Evaluation the effects of heated TC1 extract in combination with Lactobacillus Casei extract and alpha-galactosyl ceramide on mouse model of cervical cancer caused by the papilloma virus

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Research Article

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Abstract

Background: Cervical cancer is considered as the third prevalent cancer concerning the female genital tract. The prevalence of the disease is considerable in many developing countries. However, invasive cervical cancer is preventable, since it has a long pre-invasive period, a feasible screening program, and primary lesions as symptoms for starting the treatment. In this study, we are going to evaluate the effects of heated TC1 extract in combination with Lactobacillus Casei extract and alpha-galactosyl ceramide on mouse model of cervical cancer caused by the papilloma virus.

Material and Methods: Cervical cancer in the mice model was prepared by TC1 cells subcutaneous injection into the left flank of C57BL/6 among 6-8 weeks female mice (n=80). After appearance of palpable tumor, the mice with cervical cancer were randomly devoted to 8 ten-member groups. The mice in some groups were treated with PBS, TC1 cell extract, *Lactobacillus casei* extract, alpha-galactosyl ceramide, and combination of the mentioned factors and then, they were evaluated concerning the splenocyte proliferation, lactate dehydrogenase production, nitric oxide, and the respiratory burst. Moreover, IL-4, IFN-γ, and TGF-β cytokines levels of splenocyte supernatant among the mice were measured.

Result: The findings revealed that the combination therapy group (TC1 cell extract, *Lactobacillus casei* extract, alpha-galactosyl ceramide) significantly increases the splenocyte proliferation, lactate dehydrogenase production, nitric oxide, respiratory burst, and interferon-gamma ($P<0.05$); and significantly reduces production of IL-4 and TGF-β cytokines ($P<0.05$) in comparison to the control and untreated groups.

Conclusion: The study showed that combination therapy of Lactobacillus *Casei* and alpha-galactosyl ceramide is an efficient treatment for cervical cancer in the mice model.

Introduction

The Human Papillomavirus (HPV) as a double-stranded DNA virus, has over 200 various identified genotypes. The infection is assuming the most prevalent sexually transmitted infection (STI), and causes a great number of disorders, including benign lesions (anogenital condylomas) and pre-malignant lesions and different cancers [1]. Cervical cancer is one of the five top common neoplasms among women all over the world [2]. All women face the risk for cervical cancer. It is common in women of over 30. Chronic infection with some kinds of HPV is the main reason for cervical cancer. HPV is a common virus transmits during intercourse. It is present in most sexually active people; however, few women are affected by cervical cancer [3]. The TC1 cervical carcinoma can be applied as an experimental animal tumor cell model for human cervical cancer, because it is highly transplantable, tumorigenic, and invasive. Comparing with the most tumor models, TC1 cell lines can concurrently metastasize from the preliminary tumor in the cervical gland to other sites such as blood, liver, brain, lung, bone and lymph nodes just similar to human cervical cancer [4]. Recent studies show that alpha galactosyl ceramide,

glycosphingolipid derived from a sea sponge known as a combination with anti-metastasis, antitumor and immune system stimulate capability of alpha-galactosyl ceramide and increase the proliferation and activity of NK and NKT cells and production of cytokines [5]. The role of alpha-galactosyl ceramide in the changes of the rats' liver tumor is associated with the increased activity of mononuclear liver cells and NKT as well as increased production of interferon gamma. Furthermore, application of alpha-galactosyl ceramide with CpG in HER2 breast cancer treatment shows the efficient role of alpha-galactosyl ceramide stimulation [6]. Probiotics are organisms believed to improve health conditions. *Lactobacillus casei* is one of the Lactobacillus bacteria considered a safe probiotic [7, 8]. Being anticancer is the most crucial property of probiotic bacteria, along with some other inferior properties [8]. It was also proved that some strains of including *Lactobacillus Casei* may modify immune responses against solid tumors when used orally [9, 10]. For instance, in the reports the regular *Lactobacillus Casei* intake can improve the cytotoxicity of the natural killer cells and adoptive immune responses in rats bearing invasive ductal carcinoma. Moreover, the adjuvanticity potential of a killed preparation of *Lactobacillus Casei* was investigated in early surveys [11]. However, there is little data on the possible adjuvant benefits of the killed preparation of *Lactobacillus Casei* in a tumor vaccine. Therefore, this study was conducted to assess the efficacy of a new therapeutic method against cervical cancer made by mixing an extract of heated TC1 cells and a heat-killed preparation of *Lactobacillus Casei* and adding alpha galactosyl ceramide.

Materials And Methods

Reagents

The authors prepared Griess reagent kit, Dulbecco's modified eagle medium (DMEM), and Fetal Bovine Serum from Kala zist-Iran. Nitrotetrazolium blue tetrazolium (NBT), dimethylthiazol-2, 5-diphenyl tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), dioxin, and phosphate-buffered saline (PBS) were purchased from Sigma-Aldrich (St. Louis, MO). Enzyme-linked immunosorbent assay (ELISA) kits were ordered from Qiagen (Hilden, Germany).

Bacterial Strains and Growth Conditions

Lactobacillus Casei (ATCC: 393) was purchased from Pasteur Institute of Iran. A Rogosa's medium was applied to cultivate the bacteria. The process was performed at 37 °C for 24 hours, washed with PBS, heated at 56 °C for 60 minutes, and lyophilized.

Cell Culture

Pasteur Institute-Iran provided us with TC1 cells. The cells were cultured at 37°C humidified atmosphere, using 5% CO2 and kept in monolayer cultures in DMEM supplemented with 10% FBS (Figure 1). Also, 10^4 TC1 cells were exposed freeze-and-thaw (-196 °C, 30 min) and nonlethal heated shock (43 °C, 30 min), then centrifuged to be used as a killed cell extract in the treatment of tumor mice.

Experimental design, mice and Tumor Induction

C57BL/6 female mice (n=80) with an age of 6-8 weeks were purchased from Pasteur Institute in Iran. The experimental processes were in compliance with the Iran Ministry of Health regulations, and approved by the Medical Ethics Committee for Animal Studies of Islamic Azad University (IR.IAU.PS.REC.1399.265). The mice were kept under standard conditions, i.e. temperature of 22–24 °C, 12-hour light/dark cycles and standard food and water. All the mice were acclimatized to the environment for 1 week prior to the experimentations and then 1×10^4 viable tumor cells in 50 µL of PBS was injected subcutaneously in their left flanks with (Figure 2). Then, rats were randomly divided into 10 equal groups (Table 1). All groups received treatment in 100 µl volume and twice a 1-week interval. One month after treatment, Sampling was started.

Table 1

The characteristics of the studied groups

Group	Characteristics
Control	100 µL of PBS
Heated TC1	10^4 heated TC1 cells
Heated *L. casei*	2×10^8 CFU/mL of Heated *Lactobacillus Casei*
α-GalCer	5 µg of Alpha galactosyl ceramid
Heated TC1+Heated *L. casei*	Combined of Heated TC1 and Heated *L. casei*
Heated TC1+α-GalCer	Combined of Heated TC1 and α-GalCer
Heated TC1+Heated *L. casei+ α-GalCer*	Combined of Heated TC1, Heated *L. casei* and α-GalCer
Gardasil	50 µg of Gardasil vaccine

The proliferation potential of lymphocytes

The MTT assay was used to check proliferation index in the splenocyte population. The splenocytes were put in 96-well flat-bottomed plates and in a DMEM medium added by 10% FBS (1×10^5 cells/100 µl/well) and stimulated with antigens derived from the tumor cells by freezing and thawing (20 µg/mL). After 72 hours of incubation, the cultures were pulsed with 20 µl of the MTT solution (5 mg/mL) for 4 hours at 37 °C. Then, 150 mL of DMSO was added and shaken severely to dissolve formazan crystal. The optical density (OD) at 492 nm was measured using a microplate reader (Dynatech, Denkendorf, Germany). The experiments were conducted in triplicate sets.

Lactate dehydrogenase assay

Cytotoxic activity was investigated using a lactate dehydrogenase (LDH) detection kit (Takara Company, Tehran-Iran). This assay is a simple fast colorimetric medium for cytotoxicity quantification through determining activity of LDH released from the damaged cells. LDH is a stable cytoplasmic enzyme, which is available in most cells. The splenocytes were used as effector cells, and the TC1 cell lines were applied as target cells. The effector and target cells were washed with the assay medium (RPMI-1640 with 1% bovine serum albumin) and co-cultured at a ratio of 50 effector cells to 1 target cell in 96-well round-bottomed plates for 6 hours at 37 °C. Subsequently, the plates were put in centrifuge apparatus and the supernatants were transferred to 96-well flat-bottomed plates. Then, 100 µl of the LDH detection mixture was added to each well and incubated for 30 minutes at the room temperature. The OD was measured using a microplate reader (Dynatech, Denkendorf, Germany) at 492 nm.

Measurement of nitric oxide in splenocytes population

The potential of nitric oxide production was assessed by measuring the nitrite level in the splenocyte culture supernatants and using the Griess reagent. After the splenocytes being cultured, the cell free supernatants (50 µl) were collected and intermixed with 50 µl of Griess reagent (containing 0.1% sulfanilamide, 3% phosphoric acid, and 0.1% naphthyl-ethylenediamine). The resulted mixture incubated at room temperature for 10 minutes in the dark. After the incubation, microplate reader reads the absorbance at 540 nm (Dynatech, Denkendorf, Germany). The nitrite concentration is estimated based on a standard curve.

Cytokine assay

One week after the last immunotherapy, half of the mice were euthanized to measure the cytokine assay induced in splenocytes. Splenocytes were isolated from the mice in an aseptic environment, and single-cell suspensions of the splenocytes were prepared in a DMEM medium added with 10% FBS and red blood cells (RBCs) were removed by RBC lysis buffer. Next, the cell suspensions (2×10^6 cells/mL) were incubated in 24-well plates and pulsed with antigens. These antigens were derived from the tumor cells by freezing and thawing (20 µg/mL). Tumor antigen was prepared as described above. The culture supernatants were collected after 72 hours. The production of IFN-γ, IL-4, and TGF-β was evaluated by the ELISA according to the manufacturer's instructions.

Statistical Analysis

The statistical analysis was conducted using the Kruskal–Wallis test, followed by pairwise comparisons, using the Mann–Whitney U-test and with the Bonferroni adjustment. The Kaplan–Meier estimator was applied to evaluate the survival function based on the lifetime data. The results are shown as means ± SD. $p<0.05$ was considered statistically significant.

Results

Proliferation index of splenocytes

Splenocytes were cultured in the presence of TC1 antigen. The cell proliferation was measured by MTT. The results revealed that splenocytes proliferation in single-factor treatment groups was not significantly different from that in the control group (p0.05). However, splenocyte proliferation increased significantly in the combination therapy groups relative to the control group (p0.05) (Fig. 3).

Nitric oxide production rate

The findings revealed that the amount of nitric oxide production in one-factor treatment groups increased significantly in comparison to the control group ($P<0.05$). The same is true, for the combination therapy groups ($P<0.05$). The highest amount of nitric oxide production belonged to the combination treatment group with TC1 extract, bacterial extract and alpha-galactosyl ceramide (Fig. 4).

Production of lactate dehydrogenase

The findings revealed that the amount of lactate dehydrogenase production in one-factor treatment groups increased significantly relative to the control group ($P<0.05$). The same is true for the combination therapy groups ($P<0.05$). The highest amount of nitric oxide production belonged to the combination treatment group with TC1 extract, bacterial extract and alpha-galactosyl ceramide (Fig. 5).

Splenocytes supernatant cytokines

The findings of cytokine production revealed that the level of IFN-γ production (Fig. 6 A) in all single-factor and multi-factor treatment groups had a significant increase relative to the control group ($P<0.05$). The highest production amount was in the three-factor combination therapy and the Gardasil groups. The results of IL-4 production also showed that all single and multifactorial treatment groups except TC1 cell extract group had a significant decrease in comparison to the control group ($P<0.05$) (Fig. 6 B). The level of TGF-β production decrease significantly just in the group treated with bacterial extract ($P<0.05$) (Fig. 6 C). And all multifactorial treatment groups revealed a significant decrease in comparison to the control group ($P<0.05$).

Discussion

Overgrowth of cells is called cancer. Since uncontrolled cell proliferation and programmed resistance to death are the main characteristics of cancer cells, what causes death of cancer cells it may be considered as anticancer agents [12]. Resistance to chemotherapy has been a major problem in recent decades. Studies revealed that at least half of the cancers are caused by harmful compounds in the diets [13]. The use of multi-factor combination therapies has attracted the attention of researchers. In the present study, we used two agents, Lactobacillus casei (probiotic) and alpha-galactosyl ceramide (NKT cell stimulator) for this purpose. Nutritional compounds and their relationship with human health are of great importance. Probiotics as non-pathogenic microorganisms are present in the digestive system of human and have beneficial effects on the hosts. Consumption of probiotics leads to the production of a wide range of fermentation products such as high concentrations of short-chain fatty acids [14]. Among all

probiotics, bacteria of the Lactobacillus family such as Lactobacillus acidophilus, Lactobacillus casei and Lactobacillus delbrueckii are the most efficient components of the normal intestinal flora among humans and animals. The role of lactobacilli probiotics in facilitating the treatment of colorectal cancer is known and for this reason, many studies have been focused currently on investigating the cytotoxic effects of probiotic bacteria [15]. NKT cells are innate immune components restricted to the CD1d receptor and possess the simultaneous characteristics of the T cells and NK cells. INKT cells are the main group of NKT cells in humans and rats. They express the Vα24-Jα18 and Vα14-Jα18 TCRα chains in humans and rats, respectively. Activated iNKT cells rapidly secrete both the cytokines Th1 and Th2, and activate NK cells and other immune cells to stimulate antitumor immune responses [16]. Alpha-galactosyl ceramide (α-GalCer) as the main receptor ligand for iNKT cells, is a sphingolipid. It was firstly isolated from Agelas mauritianas sponge in 1994, using chloroform and HPLC purification techniques. Rats treated with α-GalCer showed strong antitumor activity against B16 metastatic melanoma cells. α-GalCer showed also antitumor synergistic effects, when used with Adriamycin as a chemotherapeutic agent. Many studies have identified α-GalCer as a non-specific (innate) immune system stimulant. The literature shows that the antitumor properties of α-GalCer are mediated by CD1d-bound iNKT cells [17]. Therefore, alpha-galactosyl ceramide is applied due to the stimulation of the innate immune arm, especially iNKT. The results of the present study revealed that the combination of multifactorial therapy (probiotic Lactobacillus casei and alpha galactosyl ceramide) and Gardasil vaccine reduce tumor volume and increases life expectancy of the rats compared to those in the control group. All the mentioned changes were statistically significant (p□0.05).

Abdolalipur et al. (2020) showed that levels of IFN-γ, IL-4 and IL-12 after treatment with GM-CSF with L. casei probiotic were significantly higher than those in other groups. Also, the apoptosis-inducing ligand associated with intra-tumor tumor necrosis factor increased significantly. Furthermore, tumor analysis showed that probiotic group therapy reduced IL-10 accumulation in the tumor microenvironment of the treated rats. Additionally, tumor volume analysis revealed that probiotic group therapy suppressed tumor growth. Abdolalipour et al. (2020) demonstrated that the combination of GM-CSF and probiotics suppresses HPV-related tumors and enhances specific antitumor immune responses [18].

Jacouton et al. (2019) determined the systemic role of T cells in protecting tumors through a negative correlation of tumor size, T cell subpopulations and increased levels of Foxp3 in tumor-bearing rats. And lastly, a negative relationship between tumor size and NK + cells, as well as local migration of NK cells and cytotoxic activity specific for BL23 treatment were observed. Studies showed that the IL-2 signaling pathway has a crucial role in antitumor effects of the probiotic strain *L. casei* BL23. They also suggest more research on probiotic strains for therapeutic applications in clinical practice, specifically for the colorectal cancer treatment [19].

Jafari et al. (2017) suggested that animals receiving probiotics face better survival curves and tumor growth rates than tumor-bearing rats and negatively controlled rats. Immunization considerably enhances nitric oxide production and cytotoxicity of natural killer cells in spleen cell culture of tumor-bearing rats. Moreover, immunotherapy increases IFN-γ secretion, while it decreases IL-4 and TGF-β secretion in the

splenic population in comparison to the splenocytes from other groups. Combined immunotherapy with heated 4T1 cells and heated Lactobacillus casei also leads to useful results in the rat breast cancer model [20]. Yazdi et al. (2009) showed that L. casei oral administration could inhibit tumor growth and increase local inflammation in DTH assays due to increase of the efficiency of immune responses. In addition, oral administration of Lactobacillus casei may regulate immune responses and upset the Th1 balance and may be efficient for cancer immunotherapy; however, further studies are necessary to investigate other mechanisms of this effect [21]. Soltan et al. (2012) showed that lactobacilli supernatant reduces cell proliferation and increases cell apoptosis. No meaningful effect on cell necrosis has been reported. On the other hand, lactobacilli extract decreases cell proliferation and increases cell apoptosis. Lactobacillus extract also leads to cell necrosis. Moreover, supernatants and cell extracts of probiotic agents reduce cell migration and invasion [22]. Zhang et al. (2019) reported that NKT cells are quasi-innate T cells with CD1d restriction that express T cell receptors and NK cell markers. The main group of NKT cells is the same among humans and rats. The most common function of iNKT cells is being potentially antitumor. Since discovery of the function from 25 years ago, the primary ligand of iNKT cells and the, α-galactosyl ceramide (α-GalCer) has been used in more than 30 antitumor clinical trials. To realize its therapeutic potential, several preclinical models have been developed in order to optimize α-GalCer-based designs and strategies. However, since there is no standard protocol for α-GalCer, we reviewed preclinical studies, focusing on the B16 melanoma model [23]. The goal was to identify the best plan for α-GalCer therapy. We then reviewed recent advances in the development of clinically relevant murine models. The new murine models with a human CD1d / iNKT cellular system was invented with new ever-emerging iNKT cellular ligands. New delivery strategies have significantly improved α GalCer therapy and preclinical models. Optimizing the new strategies, it can be hoped that there is full anti-tumor potential. The potential for α-GalCer will be realized in the near future [23]. Lee et al. (2016) evaluated the immunomodulatory effect of dead Lactobacillus plantarum with nLp size in RAW 264.7 cells and the rat primary splenocytes. nLp is the dead, shrunk, processed form of L. plantarum nF1, isolated from kimchi (a traditional Korean fermented cabbage) less than 1 micrometer in size. The nLp treatment was found out to stimulate nitric oxide production in RAW 264.7 macrophages more than in pure live L. plantarum, and the stimulatory properties may have been largely derived from its cell wall. In addition, nLp induced rat spleen cell proliferation was greater than that of pLp in particular, high-dose proliferation stimulated as much as lipopolysaccharide at 2 μg / ml. In addition, according to the results of cytokine profiles in spleen cells, nLp treatment promoted Th1 (IL-12 (p70) responses instead of Th2 responses and increased Th17. These findings suggest that dead nLp has potential application, as a functional nutrient to enhance the immune response and in particular as a means of inducing Th1 / Th17 immune responses [24]. Jafari et al. (2017) Reported that the use of the probiotic Lactobacillus casei reduced tumor volume, while increased longevity, spleen cell proliferation, lactate dehydrogenase production, nitric oxide, respiratory explosion, IFN-γ levels and lastly decreased levels of IL-4 and TGF-β relative to the control group in rats with breast cancer [20]. This study was in line with ours. Abdolalipour et al. (2020) reported that the probiotic lactobacillus casei in combination with cytokine GM-CSF reduced tumor volume, increased rat lifespan, splenocytes proliferation and cytokine levels of IFN-γ and IL-12 in rat cervical cancer model [18]. This study was in line with our study. Jacouton et al. (2019) reported that the use of

the probiotic Lactobacillus casei in C54BL/6 rats with TC1-induced cervical cancer increased cytokine IL-2 production, NK and T cell population and activity. It also reduces the population of TGF-β-producing suppressor T cells [19].

These findings was again in line with our study. Yazdi et al. (2009) reported that lactobacillus are the most known bacteria in the probiotic family that their anti-tumor properties have been studied by many researchers. The properties of these bacteria are probably due to the regulatory characters of the immune system. The researchers found that in rats receiving lactobacillus casei, the rate of increase in the 48-hour delayed response to stimulation with tumor-specific antigen was higher than that in the control group with PBS, which is a sign of stimulation of Th1 memory cells. In addition, the rate of tumor growth in mice that received lactobacillus was lower than that in the control group, i.e. the immunity of this group of rats against the tumor increased compared to the control group. Moreover, histopathological results revealed a significant increase in intra-tumor necrosis of the larynx prepared from the tumor of lactobacillus receptor rats in comparison to the control group rats [21].

This study was also in line with ours. According to Soltan et al. (2012), lactobacilli supernatant decreased cell proliferation and increased colorectal cancer cell death but had no effect on cell necrosis. However, when cancer cells are exposed to lactobacilli extracts, these extracts, in addition to reducing cell proliferation and increasing cell apoptosis, also lead to cell necrosis [24, 22]. This study was in line with our study. Falayova et al. (2017) reported that the use of the probiotic lactobacillus casei significantly decreased IL-10, IL-4 and TGF-β levels and significantly increased IL-12, IL-1 and IFN-γ levels [25]. This study was in line with our study. Qabeleh et al. (2016) claimed that the use of alpha-galactosyl ceramide as an adjuvant in the DNA of the proposed cervical cancer vaccine increased lymphocyte proliferation, IFN-γ and IL-12 cytokine levels, and inhibited tumor growth [26]. This study was in line with our study. Kim et al. (2010) reported that the use of alpha-galactosyl ceramide in combination with tumor antigen-stimulated dendritic cells increased the population of anti-tumor cell T lymphocytes and decreased tumor volume in cervical cancer rats [27]. This study was in line with our study. Ando et al. (2015) showed that the use of alpha-galactosyl ceramide in the treatment of a mice model of metastatic lung cancer increased the production of cytokine IFN-γ by spleen cells and the population of anti-tumor cell killer T cells [28]. This study was in line with our study. Li et al. (2014) explained that the use of alpha-galactosyl ceramide was effective in the treatment of B-cell lymphoma. It increased the production of IFN-γ and IL-2 by splenic cells and improving the specific pathology of B-lymphoma [29]. This study was in line with our study.

Conclusion

According to the results of the present study, it can be said that the combined treatment of rat model of cervical cancer with heated extract of TC1 and Lactobacillus casei cells with alpha galactosyl ceramide is useful and effective. In addition, the present study showed that the beneficial effects of this compound may be in part due to the diversion of immune responses from the production of anti-inflammatory cytokines such as IL-4 and TGF-β to proinflammatory cytokines such as IFN-γ and stimulation. Safety is

inherent. Also, considering that it increased the rate of respiratory explosion and nitric oxide as representatives of the innate immune system, it can be concluded that in this type of combination therapy, both arms of the immune system are activated.

Declarations

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Figures

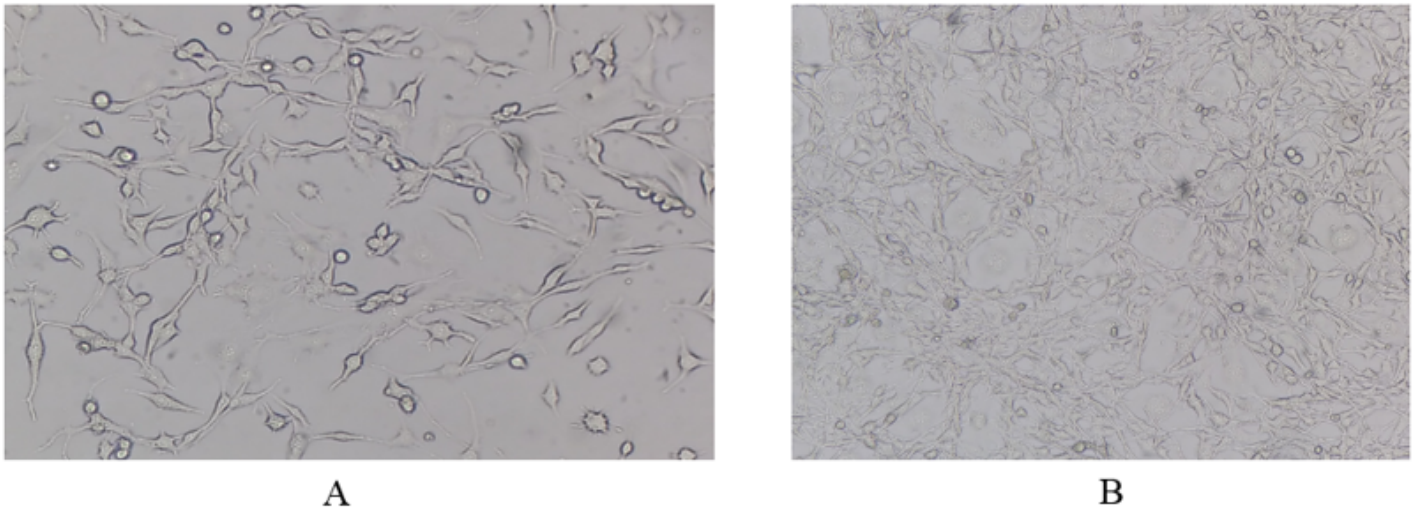

Figure 1

TC1 Cancer cell growth under cell culture condition. A) 3 days after cell culture B) 7 days after cell culture.

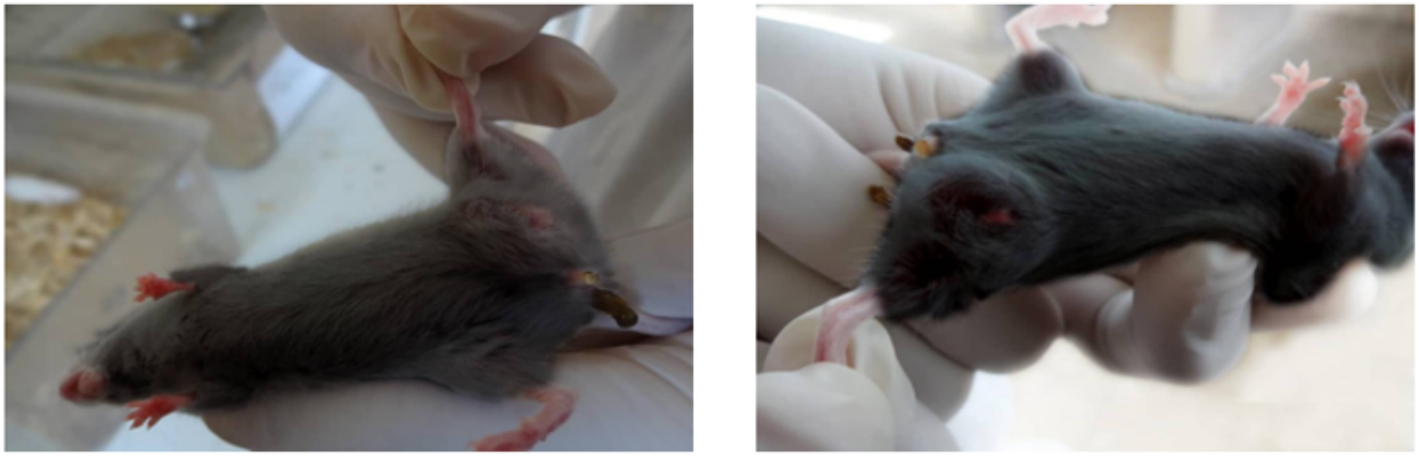

Figure 2

Tumor mice model with palpable tumor. A. 14 days after injection, B. 28 days after injection.

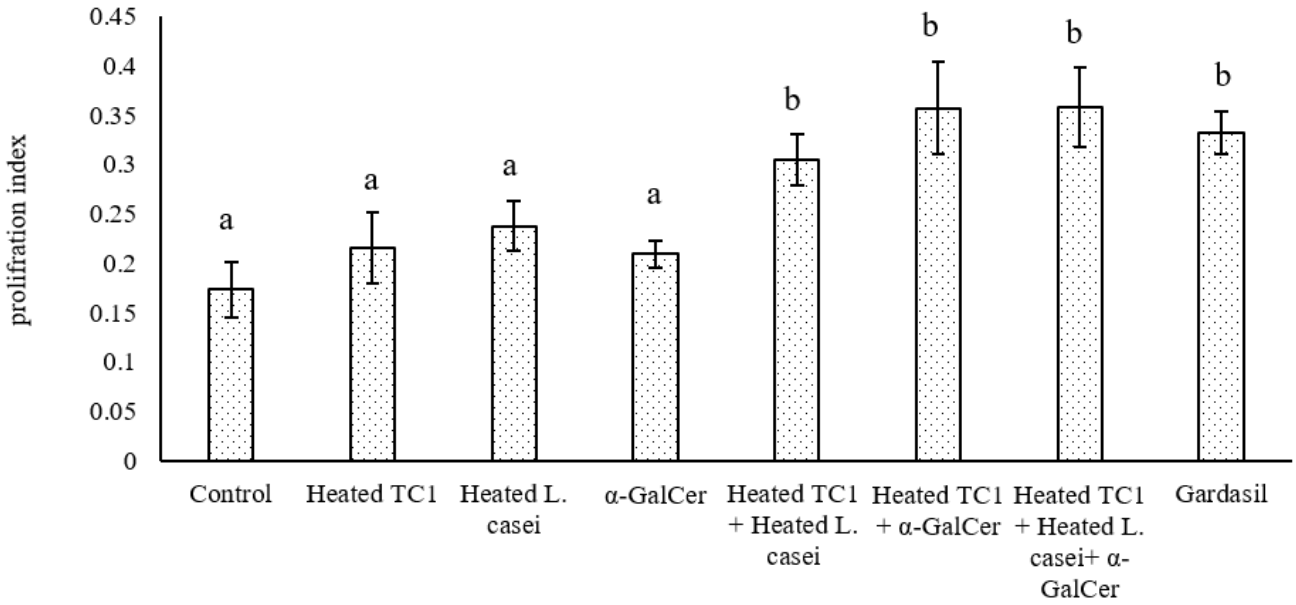

Figure 3

Effects of single and combined treatment on the proliferation of splenocytes. The values were normalized. Significant statistical differences between groups in each index are shown by the different superscript letter ($p < .05$).

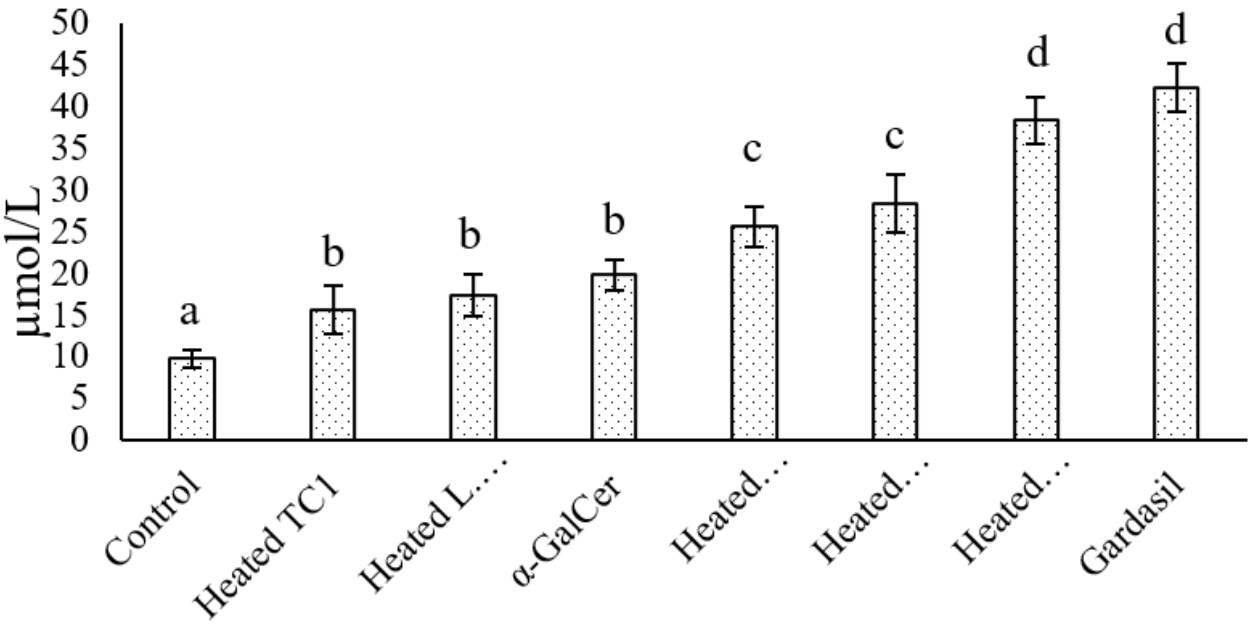

Figure 4

Effects of single and combined treatment, on the nitric oxide production of splenocytes. The values were normalized. Significant statistical differences between groups in each index are shown by the different superscript letter ($p < .05$).

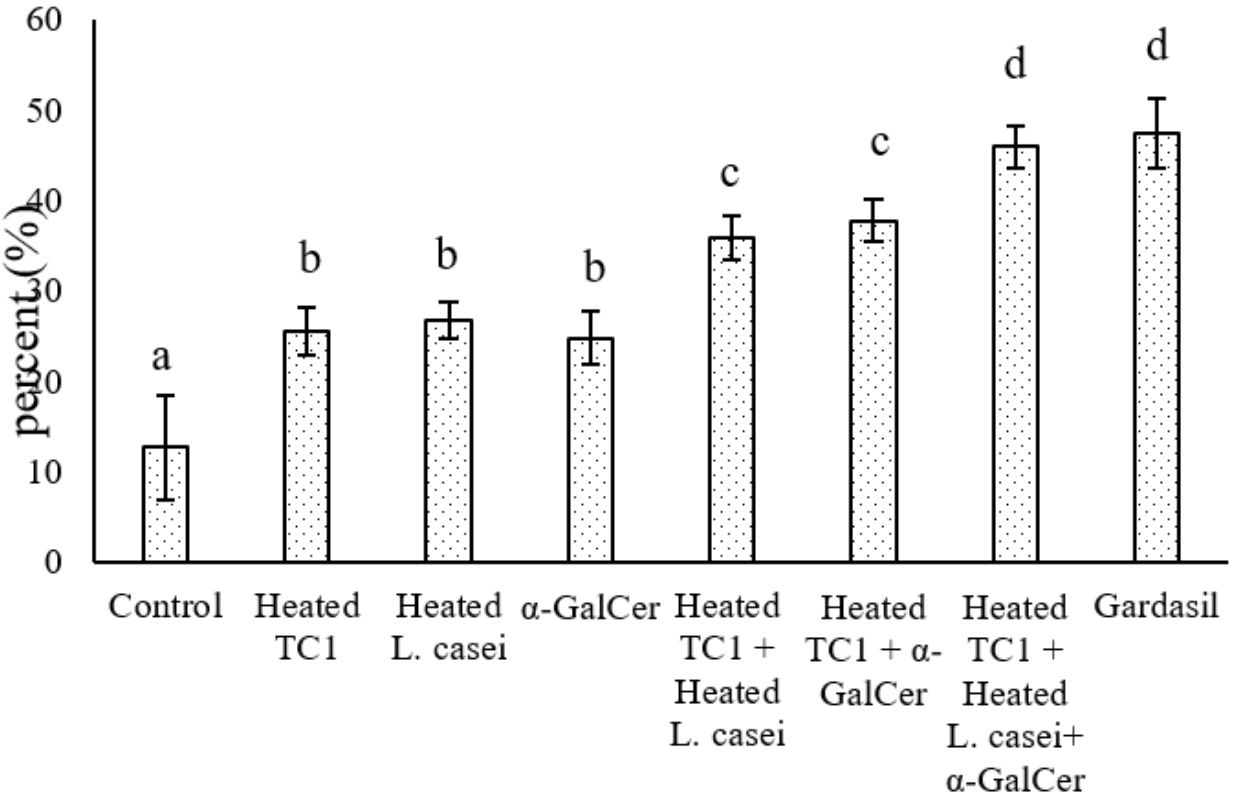

Figure 5

Effects of single and combined treatment, on the lactate dehydrogenase production of splenocytes. The values were normalized. Significant statistical differences between groups in each index are shown by the different superscript letter ($p < .05$).

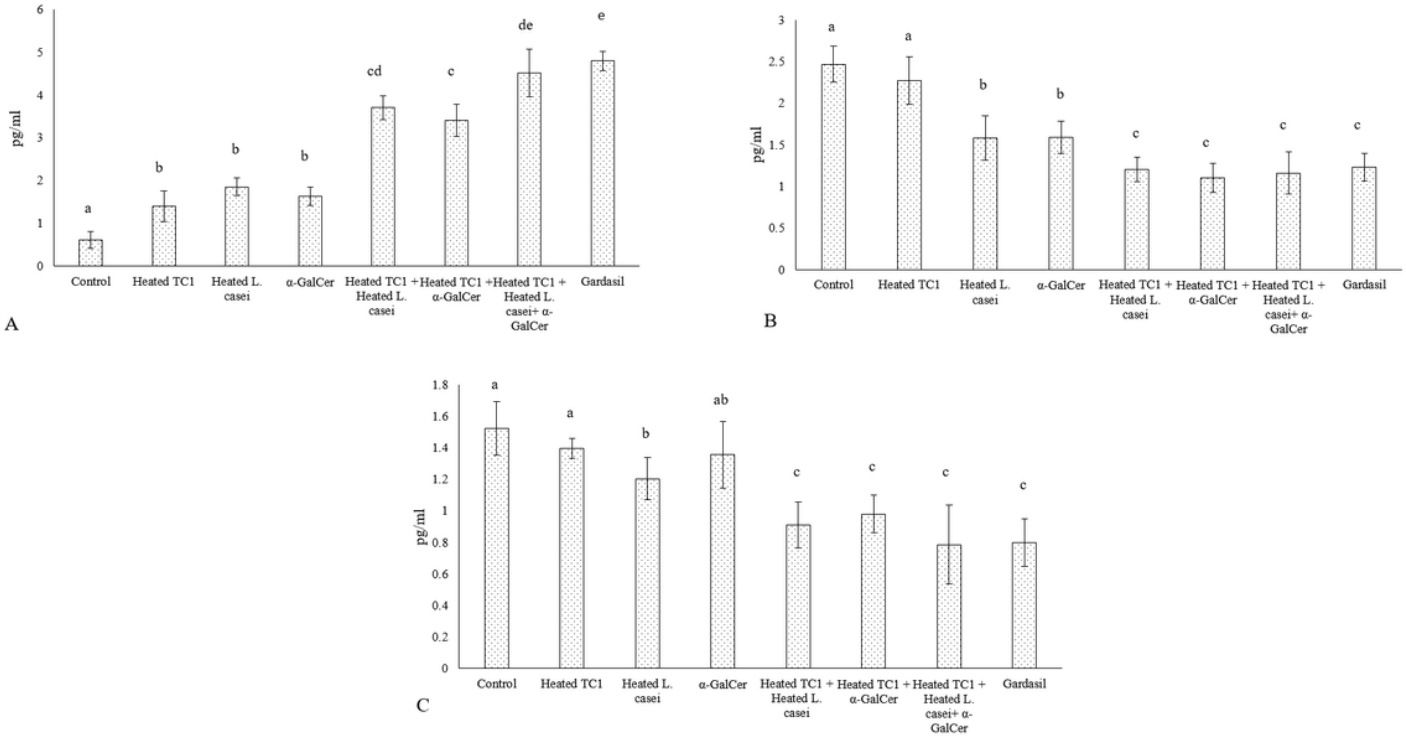

Figure 6

Effects of single and combined treatment, on the cytokine production of splenocytes (IFN-γ), B (IL-4) and C (TGF-β). The values were normalized. Significant statistical differences among groups in each index are shown by the different superscript letter (p <0 .05).