

The Role of Beetroot Extract in Overcoming Chemoresistance of Neoadjuvant Adriamycin Cyclophosphamide Regimen by Targeting Immune Response in Tumor Microenvironment: A Preclinical Study in Mammary Adenocarcinoma Rats

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Abstract

Objective: This study aims to determine the role of beetroot extract in overcoming the chemoresistance of Neoadjuvant Adriamycin Cyclophosphamide (NAC) regimens with a target immune response in the tumour microenvironment at the pre-clinical stage. **Methods:** This study was conducted on rats with 7,12-Dimethyl Benz (α) Anthracene (DMBA) induced mammary adenocarcinoma. Adriamycin Cyclophosphamide was given in 4 cycles, whereas beetroot extract was administered three times each cycle. Observations of CD8 T cells and Myeloid Derivative Suppressive Cells (MDSC) expression levels and pathological responses were carried out on tumour tissue taken at the end of the observation. **Results:** Supplementation of beetroot extract to NAC could significantly increase CD8 T cells and decrease MDSC in the tumour microenvironment. The addition of beetroot extract gave a better pathological response. **Conclusion:** Beetroot extract enhances the immune response in the tumor microenvironment so that it has the potential to overcome chemoresistance in NAC.

Keywords: Beetroot- neoadjuvant- adriamycin- cyclophosphamide- CD8- T cells- myeloid derivative suppressive

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Introduction

Breast cancer is the most common type of cancer diagnosed in women worldwide with high incidence and mortality (IARC, 2020). The currently available therapeutic modalities are surgery, radiotherapy, chemotherapy or targeted therapy. Chemotherapy is classified as systemic therapy (Miller et al., 2014). Chemoresistance is one of the obstacles to be good therapeutic outcome so causing the risk of relapse in breast cancer treatment. The mechanism of chemoresistance in breast cancer involves drug absorption on cell membranes, transporter proteins, oncogenes and tumor suppressor genes, DNA repair, stem cells, Tumor Microenvironment (TME) and Epithelial-Mesenchymal-Transition (EMT) (Ji et al., 2019; Lainetti et al., 2020). Stromal cells such as fibroblasts, immune cells, vascular endothelial cells, and other components such as the extracellular matrix present

in TME have a role in tumour response to chemotherapy. Tumour-Associated Macrophage (TAM) and Tumour-Infiltrating Lymphocytes (TIL) are immune cells in TME associated with chemoresistance (Velaei et al., 2016). Chemoresistance can be overcome through gene therapy or immune therapy approaches (Ji et al., 2019).

Neoadjuvant Adriamycin Cyclophosphamide (NAC) regimen is one of standard anthracycline-based breast cancer therapy that still recommended for patients with locally advanced breast cancer before surgery (Cardoso et al., 2017; Miller et al., 2014; Senkus et al., 2015). This regimen is given to reduce the size of the tumour so that surgery is possible. Using the Adriamycin Cyclophosphamide (AC) regimen as a neoadjuvant resulted in clinical and pathological responses of 40.1% and 13.7% and increased to 63.6 and 26.1% after adding docetaxel (Bear et al., 2003). The addition of bevacizumab to the neoadjuvant nab-paclitaxel with AC

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improves the pathologic complete response (pCR) in inflammatory or locally advanced breast cancer (Nahleh et al., 2016). The results of a meta-analysis of patients receiving anthracycline-based NAC overall showed pCR and breast-conserving surgery outcomes of 26.5% and 70.6%, respectively (Kang et al., 2021). There is currently sufficient evidence that if NAC leads to a complete pathological response, it will have a good therapeutic outcome (Masood, 2016).

Adriamycin Cyclophosphamide is a cytostatic agent with a DNA-damaging mechanism (Siddik, 2002; Tacar et al., 2013). Chemoresistance to NAC involving immune cells in TME is currently only known from TAM, which is form differentiation from immature myeloid cells, Myeloid derivatives Suppressive Cells (MDSC). Overexpression of M2, a pro-tumorigenic macrophage in TME, is associated with an absent clinical response in patients with anthracycline-based NAC (Litviakov et al., 2018). Chemotherapy doxorubicin 60 mg/m² and cyclophosphamide 600 mg/m² given every 2 weeks can increase MDSC in peripheral blood circulation (Bracci et al., 2014). Tumor-infiltrated MDSCs have been shown to correlate with clinical pathology, chemotherapy response and prognosis of breast cancer patients (Li et al., 2018). MDSCs in experimental animals such as rat can be identified through the expression of the biomarker CD11b (Condamine and Gabrilovich, 2011). TIL, primarily CD8 T cells, and MDSC cells are immune cells that act opposite in TME. CD8 cytotoxic T cell activity produces an antitumor immune response, whereas MDSC cells have an immunosuppressive role (Dieci et al., 2021). In carcinogen-induced adenocarcinoma and fibrosarcoma trials, Doxorubicin can increase CD8 T cell proliferation in lymph nodes and increase CD8 T cell infiltration through IFN- γ secretion (Bracci et al., 2014). However, there has no study of the effect of NAC therapy on CD8 T cells but CD8 TIL can be used as a predictive factor for pCR of primary breast cancer patients receiving anthracycline-based systemic therapy (Seo et al., 2013).

In recent years, the use of phytotherapy products as complementary treatment by women with a history of breast cancer has increased significantly. However, scientific evidence of its efficacy and safety is still lacking (Drozdoff et al., 2018; Lopes et al., 2017). One of the herbal products that has been empirically used with systemic breast cancer therapy is beetroot. This study aims to provide scientific evidence at the pre-clinical stage about the benefits of beetroot extract in overcoming NAC chemoresistance involving immune cells in TME. In this study revealed the efficacy of NAC after adding beetroot extract to the pathological response as the endpoint of observation. Pathologic complete response (pCR) was used as the endpoint in most studies looking at the NAC response (Parekh et al., 2015). Clinically, pCR assessment can use a variety of different standards. pCR can also be obtained from evaluating the proportion of fibrosis to tumour cells described in the absence of tumour cells and mostly fibrosis, or tumour cells are sparse and scattered throughout the fibrosis (Frentzas et al., 2019).

Beetroot which contains the main active compound betalain has bioactivity such as anti-inflammatory,

antioxidant, immunomodulatory, and cancer chemopreventive properties. The immunomodulatory activity of betalain through increased proliferation of spleen lymphocytes, phagocytic function of peritoneal macrophages, and NK cell activity in vitro, also increased the immune organ index and serum hemolysin, and improved biochemical indices in vivo (Fu et al., 2020). The results of in vivo studies on experimental animals showed that the administration of beetroot extract significantly reduced DNA damage and increased the proliferation of hematopoietic progenitor cells in C57BL/6 mice exposed to γ -ray radiation for 10 days (Cho et al., 2017). Research on the inflammatory activity related to the immune response of beetroot extract has also been carried out. The anti-inflammatory effects of beetroot betalains are well known in vitro (Reddy et al., 2005; Vidal et al., 2014) or in vivo (Asgary et al., 2016; El Gamal et al., 2014; Pietrzkowski et al., 2010; Tan et al., 2015). Betalains can reduce levels of pro-inflammatory cytokine proteins such as TNF-, IL-6, IL-8, and IL-1 β , reactive oxygen, as well as COX-2 and lipoxygenase (LOX) enzyme activities, causing a reduction in PGE2 and LOX-5. Even betacyanin and betaxanthin can decrease the activity of transcription factor activator protein and NF-kB (Clifford et al., 2015). Based on the results of this study, beetroot extract herbal can be used to overcome chemoresistance with immune cells in TME as a target, particularly by observing the expression of CD8 and CD11b biomarkers as well as the primary outcome in the form of response pathology.

Materials and Methods

Beetroot extract was obtained from Tokyo Chemical Industri, Co., Ltd, Tokyo Japan under trade name Betanine and Catalog Number B0397. Adriamycin (DoxorubicinR) and Cyclophosphamide (CytoxanR) was obtained from PT. Kalbe Farma. Animal used Sprague Dawley female rats that was obtained from National Center for Drug and Food Testing Development, Food and Drug Supervisory Agency of the Republic of Indonesia and being treated in the laboratory of Animal Research Facilities, Faculty of Medicine, University of Indonesia. Ethical approval was obtained from The Ethics Committee of the Faculty of Medicine, University of Indonesia with number: KET-756/UN2.F1/ETIK/PPM.00.02/2019.

Carcinogenesis

Carcinogenic agent for cancer induction: 7,12-dimethyl benz (α) anthracene (DMBA) from Sigma Aldrich with corn oil solvent. Cancer induction for 5 weeks at a dose of DMBA 20 mg/kg BW, orally, 2 times a week in forty days old rats.

Design Study

This was an experimental study with a Randomized Post test only Control Group Design. Twenty-four rats with nodule diameter tumor more than 10 mm were randomized allocated into 4 treatment groups, without treatment as negative control, AC only treatment at dose 5 mg/kg BW for Adriamycin and 50 mg/kg BW for Cyclophosphamide, intraperitoneally, weekly during 4

cycle, and 2 groups AC treatment with supplementation beetroot extract, orally, 3 times a week at dose 25 mg/kg BW, and 100 mg/kg BW, respectively. At the end of the 4th week, the rats were necropsied for tumor tissue for examination of CD8, CD11b expression levels and pathological responses.

Determination of CD8 and CD11b expression levels using the qRT-PCR method

Determination of CD8 and CD11b expression levels were used paraffin embedded tumor tissue slices using the qRT-PCR method. The template in this study used mRNA, where the mRNA isolation was carried out according to the Protocol for total RNA purification with On-column DNase I treatment from animal tissue from Gene AllR (GeneAll Biotechnology, 2016). The qRT-PCR amplification process used the AccuPowerR Green Star™ RT-qPCR Kit with the following primer design: CD8 forward primer sequence 5'-ACTTGTGGGGTCCTTCTCCT-3' and CD8 reverse primer sequence 5'-TCTCCCATTGACCACAG-3' also CD11b forward primer sequence 5'-ATGGACGCTGATGGCAATACC-3' and CD11b reverse primer sequence 5'-TCCCCATTCACGTCTCCCA-3'. qRT-PCR examination using Bioneer Exicycler 96™ with 40-45 cycling, begins with reverse transcription at 60°C for 15 minutes, pre denaturation at 95°C for 3-5 minutes, denaturation at 95°C for 5-30 seconds, annealing at 60°C for 5-30 seconds, and melting according to manual protocol. Data from qRT-PCR were analyze using the 2^{-ΔΔCT} method, which were presented as the fold change in gene expression normalized to an endogenous reference gene GAPDH and relative to normal mammary tissue (Livak and Schmittgen, 2001).

Pathological Response Assesment

Pathological response is a tumor response that is assessed from tumor tissue stained with Hematoxylin Eosin based on the tumor regression grade (TRG 1-5) of the assessment of the proportion of fibrosis to tumor cells on tissue slice preparations with HandE staining, as follows TRG 1 = no tumor cells and mostly fibrosis, TRG 2 = the presence of rare and scattered tumor cells throughout the fibrosis, TRG 3 = more residual tumor cells but fibrosis predominates, TRG 4 = residual cancer cells predominate over fibrosis, TRG 5 = no signs of tumor regression. The grouping of the 3 main pathological responses is as follows: Complete Response = TRG 1-2,

Partial Response = TRG 3, No Response = TRG 4-5 (Frentzas et al., 2019).

Statistical Analysis

The CD8 and CD11b expression level data were analyzed by ANOVA followed by post-hoc-test (Tukey-HSD). Pathological Response was analyzed using the Chi-Square test. The correlation test of CD8, CD11b expression levels with pathological responses was used Spearman's rho test.

Results

Characteristics of tumour tissue-based on examination of histopathological showed that tumour that occurred in the mammae of these rats was classified as mammary adenocarcinoma, meaning that the tumour caused by DMBA induction was cancer. The cancer subtypes were ductal carcinoma, cribriform carcinoma and invasive papillary carcinoma, but unfortunately the limitation of this study was that the examination of tumor type was carried out after the intervention. In the negative control group (C1), there was one rat with histopathological characteristics of fibroadenoma, so it was excluded as a study sample. In the treatment group of AC plus beetroot extract at a dose of 100 mg/kg BW (P2) there was one rat in which the nodule or tumour disappeared on the 3rd week, so no tumour tissue could be obtained. The number of final samples for the examination of molecular targets and pathological responses was 22 rats. The research CONSORT was presented in Figure 1.

Effect of addition of beetroot extract on CD8 and CD11b expression levels

The CD8 expression levels of the AC plus beetroot extract either at a dose of 25 mg/kg BW or 100 mg/kg BW treatment groups were significantly higher (p=0.000 and p=0.000) than the AC only treatment group (Figure 2). This proves that beetroot extract affects the number of CD8 cytotoxic T cells in TME. The difference in the dose of beetroot extract affected the CD8 expression level, proven by a significantly higher result (p=0.000) in the dose 100 mg/kg BW group compared to the 25 mg/kg BW group. The CD8 expression level of the AC only treatment group was higher but not significantly (p=0.305) than the negative control group, indicating that the AC at this dose did not affect CD8 expression level. Meanwhile CD8 expression level in the group given AC plus beetroot extract either at dose of 25 mg/kg BW or 100 mg/kg BW was significantly higher (p=0.000) than the negative control group. This further proves that beetroot

Table 1. Pathological Response Evaluation Results

Treatment Group	Pathologic Response (n)			Response (n)	No response (n)
	CR	PR	NR		
C1	0	0	5	0	5
C2	0	3	3	3	3
P1*	1	3	2	4	2
P2*	1	4	0	5	0

Note: Group C1, negative control, without treatment, Group C2 : AC only control, Group P1 : AC+ Beetroot extract at a dose 25 mg/kg BW treatment, Group P2: AC + Beetroot Extract at a dose 100 mg/kg BW Treatment; CR, Complete Response, PR, Partial respon

Table 2. Summary of Correlation Test Results on Pathological Response Variable

Independent variable	Corellation value (R)	P value
CD8	0,542	0,009
CD11b	-0,660	0,001

Note: Spearman's rho correlation test, significant if p <0.05. Spearman's rho correlation degree: very weak (0-0.25); enough (0.26-0.5); (0.51-0.75) strong; 0.76-0.99 is very strong; 1 perfect correlation.

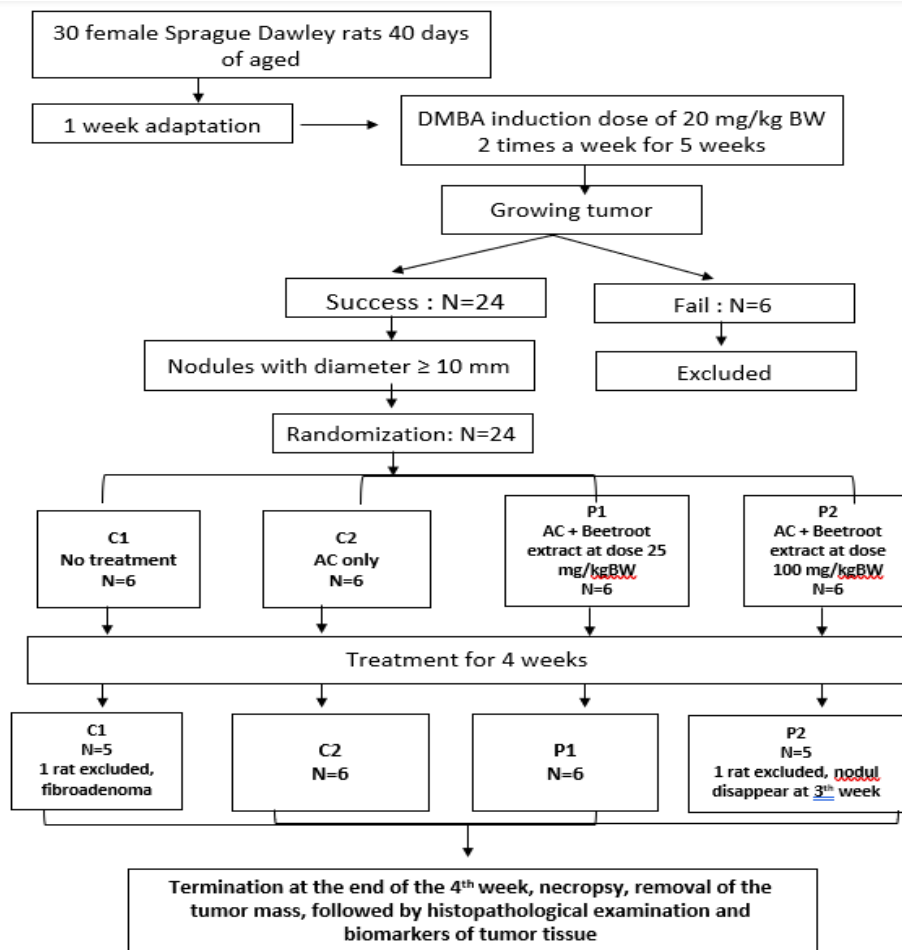


Figure 1. Consolidation of Report Trial

extract given with neoadjuvant AC regimen can affect CD8 expression level in TME.

The results showed that the CD11b expression level

in the AC plus beetroot extract treatment group was lower than the AC only treatment group, but only for the beetroot extract at a dose 100 mg/kg BW group

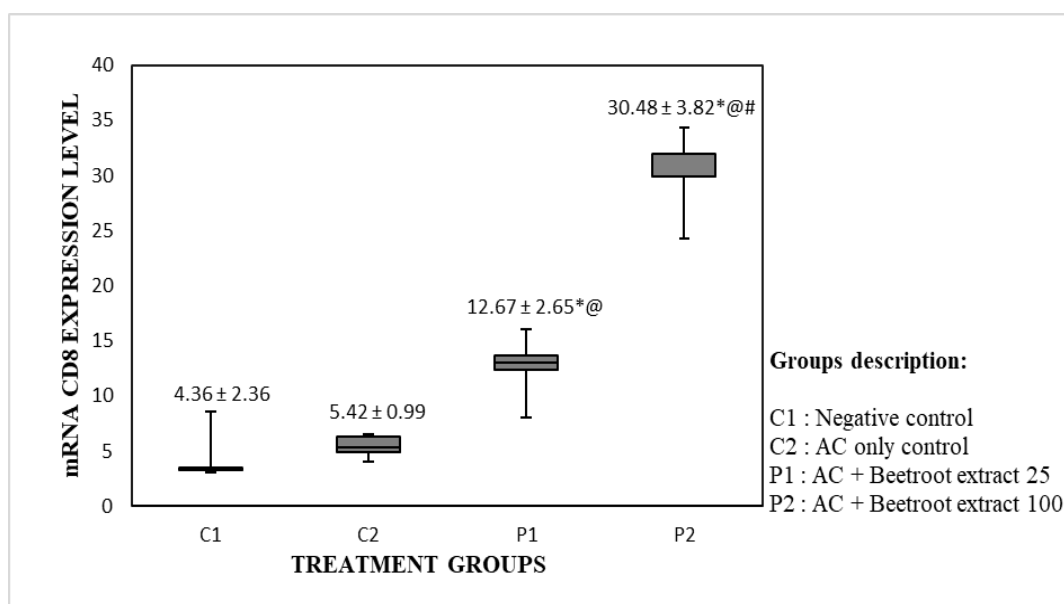


Figure 2. Comparison graph of CD8 Expression Levels (mean ± SD) between Treatment Groups. The results of ANOVA followed by Post Hoc-Test, were significant if $p < 0.05$: * : significantly different from Group C2 with p value = 0.000 for group P1 and $p = 0.000$ for group P2. @: significantly different from Group C1 with a value of $p = 0.000$ for group P1 and $p = 0.000$ for group P2. #: significantly different from the P1 group with p value = 0.000.

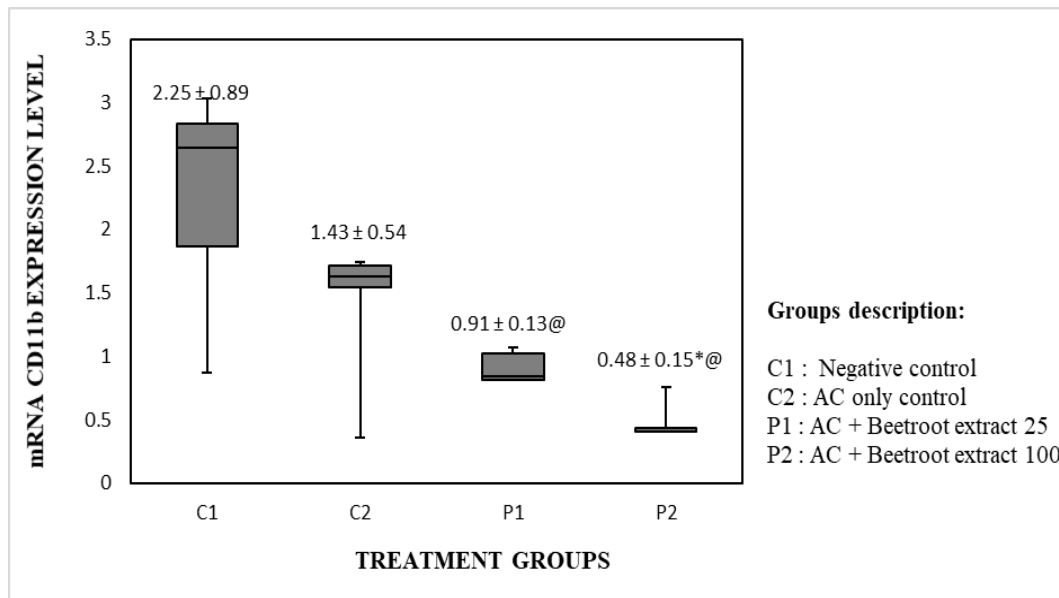


Figure 3. Comparison Graph of CD11b Expression Levels (mean \pm SD) between Treatment Groups. The results of ANOVA continued with Post Hoc-Test, meaningful if $p < 0.05$: * : significantly different from Group C2 with p -value = 0.012 . @ was significantly different from Group C1 with $p = 0.006$ for Group P1 and $p = 0.000$ for Group P2.

which was statistically significant ($p=0.012$) (Figure 3). When compared with the negative control group, CD11b expression level in the AC only treatment group showed lower but not significant ($p=0.158$), while in the group that was given AC plus beetroot extract at doses of 25 mg/kg BB or 100 mg/kg BW was significantly lower ($p=0.006$ and $p=0.000$). This proves that addition of beetroot extract to NAC affects the level expression of CD11b. However the difference in the dose of beetroot extract did not affect CD11b expression because CD11b expression level in the AC plus beetroot extract at a dose 100 mg/kg BW was lower but not significant ($p=0.249$) than a dose 25 mg/kg BW. Thus it can be stated that the addition of beetroot extract to NAC can reduce MDSC, because the CD11b biomarker is a feature of MDSC in rat.

Effect of addition of beetroot extract on pathological response

AC only treatment without beetroot extract showed a different pathological response but not significantly compared to negative control without any treatment ($p = 0.064$). The addition of beetroot extract to the AC regimen showed a different and significant pathological response compared to negative control ($p=0.022$ and $p=0.002$) (Table 1). This shows that the addition of beetroot extract to the AC regimen can improve the pathological response of tumor cells. Actually, when compared to the AC group only, the pathological response of the AC plus beetroot extract group was better but not significant statistically ($p=0.558$ and $p=0.064$). Histopathological description of mammary tumor tissue with Hematoxylin and Eosin (HandE) staining in each treatment group

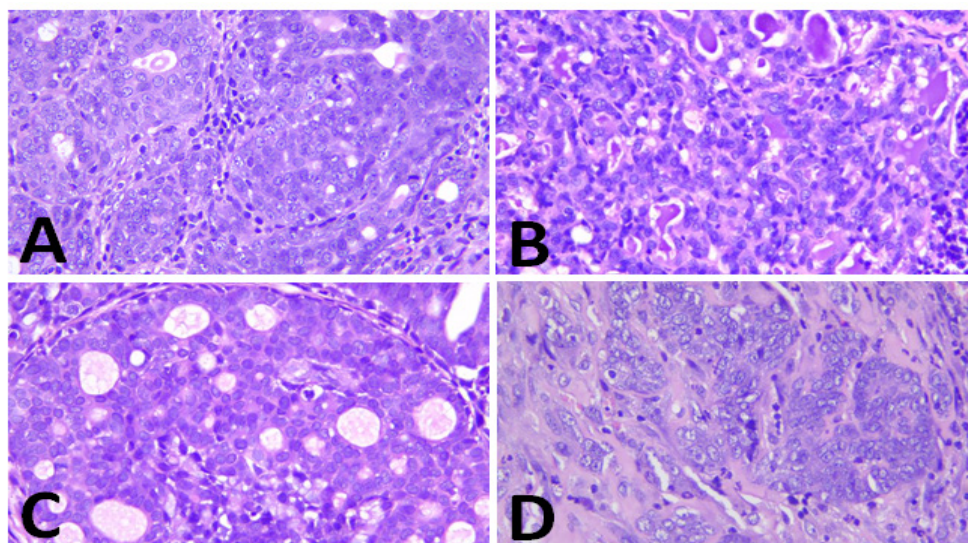


Figure 4. The Representative Image of H&E Staining from Tumor Sample in Each Group (magnification 400x). A, Group C1, negative control without treatment; B, Group C2, AC only treatment; C, Group P1, AC + Beetroot extract at a dose 25 mg/kgBW treatment; D, Group P2, AC + Beetroot extract at a dose 100 mg/kgBW treatment.

showed differences (Figure 4).

The results of the correlation test between each expression level variable CD8 and CD11b with a pathological response showed a correlation ($p < 0.05$) (Table 2). The direction of the negative relationship is shown in the expression of CD11b, meaning that the lower the expression level of CD11b make better the pathological response. A positive relationship was shown in CD8 expression, meaning that the higher the CD8 expression level, make better the pathological response. The degree of correlation between CD8 and CD11b expression with pathological response was strong.

Discussion

The low pathologic Complete Response results in the treatment of NAC in breast cancer patients could be attributed to chemoresistance. NAC chemoresistance involving immune cells in TME is associated with an increase in MDSC and overexpression of macrophage M2 (Bracci *et al.*, 2014; Litviakov *et al.*, 2018). High MDSCs in TME lead to tumor growth, because MDSC activity can inhibit the cytolytic function of CD8 T cells either through metabolic pathways that involve amino acids for T cell activity and proliferation, or inhibits T cell apoptosis (Law *et al.*, 2020). CD8 T cells are effector cells that kill cancer cells by releasing granzyme and perforin and Fas ligands for the process of apoptosis. The CD8 T cells expansion occur from the lymph nodes to the circulation and TME which its activity begins with the introduction of antigens by Antigen Presenting Cell (APC), namely dendritic cells (DC) and is presented through the MHC I complex to CD8 cells, then with the help of CD4 Th cells. Whereas MDSCs are immature myeloid cells derived from bone marrow progenitors. MDSC recruitment and accumulation into TME is mediated by cytokines and chemokines such as IL6, IL1- β , TGF-1 β , CCL2 which are secreted by tumor cells (Dieci *et al.*, 2021; Ostroumov *et al.*, 2018).

The findings in this study proved that beetroot extract can decrease MDSC and increase CD8 T cells. The ability of beetroot extract to reduce MDSC is thought to be related to its anti-inflammatory activity. Beetroot extract have anti-inflammatory activity against COX2 and PGE, also reduce levels of TNF- α , IL-6, and NFkB (El Gamal *et al.*, 2014). COX2, PGE, IL-6 and NFkB are pro-inflammatory proteins that play a role in MDSC recruitment and activity (Gabilovich, 2017; Law *et al.*, 2020; Marvel and Gabilovich, 2015). It is known that pro-inflammatory signaling is also associated with chemoresistance. Elevated levels of IL-6 expression correlate with advanced disease stage and poor prognosis in breast cancer (Das and Law, 2018). Increased NFkB activity is also associated with doxorubicin resistance (Gangadharan *et al.*, 2009). Beside being associated with a decrease in MDSC, the finding that beetroot extract can increase CD8 T cells was also associated with its immunomodulatory activity. Previous studies have shown that beetroot extract can increase the proliferation of splenocytes and hematopoietic progenitor cells in vivo (Cho *et al.*, 2017). Betalain is a constituent that is responsible for the immunomodulatory activity of beetroot extract, particularly in increasing the

proliferation of spleen lymphocytes (Fu *et al.*, 2020). CD8 T cell activity also involves other immune cells such as dendritic cells, CD4 T helper cells, TReg, but unfortunately the involvement of these immune cells has not been revealed in this study.

Pathological response is the endpoint for assessing tumor response to NAC. Complete pathological response is the expected therapeutic outcome, because it can keep the patient from relapse (Kunnuru *et al.*, 2020; Parekh *et al.*, 2015). Tumor response to NAC also depends on the molecular subtype of the cancer. Cancer with positive Estrogen Receptor (ER) was less sensitive to DNA damaging chemotherapy such as NAC than ER negative (McDonald *et al.*, 2016; Sharma, 2014). Previous research has shown that characteristics of mammary cancer formed by DMBA induction are similar to luminal A subtypes in human breast cancer, namely the presence of positive ER/PR expression and low Ki-67 (Alvarado *et al.*, 2017). Presumably because of this, the pathological response of NAC in this study was not significant compared to the untreated control. The addition of beetroot extract to NAC was proven to significantly improve the pathological response compared to the untreated control. Based on the results of a strong correlation between the expression levels of CD8 and CD11b with pathological responses in this study, it is suspected that beetroot extract can improve pathological responses due to the involvement of these immune cells, especially the decrease in MDSC and increase in CD8 T cells in TME. Nevertheless the factors that influence the complete pathological response are complex. Because the molecular characteristics of cancer in this study are still limited to the luminal subtype A, the limitation of this study is that the role of beetroot extract in improving the pathological response of NAC when given a progressive cancer molecular subtype is unknown.

As conclusion the addition of beetroot extract in the neoadjuvant AC regimen can improve the pathological response of cancer cells by increasing the immune response in the tumor environment, especially increasing CD8 T cells and decreasing MDSC. This study proves the molecular role of beetroot extract in overcoming the chemoresistance problem caused by the AC regimen as a neoadjuvant. This study provides scientific evidence about the benefits of beetroot extract when given with neoadjuvant regimen AC in cases of breast cancer in the pre-clinical trial stage. The results of the study are expected to be used as a basis for clinical research, thereby providing evidence of the effectiveness of complementary therapies between natural ingredients, namely beetroot extract and the AC regimen in breast cancer patients.

Author Contribution Statement

Substantial contributions to the conception or design of study and analysis or interpretation of data: Sri Susilowati, Catharina Suharti, Neni Susilaningsih, Ignatius Riwanto, Yan Wisnu Prajoko and Suhartono. Investigation: Sri Susilowati, Hermawan Istiadi. Writing – Original Draft Preparation: Sri Susilowati. Writing – Review and Editing: Sri Susilowati, Catharina Suharti, Neni Susilaningsih, Ignatius Riwanto, Yan Wisnu Prajoko, Suhartono.

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Approval

It is part of an approved student thesis from Doctoral Program of Medical and Health Sciences, Faculty of Medicine, Diponegoro University, Semarang, Indonesia

Ethical Declaration

Ethical approval was obtained from The Ethics Committee of the Faculty of Medicine, University of Indonesia with number: KET-756/UN2.F1/ETIK/PPM.00.02/2019.

Conflict of Interest

The authors declare no conflict of interest.

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