

RESEARCH ARTICLE

Electro-Acupuncture Therapy Increases Serum Interferon- γ Levels in Rats with 7, 12 Dimethylbenz(α)anthracene (DMBA)-Induced Breast Tumors

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Abstract

Objective: To determine the effect of electro-acupuncture (EA) treatment on serum levels of interferon- γ (IFN- γ) in rats with 7,12-dimethylbenz(α)anthracene (DMBA)-induced breast tumors. **Methods:** Twenty five female Wistar rats were divided randomly into 5 groups: normal group (N; neither DMBA-induced nor treated with EA); control group (C; DMBA-induced only); EA 3 days : (DMBA-induced + EA for 3 days); EA 5 days: (DMBA-induced + EA for 5 days); EA 10 days: (DMBA-induced + EA for 10 days) group. Animals were acclimatized from day 1 to day 7. Subcutaneous injections of DMBA 10mg/kg BW was administered every second day, from days 7 to 35. Acupuncture was performed every second day from day 42. Rats were sacrificed on the second day after the last acupuncture, breast tumors excised and stained histological sections were analysed by light microscopy. At sacrifice, blood was extracted from the heart for measurement of serum IFN- γ by ELISA. **Results:** All of the DMBA-induced rats developed tumors. Electro-acupuncture significantly increased IFN- γ levels in DMBA induced rats, when compared to control group. **Conclusions:** Our findings suggest that EA significantly increases IFN- γ levels in DMBA-induced breast tumors.

Keywords: Breast cancer- acupuncture- IFN- γ - DMBA

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Introduction

Breast cancer is the most common cancer in women worldwide and a major cause of cancer death (Torre et al., 2015). Cancer prognosis may be related to the immune status, and innate and adaptive immune responses play a role in preventing recurrent breast cancer. Assessing the immune status is a useful method of predicting risk of metastasis and recurrence of breast cancer. However, breast cancer may decrease immune function by decreasing interferon gamma (IFN- γ) levels. Interferon- γ induced NK or lymphokine-activated killer cells use perforin and Fas ligand to mediate cytotoxicity to cancer cells (Standish et al., 2008). NK cells are lymphoid cells in the innate immune system, and play a role in host defenses for preventing early and metastatic cancer (Romee et al., 2014). NK cells also produce effector molecules, such as IFN- γ , that inhibit tumor angiogenesis (Smyth et al., 2002).

Acupuncture is an ancient Chinese medicine, which is effective as adjuvant therapy in some cancer conditions (Lu et al., 2008). Adjuvant therapies such as chemotherapy can cause side effects such as nausea, vomiting, pain, poor sleep quality and anxiety. Acupuncture could be reduce side effects of chemotherapy effectively (Tas et al., 2014).

According to the Traditional Chinese Medicine theory, acupuncture promotes the flow of qi and blood and regulates visceral function. This is said to occur because

the acupuncture point (acupoint) is the visceral reaction point of the body surface (Rong et al., 2011). Acupuncture regulate the balance of Yin and Yang in the body, as well as modulating parasympathetic and sympathetic activity (Takahashi, 2011). Acupuncture can also cause the release of serotonin in the brain and stimulate endogenous opiate (β -endorphin) release, thereby reducing cancer pain and enhancing the immune response (Lin and Chen, 2008; Kim and Bae, 2010). The acupoint Stomach 36 (ST36) is an immune-enhancing acupoint that can be used to enhance immunity in cancer patients (Ma, 2004). Acupuncture of acupoint ST36 can stimulate NK cells and IFN- γ production in normal rats (Johnston et al., 2011).

The objective of this study was to determine the effect of electroacupuncture on serum levels of IFN- γ in rats with 7,12-dimethylbenz(α)anthracene (DMBA)-induced tumors. DMBA is an immunosuppressor and a powerful carcinogen, that is used in animals to generate cancer in specific organs. DMBA is a polycyclic aromatic hydrocarbon, with an active metabolite capable of damaging DNA, and thus it can act as both an initiator and promoter of carcinogenesis (Miyata et al., 2001),

Materials and Methods

Animals and Study Design

Twenty five female Wistar rats (*Rattus norvegicus*),

12-13 weeks of age (150-250 g) obtained from the Laboratory of Pharmacology, Faculty of Medicine, Brawijaya University, were used in this study. Acupuncture did not begin until DMBA induction was over and that rats were acclimatized for seven days prior to DMBA induction. The experimental design for this study was the post-test only control group design. The rats were randomly divided into 5 groups of 5 rats per group. The groups were:

(1) Normal rat (neither DMBA-induced and nor treated with EA)

(2) Control group (C; DMBA-induced only)

(3) EA 3 days group (EA 3 days; DMBA-induced+EA for 3 days)

(4) EA 5 days group (EA 5 days; DMBA-induced+EA for 5 days)

(5) EA 10 days group (EA 10 days; DMBA-induced+EA for 10 days)

Animals were acclimatized from day 1 to day 7. DMBA-induced was administered every second day, from day 7 to 35. Acupuncture was performed every second day from day 42. Rats were sacrificed at second day from the last acupuncture intervention. Tumors excised and stained histological sections from them were analysed by light microscopy. At sacrifice, blood was extracted from the heart for measurement of serum IFN- γ by ELISA.

The study was conducted in the Laboratory of Pharmacology and Laboratory of Biomedic of Faculty of Medicine, Brawijaya University, Malang, East Java, Indonesia. The study was approved by the Health Research Ethics Committee, Faculty of Medicine, Brawijaya University (No. 246/EC/KEPK/03/2014).

DMBA tumor induction

Animal models of breast cancer was performed by providing DMBA (Sigma, USA) induction. DMBA induced a dose of 10mg/kgBW given by injected subcutaneous on mamary target. DMBA performed three times in a week for 14 times. DMBA solution made by ratio 10 mg DMBA was dissolved in 1 ml corn oil , and injected subcutaneous. Repeated injections of DMBA solution was injected on between two bottom nipples, divided into two doses, for right region and the left region. DMBA-induced for tumor induction was done every second day from day 7 to 35 (Kirubha et al., 2012).

Acupuncture Procedure

Acupuncture was started 7 days after the last DMBA injection and was performed every second days for 3,5 and 10 days. A certified acupuncturist performed all acupuncture treatments. In the human, the acupoint ST36 is located 5 cm below the patella and 2 cm lateral of the anterior crest of the tibialis anterior muscle (Johnston et al., 2011). In the rat, ST36 is located 0.5 cm below the fibular head of the hind leg. Sterile single-use stainless steel needles (0.3 mm x 15 mm; Huanqui Acupuncture Needles; Huanqui, China) were used for each treatment. Acupuncture was done unilaterally at acupoint ST36 (Zusanli). The needle was inserted to a depth of 3 mm. An electrode attached in ST41 (Jiexi). ST41 is located on the dorsum of foot, at the midpoint of the transverse

crease of the ankle joint, in the depression between the tendons of musculus extensor digitorum longus and hallucis longus. Electro-acupuncture was performed using the method of Yim (2007). An electrostimulator (Yingdi stimulator, China) was used to stimulate. The handle of the acupuncture needle at acupoint ST36 and an electrode attached to ST41 was connected with electrostimulator via the cable. Then electrostimulator activated, applied for 30 minutes, with a frequency of 15Hz (Yim et al., 2007). Intensity of EA was increase until visible muscle twitches in the lower limbs of rat.

Electro-acupuncture for 30 minutes cause the increase β -endorphin (Fang et al., 2013). Beta-endorphins are neuropeptides involved in pain killer and possessing morphine like effects (Sprouse-Blum et al, 2010)

Blood and Serum Preparation

At the second day from the last acupuncture, rats were sacrificed, and tumors identified and removed for measure IFN- γ level in serum and tumor identified. After euthanasia, the chest was opened and heart blood was aseptically obtained using a 3 cc syringe and then transferred to a test tube. Tubes were centrifuged for 10 minutes at 600 rpm, serum was removed and stored at -20 °C.

Histology

At the same time by taking serum, tumors were removed and immediately fixed in 10% formalin for 24 hours. Tissues were cut and arrange in tissue cassettes, dehydrated, dried, and blocked by liquid paraffin. The block was cut in 3-5 μ m sections, placed on a slide and stained with hematoxylin and eosin. Tissues were viewed under light microscope and photographed.

Measurement of IFN- γ Level

Serum IFN- γ levels were measured using commercially available ELISA kits according to the manufacturer's instructions (IFN- γ , LegendMax™, Biolegend).

Analysis of Data

To determine the differences in IFN- γ levels between the groups, data were analyzed by the ANOVA test using SPSS (version 20). A p value of <0.05 was considered statistically significant.

Results

Of the 25 rats in this study, 20 were DMBA- induced (Control, EA 3 days, EA 5 days and EA 10 days groups). All of the DMBA-induced developed tumors. Normal group included rats that were neither DMBA-induced and nor treated with acupuncture.

Histopathology

The sample from the normal group rat shows monomorphic mononuclear mammary epithelial cells, with large nuclei. There is no evidence of abnormal proliferation and the intralobular ducts are clearly visible. In contrast, the sample from a rat in the control (DMBA-induced) group shows polynuclear,

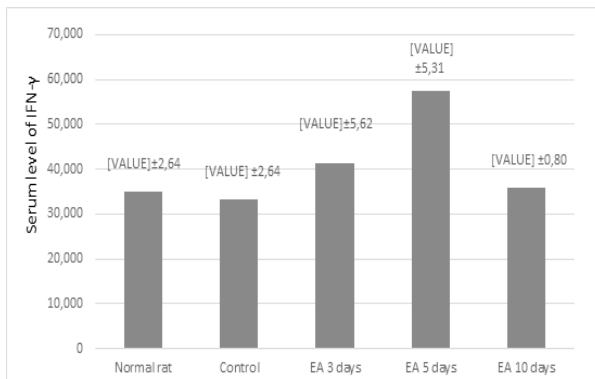


Figure 1. Level of IFN- γ in Each Group

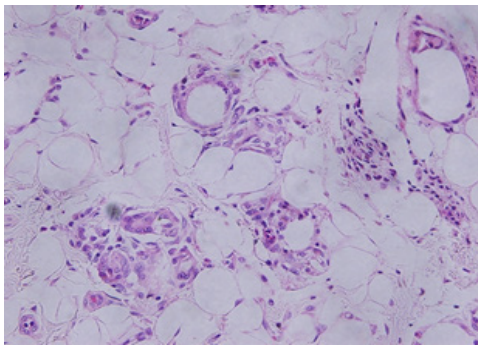


Figure 2. Histological Sections of Mammary Tissue From A Normal Rat (H and E X 400), Displaying Monomorphic Mononuclear Mammary Ductal Cells. There is no Evidence of Abnormal Proliferation

hyperchromatic, polymorphic epithelial cells with excessive proliferation almost blocking the intralobular ducts. Abnormal cells especially DCIS shows multifaceted morphologies and varies significantly with respect to nuclear atypia and architectural pattern (Pinder et al., 2010).

Repetition of electro-acupuncture causes histological changes in rat breast tissue. Electro-acupuncture for 3 days showing excessive proliferation of cells in the intralobular ducts (figure 3), there is no difference with control group. Electro-acupuncture for 5 and 10 days showing normal proliferation of cells in the intralobular ducts (Figure 4, 5).

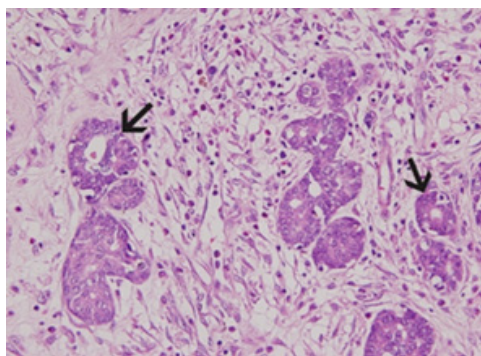


Figure 3. Histological Sections of Ductal Mammary Tissue from a Rat in the Control Group after 14 DMBA Inductions (H And E X 400), Showing Polynuclear, Hyperchromatic, Polymorphic Epithelial Cells with Excessive Proliferation Almost Blocking the Intralobular Ducts

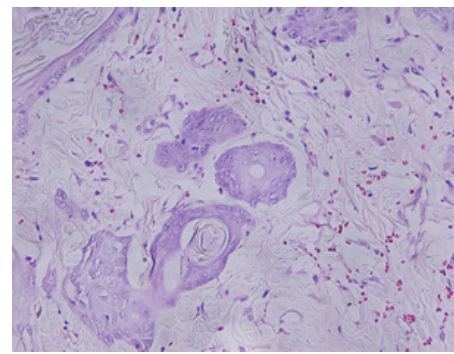


Figure 4. Histopathological Sections of Ductal Mammary Tissue from a Rat after 14 DMBA Inductions and EA for 3 Days (H And E X 400), Showing Excessive Proliferation in the Intralobular Ducts

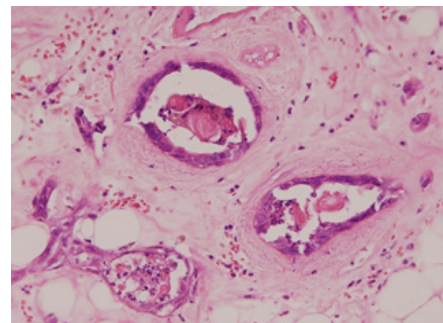


Figure 5. Histological Sections of Mammary Tissue from a Rat after 14 DMBA Inductions and EA for 5 Days (H And E X 400), Displaying Normal Proliferation

Serum IFN- γ levels

The serum level of IFN- γ was lowest in the control group (33.175 \pm 2.64 pg/ml). The level of IFN- γ was increase after AE for 3 days and 5 days. But in EA 10 days, serum level of IFN- γ was decrease (Figure 1). There was a no significant difference IFN- γ levels between the control groups and EA 3 days group ($p = 1.65$) and EA 10 days group ($p = 1.000$). There was significant difference in mean IFN- γ levels between control group and EA 5 days group ($p = 0.000$). The p value of statistical difference among group is summarized in Table 1.

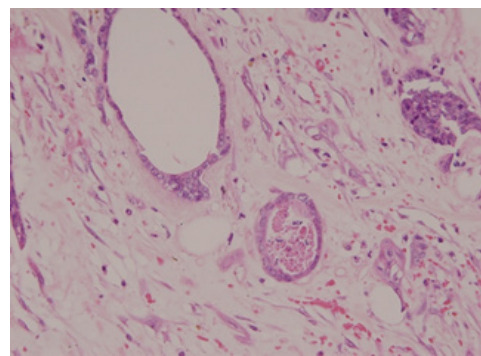


Figure 6. Histological Sections of Mammary Tissue from a Rat After 14 DMBA Inductions and EA for 10 Days (H And E X 400), Displaying no Evidence Abnormal Proliferation

Table 1. Difference level of serum in each group

Group		Mean and SD	Sig	95% CI
Control group	EA 3 days	41.41±5.62	0.165	-18.1-1.70
	EA 5 days	57.56±5.31	0.000	(-34.3)-(-14.4)
	EA 10 days	35.91±0.80	1.000	-13.2-7.80

† Bonferroni ANOVA; two sided P.value<0.05

Discussion

Breast cancer in animal can be induced with DMBA (Sihite and Endang, 2013). DMBA is a potent mammary chemical carcinogen, that is highly lipophilic and requires metabolic activation. DNA is damaged by epoxides, an active metabolite of DMBA conversion in cells. Damage of DNA in mammary cells can initiate breast cancer (Barros et al., 2004)

In this study, we used subcutaneous injection of DMBA to induce breast cancer in Wistar rats. Subcutaneous injection of DMBA increases the activation process of DMBA in the mammary gland, when compared to the sonde method. Histological examination showed excessive proliferation of cells covering the intralobular ducts, diagnostic of carcinoma in situ (Weigelt et al., 2010). In a similar study, 75% of the rats developed a tumor in the eight weeks after DMBA induction and 100% of rats developed tumors 13 weeks or 91 days after induction (Barros et al., 2004). In this study, DMBA induction was done every second day from day 7 to 35. In previous study, induction breast cancer with DMBA was given at the dose of 7,5mg/kgBW subcutaneously in the mammary region once a week for 4 consecutive weeks (Jayakumar et al., 2014). Another study, a single intragastrically dose of 80-100 mg DMBA/kgBW induced tumors with latencies that generally range between 8-21 weeks (Russo and Russo, 2011).

For acupuncture procedure, we use unilateral acupuncture in right rats leg. In historical texts, was not clear wether needling technique should be applied bilaterally, contalaterally or ipsilaterally (Sun, 2007). Another Clinical research have demonstrated that unilateral acupuncture is as affective a bilateral acupuncture (Tillu et al., 2001).

In our study, electrical stimulation was performed to needle at ST36 and electrode in ST41 as a mean we use EA. Electro-acupuncture is a form of acupuncture where a small electrical current is passed between pairs of acupuncture needles or electrode. The procedure for EA involves the needles inserted as in a traditional treatment and the needles are attached to device that sends electrical currents into the body. The purpose of EA were to strengthen the stimulation in the acupoint and influence the cells tissue and entire systems (Konopka et al., 2001).

The frequency and duration of the electricity was varies based on the condition being treated. In human, tingling or mild involuntary muscle twitches was experience during EA (Barlas et al., 2006). Selection of the duration of EA in this study is based on previous studies that EA for 30 minutes cause the increase β -endorphin (Fang et al., 2013).

Electro-acupuncture with frequency of 15 Hz cause increased β -endorphin (Taguchi, 2008). Expression of β -endorphin lead to increased production of IFN which enhances NK activity (Tache et al., 2012). In this study, EA for 5 days led to a significant increase in IFN- γ and no evidence of abnormal hiperproliferation in the intralobular ducts (Figure 1, 5). Similarly, in EA for 10 days showing normal proliferation in the intralobular ducts, while the level of IFN- γ decrease almost aqual with base line (Figure 1, 6). This phenomenon possible related to the role of IFN- γ and its ability to induce NK cell cytotoxicity in cancer cells.

In our study acupuncture result in an increase of INF- γ in EA 3 days and EA 5 days, but IFN- γ levels decreased after EA for 10 days. Another study reveal was thirty minutes of electroacupuncture daily for 3 days, in the bilateral zusanli (ST36) acupoint, increased splenic IFN- γ levels in BALB/c mice (Hisamitsu et al., 2002). In our study serum level of IFN- γ in EA 3 days group was increase but not significant. Previous study demonstrated that acupuncture in healthy human volunteers resulted in a nine times increase in IFN- γ levels (Yamaguchi et al., 2007). The slight increase in the level of IFN- γ in this study might be the measurement this cytokines were performed on cancer rat with immunodeficiency.

Acupuncture cause increasing of highest levels of IFN- γ and no evidence hiperproliferation ductal cells in the EA for 5 days group. Furthermore, in EA for 10 days group, level of IFN- γ was decline to normal value, and histopathologically show the normal proliferation in breast ductal cells of rat. Its demonstrate that acupuncture is help keep the disrupted homeostatis of internal environment normalized or recovered including regulation of cytokines in the body (Xia et al, 2012)

IFN- γ is a cytokines that can up regulate NK cell activity. The same stimulation also increased splenic β -endorphine, which may increase NK cell activity by regulating IFN- γ (Hisamitsu et al., 2002). Acupuncture enhances the immune system through control of the autonomic nervous system.(Kim and Bae, 2010) Johnston and colleagues hypothesize that stimulation at acupoint ST36 can activate neurotransmitters in the brain. Acupuncture induces nitric oxide synthase to increase the production of nitric oxide (NO) at the skin site of the needle. NO is a neurotransmitter that sends signals to the brain via the spinal neurons. Acupuncture can stimulate the hypothalamic-pituitary- adrenal axis to release the endogenous opioid neurotransmitter, β -endorphin. β -endorphin then travels from the brain via the bloodstream to locations in the body that contains immune cells. This results in activated TH-1 cells and stimulates production of IL-2. IL-2 will lead to an

activation of NK cells, the production of IFN- γ by NK cells (Levy et al., 2011) and increased production of other cytokines (Johnston et al., 2011). These cytokines possess tumoricidal or cytolytic activity against tumor cells, which can directly suppress tumor growth. As well as studies on mice and with humans, acupuncture at ST36 results in increase INF- γ and increased activity of NK cells. Our results are in agreement with other studies in mice and in humans, suggesting that acupuncture at acupoint ST 36 increases levels of IFN- γ and enhances NK cell activity (Johnston et al., 2011) 1.

This research showed that acupuncture enhances the immune system in a rat model of breast cancer by increasing levels of IFN- γ in EA 5 days. Our DMBA induced rat model may prove useful in understanding the role of acupuncture in the treatment of breast cancer.

Conflict of interest

The authors declare that there are no potential conflicts of interest with funding, authorship and/or publication of this article.

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