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Proliferative Activity of Mammary Carcinoma Cells by AgNOR Count in C3H mice Receiving Ethanol Extract of Sponge *Haliclona sp*

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Abstract. Quantification of argyrophilic nucleolar organizer region (AgNORs) was considered as one of markers of proliferative activity of cancer cells. Sponge *Haliclona sp* extract contains anticancer bioactive compounds and our previous study showed that the extract was able to improve histological grade of induced mammary adenocarcinoma in mice. The following research was conducted to study the extract administration on the proliferative activity of the carcinoma cells represented by AgNOR count in mice. This experimental study applied post test only control group design. Twenty C3H mice were divided into four groups namely C (control), H₁, H₂ and H₃. Each group was given 0, 0.15, 1.5, and 15 mg *Haliclona sp* extract respectively. After three weeks of extract administration, mice were inoculated with breast cancer cells from donor mice. The extract administration were continued for another three weeks. AgNOR count was performed on tumor sections and expressed as mean of AgNOR (mAgNOR) and percentage of AgNOR (pAgNOR). Means of mAgNOR in C, H₁, H₂ and H₃ were 4.070, 3.195, 3.450, and 3.190 respectively. Means of pAgNOR in C, H₁, H₂ and H₃ were 34,40, 25,40, 38,40 and 19,80 respectively. The lowest means of mAgNOR and pAgNOR which is an indication of lower proliferative activity of the cancer cells was found in H₃. However no significant difference can be found among treatment groups ($p > 0.05$). Using AgNOR count, the ethanol extract of *Haliclona sp* could not show significant reduction in proliferation of mammary carcinoma cells of C3H mice. This finding support the view that AgNOR alone could not be used to determine pathology of cancer cells.

Keyword : breast tumor, mammae, sea, marine, soft coral

1. Introduction

Breast cancer remains as a disease with high prevalence worldwide.¹ Chemotherapy is an important element of breast cancer treatment either as an adjuvant, neoadjuvant, or in paliatif treatment.^{2,3} In other hand there is breast cancer which is resistant to chemotherapy and attempts to discover new cancer drug with low toxicity is continuously being sought. One of novel source for antitumor substance is derived from marine biota.^{4,5} Sponge and soft coral contains bioactive compounds which



function as a defense system against natural enemies. The compounds belong to secondary metabolites and they have anticancer activity. The compounds were then being synthesized chemically⁴⁻⁶.

Previous study has shown that sponge *Haliclona* sp from Labuan Bajo waters in Flores Indonesia can inhibit L1210 cells (leukemia derived cell line).⁷ On the basis of its IC₅₀ value, toxicity studies of graded dosage of *Haliclona* sp extracts had been carried out in vivo mice.⁸ The extract of *Haliclona* sp was considered to be safe to be given by oral route. Therefore, subsequent study was conducted to test further the anti tumor activity of the extract in vivo laboratory animals. There has been no research regarding the anticancer activity of *Haliclona* sp extract against *adenocarcinoma mammae* in vivo.⁹

In the following study a graded level *Haliclona* sp extract was tested against *adenocarcinoma mammae* in C3H mice. The study used argyrophilic nucleolar organizer region (AgNOR) staining to measure the proliferative activity of cancer cells. AgNORs expression reflect the speed of cell cycle and the the doubling time of tumor cells. Proliferative activity determined the rate of tumor mass growth therefor AgNOR is considered as one of markers of proliferative activity of cancer cells.¹⁰.

2. Material and Methods

Feed pellet for CH3 mice used commercial feed (CP781) with 31 – 33 % protein, 3 - 5 % fat, 4 – 6 % crude fibre, 10 - 13 % ash, and 11 – 13 % moisture. Feed was mixed with *Haliclona* sp (Hs) extracts by weighing 150 grams for each treatment. Extracts were weighed according to three treatments namely 0.3, 3, and 30 mg extracts per mouse per day. The extracts were dissolved in 200 mL ethanol 96% until homogenous. The feed pellet was mixed with the dissolved extract and stood until it was absorbed completely. Control feed pellet was mixed with 200 mL ethanol only with no extracts. The feed pellet was dried by vacuum and heated at 40°C to complete the drying and stored in the freezer until use.

2.1. Donor cancer cells

Cancer cells were obtained from donor mice. The cancer lumps were surgically removed from the donor mouse and transferred to sterile physiological salt containing petri dish. The tissue was disintegrated using special scissor to release the cancer cells. These cells were ready to be transplanted into healthy C3H3 mice.

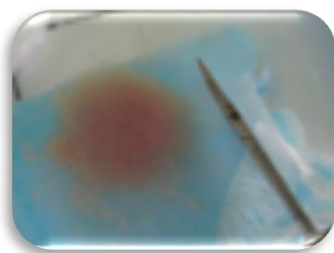


Figure 1. Donor cancer cells in physiological salt

2.2. *Haliclona* sp (Hs) Extracts administration to adenocarcinoma bearing C3H3 Mice

Mice were adapted for 7 days to the lab environment where they were kept and feed pellet and drinking water were provided in free access. Fresh feed pellet, drinking water, and cleaning were done daily. The experiment was designed as "The Post Test Only Control Group Design" [3] with one control and three treatments [4]. Twenty of 8-10 weeks of age mice with an initial weight of 20-25 gram were randomly allocated into: control group (C), receiving 0.15 mg Hs extracts (H-1), and

receiving 1.5 mg Hs extracts (H-2), 15 mg Hs extracts (H-3) per mouse per day respectively for three weeks. Cancer cells were introduced to all groups from a donor cancerous mouse. The donor cancer cells of 0.2 mL were injected to each mouse via left or right axilla and allowed to grow. The extract administration were continued for another three weeks after which termination is made on all mice. Terminations were done by ether inhalation and followed by collar bone dislocation. Finally the entire adenocarcinoma mammae tissue of each mouse was removed and fixed in a 10% buffered formalin. Histology preparation of adenocarcinoma mammae was prepared by AgNOR staining.

The proliferative activity of adenocarcinoma mammae was scored using AgNOR count according to ploton method. According to Derenzini et al in Miranti, the method was used to calculate the total interphasic AgNOR per cell in 100 cells.¹¹ The preparations are then examined under light microscope with 100x magnification.¹² AgNOR appears as a black or dark brown dot with yellowish nucleus in the background. AgNOR count was obtained by summing the points in the nucleus one by one when the points can be separated. If the points are united then they are counted as a single point.¹³ Lymphocytes are mostly present only as one point, determined by internal control reactions.¹⁴ AgNOR points from stromal cells¹², inflammatory cells¹¹, and overlapping cells or cells in necrotic areas should be removed.¹⁴ The viewing field for the observation of AgNOR point was taken from the edge and the area where the maximum number of AgNOR points indicated the most anaplastic area. Then the mAgNOR (mean of AgNOR) and pAgNOR (percentage of AgNOR) are summed from the AgNOR point count. mAgNOR is determined from the average number of AgNOR points per cell or per nucleus, whereas pAgNOR is the number of cells containing five or more AgNOR points in its nucleus in 100 cells.¹³ The microscopic reading of the preparations was performed by two observers to avoid subjective judgment.

Distribution and homogeneity of data were tested using Saphiro Wilk and Levene respectively. When mAgNOR and pAgNOR values are normally distributed and the variance is normal, they were analyzed by one way ANOVA. If both conditions are not met the data were analysed by nonparametric test using Kruskal Wallis. The data were analyzed using SPSS 15.0 for Windows program. A p value less than 0.05 indicates a significant difference.

3. Results and Dissusion

Normality test by Saphiro Wilk produced abnormal distribution results for mAgNOR and normal distribution for pAgNOR so that for mAgNOR data are described using median as the maximum concentration and minimum size as the size of spread. As for pAgNOR data is described using the mean as a centralized measure and standard deviation as the size of the spread. An overview of the number of mAgNORs and pAgNORs are shown in table 1.

Table 1. mAgNOR and p AgNOR values

Kelompok	N	mAgNOR		
		Median	Minimum	Maksimum
H1	5	3,1950	2,91	4,47
H2	5	3,4500	3,26	6,18
H3	5	3,1900	2,71	3,40
K	5	4,0700	3,45	4,35
		pAgNOR		
		Average	SD	
H1	5	25,40	13,87	
H2	5	38,40	23,68	
H3	5	19,80	4,43	
K	5	34,40	7,34	

Levene test showed that mAgNOR variance is homogeneous with p value 0.063 (> 0.005) and homogeneous pAgNOR with p value 0.000 (< 0.05). It means that in pAgNOR there are at least two groups that have significantly different data variance.

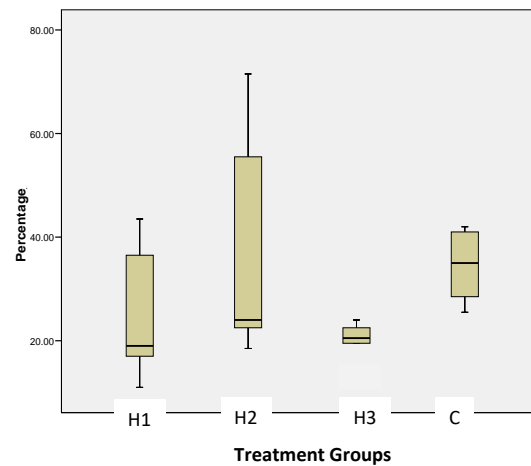


Figure 2. Box Plot Distribution of pAgNOR

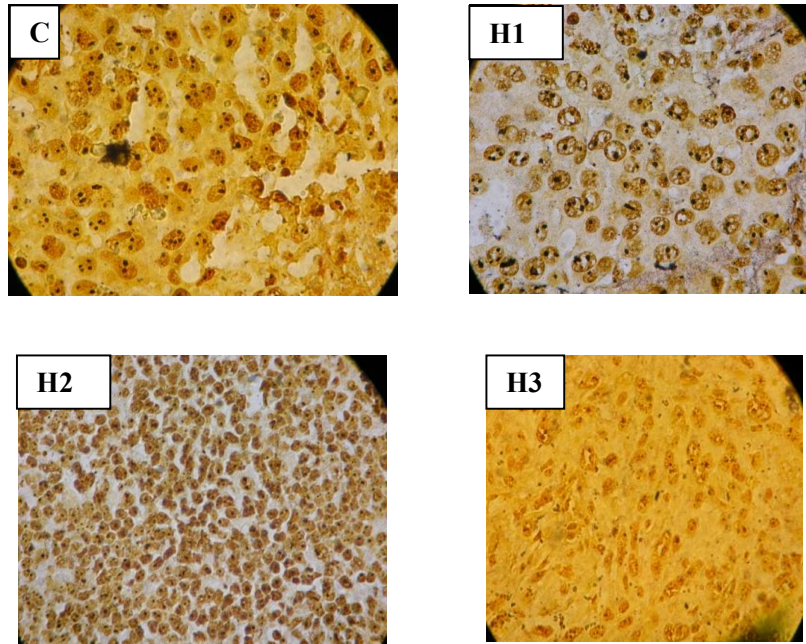


Figure 3. AgNOR staining with 100x magnification, C=control, H1, H2, and H3

Nucleolar Organizer Region (NOR) is a special part of DNA called rDNA. NOR contains the ribosome gene. Using NOR RNA polymerase enzymes encode ribosomal RNA transcription. rRNA is part of the ribosome responsible for protein synthesis. It is thought that there is a relationship between NOR and cell proliferation because protein synthesis is an important stage in cell proliferation [10].

NOR active transcription is associated with high non-histone argiophilic acid protein known as B23, C 23 (nucleolin) and RNA polymerase 1. Nukleolin and RNA polymerse 1 can be painted with silver colloidal technique so that NORs can be seen using a light microscope. NORs whose proteins are painted with silver are called argyrophilic nucleolar organizer regions (AgNOR). [15] The amount of AgNOR is directly related to the rate of ribosomes biosynthesis where cell proliferation is closely related to the duration of the cell cycle. [16,17] There is increasing evidence supporting the mechanisms that control cell proliferation by controlling the progress of the cell cycle also regulate the speed of the biosynthesis of the ribosome. Increased protein synthesis through increased regulation of ribosomal production rates is necessary in cell proliferation to ensure that cell children have sufficient numbers of cell elements to survive and function normally. [18] So the AgNOR value can measure the rate of cell proliferation. [17,18]

There are two common parameters in the AgNOR count, i.e. the average point of AgNOR per cell or per nucleus (mAgNOR) and percentage of AgNOR (pAgNOR). [13] mAgNOR is related to cell ploidy reflecting which DNA content depends on the phase in the cell cycle. The cell cycle in cancer cells is disorganized as well as normal cells so that cell cycles become out of sync within the cell (aneuploid cells) and increase cell size in phase S (DNA synthesis). In general, measuring and analyzing cell proliferation activity is based on cell cycle. In other words, the ploidy that reflects the cell's DNA content is one of the parameters for determining cell proliferation activity. [19] pAgNOR as well as mAgNOR also represents cell proliferation activity as it relates to the percentage of cells undergoing phase S in the cell cycle. [13]. The results of this study indicate that there is no significant difference in the value of mAgNOR and pAgNOR control group with the treatment group. This indicates that the *Haliclona* sponge extract has not been shown to decrease the amount of mAgNOR and pAgNOR of adenocarcinoma mammae cells in C3H mice.

Sponge *Haliclona* sp produces secondary metabolites namely *alkaloids, steroids, terpenoids, and phenols*. [9] The chemical compounds released are toxic to their environment [5], but these secondary metabolites have the potential to be anti-viral, anti-bacterial, anti-malarial, anti-inflammatory, anti-oxidant and anticancer. [4,5]. The two types of alkaloids isolated from the *Haliclona* sp species are Haliclonylamine A and Haliclonylamine B. [20] Other sources call it *halicynone A* and *halicynone B*. [21] *Haliclonylamine A* ($C_{32}H_{56}N_2$) is made up of 8 methines (4 of which are alkenes) and 24 metylene. *Haliclonylamine B* is an isomer of *Haliclonylamine A*. [20]. In 3 different fractions, *Haliclona* sponge sp showed anticancer activity against leukemia cancer cell (L1210 cell line) with IC_{50} 3,25, 2,37, and 2,90 $\mu g / ml$. [7] The results of this study showed that *Haliclona* sponge did not significantly inhibit the activity of adenocarcinoma mammary proliferation in C3H mice, although the number of mAgNOR and pAgNOR counts of the H3 group was lower than the treatment group but not significantly different. This can be caused by several things, namely the sponge extract of *Haliclona* sp which is not a specific compound (crude extract) so that the anticancer compound *Haliclona* sp is still mixed with other compounds in *Haliclona* sp which can affect the anticancer compound. Another thing that influences the results of this study is the reading of preparations by calculating the point AgNOR is still too subjective and requires the accuracy of preparatory readers, this has been tried to be overcome by using two readers or observers.

4. Conclusion

Using m and pAgNOR count, there was no significant difference in the proliferation activity of adenocarcinoma mammae cells of C3H mice given *Haliclona* sponge extracts compared to controls. This finding support the view that AgNOR alone could not be used to determine pathology of cancer cells.

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