Figure 4).

*Effect of Hyperthermia on Peripheral Blood NK Cell Killing Activity against Target Cell Lines SW-872 Heat Treated Using LDH Cytotoxicity Assay*

NK cells in combination with SW-872 cell lines were heat-treated and incubated for 12 hours and mortality was assessed by LDH assay. Results showed that the cell killing activity of NK cells treated with heating alone 6hrs after heat treatment at 39°C was increased ($P<0.05$). Moreover, heat-treated NK-target cells at a temperature of 39°C shown 12hrs after heat treatment at 39°C compared with 37°C maximum LDH released was seen (Figure 5) .

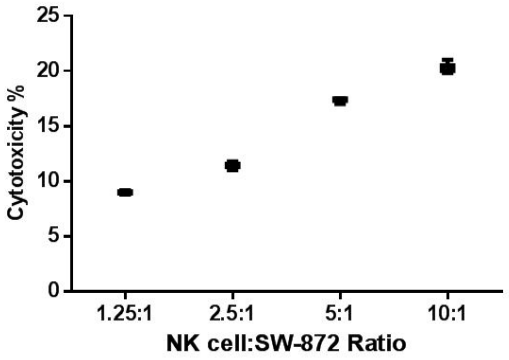


**Figure 4.** Determination of the appropriate ratio of NK cells to target cells. LDH Cytotoxicity Assay. This assay was used to compare different ratio

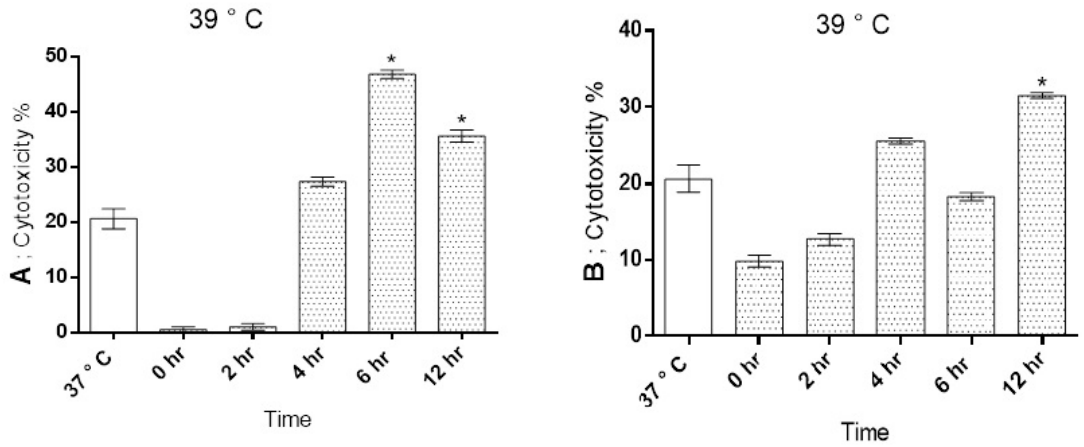


**Figure 5.** Study of NK cell cytotoxicity against target cell following thermal treatment 39 ° C NK cells treated with heating alone (A) and heat treated in combination with SW-872 cells (B).

## Discussion

Many studies have been conducted on the effects of hyperthermia on immune system, but molecular mechanisms of temperature are unknown. NK cells of the immune cells are activated by physiological stress, oxidative agents, fever and temperature. NK cells are the most effective cells against cancer cells. Many studies have shown that hyperthermia increases the activity of the immune system and that heat shock increases tumor antigenicity by enrichment of NKG2D ligands on tumor cells [11-13]. Although other studies reported diminished NK cell cytotoxicity after hyperthermia [14-16], in a study conducted by Burd R in 1998 the mice were transplanted by human breast tumor cells and treated at 39°C and 40°C. They have shown tumor growth had been suppressed at 37.5°C compared with controls [17]. Another study showed that cytotoxic activity of NK cells had increased in mice with erythroleukemia under whole-body hyperthermia 40°C and 39.5°C for a

half hour. Response rate in combination with hyperthermia and radiotherapy of tumors was 89% while it was 27% in the patients who had received only radiotherapy [18] . Also in 1994, a study on 41 patients with head and neck tumors showed the combination of hyperthermia and radiotherapy. It will lead to increased recovery of 41% to 83% [1] . In contrast with these results, some others have shown declining killing activity of NK cells to hyperthermia. In 1996 Singh.V showed reducing cytotoxic activity of NK cells at high temperature of 38.5°C [19] . Azocar J. et.al (1982) showed that heat-treated NK cells and K-562 target cells decreased cytotoxic activity of these cells at temperatures 38.5°C and 40°C [14] . In 1983, it was demonstrated that NK cells isolated from the blood of healthy people were extremely sensitive to heat. Cr-51 release test results showed a reduction in NK cell function at 42°C in comparison with 37°C [20]. NK and LAK cells cytotoxicity was evaluated by Fuggetta M. He showed decreased cytotoxicity in LAK cells receiving temperature 42ºC for one hour [12] .

To evaluate the cytotoxic activity, NK cells were treated with heating alone and co-cultured with SW-872 cells. Also, NK cells and target cells treated with heat were co-cultures. Lactate dehydrogenase enzyme (LDH) released from damaged membranes of target cells, is related to the final stages of apoptosis .The results of heat treatment in comparison with control at 37°C showed NK cells which were treated with heating alone at 39°C, increase killing activity ($P<0.05$) 6hrs after heat treatment significantly. After NK cells target cells combination heat treated at 39°C, the highest fatality rate cytotoxicity was at 12 hours after thermal treatment than 0, 4 and 6 hours ($p<0.05$). Based on these results, SW-872 cells with heating alone and then cultured with NK cells did not increase cell killing activity. Interaction between two cells in co-culture, expression of some receptors on the cell surface and cytokine secretion will affect the killing activity of NK cells. 12 hours after thermal treatment, an increase of maximum cytotoxicity activity was observed ($p<0.05$). The cause of difference between previous results and our results is related to the temperature and duration of heat treatment and tumor cell origin. The primary resistance of tumor cells to NK cells and CTL is because of the decrease in expression of most genes activating immune system at the early hours after hyperthermia [21, 22]. Interaction between tumor cells and NK cells, their mutual impact and temporary resistance are proved in this study.

## Conclusion

This study demonstrated hyperthermia would increase cell activity in vitro. To accomplish better results, experiments should be performed on laboratory animals.

## Acknowledgements

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## Conflicts of Interest

Authors have no financial conflict of interest.

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