Lycopene Prolongs the Lifespan and Enhances the Cytotoxicity of NK Cells after Ex Vivo Expansion

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Abstract — Lycopene is a nonprovitamin A carotenoid mainly found in fruits and vegetables, which has been reported to possess a variety of biological effects. The properties of lycopene on human natural killer (NK) cells after ex vivo expansion were assessed in the present study. Results showed that lycopene has a positive effect on NK cells viability and cytotoxicity. Aging and apoptosis started from the fourth week onwards in the cultured NK cells which were obtained from the peripheral blood mononuclear cells (PBMC). Supplemented with lycopene (5μM) can restore the decreased viability and cytotoxicity of NK cells and reduce NK cells apoptosis caused by aging during fourth-sixth week culture. Its anti-apoptosis effect in NK cells may be related to lycopene which can decrease the expression of caspase 3 and 9 genes. Furthermore, lycopene can enhance the IFN-γ expression in gene and protein level after 7d treatment. However, lycopene did not affect the functional receptor's (NKG2A, NKG2D, NKp30 and NKp44) expression on NK cells. These results indicated that lycopene has a positive effect on NK cells. As a health product, it may help to prolong the lifespan and enhance the cytotoxicity of NK cells after ex vivo expansion.

Key Words: Lycopene; Human natural killer (NK) cells; Apoptosis; Cytotoxicity; Caspase.

I. Introduction

As a well-known red carotenoid component of tomato, lycopene has been drawing a lot of attention because of its potential biological functions. Lycopene is recognized as the best player on antioxidant effect among carotenoids [1], its antioxidant effect is two times higher than carotene and ten times higher than tocopherol [2]. Lycopene has more potent proliferation inhibition of human cancer cells than either α-carotene or β-carotene [3]. Studies reported lycopene has ability to induce cell-cycle arrest and apoptosis in many human cancer cell lines, such as breast cancer cells [4, 5], prostate cancer cells [6], Hep3B human hepatoma cells (7), T-84, HT-29, MCF-7 and DU145 [8]. The anti-tumor effects of lycopene may be related to modulate intracellular reactive oxygen (ROS)-mediated cell growth [9]; modulate cell cycle proteins such as β-tubulin, CK8/18, CK19 and heat shock proteins [10] or affect the PI3K/Akt signaling pathway [11].

Lycopene also shows multi-immunomodulatory activity. Kobayashi *et al*. has reported that lycopene can recover the intrathymic T cell differentiation in tumorous mice than control [12]. Watzl B *et al*. assessed the effect of low-carotenoid diet supplemented with tomato (providing high amounts of lycopene) on immune functions in healthy men. Their study revealed that juice intervention has the influence on lymphocyte proliferation, NK cell cytotoxicity and IL-2 secretion by a time-delayed modulation in healthy men [13].

Natural killer (NK) cells are large granular lymphocyte which is defined by presence of CD56 and absence of CD3 on the cell surface ($CD56^{+}CD3^{-}$) [14]. NK cells comprise 5%-10% of all peripheral blood lymphocytes [15], can lyse virally infected cells or oncogenically transformed cells and secrete many kinds of cytokines without requiring prior immunization. NK cells exert cytotoxicity on oncogenically transformed cells specifically and efficiently, which make them play an important role in tumor identification and surveillance.

B-carotene is a cognate material of lycopene; many studies have confirmed the positive effect of β-carotene on NK cells. Watson *et al* reported the number of NK cells increased substantially in peripheral blood mononuclear cells (PBMC) from individuals supplemented with β-carotene [16]. Enhanced NK cell cytotoxicity was also observed in human subjects given oral β-carotene reported by Prabhala *et al* [17]. Chew and Park reported that non-provitamin A carotenoids such as lycopene, were more active than β-carotene in enhancing cell-mediated and humoral immune response in animals and humans [18]. However, a few studies have been down about lycopene effect on human NK cells.

In the present study, the viability, apoptosis, cytotoxicity, receptor expression and cytokines secretion of primary human NK cells treated by lycopene after *ex vivo* expansion were delineated to assess if there were positive effect of this kind of antioxidant on NK cells lifespan, activity and function.

II. Materials and Methods

Cells

Primary human NK cells were prepared according the methods described in Huang *et al* [19]. Briefly, peripheral venous blood (10mL) from healthy donors (n=10) was collected in heparinized tubes. Blood sample collecting procedure was conforming to the informed consent guidelines of the Ethic Committees of Northwestern Polytechnical University. Blood samples were diluted 2-fold by PBS (sterile,

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1×). PBMCs were collected by using Lymphocyte Separation Liquid (Haoyang TBD, Tianjin, China) according the instructions and washed twice with PBS(sterile, 1×), then re-suspension in RPMI-1640 media (Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), 100U/mL IL-2 (Peprotech, Rocky Hill, NJ, USA), 100μg/mL of penicillin and streptomycin (Genview, Carlsbad, CA, USA). After counted carefully, PBMCs were co-cultured with equal numbers of stimulating cells (genetically modified K562 cells, prepared according to the literature "20" and irradiated).

After 21days (3 weeks) of *ex vivo* expansion, 1×10^5 expanded cells were collected and washed 2 times with PBS (sterile, 1×). Flow cytometry (BD FACS Calibur, San Jose, CA, USA) was used to determine the percentages of NK cells ($CD56^+CD3^-$) in PBMC by staining with the CD56-PE and CD3-FITC monoclonal antibodies (mAbs) and its isotype-matched controls (IgG1-FITC/IgG2-PE) (QuantoBio, Beijing. China).

K562 cells (purchased from American Type Culture Collection, ATCC, Manassas, VA) and stimulating cells were cultured in RPMI-1640 cell media supplemented with 10% FBS, 100μg/mL of penicillin and streptomycin and routinely cultured at 37 °C in 5% CO_2 incubator.

Lycopene treatment

Lycopene was prepared to 100×storage solution (0.5mM) in DMSO. NK cells (5×10^5 each) were suspended in 990μL of RPMI-1640 and cultured in 24-well plates (Costar, New York, USA) at 37 °C in a 5% CO_2 incubator with 10μL of the 100× lycopene storage solutions for 1-3 weeks. In this assays, the working concentrations of lycopene was 4μM. Control group contain 10μL DMSO to exclude its effect on cells.

Cell viability and apoptosis

After lycopene treatment in different time interval, NK cells in each group were centrifuged, washed with PBS, and re-suspended in 600μL of RPMI-1640 media. 20μL of CCK-8 (Cell Counting Kit-8, Dojindo, Japan) was added to a 200μL cell suspension per well in a 96-well plate in triplicate for each group. The cells were then incubated for 2h at 37 °C in a 5% CO_2 incubator. The absorbance (*A*) values of each well was recorded at 450 nm in microplate reader (BioTek Synergy4, USA).

The apoptosis of NK cells was assessed by AnnexinV-FITC/PI method (Apoptosis Detection Kit, Beyotime Institute of Biotechnology, Haimen, China). After treating with lycopene in different time interval, NK cells in each group was centrifuged and washed 3 times with PBS, stained with AnnexinV-FITC and PI following the instructions and analyzed by flow cytometry.

Cytotoxicity

The cytotoxicity of NK cells were carried out according the procedure described in literature [21]. Briefly, after lycopene treatment, NK cells (8×10^5) were centrifuged and washed 3 times with PBS, then re-suspended in 400μL of RPMI-1640. 100μL of NK cells (2×10^5) were plated into each well of a 96-well plate with 100μL of K562 cells (4×10^4) in triplicate. The effector control wells contained 100μL of NK cells (2×10^5 cells) and 100μL of medium. The target control wells contained 100μL of K562 cells (4×10^4 cells) and 100μL of medium, to make the effector-to-target ratio (E:T) 5:1. Plate was incubated at 37 °C in a 5% CO_2 incubator. After 4h co-culture, 20μL of CCK-8 was added to each well and the plate was then incubated for another 2h.Recorded the*A* values at 450nm. Cytotoxicity was determined by evaluating the rate of NK cells killed K562 cells, the killing rate was calculated as following equation [22]:

Killing rate (%) = $[1-(A_{e+t}-A_e)/A_t] \times 100\%$

(*Ae*, average A_{450} of triplicate wells for NK cell control; A_t, average A_{450} of triplicate wells for K562 cells control; A_{e+t}, average A_{450} of triplicate wells for NK cells plus K562 cells).

mRNA expression analysis

After lycopene treatment, TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to isolate total RNA from NK cells (1×10^7). The mRNA levels of apoptosis related gene (FasL, caspase3, 6, 7, 8, 9, and 10), cytokines (IFN-γ, perforin, and Granzyme-B), and important functional receptors of NK cells (NKG2D, NKG2A, NKp30 and NKp44) were evaluated by relative quantification PCR (RT-qPCR) and SYBR Green random mixing method. Glyceraldehyde-3-phosphate dehydrogenase (GADPH) was used as the internal control. The $2^{-\Delta\Delta Ct}$ method was used to calculate relative changes in gene expression [23]. All primers used here were shown in Table 1. RT-qPCR was performed according to the instructions of the TransStart Top Green qPCR SuperMix kit (TransGenBiotech, Beijing, China). The reaction mixtures were incubated for 30 min at 48°C, followed by 40 cycles of PCR at 94°C for 5 s, 55°C for 15 s, and 72°C for 10 s. At the end of 40 cycles, a melting curve analysis was performed to confirm the presence of only a single amplified product of the expected size.

TABLE I. PRIMER PAIRS USED FOR RT-QPCR ANALYSIS

Primer	Sequence (5'-3')	size(bp)
IFN-γ	F: TGCAGGTCATTCAGATGTAGC R: GGACATTCAAGTCAGTTACCG	248
Perforin	F: GGGACAATAACAACCCCATCT R:GGAATTTTAGGTGGCCATGAT	174
Granzyme-B	F:AGATCGAAAGTGCGAATCTGA R: TTCGTCCATAGGAGACAATGC	138
NKG2A	F: TCATTGTGGCCATTGTCCTGAGG R: AGCACTGCACAGTTAAGTTCAGC	291
NKG2D	F: ATCGCTGTAGCCATGGGAATCCG R: AGACATACAAGAGACCTGGCTCTC	213
NKp30	F: TGAGATTCGTACCCTGGAAGG R: CACTCTGCACACGTAGATGCT	235
NKp44	F: TCCAAGGCTCAGGTACTTCAA R: GATTGTGAATCGAGAGGTCCA	163
FasL	F:5'AGTATACTTCCGGGGTCAATCT3' R: 5'CCAGAGAGAGCTCAGATACGTT3'	218
Caspase-3	F:5'CATGTATTGTTTCCATGCTCAC3' R:5' GTGGTTCTATTTGATGGAAGGA3'	192
Caspase-6	F:5'TCAAAGGAGACAAGTGTCACAG3' R:5'GCAACAGAGTAACACATGAGGA3'	211
Caspase-7	F:5'ATGAGGATAATGGGAAGAGGTT3' R:5'AGCTCACTCCATCTCAGTCAGT3'	165
Caspase-8	F:5'ATATGTCGTCCAGTTCAACTCC3' R:5'CTTGTCCAGGAAGAAGAACAAC3'	157
Caspase-9	F:5'CAGGGATTAACCTCTGCACTAC3' R:5'GGAAAGTTTTATACCCCAGGAC3'	186

Caspase-10	F:5'GTTCAAAAACCACGTTCTTAGC3' R:5'CCTATGTGAGCACCTTCCTTAC3'	244
GAPDH	F:5' TCCTGCACCACCAACTGCTTAGC 3' R:5' ACACGGAAGGCCATGCCAGTGAG3'	254

Receptors expression

After lycopene treatment for 7d, NK cells were centrifuged, washed with PBS, and divided into 4 groups (1×10^5 cells each) and stained with PE-conjugated mouse anti-human NKG2A (R&D Systems, Minnesota, USA), NKG2D, NKp30 and NKp44 (BD Bioscience, California, USA). All cells were analyzed by using flow cytometry.

ELISA assay of IFN-γ, perforin and Granzyme-B

ELISA Kits (BD Bioscience, CA, USA) was used to assess the secretion of IFN-γ, perforin and Granzyme-B. NK cells (8×10^5) in each lycopene treated group were centrifuged, washed with PBS and re-suspended in 400μL RPMI-1640 (IL-2 free), mixed with 1.6×10^5 K562 cells in 100μL RPMI-1640, then incubated for 4h at 37°Cin 5% CO_2 incubator (K562 cells were used to stimulate the cytokine secretion of NK cells according the literature [24]. Culture supernatant from each lycopene treated group was assessed by corresponding ELISA Kits. Each sample were performed twice, *A* values were recorded at 450nm. Curve Expert 13.0 was employed to create the standard curve according to the *A* values of standards. The quantities of these cytokine secreted by NK were calculated from these standard curves.

Data analysis

SPSS 16.0 statistical software (IBM, New, York, USA) were used here to perform the statistical analysis. The data were presented as the mean±SD. All results were analyzed using the analysis of variance (ANOVA). Multiple comparisons used LSD test to evaluate the significance of differences between groups. Statistical significance was defined as $p<0.05$.

III. Result

NK cells purity

Ex vivo expanded NK cells were harvested and stained with CD56-PE and CD3-FITC mAbs after 3 weeks culture. The purity of NK cells ($CD56^+CD3^-$) was shown in Fig. 1. From the one donor, the percentage of NK cell in PBMC after expansion was 91.17%, and the mean percentage of NK cells form 10 donors was 90.82%±0.39% (n=4).

NK Cells viability and cytotoxicity

There were no obvious effects of lycopene on NK cells viability in short term treatment. As shows in Fig.2A, the *A* values (reported the viable NK cells) were 0.864±0.028, 0.858±0.013 and 0.845±0.012 in 2, 4 and 7days (4^{th} week) respectively in lycopene treated group compared with 0.856±0.019, 0.857±0.014, and 0.834±0.017 in control. With time, the *A* values of NK cells in control were decreased rapidly, but which decreased slightly in lycopene treated group. The *A* values in these group were 0.524±0.018 (14d/5^{th}week), 0.329±0.1 (21d/6^{th} week) in control and 0.683±0.016 (14d/5^{th}week) and 0.47±0.041 (21d/6^{th} week) in lycopene treated group respectively. The variation tendency of NK cells cytotoxicity was similar to its viability. As show in Fig. 2B, the cytotoxicity of NK cells decreased significantly since the 5^{th} week *ex vivo* culture, which decreased to 31.4%±2.3% compared with the data (89.6%±1.1%) obtained from 2d(3^{th} week) ($p<0.05$). And in lycopene treated group, the cytotoxicity were 42.1%±2.8% in 5^{th} week compared with the control. It suggested that lycopene restore the NK cells viability and cytotoxicity in the 5^{th}-6^{th}weeks *ex vivo* culture.

NK cells apoptosis

There was no obvious apoptosis of NK cells observed under inverted phase contrast microscope in 3 weeks *ex vivo* culture, therefore the NK cells apoptosis were assessed after 7d (4^{th} week of *ex vivo* culture) of co-culture with lycopene. After co-culture with lycopene over 7d and stained with Annexin-V and PI, NK cells apoptosis were tested by flow cytometry and showed in Fig.3. It was noticed that, after 3 weeks *ex vivo* expansion, NK cells were routinely cultured in 1640 medium. Since the 4^{th} weeks, NK cells were seen to be aging and apoptosis gradually. However, lycopene show the positive effect to slow down this process. In routinely cultured (control) group, the early apoptosis ($AnnexinV^+/PI^-$) of NK cells were17%±1.46, 20.5%±1.7, and 17%±4% in 4^{th}, 5^{th} and 6^{th} week. And the early apoptosis ($AnnexinV^+/PI^-$) of NK cells in present of lycopene were 5.6%±1.1%, 17.8%±1.6%, and 15.8%±3.1% respectively in 4^{th}, 5^{th} and 6^{th} week. Meanwhile, the late apoptosis/necrosis NK cells ($AnnexinV^+/PI^+$)were 15.9%±1.1%, 20.1%±2.3%, and 56%±1.5% in control and 8.32%±1.3%, 10.6%±1.9%, and 35.4%±2.3% in lycopene treated groups in these weeks. These results suggested that lycopene could relieve the aging caused NK cells apoptosis and necrosis to a certain extent.

RT-qPCR

The expression profiles of genes involved in apoptosis and NK cell functions after lycopene treatment were depicted in Table 2 and Table 3 The mRNA level of caspase 3 and caspase 9 genes increased gradually since 4^{th} week culture compared with the 3 weeks cultured cells (data not show). And lycopene treatment can decrease this gene expression (Table 3). After 7d lycopene treatment, the IFN-γ mRNA increased significantly (8.81 fold) compared with the control ($p<0.05$). There was no significant change on NK cell functional receptors mRNA expression after lycopene treatment.

ELISA

The levels of IFN-γ and perforin proteins in the NK cells supernatants were performed in Fig.4. It was noticed that, IFN-γ level was significantly increased after 7d lycopene treatment, which was increased to 466±19pg/mL compared with the 389±17pg/mL in the control. The concentration of perforin was not changed obviously. There were about 610ng/mL perforin in lycopene treated and control groups. The ELISA result of Granzyme-B was not showed because which was hardly detected in the supernatants of NK cells.

Receptor expression

Flow cytometry assays (Fig.5.) indicated that lycopene display no significantly regulation of NK cells functional

receptor. The positive percentages of NKG2A, NKG2D, NKp30 and NKp44 were not changed obviously.

IV. Discussion

As a kind of common natural pigment in many vegetables and fruits, lycopene is a carotenoid without provitamin-A activity and a major component found in the serum and other tissues among the carotenoids [25]. Antioxidant properties of lycopene are recognized to be primarily responsible for its beneficial properties. Recently, accumulated evidence suggested that there were other bioactivities of lycopene such as modulation of intercellular gap junction communication, immune system and metabolic pathways [25].

Immune cells especially lymphocytes are particularly sensitive to oxidative stress because these highly active cells constantly generate reactive oxidative products (ROS) in their normal cellular activity [18, 26]. Accumulated ROS are highly reactive and can destroy cellular membranes, cellular proteins and nucleic acids. For this reason, antioxidants such as lycopene may help to protect lymphocyte activity and functions.

Literature shows that lycopene inhibited 7-KC-induced macrophage apoptosis by reduced the increase in ROS production, and NOX-4, Hsp 70 and Hsp 90 expression. It also limit caspase-3 activation and modulate the effects of 7-KC on AKT, Bcl-2, Bcl-xL and Bax [27]. Li *et al* reported that lycopene could protect marrow mesenchymal stems from ischemia-induced apoptosis *in vitro* by activate phosphoinositide-3 kinase (PI3K)/Akt [28]. Mills *et al* has reported that lycopene influences lymphocyte proliferation significantly through its effects on processes involved in early cellular activation [29].

In the present study, the effect of lycopene on human primary NK cells was assessed. The results show that, the viability and cytotoxicity of NK cells decreased gradually since the 4^{th} week *ex vivo* culture, and accompanied by increased early apoptosis and late apoptosis/necrosis cells, which may cause by aging and accumulated ROS. Supplement lycopene to NK cells culture system could help to relieve NK cells apoptosis via reducing caspase 3 and 9 gene expression and increase its cytotoxicity.

Lutein is another carotenoid with no provitaminA activity. Studies show that, dietary lutein increased IFN-γ mRNA expression in splenocytes and decreased apoptosis in blood leukocytes of tumor bearing mice compared to un-supplemented one [30, 31]. Our results show that, lycopene treated for 7d also significantly increases the NK cells IFN-γ expression in gene and protein levels. In conclusion, lycopene has the positive effect on NK cells by prolonging its lifespan, increasing its cytotoxicity and decrease its apoptosis as a health product.

Acknowledgment

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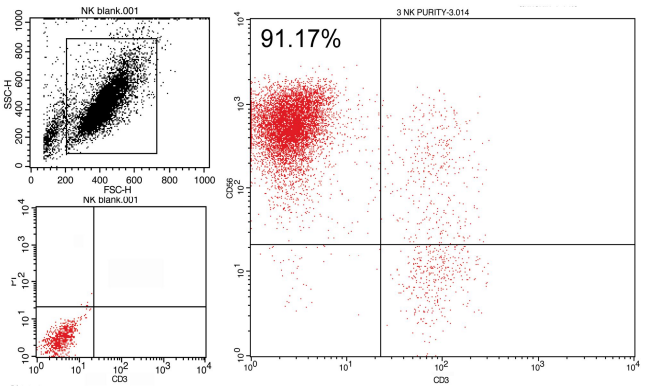
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FIGURES



Flow cytometry assay of primary human NK cells from one donor (for instance); the percentage of NK cells ($CD56^{+}CD3^{-}$) in PBMCs was 91.17% after 3 weeks *ex vivo* expansion.

Fig. 1. Flow cytometry assay of primary human NK cells.

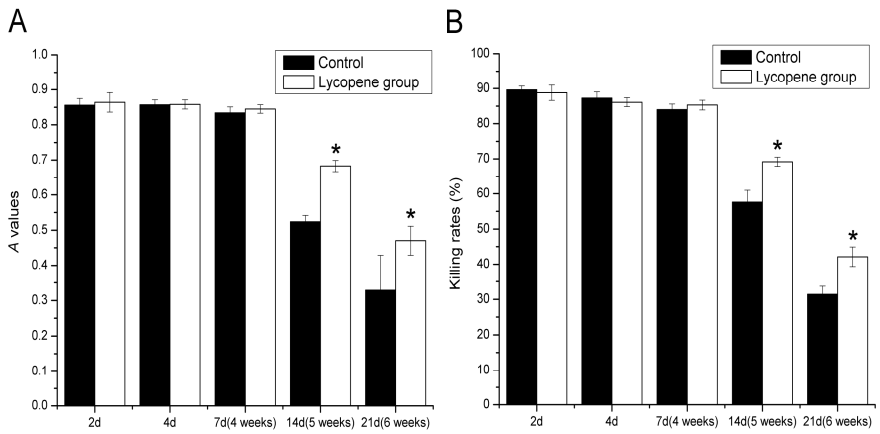


Fig.2. *A* values and killing rates of NK cells treated with lycopene
(A) *A* values of NK cells treated with lycopene for 2-21d;
(B) (B), Killing rates of NK cells treated with lycopene for 2-21d. Data represents the mean±SD of six independent experiments. One-way ANOVA and LSD tests were performed. *: $p<0.05$ compared with the control(n=6).

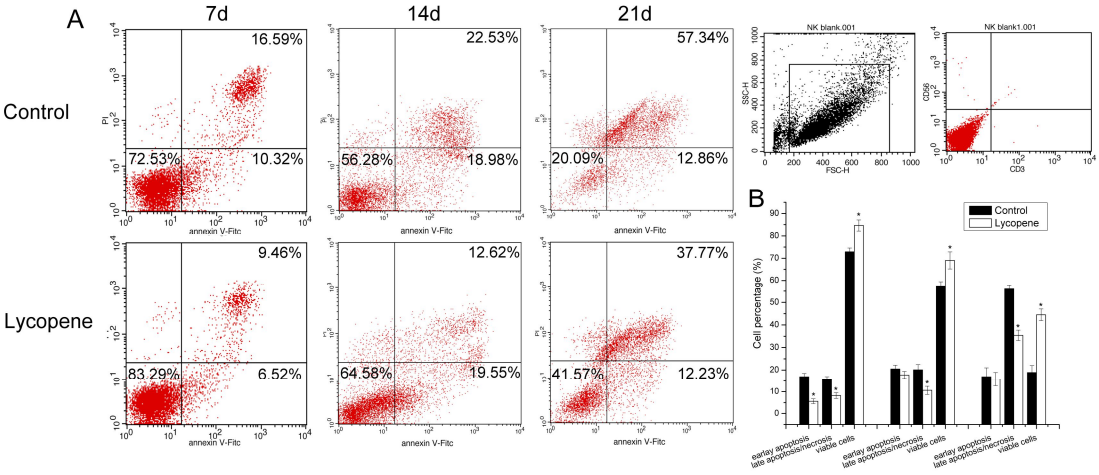


Fig.3. Apoptosis and necrosis of NK cells after lycopene treatment
(A) A representative flow cytometry plot of NK cells from one donor. The percentages of viable (Annexin V^-/PI^-), early apoptotic (Annexin V^+/PI^-), and late apoptotic/necrotic (Annexin V^+/PI^+) cells are shown.
(B) The percentage of apoptosis in these cells is summarized in the bar graph; each column represents the mean±SD from four independent experiments. Multivariate ANOVA and LSD tests were performed; *:p<0.05 compared with the control (n=4).

TABLE II. GENE EXPRESSION PROFILES OF APOPTOSIS RELATED PROTEIN AFTER 7D LYCOPENE TREATMENT

Gene	ΔΔCt(compared with control)
IFN-γ	8.81(fold)*
Perforin	1.97(fold)
Granzyme-B	0.49(fold)
NKG2A	0.79(fold)
NKG2D	0.86(fold)
NKp30	1.13(fold)
NKp44	0.66(fold)

Real-Time Quantitative (RTq)-PCR and SYBR Green Random mixing method were employed to test the mRNA of apoptosis related genes levels. GAPDH served as internal standard control. One-Way ANOVA and LSD test, *: p< 0.05 compared with control(n=4).

TABLE III. GENE EXPRESSION PROFILES OF IFN-Γ, PERFORIN, GRANZYME-B, NKG2D, NKG2A, NKP30 AND NKP44 OF NK CELLS AFTER 7D LYCOPENE TREATMENT

Gene	ΔΔCt (compared with control)
FasL	1.2 (fold)
Caspase-3	0.14(fold)*
Caspase-6	0.87(fold)
Caspase-7	0.88(fold)
Caspase-8	1.16(fold)
Caspase-9	0.12(fold)*
Caspase-10	1.85(fold)

Real-Time Quantitative (RTq)-PCR and SYBR Green Random mixing method were employed to test the mRNA of apoptosis related genes levels. GAPDH served as internal standard control. One-Way ANOVA and LSD test, *: p< 0.05 compared with control(n=4).

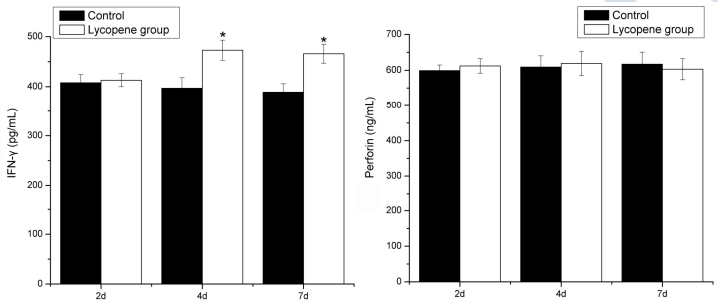


Fig.4. IFN-γ and perforin secretion of NK cells after lycopene treatment in time gradient.
Lycopene (5μM) treated NK cells were stimulated with K562 cells, supernatants were collected in different time intervals and then the concentrations of IFN-γ and perforin in them were detected. Each column represents mean±SD of four independent experiments. One-way ANOVA and LSD tests were performed, *: p<0.05 compared with control (n=4).

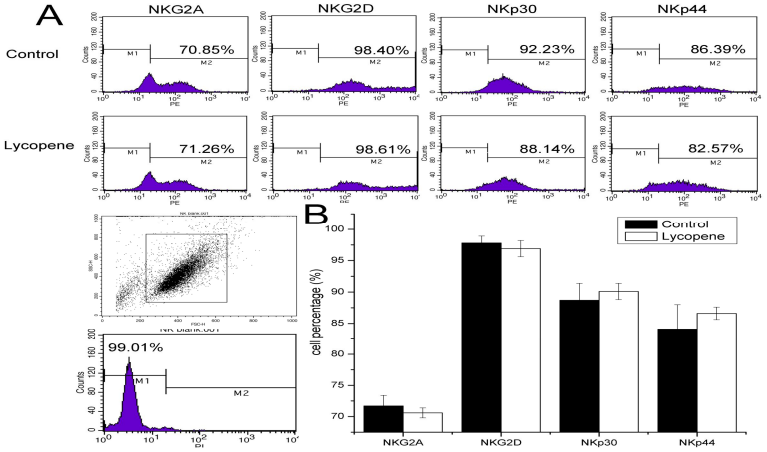


Fig.5. Protein expression profiles of the NK cell receptors NKG2A, NKG2D, NKp30, and NKp44 after lycopene treatment.
(A) NK cells were stained with PE-conjugated mAbs and analyzed by flow cytometry (for instance). (B) The bar graph represents the mean±SD from four independent experiments. One-way ANOVA and LSD test (n=4).