Multimedia Tools and Applications (2022) 81:41851–41868
https://doi.org/10.1007/s11042-022-13521-8

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Antitumor effect of infrared whole-body hyperthermia with curcumin in breast Cancer

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Received: 1 December 2020 / Revised: 29 September 2021 / Accepted: 13 July 2022 /
Published online: 7 September 2022

Abstract
Infrared Hyperthermia therapy (IHT) is a non-contacting method to elevate body temperature and treat malignant lesions such as breast cancer. Breast cancer is one of the major cancer types among females and over the years, its prevalence is increasing. Current treatments of breast cancer are surgery, radiotherapy, chemotherapy and thermotherapy via the use of IHT or combination of these. IHT is most commonly combined with chemotherapy as its effect as a stand alone treatment platform is short lasting. However, chemotherapy induced toxicity to patients. Curcumin has traditionally been used as a food additive or as a remedy in traditional medicine for its anticancer and non-toxic effects. Thus, this research proposed the combination of curcumin and IHT as an alternative to chemotherapy in breast cancer treatment. Mice were inoculated with EMT6 breast cancer cells and assigned to 4 treatment groups: (i) untreated (control), (ii) orally curcumin (CUR), (iii) whole-body hyperthermia (FRWBH), (iv) orally curcumin with whole-body hyperthermia (FRWBH+CUR). Results showed that tumor growth inhibition and body weight gain in the combination treatment group (FRWBH+CUR) are significantly different compared to control. The group also had the longest median survival time (42 days) with no mortality observed during the experiment. This result indicates that the combination treatment is well tolerated by the mice and has negligible levels of toxicity. However, frequent complications of cancer such as anaemia and thrombocytopenia are still observed in the combination treatment group. Platelet to Lymphocyte Ratio (PLR) and Neutrophils to Lymphocytes Ratio (NLR) results indicate that the combination treatment (FRWBH+CUR) has better prognosis outcome than single treatment and may become a potential alternative antitumor treatment of breast cancer.

Keywords Breast cancer · Curcumin · Infrared whole body hyperthermia

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1 Introduction

Breast cancer is a malignant tumor that originates from the cells of the lobules or the ducts and may innervate into the fatty and fibrous connective tissues of the breast [50]. It is a complex and heterogeneous disease with multiple tumor entities associated with distinctive histological patterns as well as diverse biological features and clinical appearance [87]. In Malaysia, breast cancer accounted for 32.1% of all cancers among females and the incidence trends are getting higher every year [5].

There are numerous treatments of breast cancer such as surgery, radiotherapy, chemotherapy, thermotherapy or combination of these [15]. Infrared hyperthermia therapy (IHT) is a thermotherapy method that employs infrared radiation to induce heat and elevate body temperature for malignant diseases treatment [45]. So far, it is considered as a complementary therapy in cancer treatment.

In clinical practice, the standards of IHT are whole-body (systemic), local hyperthermia and regional hyperthermia. For Whole-body (systemic) IHT, the temperature could be classified into three types – mild (37.5 °C to 38.5 °C), fever range (39 °C to 42.0 °C) and extreme range (above 42 °C). Fever range is the most popular method for breast cancer as previous studies have reported its positive impact on tumor vascular perfusion, immune function and immunogenicity [76]. IHT is commonly used as an adjuvant and combined therapy with other treatment methods such as radiotherapy, chemotherapy, surgery and immunotherapy [18, 33] as the effect of IHT alone is reported to be short lasting [31, 52, 54]. The efficacy of treatments combining radiotherapy and IHT to treat localized recurrent breast cancer of advanced stages and primary tumors were demonstrated in a series of clinical trials [30, 53, 67, 69]. Previous studies reported that there is significant tumor size reduction in combination of IHT with other treatments [10]. Optimum treatment sequence between radiotherapy, chemotherapy and IHT is reported to be the key factor to have better local control of tumors [24, 51], decrease side effects and control immune response [46] of the patients.

In combination treatment, it is also well-established that cancer cells are killed and sensitized to chemotherapy or radiotherapy within fever range temperature [22, 76]. Additionally, temperature of 39.0–40.0 °C is the most commonly used in clinical procedure due to its safety and effectiveness [9]. Another temperature range that is clinically accepted is 40–45 °C [89]. Whole-body IHT is usually conducted with mild temperature or called fever-range for 1–2 hour or 4–8 hour. These procedures are medically supervised by expert practitioners to continuously monitor the progress of IHT. Some procedures require anesthesia and several hours of aftercare treatment to ensure patient's stability. Most of the IHTs have a high proportion of short wavelengths (infrared A) close to the range of light and are radiated along with light. Significant portions of this radiation penetrates the epidermis to a depth where the blood can absorb the heat thus distributing and releasing it throughout the body.

Combinations of IHT with some chemotherapy drugs such as Lonidamine, Cisplatin, Cyclophosphamide, Bleomycin and Mitomycin C, [8, 63, 64, 67, 78, 80, 83, 88] have shown a good synergistic effect in breast cancer treatment and exhibit action against advanced and solid tumor. Additionally, Bull et al.,2008 [13] also reported that the antitumor effect of gemcitabine and cisplatin in rat tumor models increased when IHT is optimally scheduled with chemotherapy administration.

Even though chemotherapy can result in important survival advantages for many women with breast cancer, there are significant toxicities, limitations in wellbeing and competency associated with this therapy [66]. For instance, patients may develop resistance to anticancer

drugs during chemotherapy. Additionally, chemotherapy has possible adverse effects to the patient such as diarrhoea, rash, mucosal inflammation and febrile neutropenia [84]. Due to severe side effects of chemotherapy and because of the elevated death rate related to cancer, many cancer patients are opting for complementary and alternative medicines to reduce side effects and complications [44]. The presence of various compounds in plants with anticancer effects are worth it to study. The compounds such as flavonoids [92], catechins [25], betulinic acid [93], catechol [43], alkaloids, tannin [11] and curcumin [49] have been studied and exhibited with anticancer effect.

Curcumin is a hydrophobic polyphenol, a dietary phytochemical and a principal active ingredient derived from turmeric. It was first isolated in 1815 by Vogel and Pelletier and its chemical structure and synthesis was confirmed by Lampe et al. in 1910 and 1913 [37]. Molecular formula of curcumin is C21H20O6 or commonly called diferuloyl methane [1]. Commercial curcumin is a mixture of curcuminoids, containing approximately 77% diferuloylmethane (Curcumin I), 17% demethoxycurcumin (Curcumin II), and 5% bisdemethoxycurcumin (Curcumin III) [37].

Multitudes of breast cancer cell lines and animal models studies have been conducted to provide evidence of curcumin against breast cancer. A study by Bimonte et al, 2015 demonstrated that curcumin at 10 μM and 50 μM doses inhibited the migration of breast cancer cells within 48 h and enhanced apoptosis effect at 10 μM in MDA-MB-231 cell lines [11]. Moreover, curcumin inhibited MCF-7 breast cancer cells in a concentration-dependent manner (5–80 μM) with the 50% inhibiting concentration (IC50) of 40 μM/L [21]. A study by Lv et al., 2014 proved significant decrease in MCF7 and MDA-MB-231 cells viability in a time-and-dose dependent manner with effective concentration of 50 μg/kg of curcumin [43].

In vivo experiments on female BALB/C nude mice treated with 50 μg/kg and 200 μg/kg of curcumin proved that curcumin inhibited tumor growth and reduced tumor weight [68]. Similar outcome was also shown in a study conducted by Bachmeier et al., 2007 [6]. Additionally, an in vivo study using Foxn1nu/nu female mice model has verified that treatment using curcumin 0.6% imposed no toxicity to the mice and significantly decreased tumor volume within 3 to 6 weeks of treatment. Curcumin also inhibited micro vessels in tumors of the treated mice revealing its potential usage as an adjuvant agent to chemotherapy [75]. It was also observed to impose significant reduction of tumor multiplicity and prolongation of tumor-free survival in BALB-neuT female mice treated with curcumin (2 mg in 50 μl of corn oil) [77]. Another study [26] reported that curcumin treatment (45 mg/kg) twice a week in the BT-474 xenograft model for 4 weeks is effective in decreasing tumor size. Falah, Talib and Shbailat, 2017 also showed that treatment with 50 mg/kg curcumin for 14 days in BALB/C mice has caused a significant reduction in EMT6 breast cancer tumor size [21].

The anticancer effect of curcumin may also be seen when curcumin is combined with other treatment forms. The combination treatment of curcumin (200 mg/kg/day) and epigallocatechin gallate (25 mg/kg/day) for 10 weeks was proved to suppress tumor growth in female CD1 nude mice Another study also shown that taxol combined with curcumin have an antitumor effect that is comparable with taxol and herceptin treatment [21, 26]. The same study also showed that combined administration of curcumin (100 mg/kg) and mitomycin C (1-2 mg/kg) for 4 weeks produced significantly greater inhibition in tumor growth [6]. Combination of curcumin (50 mg/kg) and metformin (80 mg/kg) for 14 days in Balb/c mice inoculated subcutaneously with EMT6 breast cancer cells also showed the highest significant reduction in the tumor size [29].

Therefore, this study is conducted to investigate the effectiveness of IHT in fever range whole body hyperthermia setting (FRWBH) in combination with curcumin. Curcumin is chosen to replace the ordinary chemotherapy substances so that possible adverse effects of chemotherapy could be reduced. Anti-tumor properties in curcumin are hoped to lengthen the short lasting effect of WBH in cancer treatment.

2 Materials and method

2.1 Animal preparation

In this study, 20 female BALB/C mice ranging from 6 to 8 weeks old with around 17-25 g body weight were purchased from local university Animal Research Unit. Animals were housed in a cage of max 5 mice per cage with 12-hour dark and light cycles in a room with ambient temperature. All mice were fed with a standard laboratory diet and water ad libitum daily and wood shaving bedding were changed every two days. The mice were allowed to acclimatize for at least 7 days before experiments. Animal care and handling and all of the experimental protocols were conducted according to rules and regulations approved by the National University of Malaysia Animal Ethics Committee (UKMAEC) with approval number (UTM/2017/MAHEZA IRNA/22-NOV./884-NOV.-2017-MARCH-2019). Throughout the experiment, the body weight of each mouse was measured every alternate day.

2.2 EMT6 breast CANCER cell preparation & tumor inoculation

The EMT6 cells used in this study were supplied by Cancer Research Lab, Universiti Teknologi Malaysia. EMT6 cells were maintained in a Waymouth's Media MB 752/1 Liquid Medium (1x), Thermo Fisher [69], supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS), Thermo Fisher and 1% antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). Cells were brought up in 25 mL flasks and incubated at 37 °C in a humidified 5% CO2 atmosphere [79]. Once the cells reached 90% confluence in 25 mL flask, the cells were washed and re-suspended in Phosphate Buffered Saline (PBS), harvested using trypsin solution TrypLE™ Express Enzyme (1x) without phenol red. The cells were then centrifuged at 1500 rpm for 5 minutes and transferred to 75 mL flask at a density of 5 X 106 cells/ml [2]. Sub culturing of cells suspension was performed when the cells reached 90% confluence. In this study, each BALB/C mouse received 5 × 105 EMT6 cells in 100 μl PBS [3] injected subcutaneously at the right flank of the mice [6].

2.3 Optimization of temperature and duration of hyperthermia treatment

In this study, fever range whole-body hyperthermia (FRWBH) treatment was conducted using water-filtered infrared-A (wIRA®) Hydrosun 750 device from Medizintechnik GmbH in a prescribed temperature and duration as shown in Table 1. Fever range whole body hyperthermia is defined as the temperature in the range of 39 °C to 42.0 °C as clinical evidence shows positive impacts of IHT on tumor vascular perfusion, immune function and immunogenicity [76] as well as improved treatment sensitivity to chemotherapy or radiotherapy. Therefore, optimization of temperature and duration of FRWBH treatment was conducted to observe the viability of mice under the fever range hyperthermia regime.

4 groups of mice were tested during the temperature optimization process with each of the groups consisting of 2 mice (n = 2). Before the FRWBH treatment was conducted, mice were anesthetized with the mixture of ketamine (150 mg/kg) and xylazine (7.5 mg/kg) [34]. In this study, rectal temperature was monitored continuously in each mouse using small animal rectal thermostat probes throughout the treatment to make sure that body temperature of the mice is within the expected range of temperature.

In the first group, all mice (n = 2) were dead immediately after completion of FRWBH treatment at 40.0 °C (± 0.5) for 30 minutes. Meanwhile, the second group of mice were exposed to FRWBH treatment of 40.0 °C (± 0.5) for 15 minutes. One mouse (n = 1) died one day after the treatment and another one (n = 1) survived up to 14 days after the treatment. The result was similar in the third group in which the mice received FRWBH treatment of 39.0 °C (± 0.5) for 30 minutes. However, all the mice (n = 2) in the fourth group which received FRWBH treatment of 39.0 °C (± 0.5) for 15 minutes, survived more than 14 days after the treatment. Therefore, the temperature of 39.0 °C (± 0.5) and duration of 15 minutes was selected as optimum temperature and duration for Infrared FRWBH treatment in this study.

2.4 Curcumin solution preparation

In this study, curcumin powder of 80% curcumin and 94% curcuminoid content was purchased from Sigma Aldrich to make a curcumin solution. The curcumin solution was prepared fresh daily by adding 25 mg curcumin in 1 ml dimethyl sulfoxide (DMSO) 5% in a tube covered with aluminum foil to avoid direct contact with light. The dilution of curcumin for daily oral administration was prepared by adding on filtered water with curcumin to obtain dosage of 50 mg/kg body weight.

Literature shows that dosage ranges between 50 μg/kg to 200 mg/kg within 4 to 10 weeks are proven to have inhibitory effect on breast cancer tumor growth as reported in previous studies [6, 57, 68, 75]. In a recent study on combination of metformin and curcumin for breast cancer in mice by Falah et al., 2017, curcumin dosage of 50 mg/kg to BALB/C mice for 14 days have successfully reduced EMT6 breast cancer size and therefore, this study used the same curcumin dosage of 50 mg/kg body weight [21]. The dose was served orally on a daily basis starting on day-14 post inoculation and continued for 14 days (until day-27) of experiment [29]. This study was conducted in 28 days duration [29] as it was designed to be a 14-day repeat-dose toxicity study. Repeat-dose toxicity study concept is adopted to evaluate adverse effects of the curcumin compounds after repeated administration to the experimental animals for a period of time.

2.5 Experimental procedures

In this experiment, 20 mice were inoculated subcutaneously with EMT6 cells. After inoculation, they were divided randomly into 4 experimental groups consist of 5 mouse in each group as follows:

Group 1: Induced mice without any treatment (CONTROL)
Group 2: Induced mice treated with curcumin 50 mg/kg body weight orally (CUR).
Group 3: Induced mice treated with Fever range whole body hyperthermia only (FRWBH).
Group 4: Induced mice treated with curcumin 50 mg/kg body weight orally and Fever range whole body hyperthermia (CUR + FRWBH).

The four groups we designed so that the control group is a negative control or untreated group. On the other hand, orally curcumin (CUR) and whole-body hyperthermia (FRWBH) are positive controls. Lastly, orally curcumin with whole-body hyperthermia (FRWBH+CUR) is the treated group. Animals in the positive control groups were handled in an identical manner to animals in the treated group to ensure consistency of the final results.

Oral curcumin of 50 mg/kg body weight was given daily from day-14 after inoculation until day −27 of experiment [29]. Meanwhile, fever range whole body hyperthermia (FRWBH) was conducted twice throughout the experiment period (on day −17 and day −23 post inoculation). Rectal temperature was monitored continuously in each mouse using small animal rectal thermostat probes [13] throughout hyperthermia treatment. Blood was drawn for blood analysis on day −28 of the experiment and mice were sacrificed on day-28 of experiment. Before the exposure to infrared-A in FRWBH treatment, mice were anesthetized with the mixture of ketamine (150 mg/kg) and xylazine (7.5 mg/kg) [34]. The hyperthermia treatment was conducted at 39 °C (±0.5) and maintained for 15 minutes. FRWBH was prescribed based on the results of the temperature optimization process gathered in section C. Rectal temperature was monitored continuously in each mouse using small animal rectal thermostat probes [13] along the treatment Fig. 1.

2.6 General toxicity assessment

Body weight of the mice was measured starting from day −0 (the day of inoculation) and recorded every other day until day – 28 of experiment. It is used to indicate general health status and treatment-induced toxicity.

2.7 Tumor volume assessment

Tumor size was measured in two perpendicular dimensions by using the best formula for estimating tumor volume. 'tumor volume = $1/2(ab^2)$' in which 'a' is the longest diameter

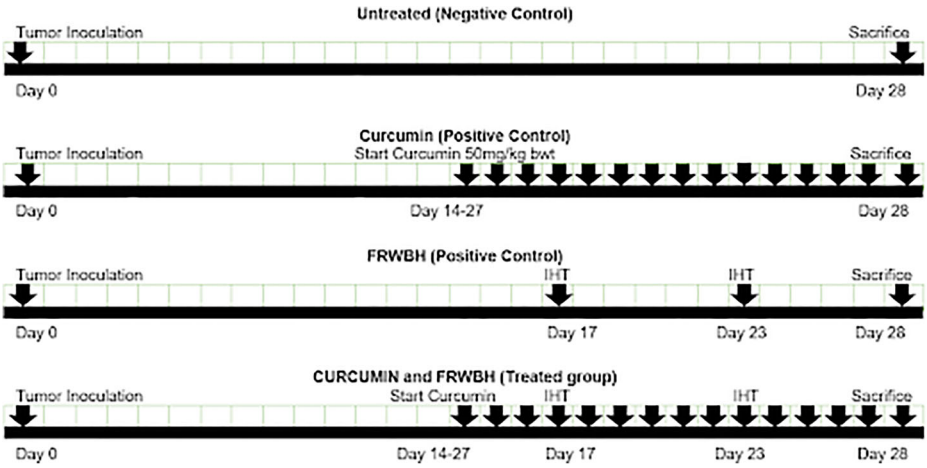

Fig. 1 Schematic of treatment received by mice during 28 days of experiment

and 'b' is the shortest diameter of the tumor [23]. It was measured and recorded when the tumor started to develop and continuously measured every 2 days until day −28 of the experiment using an electronic caliper to determine the diameter. Tumor Growth Inhibition (TGI) was calculated with the formula: TGI (%) = $(V_c - V_t) / V_c \times 100$, where V_c is the mean tumor volume of control, V_t is the mean tumor volume of treated group on day-28 of experiment [72].

2.8 Median survival rate assessment

Mice survival was monitored based on the mortality day of the mice or the day they were sacrificed when their tumor size reached a maximum size of $150mm^2$ as end point. This is the maximum size of tumor allowed by the Animal Ethics Committee to prevent over burden to the mice. Diameter of the tumor was measured and recorded from the day the tumor started to develop and continuously measured every 2 days using an electronic caliper until tumor size reaches a maximum of $150mm^2$.

2.9 Hematology assessment

Blood withdrawal was conducted using 1 ml syringe and needle 27G on day-28 of the experiment. Cardiac puncture was performed by puncturing the needle below and slightly left to the sternum. The blood was collected in an anticoagulant tube for blood analysis. Cervical dislocation was performed as a method of mice euthanasia. Blood analysis was sent and done in the animal clinic lab for complete blood counts. The following blood parameters were analysed: hemoglobin (Hb), Platelet (PLT), red blood cells (RBC), white blood cells (WBC), lymphocyte (LYM), neutrophils (NEU).

Other than that, the NLR and PLR ratio were also investigated. Neutrophil lymphocyte ratio (NLR) was calculated as the neutrophil count divided by the lymphocyte count. NLR was used as a good indicator of inflammation, tumour progression and metastasis. Patients with high NLR present shorter overall survival times and shorter disease-free periods. In a recent meta-analysis study, NLR is a good prognostic marker for breast cancer, and patients with a higher NLR have poorer prognosis [17, 47].

Platelet-to-lymphocyte ratio (PLR) is calculated as platelet counts divided by lymphocyte counts. PLR is used to identify potential prognostic value in breast cancer treatment in which higher PLR values are often indicated poorer survival times and larger infiltrated lymph nodes compared to lower PLR [4, 47]. Meta-analysis study done by Zhu et al., 2017 also showed that high PLR is associated with poor overall survival of breast cancer patients. However, a contrasting result is also reported in which there is no significant effect on survival times based on PLR in a patient with breast cancer [81, 90].

2.10 Data analysis

Statistical analysis was performed using GraphPad Prism Version 5.0 software. The data was presented as a mean ± SEM. Comparison between the control and treated group was conducted by using the unpaired t-test and One-way ANOVA (analysis of variance) with $p < 0.05$ was considered statistically significant.

3 Result and discussion

3.1 Treatment induced toxicity

In this study, loss of body weight is used as a general indicator of health status and treatment-induced toxicity. Thus, body weight is measured every other day after inoculation [39].

The results show that an increase in total body weight is observed in all groups. Figure 2 shows a significant difference in percentage of body weight gained among all groups throughout the 28 days of experiment ($p = 0.0012$). The highest mean percentage of body weight gain is recorded in the control group (5.38 ± 0.60) whilst the lowest mean percentage of body weight gain is in the CUR + FRWBH group with significant difference of (2.48 ± 0.51, $p = 0.001$). Mean percentage of body weight gain of the CUR group is 4.86 ± 0.66 with no significant difference compared to control ($p = 0.567$). Additionally, a significantly different mean percentage of body weight gain is also observed in FRWBH compared to control (2.72 ± 0.60, $p = 0.004$).

Body weight is one of the most common parameters used to indicate toxic effects of a treatment. Weight gain indicates a low toxicity and well tolerated treatment. Conversely, weight loss indicates adverse effects of a treatment and it will be significant if the body weight loss is more than 10% from the initial weight [34]. Results in this study indicate that there is body weight gain instead of weight loss after the treatments. No mortality or toxic symptoms is reported in any mice from all groups within 28 days of experiment as no weight loss is observed within the timeframe to indicate treatment toxicity. Curcumin is proven to be nontoxic either as a single treatment or in combination with other modalities. The result supports the notion that curcumin and hyperthermia have low toxicity effects [7, 39]. Similarly, fever range whole body hyperthermia (FRWBH) is a thermotherapy method with minimal toxicity [22]. However, body weight gained may also be contributed by tumor growth. Highest weight gain is observed in the control group as tumor growth at an accelerated rate without any treatment. However, the lowest percentage of body weight gain observed in CUR + FRWBH might be related to low rate tumor progression and toleration to heat effect during hyperthermia. This finding shows that the treatment received by the mice is well tolerated and has negligible level of toxicity to them [69].

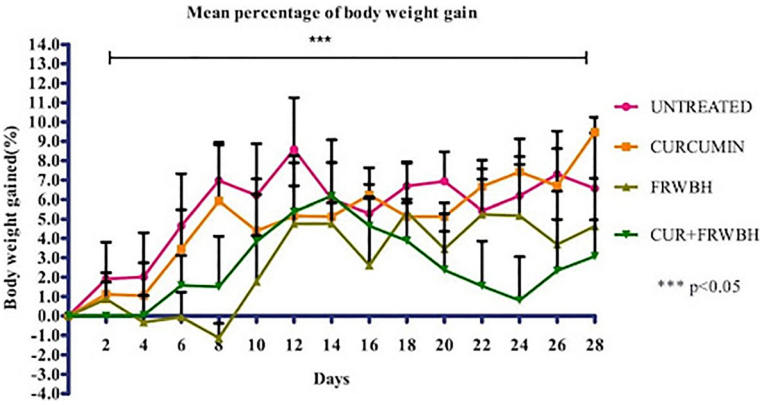

Fig. 2 Mice body weight in 28 days following treatment group

3.2 Antitumor effect

In this study, the antitumor effect of CUR and FRWBH is measured by calculating the effect of tumor volume inhibition in all groups. Figures 3 and 4 show tumor volume and Tumor Growth Inhibition (TGI) rate from the inoculation day until day-28 of the experiment. The tumor is observed to be palpable from day 7 after inoculation.

Results show that mean tumor volume of all treated groups are significantly different compared to the untreated group /CONTROL ($p = 0.0042$, $p < 0.05$). Mean tumor volume for the CUR group is significantly smaller with 69.29% tumor growth inhibition (TGI) rate. This result shows the effectiveness of CUR to reduce tumor size in mice breast cancer cells [29, 36, 48].

Mean tumor volume for FRWBH group is the smallest, with 84.50% TGI rate compared to CONTROL. Tumor volumes for FRWBH is 19.34 ± 6.40 compared to CONTROL of 139.5 ± 44.38 mm3, $p = 0.0122$). This result supports that hyperthermia is cytotoxic to breast cancer cells [73, 95]. However, there is rapid tumor growth observed a few days before the experiment ended as shown on day 24–28 in Fig. 3. Rapid tumor growth at the end of this experiment may suggest cancer recurrence due to the shortlasting response duration of cancer cells to IHT alone [52]. Many factors contributed to short response duration to hyperthermia treatment including homogeneity of heat distribution to the entire tumor, exposure temperature and exposure time. Increase in temperature, time of exposure and rate of heating will increase tumor killing and encourage better cell damage during IHT [54]. Besides, tumor microenvironments such as new vasculature may also decrease the response of tumor to heat and promote tumor recurrent [31].

Meanwhile, the TGI rate for CUR + FRWBH is 82.91%. Unlike other groups, tumor growth in the CUR + FRWBH group reached a plateau state a few days before the end of the experiment, indicating that the combination treatment has a better effect on tumor growth suppression compared to CUR and FRWBH treatment alone. This synergism effects of CUR and FRWBH kills cancer cells by inhibiting DNA, RNA synthesis and DNA repair mechanism. Heat also increases the production of heat shock protein that helps in tumor immunogenicity. Besides, hyperthermia enhances apoptosis by increasing not only oxygen free

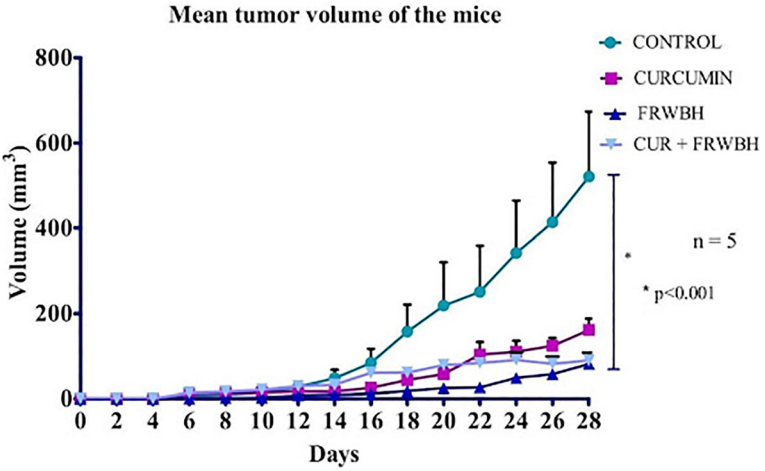

Fig. 3 Mean tumor volume of the mice during during 28 days of experiment

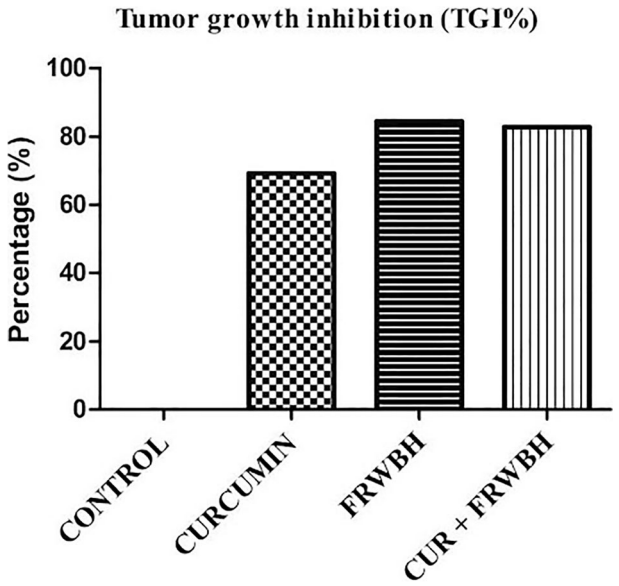

Fig. 4 Tumor growth inhibition (TGI) rate among the groups

radicals and tumor membranes permeability but also altering the expression of apoptosis genes [31]. Curcumin, on the other hand, is involved in regulating the apoptosis by modifying signalling pathways of the cancer cells through the inhibition of nuclear-factor kB (NF-kB). NF-kB is the most important player in the survival signal of cancer cells.

3.3 Median survival rate

Survival analysis is a way to estimate a cancer patient's prognosis and measure cancer burden. It is often used to compare cancer outcomes between different populations and time periods [12]. Kaplan-Meier curves are used in this study as a simple analysis of which the aim is to compare survival times of two or more small groups based on their median survival time [28, 35]. Figure 5 shows the Kaplan-Meier survival curve for the mice in CONTROL, CUR, FRWBH and CUR + FRWBH groups. As expected from tumor growth results, the longest median survival time is 42 days for the FRWBH+CUR, followed by FRWBH group with 40 days median survival time. Median survival time of the CUR group is 36 days and the shortest median survival time is the CONTROL group with 34 days. Additionally, there is a significant difference ($p = 0.025$) in median survival time between the CONTROL group and the CUR + FRWBH group. On the other hand, no significant difference is observed in the median survival time between the CONTROL group and the FRWBH ($p = 0.093$) and CUR($p = 0.349$) group respectively.

The result indicates that half of the mice in the CUR + FRWBH group probably survived at least 42 days or longer after receiving combination treatment and showed that combination of FRWBH+CUR is capable of prolong survival of the mice with breast cancer. According to Dutta and Sengupta, 2016, one human year is equivalent to nine days of mice life. This result indicated that CUR + FRWBH may prolonged the survival of human patients up to 4.6 years compared to other treatments [20].

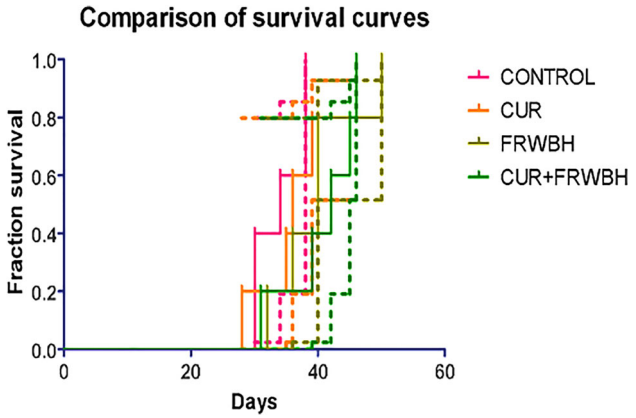

Fig. 5 Comparison of median survival curves among treatment groups

3.4 Hematology profile

Post treatment blood analysis was conducted at the end of the experiment (day-28) to predict disease severity and mortality risk [90] and to evaluate side effects and toxicity of each treatment to the mice [77]. From the result shown in Table 2, there is no significant difference in mean red blood cells (RBC) count among all treatment groups ($p = 0.27$). Hemoglobin (Hb) level is also a reliable indicator for response to treatment outcomes and survival in patients with breast cancer [19, 40]. Similarly to RBC count, there is no significant difference in mean hemoglobin (Hb) count among the groups ($p = 0.42$). However, RBC and Hb count for CUR + FRWBH group are below the normal range of RBC and Hb count. This result indicates the tendency of mice treated with CUR + FRWBH to develop anemia, a common complication in patients with cancer. Numerous factors lead to anemia such as tumor-associated bleeding, hemolysis, nutritional deficiencies and marrow damages due to metastases. However, major anemia factor for cancer patient are complex interaction between the tumor cell population and the immune system that initiates cancer-related anemia, increased level of inflammatory cytokines that induces inadequate differentiation and proliferation of erythroid progenitor cell and production of erythropoietin [42]. Survey conducted shows that 1993 patients out of 3216 breast cancer patients are anemic [42]. However, the prevalence

Table 1 Optimization of temperature and duration of hyperthermia

Temperature (°C)	Duration (Minutes)	No. of sample (n)	Result (Viability)
41.0 °C (± 0.5)	30	2	Non- viable. Both mice (n=2) died immediately after the treatment
41.0 °C (± 0.5)	15	2	Non-viable. One mouse (n=1) died a day after the treatment and one mouse (n=1) survived up to 14 days after the treatment.
39.0 °C (± 0.5)	30	2	Non- viable. One mouse (n=1) died the day after the treatment and one mouse (n=1) survived up to 14 days after the treatment.
39.0 °C (± 0.5)	15	2	Both mice (n=2) survived up to 14 days after the treatment.

depends on the level of the disease and type and duration of anticancer therapy [62, 91]. In clinical practice, the most common treatment of anemia in cancer patients are iron replacement, erythropoietin stimulating agents (ESAs), and blood transfusions [14].

In this study, the mean platelet (PLT) count for all groups is below normal range of (300–600 × 10^9/L) with no significant difference among groups ($p = 0.29$). In contrast to the mean platelet for control which is the highest, the mean platelet for CUR + FRWBH is the lowest. This result showed that all mice developed thrombocytopenia, a frequent complication of cancer and its treatment with diverse and multifactorial causes and may occur in patients with hematological malignancies [16, 27, 38, 41] [47, 59]. Platelet plays a few roles in breast cancer progression. It improves the spreading of cancer cells within the blood circulation, facilitating cancer cell adhesion to the endothelium and helps cancer penetration process into the parenchyma of remote tissues and encourage growth of tumor cells at metastatic sites [86]. Increased mean platelet volume is associated with larger tumors and higher cancer stage [27, 38]. It is also an indicator to distant metastases and a poorer prognosis in patients with breast cancer [17]. Contrarily, certain studies concluded that there is no significant difference in survival between higher and lower mean platelet volumes [90]. Hyperthermia dose reduction, the usage of thrombopoietin receptor agonists such as romiplostim and eltrombopag to stimulate platelet production and platelet transfusions are among the major treatment options offered for affected patients during the course of IHT treatment [76].

Additionally, White blood cell (WBC) count is a useful predictor for inflammatory biomarkers [65, 70, 74, 81]. It plays an important role in cancer by influencing cancer development and progression [61, 74] [74]. Elevation of total WBC count predicts poorer prognosis and is associated with mortality in cancer [70]. However, in this study, mean WBC for all groups are below normal range (4.0–19.0 × 10^9/L). This result indicates that all the mice developed leukopenia at the end of the experiment. There is no significant difference in mean total white blood cells (WBC) counts among the groups ($p = 0.44$).

Results also showed that mean LYM count for control, CUR and FRWBH are within normal range except for CUR + FRWBH that slightly falls below normal range. Thus, this result pointed out that mice treated with CUR + FRWBH has tendency to develop mild lymphocytopenia. However, there is no significant difference in LYM count among the groups ($p = 0.5223$). Systemic alterations such as a decline in lymphocyte counts and an increase in neutrophil and platelet counts in cancer patients is caused by an inflammatory response that is

Table 2 Mean hematology parameters of treatment group

Group	Unit	Normal value	CONTROL	CUR	FRWBH	CUR+ FRWBH
WBC	**× 10^9/L**	**4.0–19.0 × 10^9/L**	3.04	2.96	2.66	1.99
RBC	**× 10^{12} /L**	**4.0–19.0 × 10^{12}/L**	4.98	4.80	4.69	3.46
				p=0.83	p=0.77	p=0.13
Hb	**g/dL**	**10–19 g/dL**	13.16	12.46	11.16	9.46
				p=0.78	p=0.47	p=0.24
PLT	**× 10^9/L**	**300–600 × 10^9/L**	73.20	49.20	51.40	24.60
				p=0.45	p=0.44	p=0.08
LYM	**× 10^9/L**	**1.5–7 × 10^9/L**	2.12	2.26	1.92	1.48
				p=0.82	p=0.77	p=0.30
NEUT	**× 10^9/L**	**2.0–10.0 × 10^9/L**	1.20	0.38	0.46	0.22
				p=0.29	p=0.34	p=0.21
NLR			0.62	0.19	0.26	0.14
PLR			36.73	24.26	34.25	17.90

triggered by circulating cytokines and chemokines [4]. Lymphocytes have a significant role in tumor-derived inflammatory responses [55] and effective anti-tumor function [19, 42]. Increased infiltration of lymphocytes in tumor tissue predicted better survival outcomes in cancer patients [56, 91].

In this study, the mean for neutrophils (NEUT) count for all groups was below normal range (2.0–10.0 × 10^9/L) with no significant difference among all groups ($p = 0.29$). Mean neutrophils count for control is the highest with 1.20 ± 0.71, and the lowest count is CUR + FRWBH (0.22 ± 0.08, $p = 0.21$ compared to control). This result pointed out the tendency of mice in that group to develop neutropenia. Neutrophils play an active role in development of tumor by inducing tumor progression and have a close relationship with angiogenesis of tumor. It also induces development of metastases via the secretion of cytokines [58]. Based on meta-analysis in [71], tumor-associated neutrophils had significant statistical correlation with the rate of recurrence-free survival (RFS), cancer-specific survival, and overall survival (OS).

Assessment on NLR and PLR of the blood is used to reflect the systemic inflammation. NLR and PLR have been linked to poor outcomes in patients with early breast cancer [60, 62]. NLR is calculated as the neutrophil count divided by the lymphocyte count, and is used as a good indicator of inflammation, which shows an important role in tumor progression and metastasis. NLR is a parameter that has been studied widely in breast cancer [47]. The findings in this experiment showed that NLR for CUR + FRWBH is the lowest. Low NLR is highly desirable as a previous study reported that breast cancer patients with high NLR present shorter overall survival times and are shorter disease-free [32]. In a recent meta-analysis study, NLR is a good prognostic marker for breast cancer, and patients with a higher NLR have poorer prognosis [17, 82, 85, 94].

PLR is calculated as platelet counts divided by lymphocyte counts. In this study, there is no significant difference in PLR among all groups. However, the mean PLR for control is the highest followed by CUR, FRWBH and the lowest is CUR + FRWBH. Several studies have been done to identify potential prognostic value in breast cancer through PLR. The higher PLR in patients, the poorer survival times and larger infiltrated lymph nodes compared to lower PLR [4, 47]. High PLR is associated with poor overall survival of breast cancer patients.

4 Conclusion

This research proposed the combination of curcumin and IHT as an alternative treatment to chemotherapy in breast cancer treatment. Mice were inoculated with EMT6 breast cancer cells and assigned to 4 treatment groups: (i) untreated (control), (ii) orally curcumin (CUR), (iii) whole-body hyperthermia (FRWBH), (iv) orally curcumin with whole-body hyperthermia (FRWBH+CUR). Results showed that tumor growth inhibition and body weight gain in the combination treatment group (FRWBH+CUR) are significantly different compared to control. The group also has the longest median survival time (42 days) with no mortality during the experiment. This result indicates that combination treatment is well tolerated by the mice and has negligible levels of toxicity. However, frequent complications of cancer such as anaemia and thrombocytopenia are still observed in the combination treatment group. PLR and NLR results indicate that the combination treatment (FRWBH+CUR) has better prognosis outcome than single treatment and may become a potential alternative antitumor treatment of breast cancer.

Acknowledgements This work was supported by Research University Grant 15H87 and 15J83 and High Impact Research Grant 04G47.

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