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Addition of Beetroot Extract to Neoadjuvant Adriamycin Cyclophosphamide Regimen Increased Tumor Cell Apoptosis in Mammary Adenocarcinoma Rats

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Abstract

Apoptosis is one of the anticancer targets. Currently, the concomitant use of phytotherapy products and chemotherapy regimens is common in breast cancer patients. The purpose of this study was to examine the apoptotic effect of adding beetroot extract to the neoadjuvant Adriamycin Cyclophosphamide (AC) regimen by observing the expression levels of p53 and caspase 3 in tumor tissue from mammary adenocarcinoma rats. Twenty-four rats that succeeded in growing tumor nodules were randomly divided into 4 treatment groups: without treatment, AC only treatment, AC plus beetroot extract at dose of 25 and 100 mg/kg BW, respectively. AC was given 4 cycles in doses of 5 and 50 mg/kg body weight intraperitoneally every week. Tumor tissue was dissected at 4th week for examination of p53 and caspase 3 expression levels using the qRT-PCR method. The addition of beetroot extract at doses of 25 and 100 mg/kg BW in the neoadjuvant AC regimen showed significantly higher levels of p53 and caspase 3 expression than those with AC treatment alone. These results proved that beetroot extract has a synergistic effect with neoadjuvant AC regimen by increasing tumor cells apoptosis.

Keywords: *Beetroot extract, Adriamycin, Cyclophosphamide, apoptosis, p53.*

INTRODUCTION

Apoptosis is programmed cell death. Apoptosis is one strategy to inhibit the growth or eliminate cancer cells. There are two apoptotic pathways, intrinsic and extrinsic. The intrinsic pathway, also known as mitochondria pathway, is controlled by a caspase initiator protein caspase 9 which binds to an apoptotic protease activating factor 1 (APAF1) adapter protein. The extrinsic pathway is known as the death receptor (DR) pathway, begins with the presence of death ligands released by Natural Killer (NK) cells or macrophages which then interact with DR in the cell membrane. This

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interaction will activate pro-caspase 8 to become caspase 8. The interaction between pro caspase 8 and the death- inducing signal complex (DISC) is facilitated by the adapter protein FAS- associated death domain (FADD) or TNF receptor (TNFR)-associated death domain (TRADD). Activation of caspase 8 can act directly as an executor of apoptosis, or through activation of caspase 3 for apoptosis to occur. Caspase 3, a cysteine-aspartic acid protease is one of the executor proteins in the process of apoptosis, the others are caspases 6 and 7 (Boice & Bouchier-Hayes, 2020; D'Arcy, 2019).

The p53 protein is a transcription factor that has a central role in the response that occurs due to DNA damage. p53 activation is associated with DNA repair, cell cycle arrest, apoptosis and senescence. Related to cancer, p53 has a function as a tumor suppressor. The p53 is often referred to as the guardian of the genome because it is able to protect against tumor malignancy (Aubrey, *et al*., 2018).

Neoadjuvant Adriamycin Cyclophosphamide Regimen (NAC) is used as a standard therapy for locally advanced breast cancer. Neoadjuvant administration is intended to reduce tumor size before surgery. This anthracycline-based regimen is standardly given 4 or 6 cycles (Fisusi & Akala, 2019). Adriamycin is also known as Doxorubicin, stimulates cancer cell apoptosis by inhibiting of topoisomerase II enzymes in DNA complexes, also forming free radicals (Tacar, *et al*., 2013). Meanwhile, cyclophosphamide damages DNA by methylation, thereby inhibiting the proliferation of cancer cells (Siddik, 2002).

The use of natural products with systemic therapy in breast cancer patients is increasing. But unfortunately this complementary medicine is only based on empirical experience, there is not enough scientific evidence regarding its efficacy and safety (Drozdoff, *et al*., 2018; Lopes, *et al*., 2017). This study aims to provide scientific evidence of the benefits of adding natural products, especially beetroot extract to the response to NAC therapy by observing tumor cell apoptosis.

Beetroot extract showed cytotoxic activity on prostate and estrogen receptor-positive breast cancer cell lines (Kapadia, *et al*., 2011). Another study stated that the combination of beetroot extract and doxorubicin exerted a synergistic cytotoxic effect on human cancer cells PaCa (pancreatic cancer cells), MCF-7 (breast cancer cells) and PC-3 (prostate cancer cells) (Kapadia, *et al*., 2013). Beetroot extract could induce apoptosis through intrinsic and extrinsic pathways. It could change the mitochondrial membrane potential of MCF-7 breast cancer cells. It also activates p53, increasing protein levels of Bad protein, a pro-apoptotic protein, TRAILR4 an apoptotic receptor extrinsic pathway, Fas and can cause autophagy cell death (Nowacki, *et al*., 2015). The evidence in this study supports the use of beetroot extract to increase the response of cancer cells to the NAC regimen by increasing apoptosis by observing p53 and caspase 3 expression biomarkers.

MATERIAL AND METHODS

Beetroot extract from Tokyo Chemical Industri, Co., Ltd, Tokyo Japan with trade name Betanine and Catalog Number B0397. Adriamycin (Doxorubicin^{R}) and Cyclophosphamide (Cytoxan^{R}) from PT. Kalbe Farma. 7,12-dimethyl benz (α) anthracene (DMBA) from Sigma Aldrich. Animals 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, Jakarta, Indonesia. Ethical approval from The Ethics Committee of the Faculty of Medicine, University of Indonesia with number: KET-756/UN2.F1/ETIK/PPM.00.02/2019.

Adenocarcinoma mammary rats were prepared from female forty days of age Sprague Dawley rats induced by DMBA at a dose of 20 mg/kg BW orally 2 times a week for 5 weeks. The preparation of DMBA was dissolved with corn oil as solvent. Observation of tumor growth was

done by palpating the nodules that appear around the mammary then measuring the diameter of the tumor with a digital caliper. Rats with tumor nodule diameters above 10 mm were used for the experiment. Twenty-four rats with tumor were randomized allocated into 4 treatment groups, Group C1 without treatment as negative control, Group C2 was AC only treatment and 2 groups P1 and P2 were AC treatment with addition beetroot extract at dose 25 mg/kg BW and 100 mg/kg BW, respectively. AC treatment was given at dose 5 mg/kg BB for Adriamycin and 50 mg/kg BB for Cyclophosphamide, intraperitoneally, once a week for 4 weeks. Beetroot extract was given orally, 3 times a week for 4 weeks. Water for Injection (WFI) used as a solvent to prepare AC and beetroot extract. At the end of forth week rats were dissected, tumor tissue was removed for examination of p53 and caspase 3 expression. Determination of p53 and caspase 3 expression levels were done by qRT-PCR method.

Sample work from RNA isolation to quantitative real time-PCR carried out in the Anatomical Pathology Laboratory, Faculty of Medicine, Universitas Gadjah Mada. RNA isolation according to the Protocol for total RNA purification with On-column DNase I treatment from animal tissue from Gene All^{R} (GeneAll Biotechnology, 2016). The qPCR amplification process used the AccuPower^{R} Green Star^{TM} RT-qPCR Kit with the following primer design: p53 forward primer sequence 5'-CCTCAGCATCTTATCCGAGTGG-3', p53 reverse primer sequence 5'-TGGATGGTGGTACAGTCAGAGC-3'; caspase 3 forward primer sequence 5'-GGAAGCGAATCAATGGACTCTGG-3', caspase 3 reverse primer sequence 5'-GCATCGACATCTGTACCAGACC-3'. At this stage, RNA template, primer, 2x Master Mix, 50x ROX Dye, DEPC-distilled water were mixed. qRT-PCR examination using machine brand Bioneer Exicycler 96 with cycling protocol begins with cDNA synthesis (reverse transcription) at 60°C for 15 minutes, pre denaturation at 95°C for 3–5 minutes, denaturion at 95°C for 5-30 seconds, annealing at 60°C 5-30 seconds, and the last phase of dissociation according to the manual.

Analysis of data from qRT-PCR used a relative quantification method that correlated the PCR signal of the target transcript in the treatment group with untreated controls (normal mammary tissue did not grow tumors). In this study used the $2^{-\Delta\Delta CT}$ method, the data were presented as the fold change in gene expression normalized to an endogenous reference gene and relative to the untreated control (Livak & Schmittgen, 2001). Reference gene was used housekeeping gene GAPDH acts as an internal control.

Statistic Analysis

The p53 and caspase 3 expression level data between treatment groups were analyzed by ANOVA followed by post-hoc-test.

RESULTS

The p53 and caspase 3 are proteins involved in the process of apoptosis. Quantitative and objective examination of p53 and caspase3 in tissue can be performed based on their mRNA expression using qRT-PCR analysis. The results of study showed that the p53 expression level of treatment group with AC plus beetroot extract at a dose of 25 or 100 mg/kg BW was significantly higher (p=0.017 and p=0.001) than that of AC treatment group (Figure 1). This proves that addition of beetroot extract affects the p53 expression of tumor cells. The p53 expression level of beetroot extract group at dose 100 mg/kg BW was higher than the 25 mg/kg BW group but not significant (p=0.386). The difference in p53 expression level due to the addition of beetroot extract was not dose-dependence.

The p53 expression level of the AC alone group was higher than the negative control group but not significant (p=0.836). This indicates that the AC regimen at this dose did not affect p53

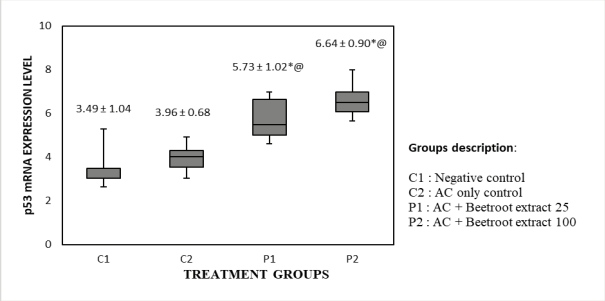

Figure 1. Comparison graph of p53 expression levels (mean±SD) between treatment groups. The results of ANOVA followed by Post Hoc-Test, were significant if $p<0.05$ (N=6): *: significantly different from Group C2 with p value=0.017 for group P1 and p=0.001 for group P2. @ is significantly different from Group C1 with a value of p=0.004 for group P1 and p=0.000 for group P2.

expression. Meanwhile, the p53 expression level in the AC plus beetroot extract at a dose of 25 or 100 mg/kg BW was significantly higher (p=0.004 and p=0.000) than the negative control group. This strengthens the evidence that beetroot extract given with neoadjuvant AC regimen can increase p53 expression levels.

The results showed that the level expression of caspase 3 in treatment group with AC plus beetroot extract at a dose of 25 or 100 mg/kg BW was significantly higher (p=0.018 and p=0.000) than the AC alone group (Figure 2). This proves that addition of beetroot extract affects the expression of caspase 3 in tumor cells. Differences in the dose of beetroot extract affected the expression of capase 3, as indicated by the result of caspase 3 expression level for the 100 mg/kg BW group was significantly higher (p=0.006) compared to the 25 mg/kg BW group.

The expression level of caspase 3 in the AC control group was equivalent to the negative control group with p=1.000, meaning that the AC regimen in this dose did not affect the expression of caspase 3 in tumor cells. Meanwhile, the expression level of caspase 3 in the AC plus beetroot extract at a dose of 25 or 100 mg/kg BW was significantly higher (p=0.026 and p=0.000) than the negative control group. This strengthens the evidence that beetroot extract given with neoadjuvant AC regimen can increase caspase 3 expression levels.

DISCUSSION

The target of the cytotoxic action of doxorubicin is topoisomerase I and II enzymes which cause inhibition of proliferation and DNA damage. The apoptotic pathway is triggered when the attempts to repair DNA damage is failed and cell growth is inhibited in the G1 and G2 phases. This apoptotic pathway involves Bcl2/Bax and p53 molecules. Doxorubicin causes downregulation of Bcl2 mRNA through increased p53 expression (Tacar, *et al.*, 2013). Doxorubicin resistance may be due to altered Bcl2 expression or loss of p53 function (Gangadharan, *et al*., 2009). The cytotoxic effect of cyclophosphamide is caused by mustard phosphoramide which is formed from drug metabolism by liver enzymes such as cytochrome P-450. Liver enzymes first convert cyclophosphamide to hydroxycyclophosphamide and then metabolize it to aldophosphamide. Aldophosphamide is broken down into the active alkylating agents phosphoramide mustard and acrolein. Phosphoramide metabolites form cross-links within and between adjacent DNA strands at the N-7 guanine position. These modifications are permanent and eventually lead to apoptosis (Ogino & Tadi , 2021).

The addition of beetroot extract at doses of 25 and 100 mg/kg BW in the AC regimen was shown to be able to increase p53 expression, although AC

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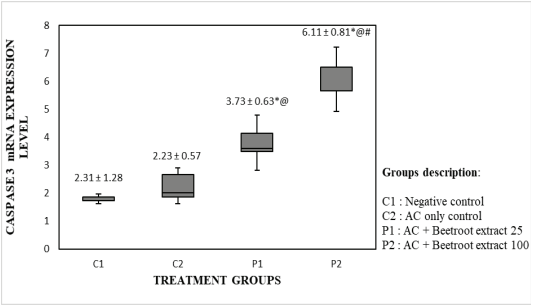

Figure 2. Comparison graph of Caspase 3 expression level (mean±SD) between treatment groups. The results of ANOVA followed by Post Hoc-Test, were significant if $p<0.05$ (N=6): *=significantly different from Group C2 with p value=0.018 for Group P1 and p=0.000 for Group P2. @ was significantly different from Group C1 with p=0.026 for Group P1 and p=0.000 for Group P2. #=significantly different from Group P1 with p value=0.006.

alone could not increase p53 expression compared to negative controls. These results were consistent with previous in vitro studies that beetroot extract increased levels of Bad, TRAILR4, FAS, and p53 proteins in MCF-7 cells (Nowacki, *et al*., 2015). The molecular type of mammary adenocarcinoma that occurs due to DMBA induction is Estrogen Receptor (ER) positive, similar to the characteristics of the MCF-7 cell line (Alvarado, *et al*., 2017).

The results showed that beetroot extract can increase the expression of caspase 3. Caspase 3 is one of the executor proteins in the process of apoptosis. The intrinsic apoptotic pathway involves the activation of caspase 9 while the extrinsic pathway involves caspase 8, both of these pathways will end with the activation of caspase 3 (Boice & Bouchier-Hayes, 2020; D'Arcy, 2019). The high expression of caspase 3 is associated with the survival of breast cancer patients and provides a good prognostic value (Pu, *et al*., 2017).

In this study, there was no control of hormonal factors due to the use of female rats as test animals which have different estrus cycles or hormonal cycles between individuals. Hormonal factors can influence carcinogenesis after DMBA induction or tumor cell response to either beetroot extract or neoadjuvant AC regimen. The response to Neoadjuvant chemoterapy may differ according to the molecular subtype of breast cancer. Breast cancer with ER negative molecular subtype is more sensitive to DNA-damaging chemotherapy such as AC regimen than ER positive type (McDonald, *et al*., 2016; Sharma, 2014). According to previous research, it was known that the molecular type of mammary adenocarcinoma that occurs due to DMBA induction in female Sprague Dawley rats is ER positive (Alvarado, *et al*., 2017). Possibly because of this, AC treatment has no effect in this research. Although not investigated in this study, it may be the molecular type of positive ER cancer that was involved in the effectiveness of the beetroot extract. This was in line with the research conducted before that beetroot extract could have cytotoxic activity on prostate and estrogen receptor-positive breast cancer cell lines and have sinergistic activity with Doxorubicin in MCF-7 cell line (Kapadia, *et al*., 2011; Kapadia, *et al*., 2013). Therefore, its efficacy on other molecular subtypes is not yet known.

CONCLUSION

The addition of beetroot extract to the AC regimen can increase tumor cell apoptosis in mammary adenocarcinoma rats by increasing the expression of p53 and caspase 3. With this synergistic effect, it proves that complementary treatment between natural ingredients and chemotherapy regimens is quite promising. It is hoped that this research can be used as a basis for

clinical research, so that scientific evidence can be obtained that can provide benefits for the treatment of breast cancer patients.

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