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MUCOXIN ENHANCED TRANSCRIPTION AND PROTEIN EXPRESSION OF P53 IN BREAST CANCER CELL LINE T47D

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ABSTRACT

Mucoxin is a type of acetogenin isolated from *Rollinia mucosa* leaves which is known to inhibit cell proliferation and induce apoptosis. However, the mechanism of mucoxin in regulating and eliminating cancer cells was not clear. This study investigated the mucoxin effect on the transcriptional-translational and posttranslational processes of p53 gene in breast cancer cells line T47D. Breast cancer cells line T47D was divided into three groups referred to hours of assays, namely hour 24th, 48th, 72nd, where each group was given mucoxin with six difference doses, namely 0.1 ng/mL, 0.5 ng/mL, 1 ng/mL, 5 ng/mL and 10 ng/mL with three replicates. Transcription of p53 gene was assayed by quantitative PCR (qPCR), whereas the expresssion of p53 protein assayed by immunocytochemistry. Mucoxin enhance p53 gene and protein in all treatment group. p53 gene transcription increased significantly in 48 h, while expression of p53 protein increased significantly in 72 h. Conclusion: Mucoxin increased transcription of p53 gene and expression of p53 protein on cell line T47D.

Keywords: Acetogenin, anticancer, cell line T47D, mucoxin, p53

INTRODUCTION

Breast cancer is one of the most frequent cancer in women [Koss and Melamed, 2006, Sumitha and Devi, 2016] and the second leading cause of death after cervical cancer [Rachmani *et al.*, 2012]. Breast cancer treatment can be done through surgery, chemotherapy, hormone therapy, radiation therapy, and immunological therapy [Kumar *et al*., 2005). Chemotherapy is one of the most common treatments. Unfortunately, chemotherapy has various side effects such as hair loss, nausea, vomiting, sleep disorders, diarrhea, skin redness and weight loss. Moreover, some cancer cells begin resistant to chemotherapy [Yuan *et al.*, 2003].

The development of new anti-cancer drugs is a strategic choice in an effort to improve the sensitivity of pre-existing therapies. One of the promising antitumor compound is acetogenin and its derivative compounds [Betancur *et al*., 1999, Yuan *et al*., 2003, Oberlies *et al*., 1997). One of the commercially type of acetogenin compound is mucoxin. Mucoxin is a non-classical acetogenin compound that was first isolated from the leaf extract of *Rollinia mucosa* by McLaughlin in (1996). This compound has a selective cytotoxic effect against pancreatic cancer cells (PACA-2) and breast cancer cells (MCF-7) (Yang *et al*., 2013). The results of our previous study showed, these compounds are able to induce apoptosis and inhibit the proliferation of breast cancer cell line T47D [Muhartono *et al*., 2016].

However, the molecular mechanisms of mucoxin in inducing apoptosis and inhibiting proliferation has not fully understood. One of the genes that play a role in the apoptosis process and proliferation is the p53 gene encoding the p53 protein. p53 is a tumor suppressor that acts as a transcription factor from genes involved in the cell cycle and apoptotic checkpoint phase in cancer cells. This causes p53 become gene target for new anticancer drugs. This study investigated the mucoxin effect on transcriptional-translational and post translational process of p53 gene in breast cancer cell line T47D. The transcription-translational process of p53 gene assayed by quantitative PCR (qPCR), which is shows the number of amplified gene products. Whereas the posttranslational process assayed by expresssion of p53 protein tested by immunocytochemistry. If mucoxin is proven to enhance p53 genes and protein, so this compound will become very potential to be used as a safe anticancer compounds.

MATERIALS AND METHODS

Material: Mucoxin was obtained from Angene International Limited with ID AG-E-32919 and CAS No. 183195995. Human breast cancer cells line T47D (ATCC HTB133^{TM}) was obtained from American Type Culture Collection (Manassas, VA 20108 USA) with a lot number 61062006.

Design: This study used a randomize block design. Breast cancer cells line T47D were divided into three groups referred to hours of assays; 24th,

48th, 72nd hours, where each group was given mucoxin with six difference doses, namely 0.1 ng/mL; 0.5 ng/mL; 1 ng/mL; 5 ng/mL and 10 ng/mL with three replication of each.

Cell Culture: The cells were grown in Roswell Park Memorial Institue Medium (RPMI1640). The media supplemented with 10% Fetal Bovin Serum (FBS) Gibco™ (Thermo Fisher Scientific Cat. 26140-079) and 0.2 mL bovine insulin (Sigma Aldrich Cat. No. 15500 and CAS RN 11070-73-8) at 37ºC in 5% CO_2.. Thawing process performed in waterbath at 37ºC for 2-4 min. Then, $5x10^4$ cells/cm^2 was taken into T-flask and incubated at 37º C in 5% CO_2. When cells density reached 80% confluent, trypsinization was done using 0.25% trypsin + 0.53 mM EDTA solution and then subcultured in new culture vessels and incubated at 37º C in 5% CO_2. Afer two times passaging, the T47D cells ready to be treated.

Mucoxin Treatment: Mucoxin preparation was made by diluting the powder of mucoxin in 1 mL of 0.1% DMSO. The stock solution then diluted further in accordance with the needs of the six treatment concentration. After subcultured for two times, cells were diluted with RPMI and seeded in 24 well plate with a cell density of $5x10^4$ cell/cm^2 in each well. Once the cell density reach 80% confluent, the cells treated with mucoxin of different concentration as follows: 0 ng/mL (K), 0,1 ng/mL (P1), 0,5 ng/mL (P2), 1 ng/mL (P3), 5 ng/mL (P4) and 10 ng/mL (P5). After being treated, the cells were incubated in accordance with the lenght of hours that have been assigned to each group, i.e. 24, 48 dan 72 h.

Gene Transcription Assays: Expression of p53 gene in T47D cells were determined by quantitative PCR (qPCR) methods using RealMOD™ Green Real Time PCR kit (Intron Biotecnology). RNA was extracted from breast cancer cell line T47D using easy Total RNA Extracion Kit (Intron Biotechnology). Primer for determining p53 expression was *forward primer* 5'–CTGAGGTTGGCTCTGACTGTACCACCATCC–3' and *reverse primer* 5'–CTCATTCAGCTCTCGGAAGCATTTGCGGTGGAC–3'. β-actin gene was used as the internal control (house keeping gene) while control sample was used as the calibrator gene. The qPCR data, analyzed using Light Cycler software from ROCHE.

Protein expression assays: After incubation period, cells were washed using PBS, then fixed in 4% paraformaldehid. The primary anti body used for P53 assay were p53 polyclonal anti body (Rabbit IgG anti p53) from Bioss USA with dilution of 1:100 in PBS with 1% FBS. The secondary anti body used were *Ultratek HRP Anti-Polyvalent* (DAB) from SkyTex Laboratories. The results were visualized using Nikon Coolpix 4500. The expression of P53 protein in each treatment was calculated by summing the number of brownish product arising from the reaction between HRP and DAB in five fields over view and then divided by the number of replicates (n=3).

Statistical Analysis: Comparison of mean values between treatment presented as mean±SD and analyzed using ANOVA followed LSD test with a 95% confidence level.

RESULTS

Effects of Mucoxin on p53 Gene Transcription

Effect of mucoxin treatment on p53 gene transcription are presented in Table 1. Based on that data, it is clear that mucoxin treatment enhanced p53 gene transcription comparing to control in all group ($p<0,05$). Furthermore, the highest transcription was exposed to mucoxin for 48 hours, at a mucoxin dose of 5 ng/mL (P4).

Effect of Mucoxin on p53 Protein Expression

Effect of mucoxin treatmen on p53 protein expression in T47D cells are presented in Table 2. Comparing to control, mucoxin treatment affected p53 protein expression in all groups. The highest mucoxin effect was found in treated groups for 72 hours, with 10 ng/mL (P5) of mucoxin. Based on this data, it can be inferred that mucoxin is able to stabilize and activate the p53 protein by some post translation modification. The inference was supported by highly significant difference ($p<0,001$) between mean values of p53 protein expression.

Table 1. Mean transcription of p53 gene in T47D cells treated with mucoxin

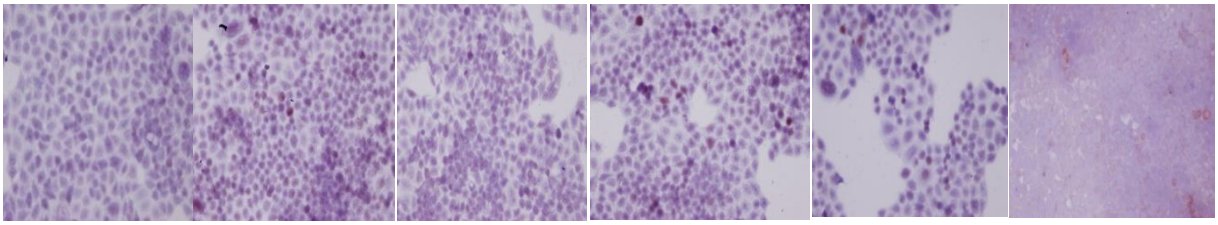
Groups (Hours)	Treatment	Transkription (Mean±SD)	p value
24	K	233,8 ± 7,45[a]	0,0001
	P1	290,2 ± 6,40[b]	
	P2	315,1 ± 9,53[c]	
	P3	374,9 ± 11,38[d]	
	P4	432,6 ± 12,18[e]	
	P5	285,1 ± 14,37[b]	
48	K	278,9 ± 11,10[a]	0,0001
	P1	535,5 ± 14,12[b]	
	P2	863,4 ± 12,94[c]	
	P3	913,7 ± 5,60[d]	
	P4	1045,2 ± 19,58[e]	
	P5	746,1 ± 11,80[f]	
72	K	235,9 ± 10,46[a]	0,0001
	P1	482,9 ± 13,52[b]	
	P2	321,6 ± 5,17[c]	
	P3	443,5 ± 22,76[d]	
	P4	375,8 ± 20,13[e]	
	P5	118,2 ± 15,61[f]	

Mean±SD values in the same hour group followed by the same superscript are not different at α 0,05 by LSD test

Table 2. Mean expression of p53 protein in T47D cell treated with mucoxin

Groups (Hours)	Treatment	Expression (Mean±SD)	p value
24	K	1,7 ± 1,53[a]	0,0001
	P1	8,3 ± 1,53[b]	
	P2	13,0 ± 3,61[c]	
	P3	11,3 ± 3,21[b,c]	
	P4	14,7 ± 3,06[c]	
	P5	14,0 ± 2,00[c]	
48	K	3,7 ± 2,52[a]	0,0001
	P1	13,7 ± 3,06[b]	
	P2	16,0 ± 2,65[b,c]	
	P3	19,3 ± 1,53[c,d]	
	P4	20,3 ± 1,53[d]	
	P5	18,3 ± 2,08[d]	
72	K	4,3 ± 3,06[a]	0,0001
	P1	18,3 ± 2,08[b]	
	P2	34,7 ± 3,51[c]	
	P3	73,0 ± 4,00[d]	
	P4	82,3 ± 6,51[e]	
	P5	114,7 ± 7,09[f]	

Mean±SD values in the same hour group followed by the same superscript are not different at α 0,05 by LSD test



K **P1.24** **P2.24** **P3.24** **P4.24** **P5.24**

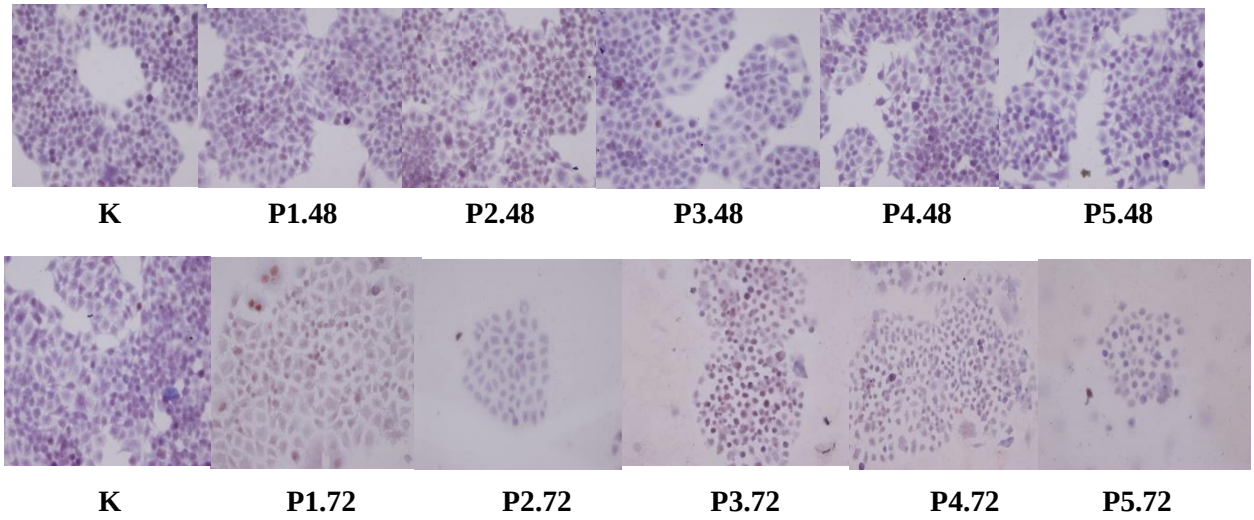


Fig. 1: Immunocytochemistry expression of p53 protein in breast cancer cells treated with mucoxin in different concentration and exposure time

DISCUSSION

The p53 is a tumor suppressor gene that plays an important role in maintaining genomic integrity.This gene is able to mediate cellular responses against cellular damage, through transcriptional regulation of genes involved in cell cycle processes, DNA repair, apoptosis and aging [Adikesavan *et al*., 2014]. Various conditions that can trigger cell damage, such as DNA damage, hypoxia and chemical induction, inducethe p53 activity [An *et al*., 1998]. p53 regulation activity, can be grouped into 2 mechanisms; transcriptional-translational mechanism and post translational mechanism. This study was conducted to determine the effect of mucoxin on both mechanisms.

The results of this study showed that mucoxin administration increased p53 gene transcription in accordance with the dose and duration of treatment (Table 1). These results are consistent with previous studies of several other acetogenin compounds, such as desacetyluvaricin and anonacin, which is showed that the acetogenin compound can increase p53 expression in some cancer cells [He *et al*., 2011, Yuan *et al*., 2003].

Increased transcription of the p53 gene by mucoxin, is thought to occur through several mechanisms. Mucoxin appears to increase or inhibit one or several transcription factors that bind to the promoter portion of the p53 gene sequence, such as HOXA 5, NF–κB, Myc/Max, C/EBPβ and RPB-*Jk* [Meyer and Targa, 2011; Reisman *et al*., 2012]. Mucoxin may also affect the p53's epigenetic mechanism. Mucoxin is thought to prevent methylation of the p53 gene promoter. Several studies have found a correlation between DNA hypermethylation in p53 gene promoters with low mRNAs formed in hepatocellular cancer and leukemia [Hervouet *et al*., 2013, Schoofs *et al*., 2014]. Mucoxin was also suspected to affect the configuration settings of p53 gene chromatin. More open chromatin configuration, it is easier to activate p53 transcription [Nicoll *et al*., 2001].

In line with its transcription, mucoxin was also able to increase p53 protein expression in T47D breast cancer cells (Table 2). These results showed that p53 protein synthesized is active and stable which is characterized by an increase in expression in accordance with the duration of treatment.

P53 protein is a very unstable and has a short half-life, so it does not affect the progress of cell cycle [Sabary *et al*., 2017] Mucoxin treatment seems that p53 protein undergoes posttranslational modification, so this protein is stable. Posttranslational modification of p53, commonly occurs by covalent modification, in which p53 proteins will undergo phosphorylation at various points, resulting in a p53 ubiquitination mission, which results in a decrease in the degradation of this protein [Gu and Zhu, 201_2].

In addition, posttranslational modifications also made p53 proteins being active [Gu and Zhu, 2012]. This occurs through changes in DNA binding activity in specific sequences of p53 gene. p53 is a transcriptional activator of a particular gene based on its ability to bind to the sequence of the gene. Normally, this binding is inhibited by inhibition of C domain terminal. When there is exposure to stress, this inhibition will be eliminated, resulting in increased DNA binding. Consequently, there is an increased activity of the p53

protein [Sakaguchi *et al*., 1999]. In addition to C domainterminals, p53 protein activation capabilities can also be induced by changes in N transactivation domain terminal.

Another mechanism of p53 activation may also occur through changes in the subcellular location of the p53 protein. Normally, latent p53 protein will reside in the cytoplasmic, when exposure to stress, p53 protein will be accumulated in the cell nucleus [Shaulsky *et al*., 1990]. As a result there is accumulation of this protein in cells, proven by higher expression of this protein along with the increase of observation time.

The p53 gene encodes the p53 protein which is a tumor suppressor. As a tumor suppressor, p53 plays a very important role in preventing excessive cell proliferation and maintaining genomic integrity [Yang *et al*., 2013]. p53 will be activated in response to the presence of stress signals originating inside or outside the cell. The presence of a stress signal, will induce various upstream mediators, such as 14ARF and Mdm2, make p53 stable and active [Christopher *et al*., 2006]. In this study, stress derived from mucoxin with various doses postulated would be a future chemotherapy agent to overcome current chemotherapy drug, which is not resistance or giving minimal side effects. The activated p53 protein will act as a regulatory protein that triggers a variety of major biological responses to cell proliferation and the process of cell apoptosis [Mollereau and Ma, 2014].

Based on the fact that mucoxin significantly increased transcription and expression of protein p53, where is the p53 are the main factor in the apoptosis dan proliferation of cancer cells, it can be concluded that mucoxin can be an aternative therapy for breast cancer.

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