Jurnal Kedokteran MEDITEK ISSN e : 2686 - 0201 p : 2686 - 1437
Artikel Penelitian
http://ejournal.ukrida.ac.id/ojs/index.php/Meditek/index

Expression of Nuclear Factor – kappa B (NF-kB) in Human Breast Cancer Stem Cells (CD 24-/CD 44+) Treated with H_2O_2 and Its Relationship with Cell Viability

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Abstract
Introduction: Breast cancer is one of the highest causes of death from cancer in women in Indonesia. This is partly due to the resistance of ROS-based therapies such as radiotherapy and chemotherapy. Breast cancer stem cells (cancer stem cells, CSCs) have a role in this resistance mechanism. Previous studies demonstrated the ability of CSC to survive oxidative stress conditions due to rotenone administration. Therefore, in this study an analysis was carried out on the transcription factor NF-kB in breast cancer cells, both CSCs and Non CSCs, related to the role of NF-kB in maintaining the survival of cancer cells under conditions of oxidative stress. Methods: The study was conducted on human breast cancer stem cells (CD24-/CD44+) and non stem cells (CD24-/CD44-) which were given H_2O_2 at concentrations of 1.1µM, 11µM, and 110µM with control cells not given H_2O_2. Assessment was carried out on the parameters of NF-kB mRNA expression, and cell viability. Results: Administration of H_2O_2 at a concentration of 11µM showed a significant increase in the expression of NFk-B CSCs mRNA compared to non CSCs ($p<0.05$). As for the viability test results, at all concentrations of H_2O_2 it appears that CSCs was able to maintain its viability compared to non CSCs which experienced a decrease in viability ($p<0.05$). Conclusion: In this study, conditions of oxidative stress due to the administration of H_2O_2 led to an increase in the expression of NF-kB mRNA in CSCs so that cell viability could be maintained.

Keywords: antioxidant, cancer stem cells, H_2O_2, NF-kB, oxidative stress

Analisis Ekspresi *Nuclear Factor kappa β (NF-kB)* Pada Sel Punca Kanker Payudara Manusia (CD 24-/CD 44+) yang Diberi H_2O_2 dan Hubungannya dengan Viabilitas Sel

Abstrak
Latar Belakang: Kanker payudara merupakan salah satu penyebab kematian tertinggi akibat kanker pada wanita di Indonesia. Hal ini diantaranya disebabkan karena adanya resistensi terhadap terapi berlandaskan ROS seperti pada radioterapi maupun kemoterapi. Sel punca kanker payudara (*cancer stem cells*, CSCs) memiliki peran pada mekanisme resistensi ini. Penelitian terhadulu menunjukkan kemampuan CSCs untuk bertahan terhadap kondisi stress oksidatif pada pemberian rotenon. Karena itu, dalam penelitian ini dilakukan analisis terhadap faktor transkripsi NF-kB pada sel kanker payudara baik CSC maupun non CSC, terkait peran NF-kB dalam mempertahankan viabilitas sel kanker pada kondisi stress oksidatif. Metode: Penelitian dilakukan pada sel punca kanker payudara manusia (CD24-/CD44+) maupun non sel punca (CD24-/CD44-) yang diberi H_2O_2 dengan konsentrasi 1.1µM, 11µM, dan 110µM dengan kontrol sel yang tidak diberi H_2O_2. Penilaian dilakukan terhadap parameter ekspresi mRNA NF-kB, dan viabilitas sel. Uji statistik dilakukan menggunakan IBM-SPSS dengan nilai $\alpha < 0.05$. Hasil: Pemberian H_2O_2 pada konsentrasi 11µM menunjukkan peningkatan yang signifikan pada ekspresi mRNA NFkB CSCs dibanding non CSCs ($p<0.05$). Sedangkan untuk hasil uji viabilitas pada seluruh konsentrasi H_2O_2 nampak bahwa CSCs mampu mempertahankan viabilitasnya dibandingkan dengan non CSCs yang mengalami penurunan viabilitas ($p<0.05$).. Kesimpulan:

How to Cite :
Kurniawan, H., Wanandi , S. I., Jusman , S. W. A. Expression of Nuclear Factor – kappa B (NF-kB) in Human Breast Cancer Stem Cells (CD 24-/CD 44+) Treated with H2O2 and Its Relationship with Cell Viability . J Kdokt Meditek, 2023: 29(2), 144–149. Available from: http://ejournal.ukrida.ac.id/ojs/index.php/Meditek/article/view/2668/version/2679 DOI: https://doi.org/10.36452/jkdoktmeditek.v29i2.2668

Kondisi stres oksidatif akibat pemberiaan H_2O_2 dapat meningkatkan ekspresi mRNA NF-kB pada CSCs sehingga viabilitasnya tetap dapat dipertahankan.

Kata kunci: antioksidan, H_2O_2, NF-kB, sel punca kanker, stres oksidatif

Introduction

Breast cancer is a type of cancer that starts in the breast and occuring almost entirely in women, although cases in men were observed.[1] Based on data from GLOBOCAN, International Agency for Research on Cancer (IARC) 2020, breast cancer happened in the highest frequency in women with 2.2 million new cases in 2020 (24.5% of all cancers). The same thing also happens in Indonesia, breast cancer is still the highest cause of death among other cancers.[2] The standard of care for breast cancer treatment includes surgical removal of the tumor and adjuvant therapies that include local irradiation and systemic therapies, such as biological agents, hormonal therapies, and chemotherapy.[3] The approach of oxidative stress in breast cancer related to treatment resistance with chemotherapy and radiation will be discussed in the present study.

Oxidative stress is a condition where there is an excessive increase in ROS (reactive oxygen species) which cannot be controlled by the antioxidant system and can damage various important components in cells such as proteins, carbohydrates, lipids, and DNA (deoxyribo nucleic acid) resulting in various effects like aging and cancer. Oxygen (O_2) has a tendency to form toxic ROS, such as superoxide anion ($O_2\cdot-$), and hydroxyl radicals (OH·), which have an unpaired electron. H_2O_2 in cells is produced from $O_2\cdot-$ which is catalyzed by antioxidant enzymes. The balance of redox homeostasis in the cell is maintained by an antioxidant system that is influenced by the levels of ROS in the cell.[4] Low levels of ROS have physiological functions in activating or modulating signal transduction pathways, modulating the activity of redox-sensitive transcription factors and functioning in the regulation of antioxidant enzyme activity.[5] whereas at high levels, ROS are toxic to cells and can trigger cell death.[6] Previous studies have shown that low levels of ROS are required for stem cells to maintain their characteristics. One of the mechanisms thought to have stem cells to maintain lower ROS levels is an increase in the expression of genes involved in the defense system against ROS such as anti-oxidant enzymes.[7] Addition of H_2O_2 in this study aims to create a state of oxidative stress, as many studies.[8]

Cancer Stem Cells (CSCs) are a small subpopulation of cells within tumors with capabilities of self-renewal, perform asymmetric division and tumorigenicity.[9] A number of cell surface markers such as CD44, CD24, and CD133 are often used to identify and enrich CSCs.[10] Cancer Stem Cells have the ability to divide asymmetrically producing cancer stem cells progeny to self-renew and to give rise to differentiated tumor cells. Therefore, CSCs are responsible for the overall organization of a tumor.[11]

The microenvironment of CSCs divided into four functional components, the stem cell niche, cancer stroma, immune cells, and vascular endothelial cells. Niche is the environmental conditions which are suitable for CSCs to maintain their properties, so they can live longer and their stemness can be maintained. Niche is also important for maintaining the plasticity character of CSCs in order to activate signaling pathways that promote survival and self-renewal.[12] CSCs reside in separate microenvironments (niches) that are different from ordinary cancer microenvironments or non CSCs.[13]

The transcription factor NF-κB plays a major role in coordinating innate and adaptative immunity, cellular proliferation, apoptosis and development.[14] dysregulations in the NF-κB activation cascade have been associated with the pathogenesis of several diseases such as cancer.[14] NF-kB plays an important role in regulating and controlling the cell viability in oxidative stress condition.[15] NF-kB inhibits programmed cell death by stimulating the transcription of anti apoptotic genes. Tumors with NF-kB activity usually show increased chemotherapy resistance.[16]

Therefore, this research was carried out to understand the regulation of NF-kB expression in CSCs and its association with the ability to survive under oxidative stress conditions.

Methodology

Materials

Tripure Isolation Kit®, Aquadest, Aquabidest, Chloroform, Isopropanol, Ethanol 75%, Ethanol 100%, 0.1M sodium sitrat, 0.3M Guanidine HCl in ethanol 95%, Diethyl

pyrocarbonate (DEPC)-treated-RNase-free water, SDS 1%, , Phenylmethylsulfonyl fluoride (Pmsf) 1M – inhibitor serin protease, H_2O_2, Phosphate Buffer Saline (PBS) 0,1 M pH 7, Na_2HPO_4, KH_2PO_4, NaCl, Bovine Serum Albumin (BSA), One-Step RT-PCR Kit with SYBR KAPA (KAPA BIOSYSTEMS,Universal, Boston-USA). Primer gen NF-kB Forward and Reverse (PT. Genetika Science, Indonesia), primer gen 18 sRNA Forward and Reverse (PT. Genetika Science, Indonesia). Kit MTS assay Promega®. Medium kultur (DMEM) plain 96 well multiplate.

Breast CSCs ($CD24^-$/ $CD44^+$) and breast non CSCs ($CD24^-$/$CD44^-$) were cultured and treated with 1.1 μM, 11 μM, and 110 μM H_2O_2

Total RNA Isolation with Tripure Isolation Kit®

Total RNA isolation from breast CSCs ($CD24^-$/ $CD44^+$) and breast non CSCs ($CD24^-$/$CD44^-$) were performed using Tripure Isolation Kit® (Roche, Indianapolis). Isolation procedure was done following protocol in the kit. Total RNA concentration was measured with a Varioskan Flash spectrofotometer ®.

Measurement of Cell Viability using MTS Assay Promega®

Determination of viability of breast CSCs ($CD24^-$/ $CD44^+$) and breast non CSCs ($CD24^-$/$CD44^-$) was performed using the Promega® MTS assay kit. Cell viability was measured to see the comparison between before and after treatment with H_2O_2. The kit consists of a solution of MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] and PMS (phenazine methosulfate) which is an electron coupling reagent. MTS is added to a cell culture that has been previously added with PMS (MTS: PMS = 20: 1), then incubated for 1 hour at 37ºC, 5% CO_2, causing MTS to be reduced by the dehydrogenase enzyme to a formazan product that is soluble in the culture medium. The dehydrogenase enzyme is only found in living cells with high metabolic activity. The formazan product, which is an indication of the MTS reduction process, is purple in color and its absorbance can be measured at 490 nm with a multi-plate reader. The amount of formazan product measured through absorbance 490 nm is proportional to the number of live cells in culture.

Analysis of NF-kB gene expression using real time RT-PCR

RNA isolation method, MTS assay and RT-PCR were performed according to the manufacturer's protocol. Analysis of the level of expression of NF-kB was conducted in breast cancer cells CD24-/CD44+ and CD24-/CD44-. The total RNA that was isolated with the Tripure Isolation Kit® (Roche) was then amplified using the Real Time PCR tool (CFX Manager 3.0, BioRad). The 18s rRNA primer used was 5'-AAC GGC TAC CAC ATC CAA G -3' for forward and 5'-CCT CCA ATG GAT CCT CGT TA -3' for reverse, (design and optimization by Hardiany 2013). For NF-kB primer, the method used is 5'-CGC TTA GGA GGG AGA GCC CA -3' for forward and 5'-TGG GCC ATC TGT TGG CAG TG -3' for reverse, (design and optimization by Arleni 2012). The 18S rRNA gene as the reference gene was treated under the same conditions as the NF-kB gene. As a negative control (NTC: non template control) aquabides (nuclease free water) was used instead of RNA to exclude false positive results due to contamination. The 18S rRNA reference gene was used as a normal, to analyze NF-kB mRNA expression. After measurement of Ct values, the expression level of the NF-kB gene in the test sample (cells with or without H_2O_2 treatment) relative to the caliber sample (untreated control cells) could be determined. Relative quantification or relative concentration of mRNA can be determined by the Livak method. Normalization of Ct values of breast CSCs test samples (CD24-/CD44+) or non CSCs breasts (CD24-/CD44-) treated with H_2O_2 against untreated Ct cells. The result of the calculation using the Livak method is the relative ratio of NF-kB gene expression in normalized samples of CD24-/CD44+ and CD24-/CD44- to untreated cells.

Statistic Analysis

Statistical analysis was conducted using a non-parametric test Mann-Whitney. The analysis was carried out at the 95% confidence level ($\alpha<0.05$). The data analyzed using SPSS was the result of 3 repetitions which were compared between concentrations.

Results

Expression of NF-kB mRNA with Real Time RT-PCR

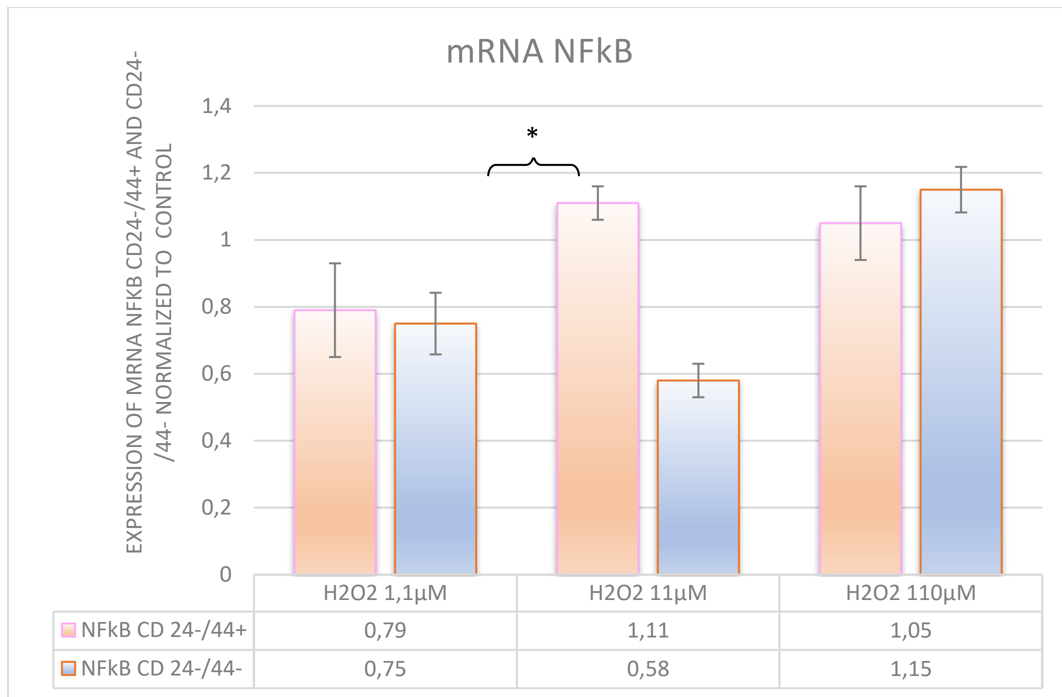

Figure 1. Comparison of CSCs and non CSCs NFkB mRNA expression(* p< 0,05)

Administration of H_2O_2 to CSCs resulted in an increase in the relative expression of NF-kB mRNA with a significant increase occurring at a concentration of 11µM. Interestingly administration of 11µM H_2O_2 to non CSCs resulted in a significant decrease in the relative expression of NF-kB mRNA. Contrary to this result, the high concentration of H_2O_2 (110 µM) in non CSCs resulted in a compensatory increase in the relative expression of NF-kB that was significantly above normal.

Analysis of the Effects of H_2O_2 on Cell Viability with MTS Assay

Viability levels of breast CSCs (CD24-/CD44+) and non CSCs breast (CD24-/CD44-) cells can be seen below

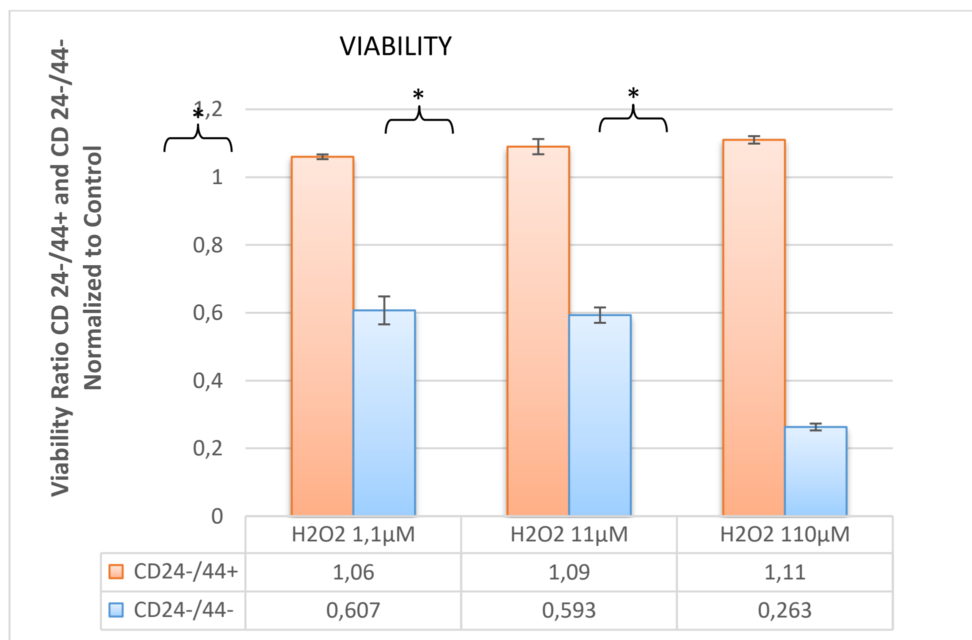

Figure 2. Comparison of CSCs and non CSCs Viability (* p< 0,05)

CSCs seems to be able to maintain its viability in the administration of H_2O_2 up to a concentration of 110 µM. While non CSCs experienced a decrease in viability starting from the administration of H_2O_2 concentration of 1.1 µM and decreased significantly when given a high concentration of H_2O_2 (110 µM).

Discussion

In this study, treatment of H_2O_2 to breast CSCs and non breast CSCs aimed to induce oxidative stress. Peroxide is one of the ROS produced by cells that has a specific function in signaling pathways. In general, low concentrations of H_2O_2 in cells act as signaling molecules that promote cell proliferation and cell viability, on the other hand, very high concentrations can induce cell death. Cell viability is regulated by NF-kB by inducing the expression of antioxidant enzymes, whereas oxidants affect the activity of NF-kB as a transcription factor.[17]

H_2O_2 administration of 11 µM and 110 µM increased the NF-kB mRNA expression, which seemed to correlate with the ability of CSCs to maintain its viability. The difference in response appeared in non CSCs where NF-kB mRNA expression surprisingly decreased at a concentration of 11 µM H_2O_2 and then compensated with a significant increase at a concentration of 110 µM. Changes in non CSCs NF-kB mRNA expression did not seem to affect the survival of non CSCs cancer cells because decreased NF-kB mRNA expression in the administration of 11 µM H_2O_2 did not cause a decrease in viability. On the contrary, a significant increase in NF-kB mRNA expression in the administration of 110 µM H_2O_2 indicated a significant decrease in cell viability. The difference in response from CSCs and non CSCs may be due to the different types of these two cells so that the NF-kB signaling pathway shows a different response.

In this study, increasing the concentration of H_2O_2 to 110 µM in non CSCs resulted in the inability of cells to maintain their viability. A study by Guangxian found that ROS levels were higher in non CSCs breast compared to breast CSCs when induced by HO^-.[18] Hence, non CSCs cancer cells can undergo apoptosis due to increased oxidative stress in their microenvironment. Clinically this can be seen from the reduction in tumor size after chemoradiation therapy. In contrast, CSCs were able to maintain its viability under conditions of high oxidative stress due to the administration of H_2O_2 up to a concentration of 110 µM. Clinically, cancer stem cells/CSCs are proven to be resistant to conventional chemoradiation therapy. Chemoresistance and radioresistance are major problems in cancer treatment, because cancer cells become resistant to the chemicals used in treatment.[19] Therefore, many cancer treatment now uses target cells (cancer stem cells) as an effective method to reduce recurrence.[20]

CSC and non-CSC have different levels of differentiation and microenvironment. This may lead to different responses to the presence of oxidants/oxidative stress and their effect on viability. In conventional breast cancer treatment with chemotherapy which causes oxidative stress, generally non-CSC cancer cells will undergo apoptosis, but CSC with its properties can survive or be resistant to oxidative stress conditions. This is what causes recurrence in most therapies based on oxidative stress such as chemoradiation therapy.

Conclusion

Conditions of oxidative stress due to the addition of H_2O_2 can increase the expression of NF-kB mRNA in CSCs which correlates with the ability of cells to maintain their viability. The role of the NF-kB signaling pathway under conditions of oxidative stress in non CSCs shows a different response that does not appear to be related to the ability of cells to maintain their viability. Further studies regarding the unique role of the NF-kB signaling pathway in different microenvironmental conditions (niches) might explain the causes of these differences in response.

Future studies may be directed to determine the role of other factors affecting cell viability, such as the role of anti-apoptotic proteins in CSCs and non CSCs. To find out more about the role of NF-kB in reducing the effects of oxidative stress, an analysis on the expression of antioxidant proteins in the NF-kB signaling pathway can be performed.

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