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Research Article

THE EFFECT OF ELECTROACUPUNCTURE AT ST36, BL18, AND BL20 ACUPUNCTURE POINTS ON INTERFERON GAMMA LEVEL, MITOSIS, APOPTOSIS, AND TUMOR MASS IN C3H MICE MODEL WITH BREAST ADENOCARCINOMA AND ITS CORRELATION WITH THE NUMBER OF INTERVENTIONS

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A B S T R A C T

Recent advancements in immunotherapy on cancer provides hope for future treatments of breast cancer. This study aimed to evaluate the effect of electroacupuncture (EA) at ST35, BL18, and BL20 acupuncture points on Interferon Gamma level, mitosis, apoptosis, and tumor mass in C3H mice model with breast adenocarcinoma. This study is a four armed randomized experimental study with 20 subjects allocated into control group, one time intervention group, two times intervention group, and three times intervention group. The data in this study is analyzed using F variance analysis test and Spearman *rank* correlation test. The results showed that there was an increase in the Interferon Gamma level in the one, two, and three times intervention groups, but not statistically significant (p=0.229). There was a decrease in mitosis in the intervention groups (p=0.009) while there was no increase in apoptosis (p=0.361) and no decrease in tumor mass. There was a correlation between two times EA intervention and Interferon Gamma level (p=0.026) and between the number of EA intervention and the number of cells undergoing mitosis (p=0.003). EA intervention reduced the mitosis in C3H mice model with breast adenocarcinoma, and correlates with the increase in Interferon Gamma level at two times intervention.

INTRODUCTION

Breast cancer is one of the most commonly found cancer. Based on the predictions of the International Agency for Research on Cancer, there will be 1.15 million new cases worldwide on 2020. 55% of deaths due to breast cancer happened in the developing countries. In Indonesia the incidence of breast cancer is 26 cases per 100,000 population. The data from the *Sistem Informasi Rumah Sakit* (SIRS) showed that from 2004 to 2007, breast cancer is one of the ten most common cancer in patients that visited hospitals in Indonesia (Rasjidi, 2010).

Although conventional treatments for cancer such as surgery, chemotherapy, radiotherapy, and hormonal therapy can increase the life expectancy of the patients, more researches is still needed to improve the treatment of breast cancer. The knowledge on the immunologic conditions of breast cancer patients is still under resarch, for example the possibilities of increasing the destruction of cancer cells by manipulating the patient's immune responses.

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Through the understanding of immunologic behaviors in cancer patients, much of the cellular and molecular reactions involved in the primary cancer cells eradication, metastasis, and the elimination of drug residue after conventional medications can be understood (Bamiceawidjaja *et al*, 2009).

Meanwhile, researches on the role of Interferon have been done for many kinds of disease treatment. Interferon is proven to be able to increase the eradication of c-line breast cancer with erB2+ when combined with 4D5, but caused various side efffects at the same time such as fever, alopecia, and reduced appetite (Yin *et al*, 2010). Researches in the role of Medical Acupuncture for the treatment of breast cancer has also been done, considering the many side efects and contraindications of drug treatments, especially in patients with organ dysfunction or geriatric patients. In this study, selected acupuncture points will be stimulated in order to determine the immunologic reactions of mice models with breast cancer. The mice model is used, besides the ability to induce breast cancer on mices in the labomiceory, because of the presence of acupuncture points similar to human in mices. Based on previous publications, the stimulation of some acupuncture points is related to various immunologic changes in human and

animal models. The acupuncture point ST36 is often used for immunologic purposes. The acupuncture point BL18, which share the same dermatome as the liver, is used to stimulate the liver; while the acupuncture point BL20, which share the same dermatome as the spleen, is used to stimulate the spleen (Lewis *et al*, 1993) (Hotaling *et al*, 1996).

Cancer cells expressed antigenic proteins that will trigger a humoral and cellular defensive immunologic reactions. Interferon Gamma is a class II cytokine in the form of water soluble protein. Interferon Gamma is produced by the Natural Killer (NK) cell in both the innate and adaptive immune responses and plays a role in the mitosis process, especially in extreme cancer cells (Lin *et al*, 2013).

Medical Acupuncture research on cancer diseases is still very limited, therefore this experimental study is done for the very first time in Indonesia in order to determine the effect of acupuncture point stimulation on the growth of breast cancer.

MATERIALS AND METHODS

This study is an experimental research with Completely Randomized Design using animal model for the case and control groups. The purpose of this study is to evaluate the changes in Interferon Gamma level, the number of cells undergoing mitosis and apoptosis, and tumor mass after Electroacupuncture (EA) stimulation at the ST36, BL18, and BL20 acupuncture points. The intervention groups is compared to the control group (without intervention) and then the correlation between the number of intervention and Interferon Gamma level and the number of cells undergoing mitosis is evaluated.

The subjects of this study were C3H mice models with breast adenocarcinoma. The number of sample needed was calculated using the Federer formula and resulted in five samples per group. In this study, the groups compared were the control group, one time EA intervention (1 x EA) group, 2 x EA group, and 3 x EA group.

The inclusion criterias in this study were C3H mice model that had been successfully induced with breast adenocarcinoma, tumor size reaching one centimeter in one of its dimensions, and no anatomical deformity on the region of the selected acupuncture points. The exclusion criteras were failure to induce breast adenocarcinoma and unhealthy mice model.

The intervention was done by inserting 13 mm acupuncture needle perpendicularly at the ST36, BL18, and BL20 acupuncture points. The needle was then connected to KWD-808 electrostimulator device. The electrostimulation was done in a continuous wave with 2 Hz frequency for 15 minutes each session. In the 1 x EA group, the intervention was done on the first day and then on the seventh day the mice models were euthanized by maximum blood drawing from the heart. In the 2 x EA group, intervention was done on the first and seventh day, and on the 14th day the mice models were euthanized. In the 3 x EA group, intervention was done on the first, 7th, and 14th day, and then the mice models were euthanized on the 21st day. In the control group, the mice models were euthanized on the 14th day.

The Interferon Gamma level was assesed using the ELISA method. Te assessments of the tumor tissue include the number of cells undergoing mitosis, the number of cells undergoing apoptosis, and tumor mass. Cellular assessment was done by fixating the tumor tissue using formaldehyde; the middle section of the tissue was then cut and fixated using methanol. The specimen obtained was then colored using Hematoxilin Eosine (HE). The cell counting was done using Image J and then confirmed manually based on the characteristics of cells undergoing mitosis and apoptosis. The tumor mass was weighted and measured in gram (g) after the tumor tissue was excised from the euthanized animal.

This study was done after obtaining the ethical clearance from the Animal Care and Use Commitee of PT. Bimana Indomedical Bogor on February 2nd 2016; the approval number was R.01-16-IR. Normality testing was done for mice model weight, tumor volume, and sex. Mice model weight and tumor volume was tested using F test (Analysis of Variance), while sex was tested using chi-square. The comparison of Interferon Gamma level, number of cells undergoing mitosis, number of cells undergoing apoptosis, and tumor mass was done using F test (analysis of Variance) and Duncan Multiple Range Test. The analysis of correlation between number of intervention and the Interferon Gamma level and cells undergoing mitosis and apoptosis was done using Spearman's Rank Correlation Coeffficient.

STUDY RESULTS AND DISCUSSION

Research on the effect of EA at the ST36, BL18, and BL20 acupuncture points on the Interferon Gamma level, mitotic picture of breast adenocarcinoma in C3H mice model, and the correlation between the number of intervention and the increase in Interferon Gamma level, the decrease in tumor mass, the decrease in the number of cells undergoing mitosis, and and the increase in the number of cells undergoing apoptosis was done on 20 *Mus musculus* mice model strain C3H which was divided into four groups: control group, 1 x EA group, 2 x EA group, and 3 x EA group. Assessment was done after 15 minutes of intervention. The first step in analyzing the data was by performing the normality test for the numerical data using the Saphiro-Wilk test, which resulted in the p value of > 0.05 for each parameter; this result showed that the data was normally distributed (Table 1). Therefore to analyze the mean difference of various measurements between each groups, Analysis of Variance (ANOVA) was used.

Table 1 Baseline data of the four groups

Baseline data	Groups				P value*
	Control (n=5)	1 x EA (n=5)	2 x EA (n=5)	3 x EA (n=5)	
1. Mice weight (g)					
Mean (SD)	23,9 (3,03)	25,4 (1,54)	27,0 (3,87)	25,9 (5,83)	
Range	19,7-28,0	24,3-228,0	23,4-32,2	18,7-34,2	0.229
2. Tumor volume (mm^3)					
Mean (SD)	14,49 (2,75)	16,72 (6,40)	20,18 (5,94)	22,07 (6,51)	
Range	10,21-17,52	11,95-27,50	11,18-27,27	11,49-27,42	0.182
3. Sex					
Male	2	3	0	1	0.190
Female	3	2	5	4	

Notes : *) based on F test (Analysis of Variance), except for sex using chi-square test.

Normality testing of the data showed that the baseline data of the mice model weight (p=0.229), tumor volume (p=0.182),

and sex (p=0.190) of the four groups was having a normal distribution (Table 1).

Table 2 Comparison of Interferon Gamma level, tumor mass, and the number of mitosis and apoptosis of the four groups

Variable	Groups				P value*
	Control (n=5)	1 x EA (n=5)	2 x EA (n=5)	3 x EA (n=5)	
1. InterferonGamma level (pg/cc) :					
Mean (SD)	5,85 (2,32)	7,02 (2,05)	8,51 (1,39)	7,12 (1,82)	0.229
Range	3,03-8,80	3,78-8,99	6,80-10,34	5,29-9,56	
2. Tumor mass (g)					
Mean (SD)	1,54 (0,56)	1,45 (0,83)	4,68 (2,64)	4,72 (2,58)	**0.016**
Range	0,90-2,30 (a)	0,21-2,30 (a)	0,90-7,80 (b)	2,20-8,60 (b)	
3. Mitosis number					
Mean (SD)	5,67 (1,58)	3,78 (11,16)	2,93 (0,79)	3,13 (1,11)	**0.009**
Range	4,22-8,00 (a)	2,89-5,78 (b)	2,11-4,22 (b)	2,11-4,89 (b)	
4. Apoptosis number					
Mean (SD)	12,13 (2,85)	11,33 (2,34)	13,53 (2,10)	10,46 (3,40)	0.361
Range	9,0-16,0	8,67-14,33	10,67-15,33	6,33-14,33	

Notes : *) F test (Analysis of Variance), mean number followed by different letters on the rows showed significant difference based on the Duncan Multiple Range Test.

Interferon Gamma was increased in 1 x EA and 2 x EA groups; and decreased in 3 x EA group, although the value is still higher than control group. The p value of 0.229 showed no significant difference between the groups. The number of cells undergoing mitosis were decreased in 1 x EA and 2 x EA groups but were increased in in 3 x EA group, although still lower than control grup. There was a significant difference in the number of cells undergoing mitosis between the groups (p=0.009). The number of cells undergoing apoptosis were increased in the 2 x EA group, but the difference is not significant (p=0.36).

Table 3 Analysis of correlation between number of EA intervention and various variables measured in the 2 x EA group

Correlation between number of EA intervention with	Correlation coefficient (r_s)	P value
1. Interferon Gamma	0,510	**0,026**
2. Number of cells undergoing mitosis	-0,762	**<0,001**

Notes: r_s = Spearman rank correlation coefficient

Table 4 Analysis of correlation between number of EA intervention and various variables measured in the 3 x EA group

Correlation between number of EA intervention with	Correlation coefficient (r_s)	P value
1. Interferon Gamma	0,213	0,366
2. Number of cells undergoing mitosis	-0,628	**0,003**

Notes: r_s = Spearman rank correlation coefficient

The Interferon Gamma level in the 2 x EA group showed a significant difference with the correlation coefficient of 0.510 and the p value of 0.026. In the 3 x EA group, there was no significant difference with the correlation coefficient of 0.213 and the p value of 0.366 (Table 3 and Table 4).

The number of cells undergoing mitosis showed a significant difference, whether in the 2 x EA group with the correlation coefficient of -0.762 and the p value < 0.001, or in the 3 x EA group with the correlation coefficient of -0.628 and the p value of 0.003. This result showed a negative correlation between 2 x EA and 3 x EA intervention and the number of cells undergoing mitosis (Table 3 and Table 4).

In this study, C3H mice model was used due to the availability of the specimen in the labomiceory of Patologi Anatomi FKUI/RSCM and the availability of a strain with breast adenocarcinoma as a donor. The tumor tissue in the donor could be used as a source of Mice Mammilary virus which would be injected into recipient mices. The other considemiceion was that C3H mices had a similar immune system to human (Bamiceawidjaja *et al*, 2009) (Kresno, 2003), therefore the effects of electroacupuncture could be directly observed. Other acupuncture researches also used mice models, such as the research by Kim SK *et al*. that observed an immunomodulatory effect on mice models that was immunisized with DNP-KLH43, and the research by Yu Y *et al*. that observed changes in Interferon Gamma, Interleukin (IL)-2, and cytotoxicity level of Natural Killer (NK) cells on healthy mice model. By using mice models, the biochemical changes related to immunologic phenomenons caused by electroacupuncture could be measured.

The acupuncture points selected in this study were ST36, BL18, and BL20. The selection was based on a study by Zhang W *et al*. In the research, 31 SD mice were divided into four groups: controal group, overexercise group, ST36 group, and SP6 group. The mice was made to swim in the water tank for 90 minutes every day until the eighth day. From the eighth to 13th day the exercise was increased to three times a day. Acupuncture intervention using fine needle was done before exercise everyday until the 13th day. A significant increase in Interferon Gamma level compared to other groups was observed in the ST36 group, while a decrease was observed in the SP6 group (Zhang *et al*, 2011).

Wenjing *et al*. performed a research by stimulating the BL20 acupuncture point on 40 SD mices which were divided into four groups: control group with chronic fatigue syndrome (CFS), ginsenoside Rg3 group (Rg3), acupuncture group (A), and acupuncture plus ginsenoside Rg3 group (A+Rg3). Fatigue in the animal model was induced by swimming in cold water everyday up to the 21st day. The acupuncture intervention given was the stimulation of BL20 acupuncture point every day for seven days. Interferon Gamma level was increased in the A group, Rg3 group, and A+Rg3 group (Zhang *et al*, 2015).

The acupuncture point BL18 was selected because the points matched the dermatome of the liver, which is a part of the reticuluendothelial system. Therefore it was expected that the stimulation of the BL18 acupuncture point could affect the activity of NK cells in the liver and increase the Interferon gamma level (Bamiceawidjaja *et al*, 2009) (Kresno, 2003) (Abbas *et al*, 2009).

Jhonston ML concluded that, based on various researches, manual acupuncture stimulation at the ST36 acupuncture point induced the activity of the enzyme Niric Oxide Synthase in the kemiceinocytes through the increase in Beta-endorphine level, causing the cells to produce Nitric Oxide (NO) which will act

as a neurotransmitter and sent signal through the spinal cord to the brain. In vitro administmiceion of β-endorphine on NK cells could increase perforin, granzym B, and Interferon Gamma level and increasing the mRNA transcription. At the same time, NO could directly stimulate the NK cells to produce Interferon Gamma (Jhonston *et al*, 2010) (Ma *et al*, 2003) (Dokur *et al*, 2005).

The stimulation method used in this study was electroacupuncture with 2 Hz frequency. This stimulation method was selected based on other researches on animal model and human subjects which showed that low frequency electroacupuncture or manual acupuncture stimulation could induce cytokines related to opioid receptors and receptors on the NK cells (Jhonston *et al*, 2010) (Yamaguchi *et al*, 2007) (Chen *et al*, 2001).

In this study, intervention was done once, twice, and three times with the range of one week between each session. This method was selected according to other experimental acupuncture research using mice models. On the site of needle penetmiceion there will be histopathological changes, such as neutrophil infiltmiceion, which will dissappear completely 72 hours after penetmiceion. The range between each intervention session was set to be seven days under the considemiceion that the weak condition of the mice models and the severity of the disease could slow down the healing process. Some researches also suggested that the ideal range between two sessions of intervention should be seven days (Pissolat *et al*, 1961) (Yun *et al*, 2005).

The Interferon Gamma measurement was done seven days after the last intervention. This was done based on a research by Yamaguchi on human subjects with manual acupuncture intervention. The Interferon Gamma level increased one day after manual acupuncture stimulation and kept increasing until reaching the peak at the seventh day (Yamaguchi *et al*, 2007).
Mitosis is one of the parameter used to determine the growth of cancer cells. Therefore a significant decrease in mitosis could be interpreted as a decrease in tumor growth. Acupuncture research on the relation of acupuncture stimulation to the mitosis of tumor tissue had not been done before, thus there are no research that can be used as a comparison.

In this study, the number of breast cancer cells undergoing apoptosis was lower in the 1 x EA group compared to the control group. The number increased in the 2 x EA group but decreased again in the 3 x EA group. In the 3 x EA group, there was also a decrease in Interferon Gamma level.

Based on a research by Ignacio Garcio-Tunon on C-line breast cancer cells, the effect of Interferon Gamma on cancer cells depended on the type of receptors on the cancer cells and the type of cancer cells themselves. In this study, the measurement of Interferon Gamma level was done without considering the type of receptors of the cancer cells or the degree of severity of the tumor.

The decrease in Interferon Gamma level in 3 x EA group was followed by a decrease in the number of cells undergoing apoptosis. This might happen due to the escape phenomenon of the tumor cells which caused the failure in preventing the tumor cell prolifemiceion. In this study, the assessment of cell apoptosis was done using the HE coloring. For further analysis, it is best to use the Tunel method (Terminal Deoxynucleotidyl Transferase dUTP Nick-end Labelling) which is more accumicee. The observation of cancer cells undergoing apoptosis had never been done before in acupuncture research. Some researches were done on subjects with cerebrovascular disease, and all of them using the Tunel coloring method.

The tumor mass was reduced in 1 x EA group, but increased in 2 x EA and 3 x EA groups. The p value of 0.016 showed that this increase is significant. This result is not the expected result. Based on the research of Oestreicher N, White E, Malose KE, and Parter PL, the prolifemiceion of tumor cells is related to Ki-67 and mitosis. Increasing body weight could increase the expression of Ki-67 but is not related to the decrease in mitosis (Oestreicher *et al*, 2004). In this study, the increase in body weight of the mice model tend to be followed by the increase in tumor mass. In mice models with the body weight of 23.9-25.4 g, the tumor mass were 0.057-0.064 % of the body weight; while in mice models with the body weight of 25.9-27.0 g, the tumor mass were 0.17-0.18 % of the body weight.

In the tumor histopathological assessment of the control group, the tumor cell prolifemiceion micee was equal to the tumor cell destruction micee, given an adequate stroma protein material. In the 1 x EA group, the tumor cell prolifemiceion was adequate, while the tumor cell destruction with protein material was minimum. In the 2 x EA and 3 xEA groups, the tomor cell histopathological assessment result was the same as in control group. The increaseing tumor weight might be affected by the mice model's body weight and the intralumen necrosis and the stroma reaction forming that showed the secondary result of tumor cell prolifemiceion. This caused the specimen to appear as if there was an increase in tumor cell prolifemiceion.

In this study, correlation analysis was done between the number of EA intervention and two measured variables: Interferon Gamma level and the number of cells undergoing mitosis. In the 2 x EA intervention, there was a significant difference on the Interferon Gamma level and the number of cells undergoing mitosis; while in 3 x EA intervention, there was no significant difference on the Interferon Gamma level but there was a significant difference in the number of cells undergoing mitosis. There was a decrease in Interferon Gamma level in the 3 x EA intervention, therefore the statistical analysis showed no significant difference. This result might be caused the condition of the mice models, as could be seen in the remaining food leftover: there were more food leftovers in the 3 x EA group compared to the 1 x EA and 2 x AE groups.

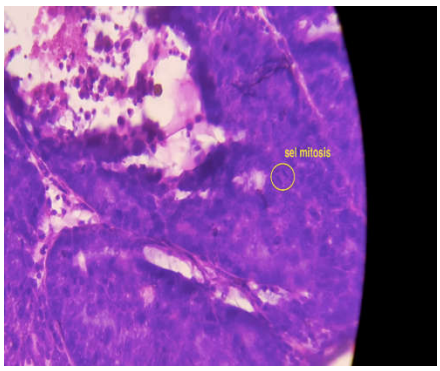

Figure 1 Cell undergoing mitosis

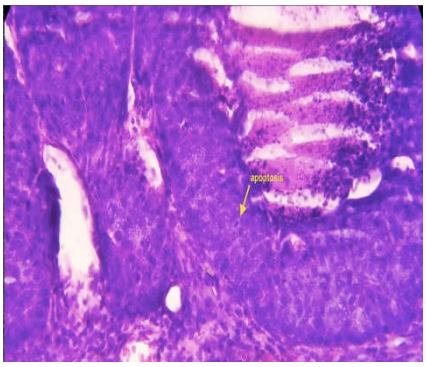

Figure 2 Cells undergoing apoptosis

CONCLUSION

In this study, it can be concluded that there is no difference in the Interferon Gamma level in mice blood after once, twice, and three times EA intervention at the ST36, BL20, and BL18 acupuncture points compared to control. There is a difference in the number of cells undergoing mitosis, but there is no difference in the number of cells undergoing apoptosis and tumor mass. There is a positive correlation between the number of EA intervention (two times) and Interferon Gamma level. There is a negative correlation between the number of EA intervention and the number of cells undergoing mitosis.

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