The Effect of Higher Temperature on the Aggressiveness of the Mice Adenocarcinoma Mammae Cells

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Abstract

The incidence of breast cancer patients was considered high both in the world and in Indonesia. The pathogenesis of breast cancer is influenced by many factors and one of the factors that would be focus on in this research is temperature.

The aim of this research is to analyze the temperature effect on the proliferation of adenocarcinoma mammae cells *in vivo*.

A true experimental design is used with C3H mice exposed to: 20-22°C, 25-27°C, and 32-34°C for 6 hours daily in 2 weeks. Two kinds of staining were used, agyrophilic nucleolar organiser region (AgNOR) for cells proliferation and Hematoxylin-Eosin (HE) for mitotic count.

AgNORs result revealed that the mean AgNORs (mAgNORs) and proliferative index AgNORs (pAgNORs) of group 32-34°C was the highest and the differences are significant with p= 0,000 through one-way ANOVA. HE result showed that there was a tendency of group 32-34°C to have a higher mitotic count compared to the other two groups but was insignificant through Mann-Whitney. The result of the AgNORs study is expected to give an idea for breast cancer non-invasive therapy by manipulating environmental temperature.

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Introduction

According to an epidemiological study in 2009, there were more than one million women diagnosed with breast cancer every year.[1] The number of patients was also high in Indonesia, noted as the most outpatient cases in 2007, recorded in Dharmais Hospital Jakarta, are breast cancer.[2]

Adenocarcinoma mammae is a cancer that start in the in the ducts or lobules of the breast.[3] The pathogenesis of breast cancer is influenced by many factors[4]. One of the factors that would be focus on in this research is temperature[5]. There are several previous studies that support the selection of this topic. A study in 1937 mentioned that frogs, injected by human red blood cells, formed antibodies when exposed to 22-27°C, but not making antibodies when they are exposed to 8-10°C.[6]Another research in vitro, comparing the cells proliferation and viability of normal human diploid cells and malignant endometrial adenocarcinoma which are cultured in 36-44°C for 24 hours for 11 days and the result was the malignant cells were drastically damaged at temperature more than 40°C with vacuolization in cytoplasm, aggregation of cells, and dispatched cells were shown.[7]

There are certain genes, according to studies, influenced by temperature. Wild-type p53 will inhibit transformation because it acts as a tumor suppressor genes in carcinogenesis. However, mutant p53 can contribute to transformation. One of the mutants is temperature-sensitive called p53val135. At 37.5°C, it can induce transformation, but suppress transformation at 32.5°C. The inhibition of both transformation and proliferation is reversible upon temperature upshift.[8]

In accordance to genes, signaling factors come along in the process of cell proliferation. Heat-induced key signaling factors such as Akt (A serine/threonine kinase), p38, extracellular signal-regulated kinase (ERK) and heat shock

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protein (HSP) may play important roles in anti-apoptosis or cellular proliferation pathway. Inhibitors of these signaling factors may be potentially beneficial for therapy because if signaling factors induce anti-apoptosis, inhibitors of the signaling factors may enhance heat-induced apoptosis.[9]

Sixteen mutants isolated from mouse mammary carcinoma FM3A cells were isolated at 39°C. Analyzed by flow microfluorometric, those cultured for 16 hours indicated that 5 clones were arrested in the G1 to S phase of thecell cycle, 6 clones were in the S to G2 phase. These 11 clones showed a rapid decrease in DNA synthesis after temperature shift-up without a decrease inRNA and protein synthesis. 2 clones were arrested in the G2 phase. The other 3 clones showed 8C DNA content after incubation for 28 hours, indicating defects in mitosis or cytokinesis.[10]

Vasodilatation of peripheral blood vessels may contribute in homeostasis of body temperature. For example, when we are exposed to hot environment, peripheral blood vessels will dilate so that there is heat loss through the skin. In normal tissue, blood flow may increase up to 44^{o}C then decrease at higher temperature or longer duration of heating. On the other hand, tumor blood flow increases only up to 40-41^{o}C, then the blood supply may be impaired due to the vulnerability of tumor blood vessels, influenced by factors such as less heat receptors and sluggish blood flow.[11] In this study we chose the environmental temperature 20-22°C, 25-27°C, and 32-34°C.

Mammary adenocarcinoma, heated for 30 minutes at temperature higher than 40.5^{o}C, showed a decrease in blood flow. Hyperthermia can reduce ATP production in the cell that will activate enzymes which can impair membrane integrity then releasing lysosomal enzymes. Lack of cellular ATP will also stimulate anaerobic glycolysis which will decrease intracellular pH that will enhance the lysosomal enzyme work causing cell death. The low pH causes erythrocytes to lose their membrane flexibility that will impair tumor blood flow. Vascular stasis results and will reduce the oxygen supply, reducing intracellular pH and hence supporting lysosomal activity.[12]

Environmental temperature is a daily life component that is important for the body to regulate homeostasis or to maintain the appropriate temperature for the cells in the body. Its role in cancer cells proliferation has been studied mostly in culture and will be analyzed in mice through this research.

Materials and methods

Research Design

A true experimental design (parallel) was used as the research design. There were 3 different temperature groups, namely 20-22°C, 25-27°C, and 32-34°C. These 3 temperature groups were given in different 3 cages and each cage consist of mice C3H strain with adenocarcinoma mammae. This exposure was given for 6 hours daily in 2 weeks. Calorimetric principle was applied with the use of thermostat for controlling the temperature in cages with lamps as the source of heat and each was set according to the 3 desired temperature groups. 20-22°C is maintained by adding portable air-conditioner and 25-27°C was chosen as the control regarding daily room temperature.

Two kinds of staining were used for analysis in order to check the cells proliferation by AgNORs and confirm the growth of adenocarcinoma mammae by Hematoxylin Eosin with a focus on the mitotic count. Parameters used in AgNORs are mAgNORs as the mean AgNORs and pAgNORs as the proliferative index AgNORs. In Hematoxylin Eosin, the criteria for mitotic count can be divided into 3, namely weak with mitosis less than 10% in 10 microscopic fields, strong with mitosis more than 20% in 10 microscopic fields, and medium in between.

Animal

There are some inclusion criteria for this research in which the mice C3H should be 3-4 months old, either female or male with weight approximately 20-30 gram, and given the same amount and type of daily food intake.

Temperature exposure

The 4 cages used the principle of calorimetry, in which the heat lost by the system would equal to the heat gained by the surroundings.[13] The temperature was set in each cage by controlling the lamps heat up to 32-34°C. The 25-27°C was obtained with the same way, while the 20-22°C was maintained by combining the heat from the lamp and the cool temperature from the portable air-conditioner in front of the

cage. After the temperature stable, the mice were put into the cages and monitored for 6 hours each day in 2 weeks duration. We chose the temperature based on the environmental temperature. Tropical climate is one with an average temperature of above 18 degrees Celsius.

Tumor transplantation

In order to have mice C3H with adenocarcinoma mammae, donor was needed from the same mice strain which had already got a tumor. With sterilized tools and equipment prepared, mice were anesthetized with ether. Dissection was done to obtain the gel-like tumor tissue. The tumor tissue was rinsed with PBS solution and cut into small pieces in a petri dish. After being put on ice, it was aspirated with 18g x 1 ½" syringe and injected to the recipient mice.

Tumor measurement

Mice were weighed twice. First measurement was done before transplantation. At that moment, the average weight of the mice was almost the same. After the transplantation and exposure to three different temperatures, the second measurement was conducted.

AgNORs staining

Each of the paraffin blocks was deparaffinized with xylol I and II, followed by rehydration process with decreasing concentration of alcohol solution (100%, 95%, 70%). Then it is deionized by aqua. All the steps took about 2-5 minutes. Mix gelatin with silver nitrate with the ratio of 1:2 and put a drop into the slides for 60 minutes at room temperature or 40 minutes in a dark room. Rinse with aqua. Next, counterstain with Hematoxylin Mayer 1-2 minutes and rinse once again with aqua, alcohol 10%, and xylol respectively. Finally, cover it with covering glass containing Entellan.[14]

Hematoxylin-Eosin staining

Each of the paraffin blocks was deparaffinized with xylol, xylene twice for 5 minutes. Hydrate with decreasing concentration of alcohol solution: ethanol 1 minute, 96% alcohol 1 minute, 70% alcohol 1 minute, and aquadest 1 minute. Stain with Hematoxylin Mayer 5-10 minutes. Next, rinse with tap water for 5-10 minutes and 80% alcohol for 1-2 minutes. Then, stain with eosin for 15 seconds until 2 minutes. Dehydrate with increasing concentration of alcohol solution (95% alcohol and 100% alcohol twice, each for 2 minutes). Put xylol/xylene twice, each for 5 minutes. The final step was covering it up with cover glass containing Entellan.

Data calculation and analysis

After collecting mAgNORs and pAgNORs, AgNORs data were analyzed through one-way ANOVA test. Mitotic count, achieved from Hematoxylin Eosin analysis, was analyzed through Mann-Whitney test.

Results

AgNORs

The 2 parameters in AgNORs study were obtained as the results. The immunohistochemistry staining of AgNORs is shown in Figure 1. mAgNORs is calculated by taking the average of the summation of intranuclear AgNORs granules of 100 cells divided by 100 (Table 1), while pAgNORs is calculated by taking the percentage of the total amount of each cell which has more than 5 granules, in which p>5 is significant (Table 2).

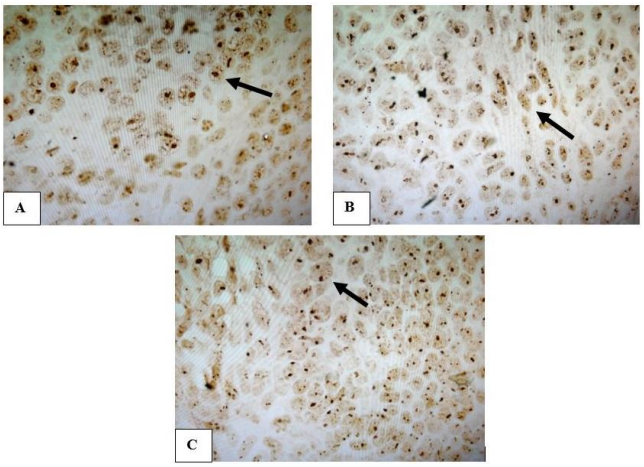

Figure 1. A) AgNORs (temperature 20-22°C), showing cell with 3 AgNORs granules**; B)** AgNORs (temperature 25-27°C), showing cell with 6 AgNORs granules. C.AgNORs (temperature 32-34°C), showing cell with 8 AgNORs granules. (10x100 magnification with light microscope).

Mice	Temperature		
	20-22°C	25-27°C	32-34°C
1	3,11	4,32	7,02
2	2,99	4,61	7,36
3	2,71	3,74	6,98
4	2,8	4,53	6,11
5	2,8	3,24	7,31
6	3,48	3,02	7,65

Table 1. mAgNORs for Each Group.

Mice	Temperature		
	20-22°C	25-27°C	32-34°C
1	10	27	67
2	13	30	85
3	13	27	65
4	11	31	62
5	2	15	76
6	17	16	79

Table 2. pAgNORs (p>5) for Each Group.

After data from microscopic view had been collected, the next step after getting AgNORs data was statistical analysis. One-Sample Kolmogorov-Smirnov test was done for determining the distribution whether it was normal or not. From the result, mAgNORs show p = 0.549 and pAgNORs with p = 0.268, meaning that both have p> 0.05 thus a null hypothesis that stated distribution is not normal was rejected while alternative hypothesis that stated distribution is normal was accepted. In conclusion, both mAgNORs and pAgNORs data distribution are normal.

As the distribution of data was normal, homogeneity of variants was checked by Levene test. The result showed the variants in mAgNORs data (p= 0.088 or p>0.05) and pAgNORs data (p = 0.094 or p>0.05) were homogenous. Both data were normally distributed and homogenous, thus, the statistical analysis was continued using a parametric test one-way ANOVA. The result showed there was a significant difference of mAgNORs between three groups (p=0.000). For pAgNORs, there was also a significant difference between three groups (p = 0.000).

Further analysis was carried out using Duncan test. The result showed mAgNORs was different between group 20-22°C, 25-27°C, and 32-34°C. For pAgNORs, there were differences between group 20-22°C, 25-27°C, and 32-34°C.

Hematoxylin and Eosin

Hematoxylin and eosin staining used in order to get a descriptive result as well the statistical result (Figure 2). There are 4 components, mitosis is the focus of this report (Table 3).

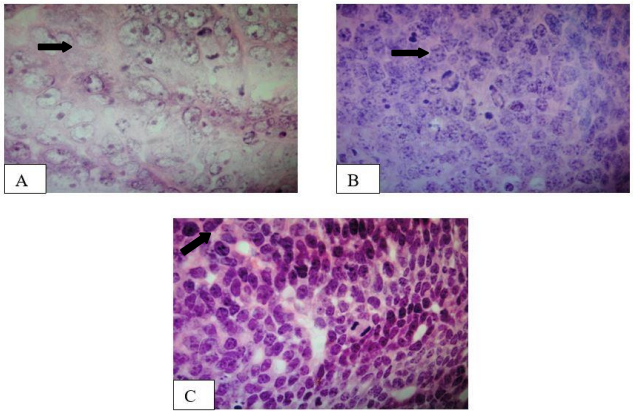

Figure 2. A) Mitosis (temperature 20-22°C); **B)** Mitosis (temperature 25-27°C), C. Mitosis (temperature 32-34°C). HE staining with 10x100 magnification light microscope.

		mAgNORs	pAgNORs (p>5)
N		18	18
Normal Parameters*	Mean	4.6544	35.89
	Std. Deviation	1.86767	27.937
Most Extreme Differences	Absolute	.188	.236
	Positive	.188	.236
	Negative	-.171	-.158
Kolmogorov-Smirnov Z		.797	1.002
Asymp. Sig. (2-tailed)		.549	.268

*Test distribution is Normal

Table 1. One-Sample Kolmogorov-Smirnov Test.

The statistical analysis using Mann-Whitney showed the relation between control and group 20-22°C was not significant (p= 0.138). Furthermore, the relation between control and group 32-34°C was also not significant (p= 0.575).

The graph illustrates the differences in mitotic count in 3 groups, namely 20-22, 32-34, and 25-27 as a control (Figure 3). The mitotic count in each group was further classified into 2 types, weak and strong. Weak means there is less than 10% of mitosis in 10 microscopic fields, whereas strong indicates there is more than 20% of mitosis in 10 microscopic fields. In comparison with the control, the group 20-22 has a tendency to have a lower mitotic count because mitosis in all slides is less than 10% in 10 microscopic fields and the group 32-34 has a tendency to have higher mitotic count compared to the other

two groups because it has more slides with more than 20% mitosis in 10 microscopic fields.

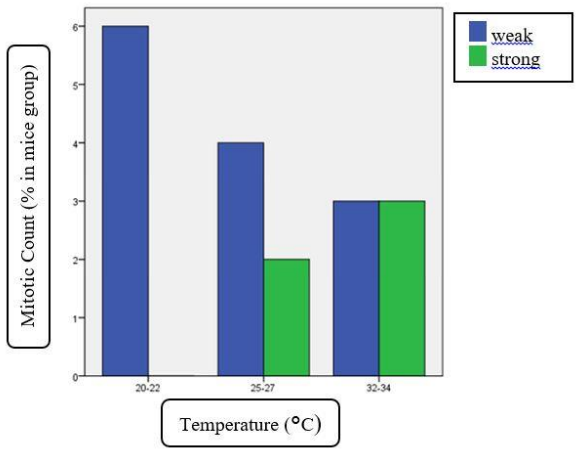

Figure 3. The relationship between temperature and mitotic count.

Discussion

According to the results that have been described, the discussion can be divided into two, based on the type of staining of mice adenocarcinoma mammae cells, which are AgNORs and Hematoxylin Eosin.

From the AgNORs aspect, based on the rough examination before statistical calculation, the nuclei have a tendency to have more granules in mice exposed to 32-34°C and fewer intranuclei granules seen in mice exposed to 20-22°C. As a matter of fact, the AgNORs is used to detect granules that indicate the cell proliferation. Supported by statistical analysis One-way ANOVA with p= 0.000, the differences in the three groups of temperature are significant. In other words, the mice have more aggressive cells proliferation in exposure to 32-34°C while those with 20-22°C exposure have less aggressive cells proliferation. The underlying mechanism of the effect of temperature to the cells proliferation is possibly related to the temperature-sensitive genes that regulate the apoptosis and controlling the checkpoints in cell cycle, as it has been stated in previous study, about p53. Mutations in this gene, induced by temperature, could change the original function as tumor suppressor to become tumor inducer.[15]Another research mentioned that tumor cells are more sensitive in high temperature compared to normal cells.[7]It is possible that this sensitivity makes them to be more aggressive in the proliferation of their cells. A study of frog gave a result that they formed no antibodies when they are exposed to low temperature, meaning that it is possible there is a decrease in immune response that will make less cell proliferation. However, since frog is a poikilothermic, not like mice, the immunity theory should be further investigated. High temperature can also induce stress, as some proteins in the body are heat-shock proteins that will release oxidants that will cause anti-apoptosis signal thus making cells exposed to heat proliferate more.[16]In conclusion, from the statistical analysis of AgNORs, it can be highlighted that there is an effect of temperature to the mice C3H adenocarcinoma mammae cells proliferation, but in order to identify the exact etiology and mechanism, further studies are needed.

One of the morphological parameters used for cancer grading with Hematoxylin Eosin staining that is discussed in this research is mitosis. From the descriptive analysis, seen through light microscope, it can be seen that there are many mitotic figures and the quantity is the highest in mice with exposure to 32-34°C. Beside the pictures, the graph describes that the mice in 32-34°C have a tendency to have more mitotic count compared to the other two groups. In accordance to the theory, mitosis goes hand-in-hand with cell proliferation because the basic of the cell proliferation itself is the mitosis, thus the more the mitosis, the more the cell proliferation will be. However, based on Mann-Whitney test, it is showed that the relation of temperature and mitosis is not significant for both 20-22°C (p= 0.138) and 32-34°C (p= 0.575) in comparison with control. This insignificance is probably because the mechanism of mitosis in cancer is not only induced by temperature, but also genes or other factors. As a previous study of mouse mammary carcinoma FM3A cells in vitro mentioned that there was a change in DNA-synthesizing ability that will influence the cell cycle or the mitosis when cells are exposed to temperature shift-up.[7] Concerning the samples, because the group 37-39°C was discontinued, the samples should have been added into the 3 groups so that more samples are expected to give better results regarding the significance.

The other point to be mentioned is the failure of mice in exposure to 37-39°C to survive. Previous study in vitro reported that normal cells were damaged in exposure of more than

42°C.**Hata! Yer işareti tanımlanmamış.** It is possible that 37-39°C is already too extreme in which the mice normal cells are also damaged.

Another note is the measurement of the core temperature of the mice had not been done, yet it is an interesting topic that may lead to possible treatment for patients with breast cancer. The analysis of the mice' organs after exposure to temperature would be a sophisticated study, for example liver or spleen and the possible relation with antibodies or cell destruction. Therefore, the author hopes this research will make opportunities to researchers to develop or continue this study.

Conclusions

The hypotheses "Temperature can affect the mice adenocarcinoma mammae cells proliferation is accepted because based on AgNORs analysis, the high (32-34°C) and low (20-22°C) temperature showed a significant different compared to control in relation with cell proliferation.

Based on the HE analysis the hypotheses "High temperature will cause high mitotic count" is rejected because although from the graph there is a tendency that mitotic count is higher in high (32-34°C) temperature, the result from Mann-Whitney test is insignificant.

Further studies are needed with the help of more advanced facilities (e.g. design of cages) in order to confirm the effect of temperature on the cell proliferation. Immunobiochemistry study, such as Ki-67 or PCNA would probably give more detailed results in identifying the cause or mechanism.

Declaration of Interest

The authors report no conflict of interest.

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