

Long Acting Propranolol and HSP-70 Rich Tumor Lysate Reduce Tumor Growth and Enhance Immune Response against Fibrosarcoma in Balb/c Mice

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ABSTRACT

Background: Noradrenaline (NA), the principal neurotransmitter released from sympathetic nerve terminals, influences T-cell maturation, not only directly in developing T cells, but also indirectly, by acting on the thymic nonlymphoid cells. In vitro and in vivo studies have demonstrated the anti-proliferative, anti-migratory, anti-angiogenic and cytotoxic properties of propranolol, β -AR blocker, against various cancers. **Objectives:** To evaluate the effect of propranolol on efficacy of HSP-70 rich lysate vaccine in immunotherapy of fibrosarcoma. **Methods:** Mouse fibrosarcoma WEHI-164 cells were used to immunize tumor-bearing mice with or without propranolol and HSP-70. Splenocytes proliferation, cytotoxic activity of the splenocytes, naturally occurring CD4⁺ CD25^{high} T-reg cells and IFN- γ and IL-4 secretion as well as tumor size, were assessed to describe the anti-tumor immune response. **Results:** A significant increase in the level of IFN- γ in the mice vaccinated with WEHI-164 cells enriched with HSP-70 and co-treated with propranolol was observed compared to controls. However, HSP enrichment or propranolol treatment alone did not enhance the immune response as measured by the level of IFN- γ . Likewise, a decrease in tumor growth in the test group ($p < 0.01$) and a significant increase in CTL activity ($p < 0.05$) was observed. **Conclusion:** HSP enriched vaccine shows anti-tumor activity, probably due to the modulation of immune responses.

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Keywords: IFN- γ , IL-4, Immune Modulator, Propranolol, T regulatory cell

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INTRODUCTION

It has been well documented that the sympathetic nervous system, a major component of the autonomous nervous system, innervates primary lymphoid organs (1-3). The expression of β -ARs has been discovered on the surface of both thymocytes (4,5) and thymic nonlymphoid cells (2,6-8). Accordingly, it has been suggested that noradrenaline (NA), the principal neurotransmitter of sympathetic nerve terminals, influences T-cell maturation, not only directly via β -ARs, but also indirectly, by acting on the thymic nonlymphoid cells via β -ARs (8). However, knowledge about the role of β -AR-mediated signaling in the modulation of intrathymic T-cell development is still extremely limited. Since it has been shown that thymocytes express a significantly lower number of β -ARs on their surface in comparison with circulating peripheral T cells (9,10), it is assumed that the surface expression of β -ARs increases during T-cell maturation.

A number of *in vitro* studies have demonstrated the anti-proliferative, anti-migratory and cytotoxic properties of propranolol, particularly against lung adenocarcinoma (11,12) colon (13) breast (14) nasopharyngeal (15) ovarian (16) pancreatic (17-19) and gastric cancer cells (20). Propranolol is also found to exert potent anti-angiogenic effects *in vitro* through direct mechanisms affecting vascular endothelial cells (21, 22) and by decreasing pro-angiogenic signaling in both stromal (23) and cancer cells (15,24-27). Some of these promising anti-cancer properties have been confirmed *in vivo* using different animal models of human cancers. Propranolol was thus found to exert potent cancer preventive effects in models of chemically-induced lung and pancreatic cancers (28,29). Furthermore, innovative pre-clinical models of breast and ovarian cancer have showed that propranolol is able to specifically inhibit stress-induced tumor growth and metastatic spread through anti-angiogenic and immuno-stimulatory mechanisms (30,31).

Several experimental vaccine strategies have been developed to enhance cell-mediated immunity against tumors. In addition, several phase I and II clinical trials using these vaccine strategies have shown extremely encouraging results in patients (32). Heat shock proteins (HSPs) are intracellular molecules that act as antigen chaperones. When a cell is subjected to temperature changes, heat shock proteins bind to intracellular peptides and chaperone a large number of non-defined antigenic peptides derived from the cells (33,34). Some HSPs derived from tumor cells have been found capable of effectively initiating specific immunity against the tumor (35-39). Clinical trials of tumor derived HSPs have been conducted in patients with a broad range of malignancies including lymphoma, renal cell carcinoma, melanoma, colorectal, gastric, pancreatic and breast cancer (40,41).

The aim of this study was to evaluate the effect of propranolol on efficacy of HSP-70 rich lysate vaccine in immunotherapy of fibrosarcoma.

MATERIALS AND METHODS

Mice and Tumor Models. Female inbred BALB/c mice (6 to 7 weeks old) were purchased from the Pasteur Institute, Tehran, Iran. They were given sterilized water and autoclaved standard mouse chow ad libitum throughout the study. BALB/c mouse fibrosarcoma cells (WEHI-164) were purchased from Pasteur Institute, Tehran, Iran and

propagated in DMEM (GIBCO) supplemented with 10% FBS (GIBCO) and incubated in 5% CO₂ and 95% humidity at 37°C until sufficient numbers were obtained. A single aliquot of WEHI-164 (5×10^5 cells/100 μ l) was injected subcutaneously into the right flank of the inbred BALB/c mice to establish a tumor model (18). Palpable tumors started to develop after 7 days from which mice were divided into five groups of treatment.

Group 1: 13 mg/ml lysate fibrosarcoma cells enriched with HSP-70 along with 3 mg/kg propranolol (co-treatment group)

Group 2: 13 mg/ml lysate fibrosarcoma cells enriched HSP

Group 3: 3 mg/kg propranolol

Group 4: 13 mg/ml lysate fibrosarcoma cells non-enriched HSP

Group 5: tumor bearing mice receiving only PBS

Each group received the injections intraperitoneally on a six hour basis. Tumor growth was monitored using digital Vernier calipers after tumor challenge until the experiment was completed. Tumor volume (mm^3) was calculated by the formula: $\text{length} \times \text{width}^2 \times \pi / 6$ (19).

Cell Culture and Vaccine Preparation. The WEHI-164 cells were cultured in DMEM supplemented with 10% FBS. WEHI-164 cells in the logarithmic growth phase were heated by direct immersion of the cell culture dishes in a waterbath, with a controlled temperature of $42 \pm 0.1^\circ\text{C}$ for 60 min. After the heat treatment and incubation periods of 8 and 12 h, the cells were collected with trypsin/EDTA (GIBCO), washed 3 times in PBS and re-suspended in PBS (5×10^5 cells /100 μ l). Tumor cell lysate was prepared following a previously published method (20,21). Briefly, the cell suspensions were disrupted by 5 cycles of freeze-thaw using liquid nitrogen and a 37°C waterbath. The large particles were removed by centrifugation (20 min, 3000 \times rpm) and the supernatants were passed through a 0.2- μ m filter. The filtered supernatant was used as the HSP-70 enriched vaccine. The concentration of HSP-70 was measured using HSP-70 ELISA measurement kit.

Tumor Antigen Preparation. Tumor antigen was brewed using WEHI-164 tumor cell lysate as prepared above. Lysate from 1×10^7 cells were then subjected to sonication (60 HZ, 0.5 Amplitude) after 5 times of freezing and thawing. PMSF (1 mM) was added to the cell lysates to inactivate proteinases. The protein concentration was determined using the Bradford method.

Splenic MNCs Separation and Splenocyte Proliferation Index. Animals were sacrificed to remove their spleens. Splenocytes were isolated using the needle perfusion method and sterile cold RPMI-1640. Erythrocytes were lysed at room temperature using the ACK lysis buffer (NH₄Cl, KHCO₃, Na₂EDTA). Cells were counted and the viability test was carried out using the Trypan blue dye exclusion. The splenocytes were cultured at a concentration of 3×10^5 cells/well in 96-well plates in the presence of 25 μ g/ml prepared antigen in a total volume of 200 μ l. The plates were incubated for 36 h at 37°C in a humidified 5% CO₂ atmosphere. Cell proliferation was defined with Bromodeoxyuridine (BrdU) labeling solution. The uptake of BrdU was detected using the cell proliferation ELISA BrdU kit (Roche Diagnostic GmbH, Mannheim, Germany) and expressed as the stimulation index (S.I.): $\text{S.I.} = [(T-N)/(P-N)] \times 100$

Splenic MNCs Separation and Measurement of Cytokines by ELISA. To evaluate the effect of HSP enriched and non-enriched lysate vaccine on the cytokine production of splenic MNCs, after treatments, the spleens were removed under sterile conditions; and single cell suspensions were prepared in RPMI 1640 as above. The MNCs were isolated by density centrifugation (700g, 15min, 20°C) using ficoll hypaque (Baharafshan, Iran). The layer was removed and washed twice with PBS for 10 min in 360×g and 4°C. The precipitated cells were re-suspended in RPMI 1640 containing 10% FBS. The cell viability was more than 90%. 4×10^5 cells/well was dispensed into 96-well micro plates and to stimulate the cells, the lysate antigens were added at 5µg/ml final concentrations. The mixture was then incubated for 72 hours. The supernatants were collected and stored at -70°C until use. An ELISA kit (R&D Systems, USA) was purchased to measure IFN-γ and IL-4 levels. Briefly, after washing the wells with buffer, the standard samples were added to each well, followed by the addition of biotin conjugates and then incubation for 2 h. The microplates were washed three times with washing buffer, and Streptoavidin-HRP was added. The plates were incubated for 1 hr at 37°C, and then washed with washing buffer. The TMB substrate solution was dispensed for 15 min; afterwards the stop solution was added. An ELISA reader (450 nm filter) was used to read the results.

Flow Cytometric Analysis of Regulatory T- Lymphocytes Subpopulation in Spleen. Spleen cell suspension was prepared. The cells were washed twice and labeled with monoclonal antibodies. The freshly prepared cells were analyzed using a direct immunofluorescence staining. Mouse regulatory T cell staining kit (eBioscience, UK) was used. The staining was performed in a washing buffer consisting of PBS supplemented with 1% FBS, 0.1% sodium azide (Sigma, USA), and 2 mM EDTA (Sigma, US). After determining the cell viability using trypan blue, cells were washed twice in a washing buffer. Each sample was immunostained with antibodies for 45 min at 4°C. The cells were washed in the washing buffer and fixed with 2% paraformaldehyde. Flow cytometric analysis was performed in an EPICS flow cytometer (Coulter, UK). Focusing on the lymphoid areas of forward and side scatters, and using the Coulter software, the double stained cells were analyzed.

Measurement of the Tumor Volume Following the Vaccine Therapy. The tumors grew for approximately 2 weeks, after which animals were divided into groups of 5 mice. Experimental groups were injected intraperitoneally with vaccine in a total volume of 0.1 ml as mentioned above. The control group received PBS in the same route and volume. Tumor-bearing mice were treated for 20 consecutive days. Tumor volume was measured daily using a digital vernier calliper (Mitutoyo, Japan) and the following formula:

$$V = 1/6 \pi LWD$$

Where L = length, W = width, and D = depth.

Measurement of the Level of HSP-70 Expression in Splenocytes after Treatment by ELISA method. One week after final immunization, a total number of 1×10^7 of spleen cells were lysed with one milliliter of WBC lysis buffer with 10 mM of PMSF. The lysate of each experimental mouse were estimated for HSP-70 by ELISA Kit (Quantikine, R&D Systems, USA) according to the manufacture's instruction. The concentration (pg/ml) of each sample was calculated according to the standard curve.

Cytotoxic T-Lymphocyte (CTL) Activity Following Vaccine Therapy. Splenocyte suspensions were prepared as above in RPMI 1640 (containing 2% bovine serum albumin (Sigma, USA) and used as effector cells; mouse WEHI-164 (H-2^d) cells were prepared for use as target cells. Briefly, 2×10^4 effector cells (in 100 μ l/well) were incubated in 96-well plates and pulsed overnight with a 20 μ l aliquot of tumor antigen (containing 20 μ g tumor antigen/ml). Thereafter, for the CTL assay, fixed volumes of effector cells were transferred to wells containing 100 μ l of target cells to establish effector:target (E:T) ratios of 100:1, 50:1, and 25:1. The plates were then gently centrifuged (250 \times g, 10 min) and placed in a 37°C incubator for 4 hr. The plates were then centrifuged and 100 μ l supernatant from each well was transferred to a 96-well flat-bottom plate; the extent of cytotoxicity that had occurred was then determined by assaying LDH release with an LDH kit (Takara Company, Tehran, Iran) according to manufacturer protocols and absorbance measurements at 492 and 620 nm in the MultiScan plate reader. Specific lysis (%) was calculated as: $100 \times (\text{LDH release in sample well} - \text{spontaneous LDH release by effector cells} - \text{spontaneous LDH release by target cells}) / (\text{maximum LDH release by target cells} - \text{spontaneous LDH release by target cells})$. All determinations were performed in triplicate. Maximum lysis was determined from supernatants of cells lysed with 1% Triton X-100; spontaneous release was determined from target cells incubated with RPMI 1640.2% BSA only.

Statistical Analysis. The results were depicted as the mean \pm standard deviations of triplicate determinations. Statistical analysis was performed using one way ANOVA and two-tailed Student's *t*-test. A *p* value less than 0.05 was considered to be statistically significant.

RESULTS

HSP-70 Expression by the WEHI Cells Treated with Heat. Using an ELISA assay the level of HSP-70 in the lysate of the heat treated and non-treated WEHI-164 cells at 42°C for 60 min were assessed after removal of the pellet. Non-treated lysate of WEHI-164 cell was used as control. Figure 1 illustrates a significant increase in the level of HSP-70 accumulation in cells after heat treatment compared with non-heat shocked control cells (*p*<0.05).

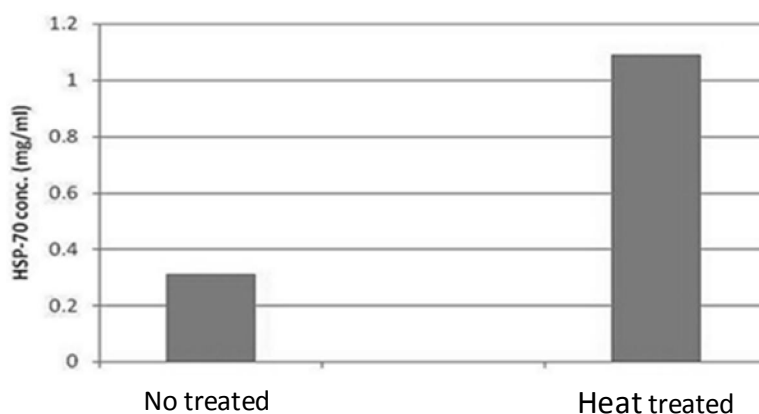


Figure 1. Induction of HSP-70 following heat treatment of cultured cells at 42°C for 60 min was evaluated using ELISA. As illustrated, the heat treatment was able to induce the expression of HSP70 (i.e., 0.31 vs. 1.09 mg/ml) in the cells.

Measurement of the Lymphocytes Proliferation Index Following Vaccine Therapy.

In order to assess the lymphocyte proliferation index in the animals treated with HSP enriched and non-enriched fibrosarcoma, 25 female mice in five groups were used. Spleen cells were collected 6 days after the final injection and re-stimulated with the lysate antigens. The results, depicted in Figure 2, indicated that, no significant differences were noticed among treatment groups ($p>0.05$).

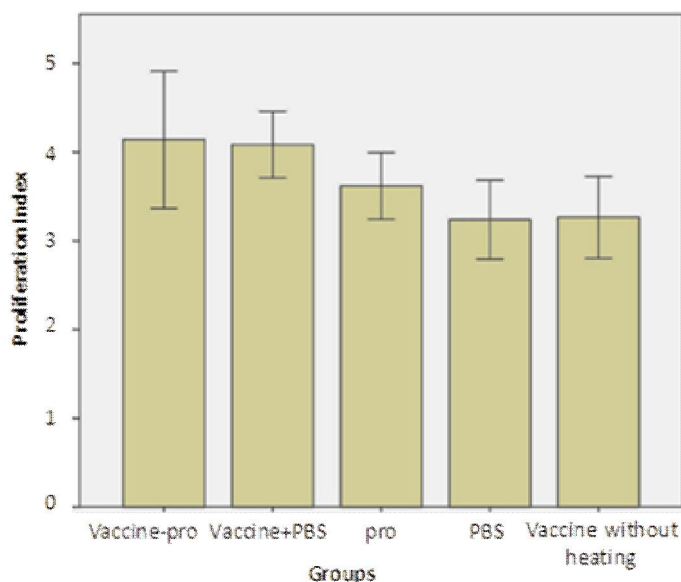


Figure 2. Splenocytes were recovered from tumor-bearing mice that were treated four times per day with HSP70-enriched lysate (1.3 mg/mouse), HSP70-enriched lysate and 3 mg propranolol/kg, 3 mg propranolol/kg only, PBS only, or non-enriched lysate (1.3 mg /mouse). All values are derived from BrdU ELISA measurements. Values shown are mean \pm SD from 5 mice/group and experiments were repeated 4 times. Splenocyte proliferative responses in propranolol-treated animals showed a relative increase to that by cells from mice receiving non-HSP70-bearing lysate (alone or with propranolol), though the changes were not significant.

Cytokine Shift Following Vaccine Therapy. In order to assess the Th1/Th2 cytokine shift in the animals treated with HSP enriched and non-enriched fibrosarcoma, 25 animals in five groups were treated. Animals were sacrificed and their splenocytes was obtained. Our results revealed that HSP-70 did not influence the induction of IFN- γ but the co-treatment is highly more effective in augmentation of IFN- γ . The level of IFN- γ was somewhat similar in both HSP enriched and non-enriched lysates. Treatment with propranolol alone did not show the same effects as the co-treatment (Figure 3).

Frequency of CD4⁺CD25⁺Foxp3⁺ Regulatory T Cells. To define the percentages of intra-tumoral and splenic CD4⁺ CD25⁺ FoxP3⁺ T cells, 25 tumor-bearing mice (five groups, each containing five mice) were used. After the isolation of lymphocytes from the spleens and tumor tissues of animals, the three-color staining and flowcytometry analysis were performed. Using WinMDI software, lymphocytes and then CD4⁺ cells were gated; dot plots were depicted for the co-staining of CD25 and Foxp3 markers on the CD4⁺ lymphocytes gating (Figure 4).

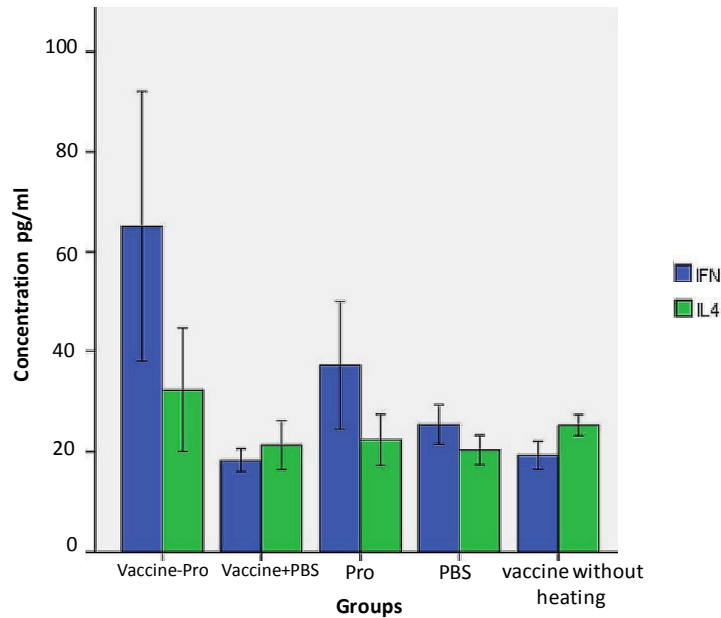


Figure 3. IFN- γ and IL-4 levels in cultures of splenic MNC recovered from tumor-bearing mice that were treated four times per day with HSP70-enriched lysate (and with or without co-treatment with propranolol), non-enriched lysate, propranolol only, or PBS. Values shown are mean (\pm SD) from 5 mice/group. All tests were evaluated in triplicate. Co-treatment with propranolol was able to induce significant ($p=0.015$) increases in IFN- γ production relative to that by MNC from mice that received HSP70-enriched lysate only, as well as from mice in all the other groups. There were no significant differences among the MNC from the other treatment groups. Decreases in IL-4 production were evident in MNC from mice that received propranolol only, although the drop was not significant. There were no significant differences among other four groups.

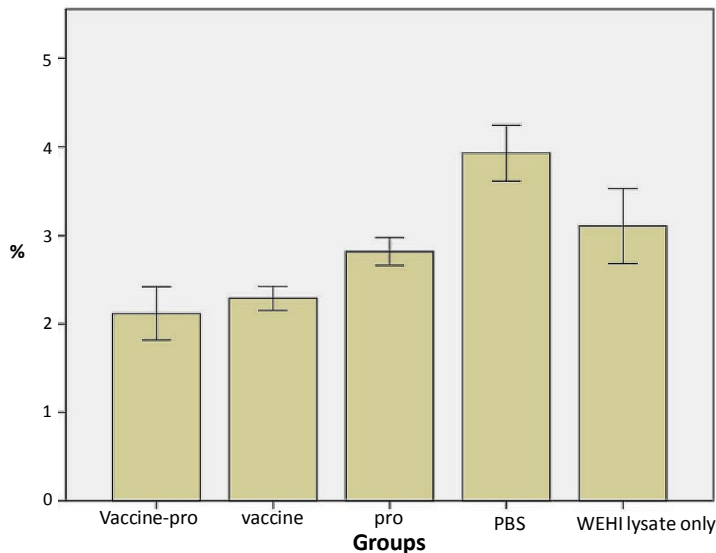


Figure 4. Freshly-prepared spleen cells were stained with FITC-conjugated anti-CD4, PE-conjugated anti-CD25, and PeCy5-conjugated anti-Foxp3. Based on dot-plots, CD25 and Foxp3 co-positive cells gated in lymphocytes and CD4⁺ cells, it was determined that all the experimental groups had fewer numbers of splenic T_{reg} cells relative to levels in mice that had received the PBS only treatment; with mice that received the heat-activated cell lysate, the difference from the control was significant ($p=0.02$).

The data were computed with Mann-Whitney non-parametric test to determine the statistical significance. Significant difference was observed between the percentage of splenic T-reg cells in the animals treated with HSP enriched vaccine and PBS only ($p=0.029$). No statistically significant difference was observed among other groups.

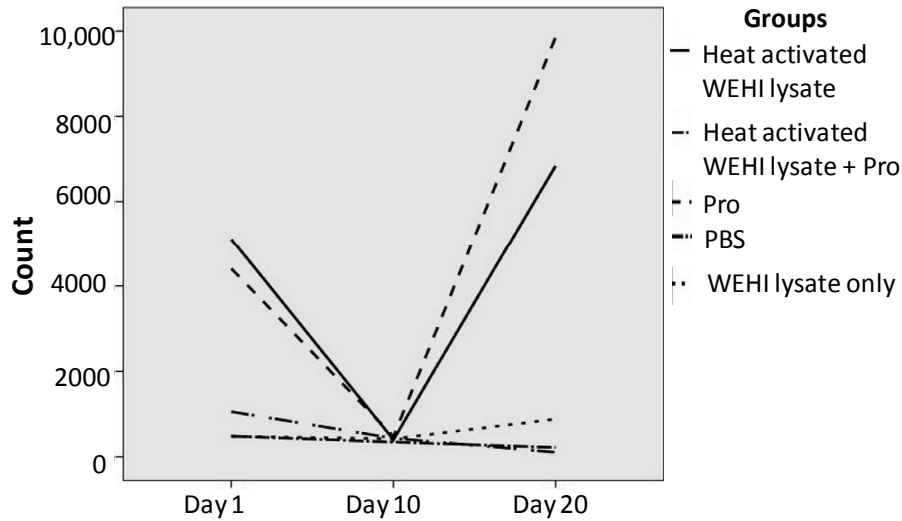


Figure 5. Tumor volume (in mm^3) was calculated and monitored throughout the experiment. The results indicate that tumors in mice receiving HSP70-rich lysate \pm propranolol grew more slowly than those in the control groups. Co-treatment (HSP70-enriched lysate + propranolol) caused a more effective growth control over the 20-d monitoring period compared to either single treatment (lysate or propranolol alone) or no treatment (PBS or lysate only). Propranolol itself was able to partially control growth until Day 10; however thereafter, tumor growth progressed.

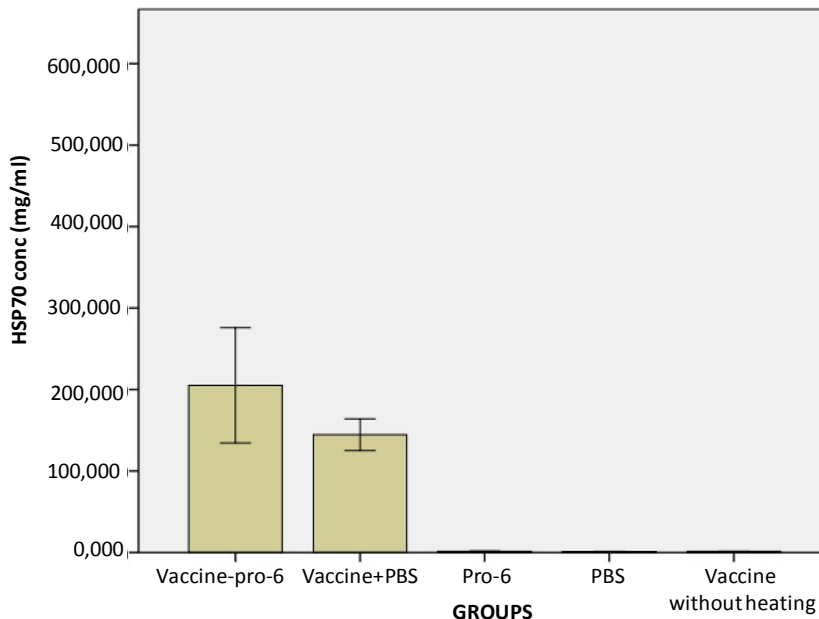


Figure 6. Blood was drawn at sacrifice and serum was obtained and assayed for HSP70 by ELISA. Values shown are mean (\pm SD) from 5 mice/group. All samples were evaluated in triplicate. *Value significantly different from controls receiving PBS and also from mice treated with non-enriched lysate ($p<0.01$). No significant differences were observed among treatment Groups 1, 2 and 3. Injections were repeated 4 times per day.

Measurement of the Tumor Volume Following Vaccine Therapy. The changes in the tumor volume in the five groups of mice were assessed as shown in Figure 5. The results indicate that the tumors in the test group grew more slowly than those in the control groups. Immunization of the mice with the lysate of heat shocked tumor cells (vaccine) significantly suppressed the tumor growth comparing to the control groups ($p < 0.01$). Similar results were obtained in two separate experiments.

HSP Levels in Splenocytes Following the Vaccine Therapy. In order to assess the HSP microenvironment in the spleen of animals treated with HSP enriched and non-enriched fibrosarcoma and propranolol, 25 mice were used and the mentioned protocol was applied. The results in Figure 6 indicate that the level of HSP was significantly increased in the vaccine enriched "HSP + propranolol" and vaccine enriched "HSP" compared with control group ($p = 0.02$).

Cytotoxic Activity of T Lymphocytes Following the Vaccine Therapy. The level of cytotoxic activity of the T lymphocytes in the vaccinated mice was assessed using LDH kit (Takara Company, Tehran) according to manufacturer protocols. As shown in Figure 7 the splenocytes from the mice in all of the test groups in the 100 and 50 ratio showed a significant increase in CTL activity against the target WEHI-164 cells compared with control groups ($p < 0.001$).

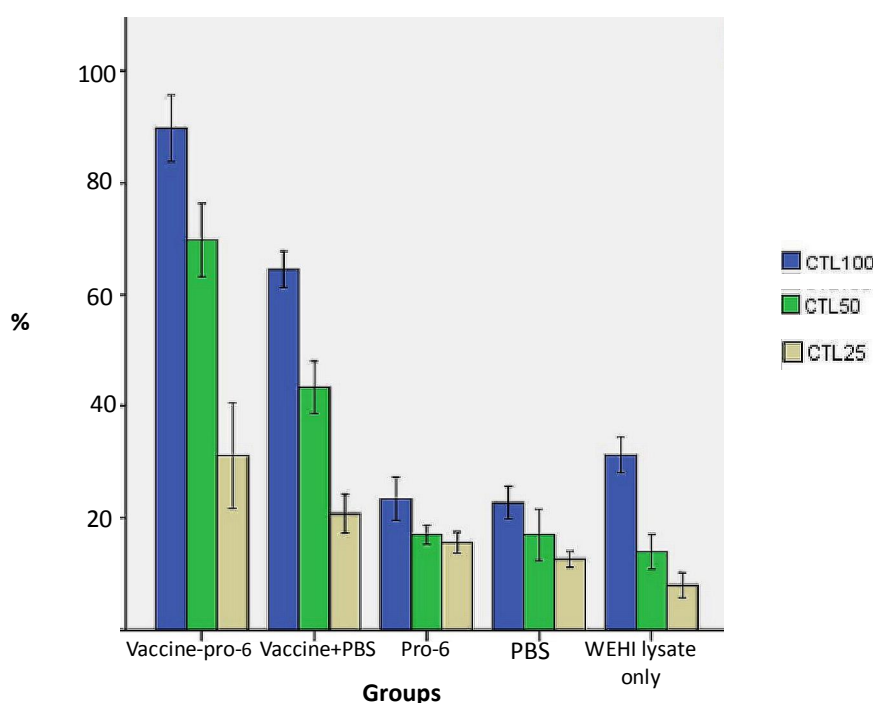


Figure 7. Splensens were recovered from tumor-bearing mice in each treatment group and assessed for CTL activity. All values are derived from measures of target cell LDH release. Values shown are mean (\pm SD) from 5 mice/group. All samples were evaluated in triplicate. Differences were significant in 1:50 and 1:100 ratios with the group receiving co-treatment showing the greatest cytotoxicity compared to other treatments at same E:T ratio ($p < 0.01$).

DISCUSSION

Propranolol is generally a safe drug, but it has been associated with adverse events such as bradycardia, hypotension, hypoglycemia, and bronchospasm (42). Propranolol has

recently been found to be efficacious in treating problematic infantile hemangiomas (43). Our results indicated a significant increase in the level of interferon gamma in the animals vaccinated with WEHI-164 cells enriched with HSP and co-treated with propranolol comparing with control groups. The cytokine profile secretion divides T-helper cells into two subpopulations with different roles: the Th1 subset that secretes interleukin-2 and interferon γ (44,45) and the Th2 subset that produces IL-4 and IL-5 (45). IL-4 and IFN- γ have modulatory effects on macrophages, which are in some cases coincidental and in others opposing (46). Our results revealed that the co-treatment is much more effective in augmentation of IFN- γ than administration of each one alone. Apparently, the level of IFN- γ was somewhat similar in both HSP enriched and non-enriched lysates. Therefore, it seems that HSP-70 does not influence the induction of IFN- γ . However, treatment with propranolol alone did not show the same effects as the co-treatment, thus there must be a synergistic effect between HSP-70 and propranolol. *In vitro* and *in vivo* studies have shown that catecholamines are capable of inhibiting IFN- γ production from spleen cells and are responsible for acute immunodeficiency. Accordingly this inhibition was prevented by propranolol (47,48). Therefore, it seems appropriate to state that by inhibiting the sympathetic nervous system, propranolol was able to augment the immune response in our model of cancer therapy.

On the other hand, our results showed a significant decrease in the number of T-reg cells in the animals vaccinated with WEHI-164 cells enriched with HSP and injected with propranolol compared with control groups. Animals injected with WEHI-164 cells lysate showed a decrease in the number of T-reg although not significant, while propranolol injected animal showed decrease in the level of T-reg comparing with PBS and WEHI-164 cells lysate group. T-reg cells are specialized in the control of responsiveness to self. They are composed of subsets with distinct ontogeny and functions. Naturally occurring CD4⁺CD25^{high} T-reg cells are produced in the thymus (49) and express FoxP3 (50,51). Their depletion results in autoimmune diseases (52,53). T-regs are also generated in the periphery from non-regulatory T cells (52-53). These include regulatory type 1 (Tr1) (54) and Th3 (53) cells, both of which preferentially secrete regulatory cytokines, IL-10, and/or TGF and do not express FoxP3 (54,55).

It is well established that T-regs recognize HSP70 self-antigens, enabling selective activity in inflamed tissues (56). Also HSP70-treated T-regs inhibit the proliferation of CD4(+)CD25(-) target cells and downregulate the secretion of the proinflammatory cytokines IFN- γ and TNF- α and increased the secretion of T-reg suppressor cytokines IL-10 and TGF- β (57). These are all indications of HSP70 applications in autoimmune and allograft transplantation. However, the mechanism by which the HSP70-enriched vaccine was able to lower the number of T-regs in our study, is to be elucidated. We postulate that the probable represented antigen might have affected the result. Whether the responses arising from presenting tumor antigens differ from self antigens might open-up some new horizons. It has been shown that the number of T-regs drop in lethal infections (58). Although responses to cancer and infection are not comparable, it can be a possible explanation on how our vaccine was able to reduce the numbers of T-regs. Another study utilizing HSP-70 pulsed DCs, co treated with COX-2 inhibitors, showed similar effects in reducing the frequency of *T-regs* (59).

Our results demonstrate a significant increase in the level of HSP in the vaccinated animals and injected with propranolol. HSP was elevated in the circulation. Accumulating data demonstrating the immunomodulatory effects of HSP and its

potential for therapeutic use, it is important to understand its endogenous regulation. Beyond the fact that HSP can be released during necrotic cell death and in the absence of detectable cell death, little is known about the signals that stimulate the release of *in vivo* HSP or the cell types that release. Research in our laboratory has focused on the *in vivo* releasing HSP during times of stress or exposure (60).

Our result showed a significant decrease in the tumor size in the animals treated with propranolol and also vaccine-HSP+ propranolol. A number of *in vitro* studies have demonstrated the anti-proliferative, anti-migratory and cytotoxic properties of propranolol, particularly against lung adenocarcinoma (57,61), colon (62), breast (63), nasopharyngeal (64), ovarian (65), pancreatic (66,67) and gastric cancer cells (68). Propranolol was also found to exert potent anti-angiogenic effects *in vitro* (69). These results revealed that propranolol has antiproliferative and apoptotic effects on multiple myeloma cells. Being supported with *in vivo* analyses, propranolol can be a good and economical way to treat multiple myeloma patients.

Our results showed a significant increase in the cytotoxicity against tumor cells after vaccination with HSP enriched lysate and propranolol. In conclusion, it is proposed that a possible mechanism for anti-tumor activity of HSP enriched vaccine may be due to the modulating of immune responses; However, its anti-tumor activity appropriately requires further study.

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