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Modulated electro-hyperthermia therapy combined with Korean mistletoe extract treatment exerts a strong anti-tumor activity by enhancing cellular and humoral immune responses in mice

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ABSTRACT

Electro-hyperthermia therapy (EHT) has been known to cause temperature-dependent cell death and enhance the effects of conventional antitumor treatments, such as chemotherapy and radiotherapy. Furthermore, EHT modulates the innate and adaptive immune systems. Mistletoe is one of the most broadly studied complementary and alternative therapeutic agents for cancer treatment due to its ability to stimulate the immune systems. This study aimed to investigate the effects of EHT and mistletoe therapy combination on immune responses. Tumors induced by B16-BL6 melanoma cells were treated twice with modulated EHT (mEHT) (43°C for 10 or 20 min) and with intravenous injection of a Korean mistletoe extract (KME). We examined the level of interferon (IFN)-γ, granzyme, interleukin (IL)-2, IL-10, and tumor-specific antibodies using enzyme-linked immunosorbent assay methods to further study the immunological responses in the combination of mEHT and KME. Additionally, cytotoxic T lymphocyte (CTL) activity is investigated. In this study, we revealed a significant anti-tumor immunological activity elevation in tumor-bearing mice by combined mEHT and KME therapy. Specifically, the combination of mEHT and KME treatment was effective in inhibiting tumor growth in mice. The combination treatment elicited CTL immune response and increased IFN-γ and granzyme secretion. Particularly, the co-treatment appeared to efficiently suppress the immune signal related to tumor-associated macrophage differentiation. Importantly, tumor cell-specific antibodies could be induced in mice after mEHT-treated tumor cell immunization, which represent a promising cancer vaccine strategy. Thus, our results indicate the therapeutic actions of KME as a feasible partner of mEHT, suggesting its potential candidate for cancer immunotherapy.

Abbreviations: APC, Antigen-presenting cell; CTL, Cytotoxic T lymphocyte; EHT, Electro-hyperthermia therapy; ELISA, Enzyme-linked immunosorbent assay; HSP, Heat shock protein; KME, Korean mistletoe extract; NK, Natural killer; PBS, Phosphate-buffered saline; QOL, Quality of life; RF, Radio-frequency; TAM, Tumor-associated macrophage

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Introduction

Electro-hyperthermia therapy (EHT) is a form of cancer therapy that induces temperature-dependent damage to cell proteins and structures that leads to cell death (van der Zee 2002). Various changes occur inside the cells when tumor cells are heated at 41°C – 42°C, which then renders the cancer cells more vulnerable to other anti-tumor treatments (Hildebrandt et al. 2002). Therefore, EHT is commonly used with other cancer treatment, such as chemotherapy and radiation therapy (Wust et al. 2002). Modulated EHT (mEHT), also known as oncothermia, is a hyperthermia device that is used for treating various cancer types. Malignant lesions are preferentially heated relative to adjacent normal tissues in clinical mEHT application (Andocs et al. 2009; Hegyi et al. 2013a, 2013b). mEHT, combined with classical or

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targeted chemotherapeutic agents, has been demonstrated to be markedly effective in clinical trials (Wismeth et al. 2010; Gadaleta-Caldarola et al. 2014).

Whole-body mEHT at fever range temperatures has shown general immune system stimulation in the hosts (Evans et al. 2015), and local mEHT of tumors elicit anti-tumor immunity (Wang et al. 2001a; Skitzki et al. 2009). The mechanisms underlying the heat-induced stimulation of immune response include heat shock protein (HSP) generation and antigen-presenting cell (APC) activation (Skitzki et al. 2009). HSPs that are produced in response to hyperthermia treatment act as a signal to elicit immune responses (Wang et al. 2001a; Schmitt et al. 2007). HSP-derived tumor peptide complex is effectively endocytosed by APCs through several HSP receptors, and it activates dendritic cells, which are the most potent APCs (Matsumoto et al. 2011). Notably, mEHT treatment has been reported to create a more favorable microenvironment for immune response induction than conventional hyperthermia, thereby inducing tumor cell apoptosis (Cha et al. 2015; Tsang et al. 2015a).

Mistletoe is a semi-parasitic plant that has been utilized as a traditional medicine in many countries to treat various human illnesses (Adesina et al. 2013; Moghadamtousi et al. 2014). Mistletoe is widely used in Europe where diverse mistletoe extracts are manufactured as injectable anti-cancer drugs or supplements (Galun et al. 2015). The anti-cancer effect of Korean mistletoe (Viscum album coloratum) has been widely studied in the past few decades. Reportedly, the Korean mistletoe extract (KME) enhances the host defense system against tumors via macrophage and natural killer (NK) cell activation, which indicates that KME is a potential anti-cancer immunotherapy agent (Yoon et al. 1998b; Yoon et al. 2001; Yoon et al. 2003; Kim et al. 2014).

This study aimed to delineate the anti-tumor immune response elicited by the combination of mEHT and KME treatments. The combined therapy effectively inhibited tumor growth by significantly increasing the T lymphocyte activity, interferon (IFN)-γ, and granzyme secretion. Consistently, the combined treatment of mEHT and KME inhibited tumor-associated macrophage (TAM) differentiation. Furthermore, mEHT-treated tumor cells were observed to be potentially useful as a tumor vaccine.

Materials and methods

Cell culture

The mouse melanoma cell line B16-BL6 and colon carcinoma cell line 26-M3.1 were grown in DMEM (GIBCO-BRL; Life Technologies, Rockville, MD, USA) supplemented with 10% fetal bovine serum and 0.03% l-glutamine. Cultures were maintained at 37°C in a humid atmosphere of 95% air and 5% CO_2.

Treatment with mEHT

The Lab-EHY device (OncoTherm, Hungary) was used for mEHT treatment *in vitro* and *in vivo* following the instruction manuals (https://www.oncotherm.com/web/phy/EHY%20Lab.pdf). In the case of mEHT, the Lab-EHY machine was set up at each temperature. Tumor cells and tumors were also heated with water bath.

Preparation of KME

The leaves of Korean mistletoe were grounded and homogenized with 10 volumes of distilled water. The suspension was stirred overnight at 4°C and centrifuged at 15,000×*g* for 30 min. Then, the supernatant was filtered and lyophilized. The obtained brown powder was dissolved in phosphate-buffered saline (PBS) and stored at 4°C until use.

Animals and tumor induction

Female C57/BL6 mice of 5–7 weeks old were used for the experiments (DBL, Incheon, Republic of Korea). The animals were housed (4–5 mice per cage) in a temperature-controlled room. After 7 days of acclimatization, each mouse was intradermally inoculated on the back with 1×10^5 B16-BL6 melanoma cells in 0.05 ml of saline. Mice were anesthetized by intraperitoneal injection of 250 µl of solutions containing 1:1 mixture of avertin stock and 5-fold diluted Rompun. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Dongguk University (IACUC No. 2021-020-3).

Cytotoxic T lymphocyte (CTL) assay

CTL assay was performed as previously described (Rohrer et al. 2006). Briefly, spleen cells were isolated from control and treated mice and were minced with a homogenizer and washed with PBS. After removing the erythrocytes, the splenocytes were cultured with B16-BL6 melanoma cells in a 37°C incubator under humidified 5% CO_2 atmosphere. Cytotoxicity assays were done by incubating spleen cells (effector cells [E]) with 1×10^5 melanoma cells (target cells [T]) at an E:T ratio of 100:1, 50:1, or 25:1. The specific cytotoxicity

was measured using the LDH assay kit (Promega, Madison, WI, USA) after 6 h of incubation.

Measurement of tumor-specific antibody titers

Mice were immunized by subcutaneous injection of 2×10^5 tumor cells. The serum antibody titers from the immunized mice were measured by ELISA. Briefly, approximately 1×10^5 tumor cells were plated on a flat-bottomed microtiter plate and fixed with 70% methanol. The plate was then blocked with 1% skim milk and washed with PBS. Serum was diluted 100-fold, added to the wells, and incubated for 2 h. Antibody titers were measured using HRP-conjugated rabbit secondary antibody to mouse immunoglobulin (Ig)G (R&D Systems).

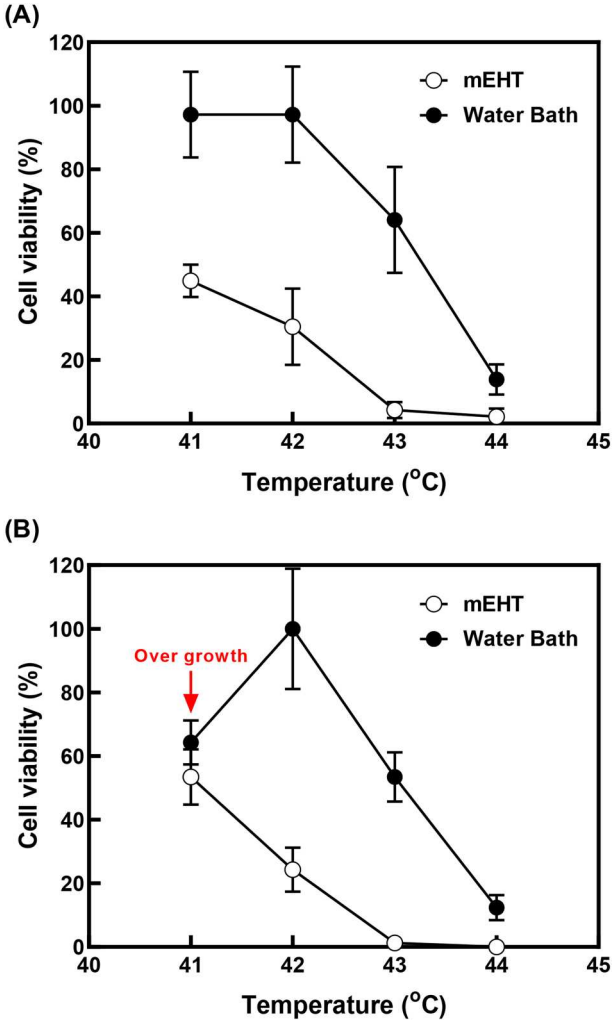

Figure 1. Comparison of the cytotoxic effects of heating with mEHT and that with a water bath. The B16-BL6 cells were heated with mEHT or a water bath for 20 min, and the cellular viability was measured after incubation for 24 h (A) or 72 h (B) at 37°C.

Statistical analyses

All graphs were generated using GraphPad Prism 6.0. Statistical analyses were conducted using one-way analysis of variance (ANOVA), and significant differences between treatments were identified using Bonferroni's post hoc test. All results are shown as mean ± standard deviation from at least three independent experiments. *, **, and *** indicate significance at $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$, respectively.

Results

Determination of optimal temperature for EHT

The B16-BL6 tumor cells were treated in suspension with mEHT at 41°C to 44°C for 20 min to determine the optimal temperature for mEHT. The control group was heated at the same temperatures with a water bath. After the treatments, tumor cells were plated on a 96-well plate and incubated for 24 or 72 h, and the viability of the cells was determined by the improved MTT method (Sladowski et al. 1993). We found that mEHT treatment at 41°C for 20 min reduced the viability to 45% to 50% and mEHT at 42°C to 43°C for 20 min reduced the viability to almost 0%. Meanwhile, approximately 50% to 60% of cells were viable after heating at 43°C for 20 min with a water bath (Figure 1(A)). Our result that mEHT treatment significantly reduced cell viability suggests a potential role of mEHT in cell death induction; however, further apoptosis and necrosis assays are required to confirm this effect.

Effect of mEHT treatment time on tumor growth in an animal model

The effects of mEHT in combination with KME treatment (intravenous) on the intradermal B16-BL6 melanoma that is grown in the back of C57/BL6 were investigated. Tumors were treated twice with mEHT on 7 and 11 days after melanoma cell inoculation. The tumor size on day 7 was 0.6–1.6 mm in diameters. The mEHT was done with 1.8–2.4 W RF, which raised the tumor temperature to 43°C, for 10 or 20 min. Tumors were also heated with a water bath at 43°C as the control. The host mice were treated with an intravenous KME injection simultaneously with tumor cell inoculation. The KME treatment was repeated five more times beginning the next day after tumor cell inoculation at 4-day intervals, in a total of six treatments (Figure 2). We found that the treatment of mEHT alone for 10 min did not show the reduction of tumor size whereas KME alone treatment significantly inhibited the tumor growth. The

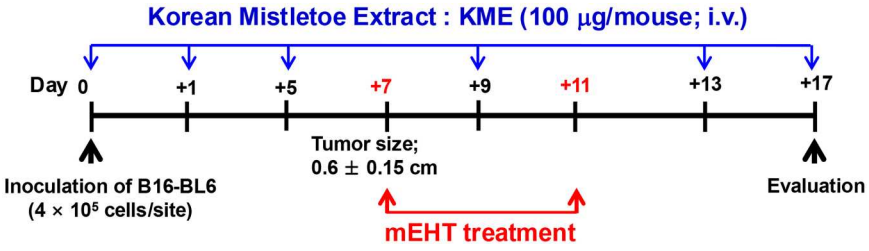

Figure 2. Schedule of mEHT and KME treatment.

combinational treatment of mEHT and KME was slightly more effective than KME alone for tumor growth inhibition (Figure 3(A)). However, the treatment of mEHT for 20 min with KEM combination treatment showed the excellent effects in suppressing tumor growth (Figure 3(B, C)). These finding suggest that the co-administration of mEHT and KEM has strong effect on inhibiting tumor growth.

Effect of mEHT treatment on the mode of cytokine production by TAMs

Macrophages have two major groups that are classified into M1 and M2 (Mosser and Edwards 2008). M1-type macrophages are activated by LPS or IFN-γ and produce high IL-12 concentrations, which cause inflammatory responses and suppress tumor growth and metastasis. Contrastingly, M2-type macrophages secrete high IL-10 levels and low IL-12 levels and are involved in the anti-inflammatory response, wound healing, and tumor development (Chanmee et al. 2014). Solid tumors are composed of not only malignant cells but also immune cells recruited from the blood circulations (Martinez and Gordon 2014). Among these immune cells in tumor tissue, macrophages are common, and most macrophages are tumor-associated macrophages (TAMs). These TAMs are mainly of the M2 type and actively promote tumor growth (Pollard 2004; Galdiero et al. 2013; Noy and Pollard 2014).

We treated the macrophages with mEHT at 43°C for 20 min and then incubated them at 37°C to investigate the effects of mEHT on the macrophage differentiation pattern. We found no notable changes in the IL-12 levels, which is a typical cytokine that is produced by M1-type macrophages, following mEHT treatment (Figure 4(A)). Meanwhile, the pro-inflammatory cytokine levels, such as TNF-α, were increased by mEHT (Figure 4(B)). IL-10 is a typical cytokine that induces macrophage polarization to M2 type in macrophage differentiation that is affected by tumor cells (Mantovani and Locati 2013). We found no noticeable IL-10 level changes when macrophages were stimulated with viable tumor cells in a heat-treated dead tumor or mEHT-treated tumor cells (Figure 4(C)). Contrastingly, the IL-10 production was significantly lowered when KME was added to the medium, which indicates that KME is capable of effectively inhibiting macrophage differentiation (Figure 4). Overall, these results indicated that mEHT and KME co-treatments inhibited the M2 macrophage differentiation, which are associated with tumor growth and progression.

Combined effect of mEHT and KME treatment on the tumor-specific immune response

Lymphocytes obtained from spleens were co-incubated with B16-BL6 melanoma cells *in vitro* to investigate whether mEHT can increase the cytotoxic T lymphocytes (CTLs) activity. The CTL activity showed a slight increase compared to the non-treated controls in both groups treated with mEHT or KME alone. However, the combined mEHT and KME treatments were more significantly effective in increasing the cytotoxicity (Figure 5(A)). Cytotoxic T cells act on tumor cells by releasing cytokines, such as IFN-γ, TNF-α, and TNF-β (Schroder et al. 2004). Especially IFN-γ, which is produced by $CD4^+$ T helper cells, enhances the cytotoxic capacity of T lymphocytes on tumor cells (Pardoll and Topalian 1998). Concurrently, cytotoxic T cells produce IFN-γ in the process of killing tumor cells. Thus, we analyzed IFN-γ in the obtained supernatant from the mixed cultures of mouse spleen cells and tumor cells. Figure 5(B) shows that IFN-γ production was the highest in the mEHT + KME treatment group compared to the control and other treatment groups.

We further investigated the alteration of granzyme, which is a serine protease that is released by cytoplasmic granules within cytotoxic T cells. Granzyme-induced apoptotic cell death is one of the main mechanisms underlying the tumor cell elimination by the cytotoxic T cells (Trapani and Smyth 2002). Consistent with IFN-γ production, the combination treatment of mEHT and KME released the greatest amount of granzyme compared with other groups (Figure 5(C)). These results

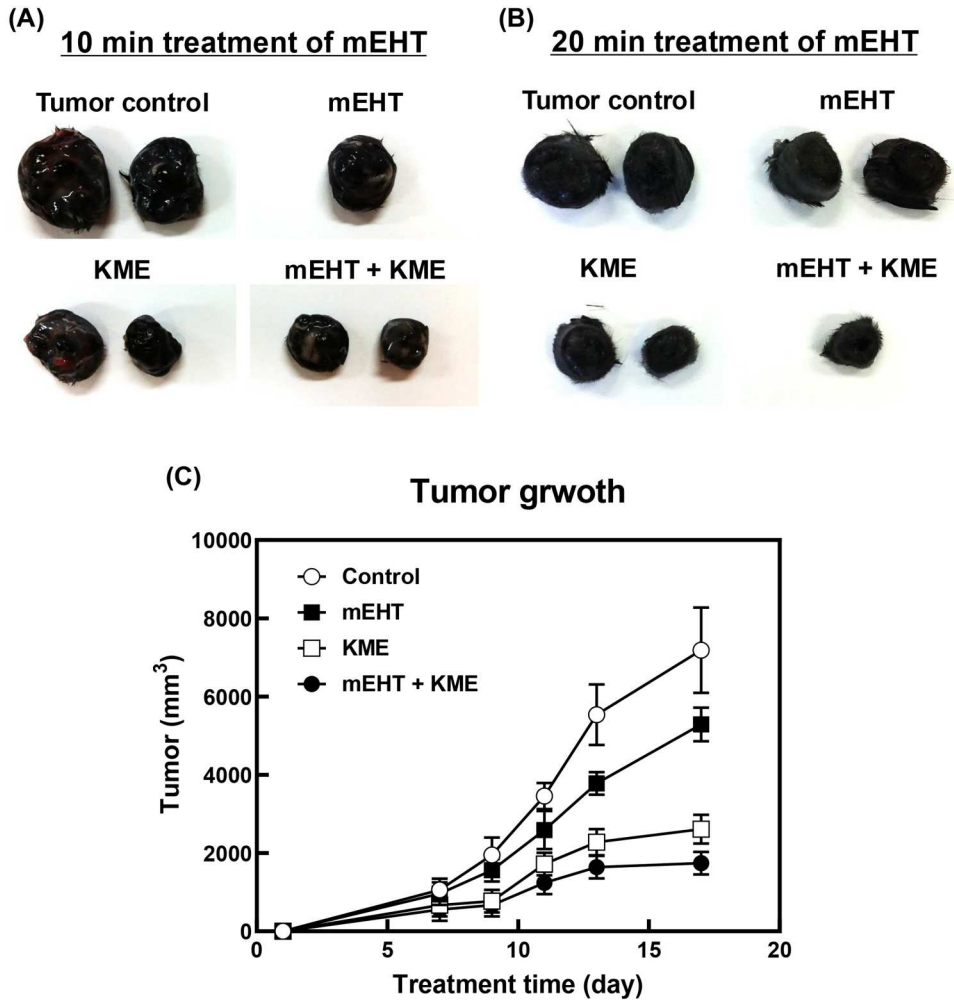

Figure 3. The inhibitory effect of mEHT and KME on tumor growth. B16-BL6 melanomas were obtained from the control (water bath heating), mEHT, KME, and mEHT + KME-treated groups. (A) the mEHT-treated group had 10 min on each indicated day. (B) The mEHT-treated group had 20 min. (C) Tumor size of the control, mEHT, KME, and mEHT + KME-treated B16-BL6 melanomas was measured. Five mice were used in each group.

support that mEHT treatment enhances the cytotoxic activity of T cells, resulting in an increased anti-tumor effect by combined therapy with Korean mistletoe extracts.

Combined effect of mEHT and KME treatment on antibody production

Tumor antigen-specific antibodies play an important role in anti-cancer immune response because they mediate antibody-dependent cellular cytotoxicities (Scott et al. 2012). Additionally, tumor antigen-specific antibodies bind to tumor cell surface receptors and induce apoptotic tumor cell death (Scott et al. 2012). Therefore, we investigated whether mEHT treatment influences tumor-specific antibody production. Mice were immunized by injecting melanoma tumor cells treated with mEHT alone or in combination with KME. Three days after injection, the tumor-specific antibody levels were checked in the serum samples of mice using an ELISA. We found that the mEHT treatment alone enhanced the antibody production against melanoma. The combined treatment of mEHT and KME was significantly more effective than mEHT alone in increasing antibody production (Figure 6).

Induction of anti-tumor immune response using the mEHT-treated melanoma as a tumor vaccine

The potentiality of mEHT-treated tumor cells as a tumor vaccine to induce specific anti-tumor immunity in mice was investigated. Mice were immunized with mEHT-treated B16-BL6 melanoma cells. Mice were injected with viable tumor cells 2 weeks after immunization. Then, 2×10^5 tumor cells were subcutaneously injected into the back of mice after 24 h of incubation. The

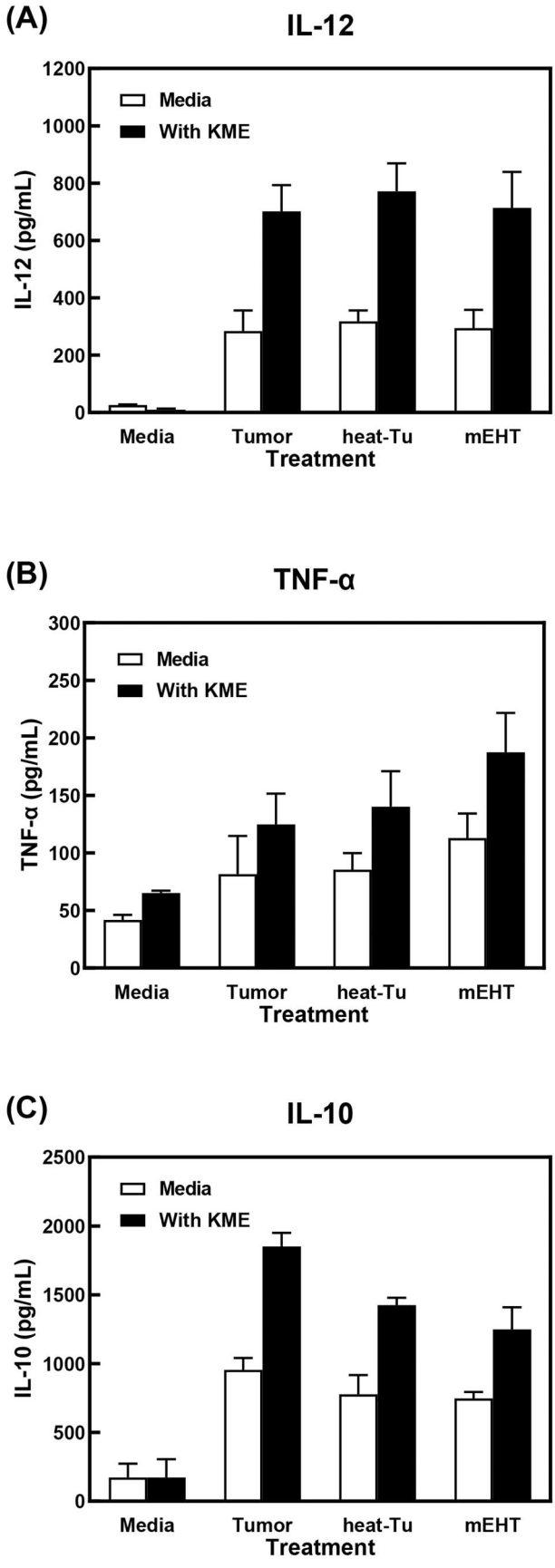

Figure 4. Effect of mEHT treatment on cytokine production by the stimulation of macrophages (A) IL-12, (B) TNF-α, and (C) IL-10.

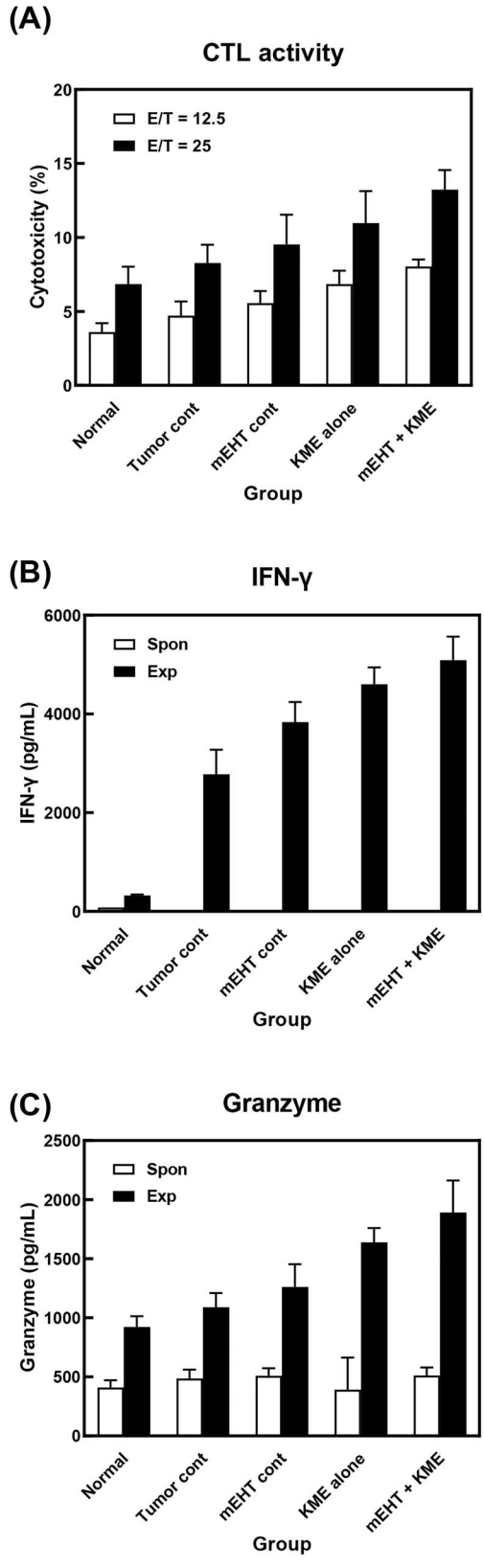

Figure 5. Effects of mEHT and KME treatment alone or in combination on tumor-specific immune responses. (A) T cell cytotoxic activity against B16-BL6 was measured at ratios of effector/target cells (E/T ratio). The IFN-γ (B) and granzyme (C) levels were measured in the obtained supernatant from the mixed cultures of mouse spleen cells and B16-BL6 tumor cells.

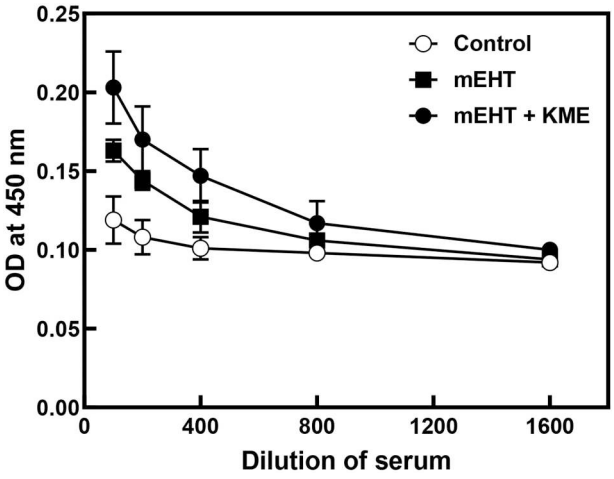

Figure 6. Detection of tumor-specific antibody production in mouse serum samples using ELISA. Mice treated with mEHT alone or mEHT and KME had significantly higher IgG levels.

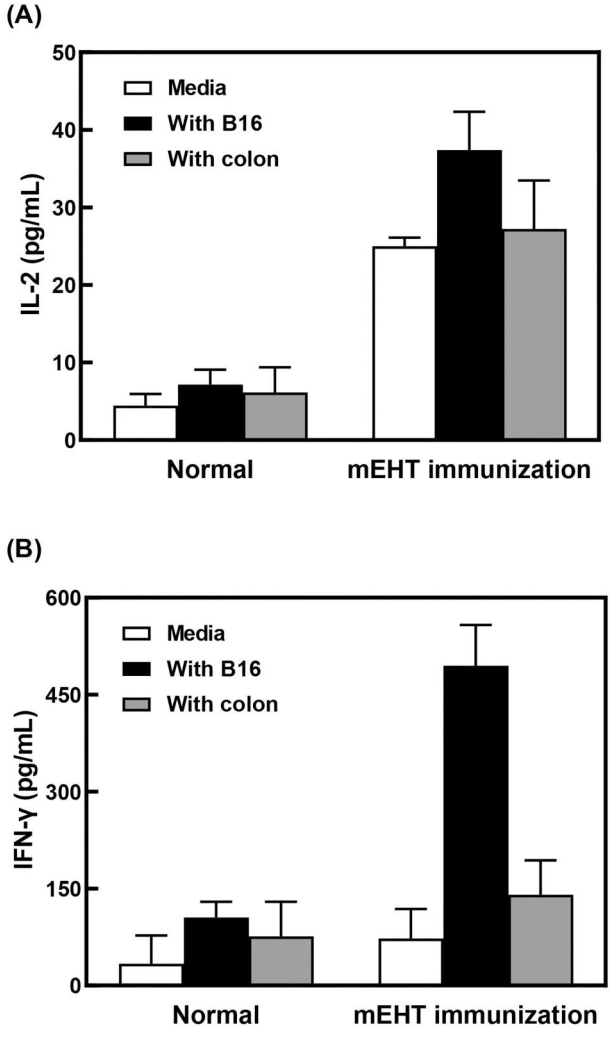

Figure 7. Production of tumor-specific cytokines by immunization with mEHT-treated tumor cells. IL-2 (A) and IFN-γ (B) levels are measured from the splenocytes of normal mice and mice immunized with mEHT-treated tumor cells.

production of IL-2 and IFN-γ in splenocytes, which are typical Th1-type cytokines that are involved in enhanced cellular immune responses (Schroder et al. 2004; O'Garra and Vieira 2007), was then examined after 2 weeks of tumor injection. We observed a significantly increased IL-2 level in the spleen cells of the immunized mice without re-stimulation. Additionally, IL-2 production was further increased when re-stimulated with syngeneic melanoma B16 cells. However, no significant change was found in the IL-2 production in the splenocytes from mice that are re-stimulated with allogenic colon 26-M3.1 carcinoma cells (Figure 7(A)). IFN-γ production was increased when re-stimulated with syngeneic melanoma (Figure 7(B)). Thus, this result suggested that mEHT might enhance the cancer-specific immune response.

Discussion

EHT is a powerful enhancer of chemotherapy and radiotherapy (Horsman and Overgaard 2007). Additionally, recent reports strongly indicated that mild hyperthermia or fever range whole-body heating is an effective enhancer of the immune responses against infection and cancers (Wang et al. 2001b; Skitzki et al. 2009a; Evans et al. 2015). We have been interested in the possibility that EHT may enhance the anti-cancer effects of various complementary and alternative therapies. To our knowledge, little has been explored about the potential usefulness of combining EHT with alternative anti-cancer therapy with natural compounds that have been known to improve anti-cancer immunity. Mistletoe preparations are used as immuno-stimulating agents in cancer treatment and have been shown to improve the quality of life (QOL) mainly by increasing the immunity in several clinical trials over the years (Bussing et al. 2012; Kim et al. 2012; Troger et al. 2014). Therefore, we hypothesized that a combination of EHT and mistletoe therapy would significantly enhance the immunity of patients with cancer, resulting in an improved QOL.

In recent years, the anti-tumor effects of EHT have become increasingly evident due, at least in part, to the improvement of the immune responses (Frey et al. 2012). TNF-α, which is a pro-inflammatory cytokine, affects the class II MHC expression together with IFN-γ (Trinchieri 2010). Accordingly, the enhanced TNF-α production by mEHT treatment can be concluded to be associated with antigen-presenting T cells after the processing of dead cancer cells by macrophages. In tumor progression, TAMs are generally converted to M2-type macrophages, which are characterized by high IL-10 levels and low tumoricidal activity (Mantovani and Sica 2010; Jeong et al. 2023). Such TAMs have been shown

to support a favorable microenvironment for tumor growth and tumor cell survival (Pollard 2004). Therefore, IL-10 secretion inhibition is helpful for suppressing the macrophage differentiation to M2 type (Ahn et al. 2024), resulting in anti-tumor efficacy of mEHT and KME combined treatment (Figure 3). Our data indicate that mEHT and KME combination therapy suppresses IL-10 production and increases TNF-α secretion, suggesting a shift from the M2 immunosuppressive phenotype toward the pro-inflammatory M1 phenotype (Martinez and Gordon 2014). This shift in macrophage polarization enhances tumor immune surveillance and contributes to the observed inhibition of tumor growth.

The combination of mEHT and KME was effective in inhibiting tumor growth and increasing CTL activity (Figure 4). Additionally, the immunization of mice with tumor cells treated with mEHT and KME significantly boosted the antibody production against tumor cells and increased IFN-γ and granzyme secretion. Thus, immunization with mEHT-treated tumor cells mainly contributes to T cell-mediated immunity activation. Importantly, we have observed a significantly increased immune response to tumors after pre-immunization with mEHT-treated tumor cells. The antibody titer against mEHT-treated melanomas, which act as antigens, was also examined (Figure 6). The use of cancer vaccines is based on the expectation that the host immune systems recognize the attenuated tumor cells as an antigen and develop immunity. However, tumor cells are often poorly immunogenic. Thus, developing an adjuvant to increase the immunogenicity of tumor cells is necessary to overcome this drawback. The combination of mEHT and KME treatment for the tumor cells may be used to augment the tumor cell immunogenicity and increase the anti-cancer vaccine effectiveness.

Heated tumor cells generate HSPs, which initiate increased tumor antigen presentation, resulting in NK cell and APC activation (Multhoff 2009a). The induction of tumor cell apoptosis by mEHT increases HSPs release, thereby contributing to the tumor-specific immune response (Tsang et al. 2015b). Therefore, examining the effect of combined therapy on HSP production would be interesting. Additionally, detailed studies about the synergistic effect of combined treatment on NK cell activation are intriguing because both EHT and mistletoe extract show the NK cell enhancement function (Yoon et al. 1998a; Multhoff 2009b).

In summary, we have found that the combinational treatment of mEHT and KME improved the anti-cancer activity and immunological response in mice, which suggests the potential of mistletoe preparation as a partner of EHT for cancer therapy. Our findings suggest that this combination therapy may exert its effects through multiple mechanisms, including enhanced antigen presentation, modulation of the tumor microenvironment, increased tumor-specific antibody production, and induction of immunogenic cell death (Kim et al. 2024). Future studies should focus on elucidating the precise molecular pathways underlying these effects, as well as investigating the potential application of mEHT-treated tumor cells as a cancer vaccine. Thus, our findings contribute to a deeper understanding of how mEHT and KME synergistically enhance antitumor immunity and pave the way for novel therapeutic approaches in cancer treatment.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Ethics approval

All experimental experiments were approved by the Institutional Animal Care and Use Committee of Dongguk University, by approval number IACUC-2021-020-3.

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