

RESEARCH ARTICLE

**BALB/C MICE OPTIMIZATION AS TRIPLE NEGATIVE BREAST  
CANCER MODEL WITH 4T1 CELLS AND 4T1 CELLS 3D  
CULTURE INOCULATION METHODS**

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**ABSTRACT**

**Background:** Triple Negative breast cancer (TNBC) is a subtype of breast cancer with negative expression of Progesterone Receptor (PR), Estrogen Receptor (ER), and Human Epidermal growth factor Receptor 2 (HER-2). Among other types of breast cancer, TNBC has a high rate of malignancy and recurrence, invasive, distant metastases, and resistance to conventional therapy. An optimal TNBC model is needed for drug discovery research in targeted therapy. However, optimizing the TNBC animal model by inoculating the 4T1 tumor cell line has a number of challenges and it is quite hard to be developed, especially in Indonesia.

**Methods:** This quasi-experiment study used 28 Balb/c mice divided into four groups (7 mice respectively), group A (injected with basal medium), group B (injected with 4T1 cell), group C (injected with 4T1 cell + Geltrex), and group D (injected with 3D Culture 4T1 cell + Geltrex). After being injected, mice were followed up for 35 days. Once a week they had to be observed by measuring body weight and

tumor volume. After 35 days, mice were sacrificed. Liver, lung, brain, and mammary gland were analyzed histopathologically with Hematoxylin-eosin (HE). Chi-square statistical analysis was done to compare intergroups.

**Result:** Histopathologically, there was a group that found positive samples of Invasive Ductal Carcinoma (IDC)-like with a percentage of 28.57% (2 out of 7), namely group C. Meanwhile, group B and D did not find any positive samples of TNBC, but in the mammary tissue, there was inflammation and epithelial hyperplasia. No distant metastases were found in the liver, lungs, and brain, however, there was inflammation in these secondary organs. Statistically, there was no significant difference between treatment groups with p-value = 0.41.

**Conclusion:** The 4T1 cell inoculation method can produce IDC-like on HE with an incidence of 28.75% (2 of 7 individuals) in group C.

**Keywords:** TNBC, 4T1 cells, IDC, Tumorigenesis, 3D Culture

**INTRODUCTION**

According to the Canadian Cancer Society, around 25-30% of new cases of breast cancer happen to women in the world, 13-15% of them cause death. As one type of breast cancer, Triple Negative Breast Cancer (TNBC) is the most aggressive breast cancer with 12-20% of the total prevalence of breast cancer. It is marked by negative expressions of Estrogen Receptor (ER), Progesterone Receptor (PR), and

Human Epidermal Growth-factor Receptor 2 (HER2).<sup>1</sup> Epidemiological study revealed that TNBC happened in premenopausal women (under 40 years old) with 15-20% prevalence of total breast cancer patients. Compared with other breast cancer types, TNBC patients have a low survival rate with a 40% death risk since the first diagnosis within 5 years. Due to its special molecular phenotype, TNBC is not sensitive to endocrine therapy and weak of chemoradiotherapy conventional efficacy.<sup>2</sup> In Indonesia, especially in the Special Region of Yogyakarta, there were 20-25% of

the total cases of breast cancer reported from Dr. Sardjito General Hospital.<sup>3</sup>

Breast cancer is a heterogeneous disease and has a number of sub-types that can be distinguished based on its histology profile and molecular expression.<sup>4</sup> The poor clinical manifestation due to late cancer detection triggers the advance of research for understanding cancer biology to detect the disease history, screening, diagnosis, prognosis, and therapy.<sup>5</sup> Hallmarks of Cancer are important for understanding breast cancer carcinogenesis, which illustrates the complexity of cancer biology which is divided into several characteristics that develop very quickly, including self-signaling, insensitivity to growth factors, avoidance of apoptosis, continuation of angiogenesis, tissue invasion and metastasis, evasion of the immune response, metabolic reprogramming, genome instability, mutations, tumor-inducing inflammation, immortal replication, promotion of inflammation by tumors, and sustained proliferative signaling. In 2022, the Hallmarks of Cancer will be enriched with phenotypic plasticity, non-mutational epigenetic reprogramming, senescence, and polymorphic microbiomes.<sup>4,6,7</sup>

Tumor Microenvironment (TME) is a part of Hallmarks of Cancer and has a critical role in the immunity mechanism to eradicating cancer cells. TME refers to tumor cellular environment and Cancer Stem Cells (CSCs), including immune cells, blood vessels, endothelial cells that protect cancer cells from the immune system, extracellular matrix, fibroblast, lymphocytes, bone marrows, and signaling molecules that interact between benign and malignant tumor cells, so it can affect progression and development of cancer.<sup>8</sup> TME became one of the targeted therapies in eradicating breast cancer, including Tumor-Infiltrating T-cells, Cancer-Associated Fibroblasts (CAFs), Tumor-Associated Macrophages (TAMs), Tumor Associated-Neutrophils (TANs), Tumor-Promoting Chronic Inflammation, tumor angiogenesis, adaptive immune cells, adipocytes, Mesenchymal Stem Cells (MSCs), and exosome.<sup>9</sup>

Due to that, the representative TNBC model is needed to study carcinogenesis as well as the mechanism of drug delivery with a molecular approach such as the use of biomarkers which have been widely developed to welcome the era of precision medicine. TNBC has a more aggressive phenotype, distant metastases, and high recurrence than other breast cancer subtypes. However, until now the TNBC transplant model is still not optimal for clinical research on TNBC. This study aimed to discover a representative model of TNBC from inoculation methods of 4T1 cells. Therefore, this study made a TNBC experimental animal model using Balb/c mice with three methods of inoculation of 4T1 cells injected in vivo through a mammary fat pad so that they can target the location of tumors precisely because there are stromal compartments similar to conditions in humans.<sup>10</sup> The

inoculation methods were DMEM basal medium injection, 4T1 cell injection, 4T1 cell injection + Geltrex, and 4T1 cell 3D Culture + Geltrex. 3D culture was chosen based on the consideration that 3-dimensional culture better represents the in vivo environment than 2-dimensional as well as it is possible to reveal the complex mechanisms related to cancer progression and metastasis.<sup>11,12</sup>

## METHODS

4T1 cells were thawed in BSC Level 2. Therefore, 500  $\mu$ l cells were seeded to a 10 cm petri dish with a 10 ml complete medium. Within 24 hours, cells were harvested. The first step was to wash 4T1 cells (6 cm in diameter in a dish culture) with 2 ml of PBS, and then they were discarded. Then, 500  $\mu$ l of trypsin was given and incubated for 3 minutes. Therefore, 5 ml of complete medium was added to the dish culture while shaking. Next, the complete medium solution and the cells were transferred to the conical tube to be centrifuged for 5 minutes.

The next step was to remove the supernatant and leave the pellet at the bottom of the conical tube. Next, 1 ml of complete medium was added and resuspended with the pellet. Next, 10  $\mu$ l of cells were taken and dissolved with 90  $\mu$ l in a microtube. Once dissolved, 10  $\mu$ l was added with 10  $\mu$ l trypan blue and placed in a hemocytometer. Therefore, cells were counted under a microscope using a counter by counting the number of luminescent cells visible in the field of view. The number of cells is calculated using the following formula:

$$x = \frac{\sum \text{spreading cell}}{4} \times 10^4 \times \text{dilution}$$

x = number cells

After the cells were counted up to  $4 \times 10^6$  cells/ $\mu$ l, the next step was seeding by taking 25  $\mu$ l of cell suspension in a conical tube, then dropping it on 5 ml of complete medium (which has been provided) in a new petri dish with a diameter of 6 cm. Then incubated for 72 hours. These cell cultures were then used for injection in mice after the incubation period.

Next step was culturing 4T1 cells with 3D culture method. When it was being seeded, 4T1 cells were dropped onto the lid of the petri dish in an upside-down position (hanging drop). We dropped about 80-100 drops (each drop contains 100 cells/ $\mu$ l) onto the petri dish lid and the petri dish must be contained with PBS (Phosphate Buffer Saline) to prevent dehydration of the droplet and the cells hang due to surface tension. The cells accumulate at the tip of the drop at the air-liquid interface, spontaneously aggregate, and finally form spheroids.<sup>13</sup>

Balb/c mice were weighed, then anesthetized with injection of Ketamine 1 mg/kg body weight and Xylazine 35

mg/kg of body weight, using a 100 cc low dead space syringe. A total of 5µg/g body weight. After anesthetizing, the mice were placed on the board, and the area adjacent to the inguinal 7 gland was cleaned with 70% alcohol. Spring-loaded scissors were used to cut the mice's teats while gently holding the base firmly. Small scissors and ball-tip scissors are used to make an inverted Y shaped incision across the abdomen near the nipples. Then a cut is made between the 4<sup>th</sup> nipple.

For group A, 100 µl DMEM basal medium was injected into each mice with a 100 cc low dead space syringe through 4<sup>th</sup> nipple, the syringe was held in the same direction from the canal. For group B, 100 µl cells from the pellet suspension in a microtube containing a concentration of 4x10<sup>6</sup> cells were injected into each mice using a 100 cc low dead space syringe through 4<sup>th</sup> nipple, the syringe being held in the same direction from the canal. For group C, the injection was the same as the treatment in group B, but at the final stage of harvesting 4T1 cells, a concentration of 4x10<sup>6</sup> cells was made in 50 µl of 4T1 cells and mixed with 50 µl of Geltrex in a microtube so that the total volume of the suspension became 100 µl, then injected via the intramammary route at nipple number 4 of each mouse with a 100 cc low dead space syringe. For group D, the 3D culture cells were harvested within checked the morphology of the spheroid shape below the microscope (Figure 1).

After that, the cells were removed from the petri dish lid by dropping 20 µl of basal medium. Then, the cells were put into a conical tube for centrifuge at 1000 g (5 minutes). The supernatant was separated from cells, and 50 µl of basal medium was mixed. Then, it was mixed with 50 µl of Geltrex in a microtube. The Geltrex was taken from freezer and kept on -20°C.

Five weeks after injection, observations were made by weighing the mice's body weight per week using a digital balance and palpating the tumor by feeling the nipple that had been injected to find out whether a tumor lump had formed or not. The palpable tumor was measured using a digital caliper in the nipple area where there is a tumor vertically and horizontally. After that, the total tumor volume was calculated using the following formula:<sup>14</sup>

$$v = \frac{a \times b^2}{2}$$

V = tumor volume (mm<sup>3</sup>)  
a = maximum diameter  
b = minimum diameter<sup>8</sup>

During the observation, the mice cages were replaced every 4 days, and then cleaned using sodium hypochlorite, the mice were given food, and given Reverse Osmosis (RO) water.

Before surgery, the mice were weighed first and the tumor volume was measured. Next, the mice were injected

with Ketamine 1 mg/kg body weight and Xylazine 35 mg/kg of body weight via the intraperitoneal route and waited until they fainted. After the mice have fainted, place them on the styrofoam board in the supine position. To ascertain whether it was completely anesthetized, the toes of the mice were pinched with tweezers. If there is no reflex movement, then the tips of the mice's legs are fixed with pins. Next, incise the caudal and abdominal sections transversely until the skin is exposed. After that, stretch the skin on the right and left sides, then fix it with a pin. Mammary tissue was taken 2.0-0.5 cm from 4<sup>th</sup> nipple. Transversely, the nipple was cut, but not all of the cross-section was exactly perpendicular to the length of the mouse. The skin of the chest and abdomen and under them the mammary glands were bluntly dissected from the muscles forming the thorax and abdominal wall. The mammary glands and the skin above them were placed in a sample pot containing 10% formalin.<sup>15</sup> Mammary tissue, lung, liver, and brain organs were also taken and preserved in 10% formalin to determine whether there were metastases or not. After being preserved in formalin solution, the tissue was examined histopathologically using hematoxylin eosin (HE) staining, which was carried out at the Anatomical Pathology Laboratory, Faculty of Medicine, Public Health and Nursing, UGM.

Analysis of the three methods of inoculation of 4T1 cells in Balb/c mice was carried out by differentiating normal tissue and cancerous tissue using a microscope by observing alteration of the lymph nodes, inflammatory cell infiltration, and the formation of lobules in the mammary tissue. Apart from mammary tissue, other organs such as the lungs, liver, and brain are also examined to evaluate the presence of metastases. Statistical data analysis was performed using the Jamovi application with the Chi-Square test. The difference is significant if the result is p value = 0.05.

## ETHICAL CLEARANCE

This study has been approved by the Medical and Health Research Ethics Committee (MHREC), Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada/Dr. Sardjito General Hospital (Ref. No. : KE/FK/0725/EC/2022).

## RESULTS

### *Progression of tumor development based on tumor measurement data*

After injecting mice with 4T1 cells, the progression of their tumor was observed by palpation of the injected nipple area each 7 days a week for 5 weeks. Palpable tumors were measured vertically and horizontally using a digital caliper. In group A, no palpable tumor was found. Overall, in group B, the number of samples with palpable tumors was 2 out of 7 (28.57%) with a mean volume of 6.102 mm<sup>3</sup> (SD ± 4.89), the rest were not detected at all. Whereas in group C, there was a sharp increase in tumor growth in the second week, then

shrank and disappeared in the fifth week. In group C, the number of samples with tumor palpation was 5 (71.42%) with a mean volume of 32.56 mm<sup>3</sup> (SD ± 15.32). In group D, the number of samples with palpable tumors was 2 out of 7 (28.57%) with a mean volume of 0.948 mm<sup>3</sup> (SD ± 1.33). However, from the presence of samples that palpated the tumor, none was declared cancer. The tumor condition of group A, B, C, and D mice can be seen in Figure 2.

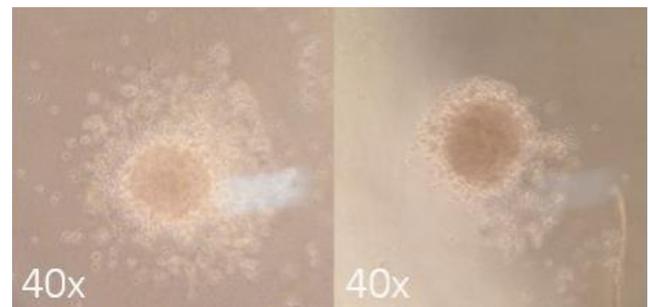
### **Histopathological evaluation of tumorigenesis in mammary tissue**

To investigate the presence of tumors, 28 samples were examined histopathologically with HE staining and were analyzed by a pathologist expert at the UGM Faculty of Veterinary Medicine. Histopathological examination included mammary tissue, lung organs, liver organs, and brain organs with 40x and 100x magnification under a binocular microscope, then images were taken using the Optilab software. Based on histopathological examination, of the 28 samples of mammary tissue examined, there were 2 samples that tested positive as Invasive Ductal Carcinoma (IDC)-like, that was samples number 1 and number 5 in group C. IDC is a histological subtype of TNBC characterized by non-infiltrative extensive areas of necrosis, lack of differentiation nuclear pleomorphism, high mitotic activity, lobular-lobular formation, and rapid inflammation. Table 1 is the result of histopathological examination of tumorigenesis in mammary tissue in each sample of each group conducted by a pathologist from the UGM Faculty of Veterinary Medicine. Figure 3 presents a histopathological comparison of four groups of mice inoculated with 4T1 tumor cells and observed for five weeks, namely group A (injected with DMEM basal medium/control), group B (injected with 4T1 cells), group C (injected with of 4T1 cells + Geltrex), and group D (3D culture of 4T1 cell + Geltrex). Due to the data obtained were in the form of IDC positive and negative event data, the statistical test performed was the Chi-Square statistical test using Jamovi Vers 2.3 software to

see the significance of differences between treatment groups. The results can be seen in Table 2 with  $p=0.41$  which means that the differences between groups are not significant.

### **Histopathological evaluation of metastases in the lung, liver, and brain**

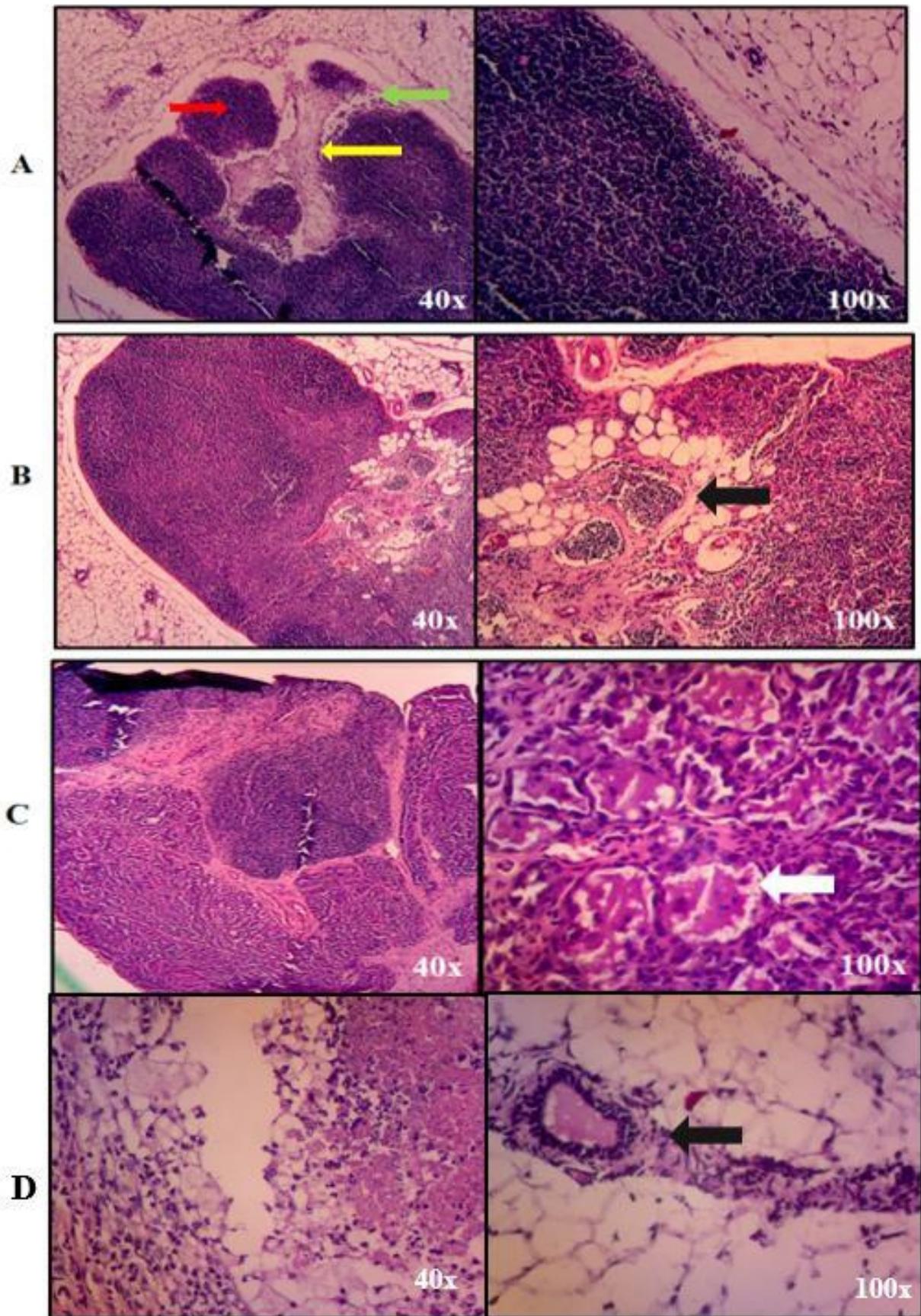
Examination of the organs of the lungs, liver, and brain organs aims to determine the tumorigenesis of 4T1 tumor cells that can metastasize distantly. Metastasis is an advanced stage of the spread of tumor cells from the initial site to various secondary organs or tissues. Macroscopically, there were no signs of metastases in the liver, lungs, and brain. This can be seen in Figure 4. Although macroscopically there appears to be no indication of metastases, through histopathological examination using HE staining which was carried out at the Anatomical Pathology Laboratory in the Faculty of Medicine, Public Health, and Nursing UGM and analyzed by a pathologist at the Faculty of Veterinary Medicine UGM, found signs of tissue abnormalities such as inflammation, congestion, epithelial hyperplasia, and peri arthritis, especially in group B (4T1 cell injection), group C (4T1 cell injection + Geltrex), and group D (3D culture of 4T1 cell + Geltrex) compared to the normal tissue of group A (control/DMEM basal medium injection). The results of the examination can be seen in the Table 3.



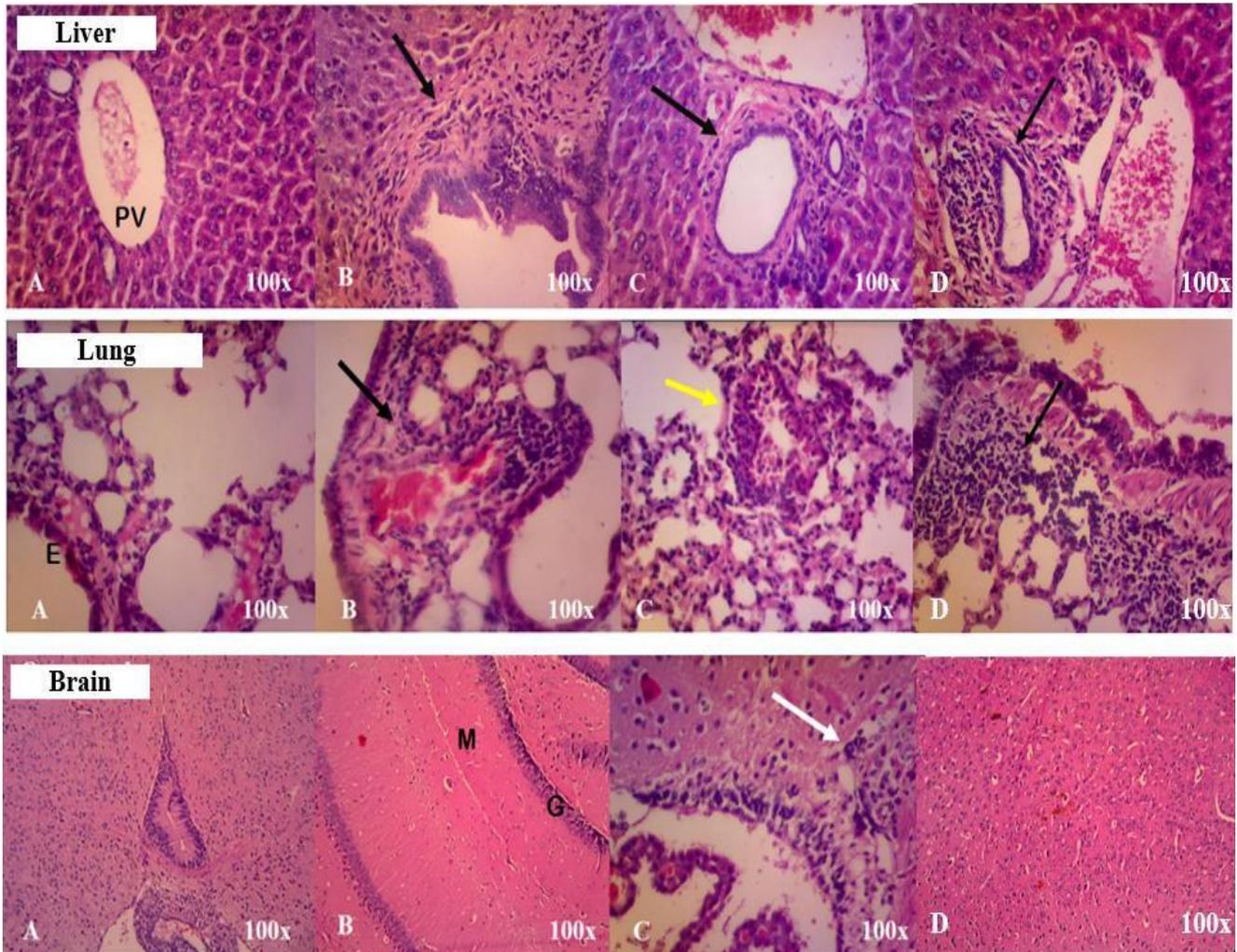
**Figure 1.** 7 days of three-dimensional spheroid formation under microscope



**Figure 2.** 35 days of mice after necropsy who presented with mammary fat pad condition. None tumor was found in all groups, except group C. The tumor nodule is directed by white circle. A) Group A was injected with DMEM basal medium (control group). B) Group B was injected with 100 µl of 4T1 cells. C) Group C was injected with 50 µl of 4T1 cells and 50 µl of Geltrex. D) Group D was injected with 50 µl of 3D culture 4T1 cells and 50 µl of Geltrex.



**Figure 3.** Histopathology image of mammary tissue, stained with Hematoxylin Eosin (HE) from from group A (with DMEM basal medium (control group), group B (injected with 100 µl of 4T1 cells), group C (injected with 50 µl of 4T1 cells and 50 µl of Geltrex.), and group D (injected with 50 µl of 3D culture 4T1 cells and 50 µl of Geltrex)



**Figure 4.** Histopathology image of liver, lung, and brain, stained with Hematoxylin Eosin (HE) from from group A (with DMEM basal medium (control group), group B (injected with 100 µl of 4T1 cells), group C (injected with 50 µl of 4T1 cells and 50 µl of Geltrex.), and group D (injected with 50 µl of 3D culture 4T1 cells and 50 µl of Geltrex)

**Table 1. Histopathological test result of mice mammary tissue from group A**

Group	Result	TNBC Subtype	Total Number of TNBC Positive	Percentage
A	-	-	0/7	0
B	-	-	0/7	0
C	Available	Invasive ductal carcinoma (IC)	2/7	28.75
D	-	-	0/7	0

(with DMEM basal medium (control group), group B (injected with 100 µl of 4T1 cells), group C (injected with 50 µl of 4T1 cells and 50 µl of Geltrex.), and group D (injected with 50 µl of 3D culture 4T1 cells and 50 µl of Geltrex)

**Table 2. Chi-square statistical result using Jamovi Vers 2.3 (p value=0,411)**

	Value	df	P
X <sup>2</sup>	28	27	0.411
N	28		

**Table 3. Histopathology analysis result of liver, lung, and brain from group A**

Goup	Liver	Lung	Brain
A	Normal	Normal	Normal
B	Inflammation	Inflammation	Normal
C	Inflammation	Inflammation, ephitelial hyperplasia	Inflammation, congesti
D	Periarthritis	Inflammation	Normal

(with DMEM basal medium (control group), group B (injected with 100 µl of 4T1 cells), group C (injected with 50 µl of 4T1 cells and 50 µl of Geltrex.), and group D (injected with 50 µl of 3D culture 4T1 cells and 50 µl of Geltrex)

## DISCUSSION

Based on the histopathological examination conducted by a pathologist at the Faculty of Veterinary Medicine UGM, of the four groups of the 4T1 tumor cell inoculation method, only one group was found to have TNBC with the IDC subtype, namely group C (4T1 cell injection + matrigel). The discovery of IDC in group C (shown by white arrows in Figure 3) was supported by the addition of a scaffold, namely, the Geltrex. Geltrex is a special extracellular matrix from mice that forms bonds between endothelial, epithelial, muscle, or nerve cells, and stroma continuously so that it can affect a number of cellular functions, such as proliferation, adhesion, migration, differentiation, and polarization. In the process of tumorigenesis, 4T1 tumor cells with the addition of the Geltrex form an alveolar-like structure<sup>15</sup>. In addition, the tumor size in group C was larger than group B due to the rapid formation of spheroids due to the addition of matrigel stimulated by its composition. Geltrex was extracted of rat Engelbreth-Holm Swarm sarcoma (EHS) and contains laminin, collagen IV, and enactin, which are high proteins and contain growth factors. Matrigel also has properties in maintaining pluripotency and stem cell renewal.<sup>16</sup> Research by Katsuta et al., (2016) also proved that 4T1 tumor cells mixed with matrigel formed larger tumors compared to 4T1 cell injections without matrigel. However, the addition of an extracellular matrix caused changes in the tumor microenvironment (TME) and was able to activate CD4+ T cells. This could be a limiting factor for the development of the TNBC animal model because the Geltrex contains mouse antigens so that it can stimulate an immune response when 4T1 tumor cells mixed with Geltrex are injected into mice.<sup>17</sup> Therefore, the success in making the TNBC model in group C was only 2 out of 7 samples or 28.57%.

To overcome these limitations, several studies have developed a TNBC model by inoculating a 3-dimensional culture of 4T1 tumor cells. The 3-dimensional culture model is able to form aggregates and spheroids, which allow for good inter-cell communication. In addition, the morphology of 3D cell culture is also more similar to tumor conditions in vivo compared to 2D cell culture.<sup>18-20</sup> However, this study did not show cancer in all samples in the 3D culture group due to

several factors, such as the mechanical weakness of reversible scaffold (Geltrex) and complexity in maintenance process of 3D culture via scaffold degradation.<sup>21</sup> Not all TNBC are basal-like carcinoma types (tumors expressed on myoepithelial cells) have similar histopathological characteristics and prognostic features. TNBC neoplasms cannot always be determined as tumor cell aggressiveness because it depends on the level of the heterogeneous lesion, from low grade to high grade.<sup>16</sup> Group C in Figure 3 shows signs of IDC, including neoplastic invasion that penetrates the basement membrane and then enters the stroma, the presence of a number of ductal differentiation, pleomorphism of tumor cells, and the presence of mitosis.<sup>22,23</sup> The largest tumor growth on average occurred in the second and third week, either in groups B, C or D, then the tumor shrank and disappeared. The disappearance of tumors after the third week is related to Disseminated Tumor Cells (DTC) or the spread of tumor cells. When 4T1 tumor cells are injected into mice, metastatic colonization occurs to various secondary organs, resulting in DTCs being eradicated by various mechanisms, both in the circulation and in other organs. secondary, including evasion of the immune response by DTCs in primary organs, becoming inactive, and being eradicated by the innate and adaptive immune systems. This is reinforced by previous studies regarding immunotherapy in TNBC, namely the rejection of the immune system by mice.<sup>24,25</sup> Significant tumor growth on the seventh day and a decrease in the following weeks was also found in a study conducted by Katsuta et al. (2016).<sup>26</sup> The study revealed that there was a rejection reaction from the immune system of the experimental animals to the 4T1 cells injected into the mice's bodies. Tumor rupture in the fourth and fifth weeks, especially in group B, was also associated with granulomatous inflammation. This supports the results of histopathological examination that in group B, none of the samples tested positive for TNBC. However, the mammary tissue in group B showed granulomatous inflammation, which was characterized by the presence of lymphocytes, plasmacytes, and leukocytes that infiltrated the stroma in the mammary tissue (Figure 3). Granulomatous inflammation of the breast is an inflammation of the breast that has characteristics similar to breast cancer. The pathogenesis of granulomatous inflammation of the breast is still not known

with certainty, but there are three hypotheses that cause granulomatous inflammation of the breast, namely autoimmune, infection, and hormonal disorders.<sup>27</sup> This was proven histopathologically that most of the mammary tissue examination in the treatment group contained inflammatory cells.

With regard to the success in tumorigenesis of experimental animals inoculated with 4T1 tumor cells, a study revealed that mice that had developed tumors on the 14<sup>th</sup> day underwent necropsy, so that at that time, the tumor could be examined to confirm the formation of TNBC.<sup>26</sup> In contrast to this study, which used orthotopic mouse models, Murayama and Gotoh's (2019) study used the Patient-Derived Xenograft (PDX) mouse model, where the PDX model has a deficient immune system and lacks a stromal component, so it is not easy to eradicate tumor cells injected into the body of experimental animals.<sup>28</sup> In addition, the use of animal models that are immunocompromised can be recommended as animal models for TNBC by first weakening the immune system of the experimental animals, such as eliminating the thymus, reducing the function of B/T lymphocytes so that they become Severe Combined Immunodeficient (SCID) mice, and using mice Balb/c strain Rag-2 null /IL2R $\gamma$  null which is immunocompromised with the characteristics of lower macrophage-mediated phagocytosis making it suitable for tumor cell transplant recipients.<sup>29</sup> In group B and D, no samples were found that tested positive for IDC, and in Figure 3, an incomplete section of mammary tissue is seen; however, inflammation can be seen due to the injection of 4T1 tumor cells in mammary fat. Histopathologically, the inflammatory symptoms in Figure 4 are characterized by diffuse dermal lymphatic invasion due to the presence of carcinoma cells, namely 4T1 cells, indicated by black arrows. Inflammatory carcinoma that occurs in group B is classified into class A according to Bonnier in the histopathological classification system of breast cancer patients. The class A group has signs, namely the presence of inflammation but no palpable tumor (sample B with code B1). Then, other signs are sub-dermal lymphovascular invasion by tumor cells 4T1.<sup>30</sup>

Histopathological examination of the liver, lungs, and brain was also carried out to determine the presence of distant metastases. From these results, no metastases were found in three organs from all groups, both macroscopically and microscopically. However, based on Figure 4, there are signs of abnormality in the three organs of group B, C, and D, such as inflammation, epithelial hyperplasia, and congestion. The failure of distant metastases to three secondary organs in all groups of mice was caused by the eradication of DTCs by the innate immune system and the adaptive immune system.<sup>24</sup> Natural Killer Cells (NK cells) and T cells play an important role in the regulation of metastasis. One study revealed that NK cells effectively limit the spread of tumor cells to the lungs, while T cells play a role in limiting metastatic growth in the lungs. Although

metastases in the lung are not found in NK cells, NK cells play a role in stimulating the recruitment of T cells to the metastatic center. The liver is the dominant secondary organ where NK cells eradicate tumor cells to limit their spread to the lungs.<sup>31</sup>

Inflammation that occurs in the liver and lungs of groups B, C, and D is caused by a reaction of immune cells, especially neutrophils, which produce Neutrophil Extracellular Traps (NETs) due to the influence of tumor cells injected into the mammary glands. NET can enter the circulation and cause endothelial damage, resulting in inflammation, and then NET also activates the NF- $\kappa$ B signaling pathway, which acts as a chemotactic factor.<sup>32</sup>

## CONCLUSION

Two methods of 4T1 tumor cell inoculation resulted in palpable tumors and volume measurements using a caliper in the second to fourth weeks. There was a single nodule produced from two 4T1 cell inoculation methods, namely group B and group C. However, the resulting nodule shrank and disappeared in the fourth and fifth weeks. Histopathologically, there were 2 samples in group C that were declared IDC because there were a number of characteristics that characterize IDC. Based on the Chi-Square test, the differences between treatment groups were not significant.

## IMPLICATION

This study contributed to drug discovery research, especially for triple-negative breast cancer (TNBC). The optimal animal model is needed as a representative of TNBC patients with severity levels. Furthermore, 3D cell culture can be used as an advanced method to investigate how cancer cells work within their proliferation, signaling mechanisms, and even their progression through tumoroid development with a spheroidal shape in order to mimic human conditions.

## STRENGTHS AND LIMITATIONS

Due to the importance of drug discovery research, this study has the strengths of a novel animal model from spontaneously injected mice with breast cancer cell line (4T1) in Indonesia, with modified techniques of culturing cells, such as 3D Culture and the addition of Geltrex to 4T1 cell culture. It would become a potential method for improving the quality of drug discovery research in Indonesia. However, this research also has some limitations, especially in determining inclusive and exclusive criteria for mice. The failures of this research were also caused by the quality of mouse selection. Aside from that, an immunohistochemistry assay was not performed in this study, so we could not rule out the possibility of malignancies or tumor prevalence due to the absence of further examination, such as immunohistochemistry.

## CONFLICT OF INTEREST

None

## ACKNOWLEDGMENTS

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## DECLARATION OF USING AI

The authors affirm that artificial intelligence (AI) tools were used to assist in the writing process solely for language enhancement purposes, such as grammar checking, paraphrasing, and improving clarity. No AI tools were employed to generate original content, conduct data analysis, or interpret research findings. The authors take full responsibility for the content, interpretations, and conclusions presented in this manuscript.

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