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Deriving Breast Cancer's Primary Cultures from Patients' Tumor Biopsies in Indonesia Using Explant and Enzymatic Methods

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Abstract: Breast cancer is the most common cause of death in women globally with high rates of heterogeneity. The development of breast cancer treatment is still constrained because of the different responses to its therapy. The use of primary breast cancer culture is invaluable because it provides the same properties as breast cancer itself. Primary culture is used as a tool to determine the proliferative ability of breast cancer cells. However, the use of optimum methods in cultivating primary culture must be evaluated because of the unstable nature of primary culture. In this study, we investigate the optimum method for culturing cells derived from the tumor biopsies of breast cancer patients in Indonesia by comparing explant and enzymatic methods. Breast cancer tissues were obtained from five breast cancer patients, who underwent surgery and incision biopsies. Tissues were further cultured by explant and enzymatic methods. The cell cultures were observed daily using a microscope for up to 30 days. The results showed that the cells cultured using the enzymatic method for more than 16 h were susceptible to microbiome contamination in the following days after enzymatic digestion, while those cultured using the explant method grew well for 30 days. The findings of this research suggested that the explant method gave better results compared with the enzymatic method. The findings of this study provide insight into the optimum conditions for the primary culture of breast cancer cells.

Keywords: breast cancer, enzymatic culture, explant culture, in vitro, primary culture.

使用外植体和酶促方法从印度尼西亚患者的肿瘤活检中获得乳腺癌的原代培养物

摘要：乳腺癌是全球女性最常见的死亡原因，具有很高的异质性。由于对其治疗的不同反应，乳腺癌治疗的发展仍然受到限制。原发性乳腺癌培养物的使用是无价的，因为它提供了与乳腺癌本身相同的特性。原代培养被用作确定乳腺癌细胞增殖能力的工具。然而，由于原代培养的不稳定性质，必须评估在培养原代培养中使用最佳方法。在这项研究中，我们通过比较外植体和酶促方法来研究培养来自印度尼西亚乳腺癌患者肿瘤活检的细胞的最佳方法

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。乳腺癌组织取自五名接受手术和切口活检的乳腺癌患者。通过外植体和酶促方法进一步培养组织。每天使用显微镜观察细胞培养物长达30天。结果表明，酶法培养16 h以上的细胞在酶消化后的几天内易受微生物污染，而外植法培养30 d的细胞生长良好。该研究结果表明，与酶法相比，外植体法的效果更好。这项研究的结果提供了对乳腺癌细胞原代培养的最佳条件的深入了解。

关键词：乳腺癌，酶培养，外植体培养，体外，原代培养。

Introduction

In 2020, breast cancer was the leading cause of death in women globally and ranks fifth in terms of cancer mortality [1]. Based on Globocan 2020, the cases of breast cancer in Indonesia were the highest among all cancers [2]. More than 90% of breast cancer deaths are caused by metastasis, with the most common spread to the bones, liver, lungs, and brain [3–4].

Breast cancer is a heterogeneous disease due to genetic changes in breast epithelial cells [5]. The heterogeneous nature characterizes the malignancy of the disease and becomes a challenge for the response to treatment and therapy for breast cancer because it can cause resistance [6].

To date, one of the commonly used treatments for breast cancer is chemotherapy. Nevertheless, it has also been reported that chemotherapy over a long period causes adverse drug resistance and metastatic effects [7]. Development in treating breast cancer continues to be leveled to this day, but high heterogeneity and metastatic breast cancer become obstacles in treatment [8, 18]. Primary culture is a potential tool in clinical approaches for treating cancer.

Research has been carried out to investigate cancer characteristics, genetic changes, molecular mechanism, metastasis, diagnosis, and drug exploration for therapy. The cell line was used in some studies due to its practical handling. However, the use of primary cultures provides more advantages because it is closer to the native state of the cancer cells in vivo compared to the cell line [9]. Explant and enzymatic methods can be used to conduct the primary culture. Additionally, they have both advantages and disadvantages [10]. Although primary cultures have been used in some studies, proper optimization in the development of primary cultures of breast cancer, especially in Indonesia, has never been reported. Furthermore, the primary culture is challenging due to its unstable nature; thus, further research must find the optimum method for developing breast cancer primary cultures.

1. Materials and Methods

1.1. Collection of Samples

Breast cancer tissues were obtained from five breast cancer patients, who underwent surgery and incision biopsies. Sampling was conducted with the patient's consent and has been through ethical approval (Ethics Approval Committee of the Faculty of Medicine, University of Indonesia # KET-1058/UN2).F1/ETIKA/PPM.00.02/ 2021).

1.2. Explant Culture Method

The tissue was separated using scissors and a scalpel from debris and fat. Once the blood vessels and debris from the sample were separated, the tissues were then cut into smaller fragments that were approximately 1 mm^3. The sample was rinsed with 5 drops of povidone-iodine in 1 mL of Dulbecco's modified Eagle medium (DMEM) medium for 20 seconds. Every two or three small pieces of each tissue were placed in a 24-well plate in duplicate/triplicate and air-dried for 20 min. Approximately 1–2 drops of complete culture media of DMEM: Ham's F12 medium (1:1) consisting of 10% Fetal Bovine Serum (FBS) [Gibco], 100 U/mL penicillin, 100 µg/mL streptomycin, 6 mM L-glutamine, were added to each cell culture plate. On the other plates, an approximately equal volume of Mammocult [STEMCELL Technologies] was added to the plates. Tissues were cultured at 37°C with 5% CO_2 for approximately three weeks to achieve a 70%-80% confluence. Cell cultures were observed daily using a microscope, and the medium was replaced every 3 or 4 days.

1.3. Cell Isolation Using an Enzymatic Culture Method

Breast cancer tissues were mechanically disaggregated into smaller pieces and rinsed using similar methods as explant cultures. Samples were placed into a 15 mL centrifuge tube containing 1% of type 1 collagenase (Gibco, Thermo Scientific, USA). DMEM at 37°C for 16 h and enzymatic reactions were stopped by adding 1:1 of complete DMEM medium, followed by centrifugation at 350 × g for 5 min afterward. The supernatant was discarded, and pellets were resuspended in two different media as stated previously in the explant culture method sections. Primary cell cultures were incubated at 37°C with 5% CO_2 for approximately three weeks and observed daily.

2. Results and Discussion

2.1. Observation of the Enzymatic and Explant Methods

In this study, two different culture methods were evaluated, i.e., the enzymatic and explant methods. Samples that can be further observed in this primary culture study are sample (H01) representing high-proliferative breast cancer and sample (L02) representing low-proliferation breast cancer. Clinical assessment data were obtained (Table 1).

Table 1 Clinical assessment

Sample	Clinical assessment	
	Histological assessment (EHR results from pathology)	**Tumor Marker Expression**
H01	Lesion, UDH, and ALH	LCA + . Vimentin + . Ki-67 + 10% (low proliferation)
L02	NST Grade III	ER - . PR - . Ki-67 + 30% (high proliferation)

Based on the results of the study, the explant method was more effective for the primary culture of breast cancer. This is because the explant method is simpler, more efficient, and relatively faster. In contrast, the enzymatic method requires type I collagenase enzymes in the lysis process and is more susceptible to contamination. In the enzymatic method, an overnight incubation is carried out for 16 h, and then the cancer cells can be observed. The results of cell culture in the enzymatic method can last for up to 5 days until the cells finally die and fungal contamination occurs. While in the explant method, cells leave the tissue and spread after 1–2 days. The enzymatic method is more efficient for obtaining faster cell yields but is more susceptible to contamination.

The use of the enzymatic method with an incubation of 16 h can decrease cell viability and tissue contamination. In melanoma cancer cultures, the required incubation time is four hours, and one day after the incubation period, the cells are already attached to the substrate [11] The trypsin-EDTA content in the enzymatic method induces a loss of cell membrane capability and cell viability. The ability of cell membranes can decrease when induced by trypsin after 20–60 minutes, so it takes a minimum time for cell isolation [12]. Therefore, a shorter incubation time (3–4 hours) can be considered to prevent contamination. The contamination is prone to occur in the enzymatic method using the trypsin-EDTA enzyme. The trypsin-EDTA composition contains animal matter, i.e., pancreatic secretions of cattle or pigs. The use of trypsin-EDTA can trigger an immunological response due to the contamination with foreign substances such as viruses and fungi.

Research using the explant method in primary cancer cultures showed a more stable condition with higher observed cell numbers and proliferation. Primary cultured cells can last up to 45 days. In the explant method, there is no need for enzyme induction to accelerate the occurrence of lysis, so it takes a longer time, namely two days for cancer cells to be observed. Cancer tissue cut into small segments releases cytokines and growth factors into the medium that stimulates natural cell growth. This is triggered when tissue is cut, causing tissue injury and stimulating the production and release of growth factors by injured cells. The explant method is simple, reasonably priced, and more suitable if used for clinical purposes [13].

2.2. Morphology and Proliferation of Cultured Cells Using the Explant Method

The difference in morphology and proliferation in breast cancer cell cultures can be seen in (Fig. 1). In the enzymatic method, the spheroid (3D) was found after 16 h of incubation (Fig. 1A) and the explant method was found after 48 h (two days) after incubation (Fig. 1D). Spheroidal cells are spherical in shape and do not stick to the surface of the flask. Spheroids were found in H01, which is a sample with Immunohistochemistry (IHK) results that show high proliferation and is a HER2-positive breast cancer subtype. However, observations of cell growth could not be continued because the samples were contaminated. Furthermore, at L02, mammosphere and fibroblast morphology were found. In this study, fibroblast cells were found only in cells using the enzymatic method [14]. The use of the explant method in primary culture did not find any fibroblast contamination; in contrast, the enzymatic method found fibroblasts.

Spheroid cells (3D) were found spontaneously in H01 with high proliferation characteristics and not in L02 with low proliferation characteristics. Melanoma cell cultures derived from spheroid cells also have high proliferation characteristics [14]. Melanocytes isolated from spheroid cells have the characteristics of mature stem cells and exhibit high proliferation properties. Spheroid cells can be formed due to environmental conditions containing a small amount of serum [15]. Spheroidal cells were assumed to differentiate into epithelial and grape-like cell morphologies and migrate away from the primary tissue to the flask surface (Fig. 1E).

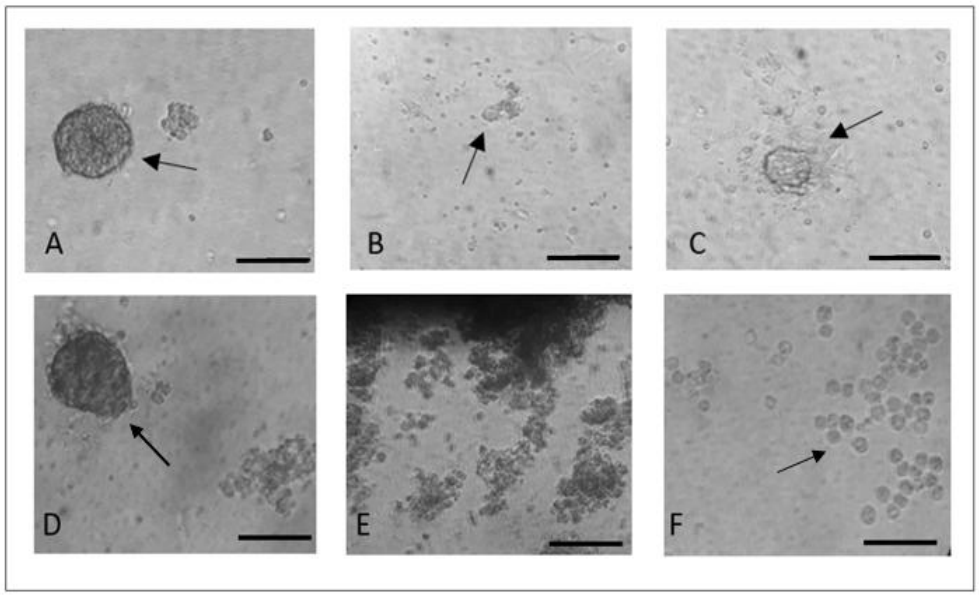

Fig. 1 Comparison of morphology and enzymatic proliferation in breast cancer cultures. Images (A-C) of enzymatic cultures and images (D-E) of explant cultures. The arrows indicate the morphology of breast cancer. The arrows in Fig. A and D show the morphology of the spheroid cells by the enzymatic and explant methods. Fig. (B) arrow shows epithelial cells. Fig. (C) arrow shows the morphology of the mammosphere. Image (E) of cancer cells leaving the primary tissue. Fig. (F) arrows show morphology of cells like grapes and already proliferating. Scale: 100

H01 cell proliferation in the explant method looks more aggressive than L01 cell proliferation in the explant method. The H01 cells on the 5th day had come out of the original tissue and the cell colonies began to spread to the surface of the flask. While on H02 the cells had left the original tissue and only one colony appeared. L01 cell proliferation continued for up to 45 days, quite high proliferation occurred until day 14 of primary culture. Cell proliferation decreased after cells were cultured for 7 days and formed a confluence of ~50% [16]. Decreased proliferation may occur because cells in culture undergo replicative senescence after certain cell divisions, where they enlarge and eventually stop proliferating. Other factors that cause cessation of cell proliferation are oxidative stress and chromosomal aberrations.

Decreased cell proliferation in breast cancer can be caused by the downregulation of *EpCAM* and down-regulation of *EpCAM* decreasing proliferation and migration. Based on this, the function of *EpCAM* is required for the proliferation and differentiation of cancer cells [17]. L02 cell proliferation was minimal, until the 7th day of primary culture, cell colonies did not develop like H01. The expression of tumor markers (Table 1) is believed to affect the rate of cell proliferation, H01 is a high-proliferation breast cancer and L02 is a low proliferation type. It is also consistent that stem cells are characterized by high proliferative potential and self-renewal capacity.

3. Conclusions

This study showed that primary culture using the explant method gave better results than the enzymatic method. Explant culture can maintain the environmental conditions of breast cancer that are similar to those of the original tissue. Primary cultures can be used to classify tumors as benign or malignant by observing the proliferation of cancer cells. Additionally, the spheroid cells were also found that can be used as an indication of cancer with a poor prognosis. The findings of this study are expected to be used to observe the response to cancer therapy in vitro, especially in Indonesia. The main challenge in this study was the difficulty in maintaining cells in primary culture, especially using the enzymatic method. This is due to the limited number of specimens and the unstable primary culture method protocol. Further research must determine the most optimal conditions for maintaining primary culture cells and evaluating the correlation between spheroid cells and cancer malignancy.

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