Suppression of MnSOD by Andrographolide and its Relation to Oxidative Stress and Viability of Breast Cancer Stem Cells Treated with Repeated Doxorubicin Administration

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Abstract: Cancer stem cells (CSCs) are chemoresistance and could be a preferential target for reversing resistance. Doxorubicin is one of the most effective chemotherapy agents, but resistance still occurs. Doxorubicin generates reactive oxygen species (ROS). The increased specific activity of MnSOD has been suggested as one possible mechanism for breast cancer stem cells (BCSCs) to maintain stemness and survival. The study performed to determine the effect of andrographolide on oxidative status and its relation to the viability of BCSCs given repeated doxorubicin. CD24-/CD44+ BCSCs were grown in DMEM/F12 medium with 0,1µM doxorubicin for 14 days, then treated with a combination of 0,1µM doxorubicin and 0,285 mM andrographolide until day 22. MnSOD activity was suppressed on day 4 when BCSCs were still sensitive to doxorubicin treatment. Viability significantly increased on day 20 along with increased MnSOD activity. Andrographolide restored the sensitivity of BCSCs to doxorubicin, which correlated with MnSOD activity but not catalase. There was no change in MDA levels in all days of treatment which means BCSCs can maintain oxidative stress level. Andrographolide supplementation can decrease MnSOD activity but not catalase and is closely related to decreased cell viability with low sensitivity to doxorubicin.

1 INTRODUCTION

Breast cancer is the most frequently identified cancer and the leading cause of death from cancer in women worldwide. (Bray et al., 2018) There are several treatment options to cure breast cancer, including mastectomy, chemotherapy, hormone therapy, radiotherapy, and other therapies. (Sterba et al., 2013) An anthracycline, doxorubicin, is considered one of the most effective and most used chemotherapy agents to treat breast cancer and several other cancer types. However, resistance to chemotherapy still occurs both intrinsically and resistance that develops during treatment. (Austreid et al., 2014) One of the causes of doxorubicin resistance is the reduced amount of the accumulated drug in the nucleus resulting in a decrease in DNA damage. Failure to accumulate drugs is caused by active efflux through the ATP binding cassette (ABC) transporter family. The upregulation of this transporter is related to drug resistance. (Shiraga et al., 2001)

Cancer stem cells (CSCs) represent a subpopulation of cancer cells with close characteristics to normal stem cells, namely pluripotency, self-renewal ability, and differentiation.

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CSCs can originate from normal cells/progenitors with mutations or environmental changes and can also come from normal somatic cells that experience genetic changes. High CSCs indicate a poor prognosis because cancer stem cells have a slow rate of division, better DNA repairability, and a lower ability to experience apoptosis than cancer cells in general. The causes of cancer stem cells to be more resistant to cancer therapies such as chemotherapy and radiation have been proven in vitro studies using CD24-/CD44+ cancer stem cells. (Phillips et al., 2006)he

Oxidative stress is a phenomenon that appears when an imbalance occurs between reactive oxygen species (ROS) formation and the capability of cells to detoxify them. The lipid peroxidation is one of the results of oxidative stress, which will lead to malondialdehyde (MDA) formation. MDA is one of the most reliable markers to determine oxidative stress. (Giera et al., 2012) Oxidative stress is remarkably known to play an important role in damaging lipids, protein, and DNA molecule, alter signalling pathways and impact cancer progressions. (Lee et al., 2017) ROS also has a role in stem cell renewal and differentiation. Breast cancer stem cells can maintain lower ROS levels than differentiated cells by increasing antioxidant capacity. The condition can be an obstacle for treatments that use oxidative stress like chemotherapy and radiotherapy. (Gorrini et al., 2013) The conditions doxorubicin was known to generate ROS, although the other mechanisms, namely through intercalation of DNA and poisoning topoisomerase II, constitute their cytotoxic actions. (Lazo et al., 1998)

Previous studies in our laboratory showed that doxorubicin could reduce the viability of CD24-/CD44+ breast cancer stem cells (BCSC) at initial exposure of doxorubicin MnSOD levels were maintained, thus high ROS levels. After more extended doxorubicin administration, the viability of BCSCs increased, and the MnSOD activity was significantly high, which caused the ROS levels to decline. Breast cancer stem cells CD24-/CD44+ maintain survival by increasing the activity of the MnSOD. (Syahrani et al., 2019)

Andrographolide (ANDRO) is a diterpene lactone derived from the extract of the plant bitter (*Andrographis paniculata*), and it has long been known for its antioxidant (Xu et a;., 2019), anticancer (Siripong et al., 1992), immunostimulatory (Puri et al., 1993), anti-inflammatory (Abu-Ghefreh et al., 2009), and anti-viral (Manjula et al., 2018) activities. Andrographolide has demonstrated in promoting apoptosis in BCSCs by inhibition of anti-apoptotic protein survivin. (Yunita et al., 2017) Initially, andrographolide was reported to be a ROS scavenger (Xu et al., 2019), but natural compounds may demonstrate both antioxidant and prooxidant characteristics that depend on the concentrations and exposure. (Sznarkowska et al., 2017) In a parallel study, it showed that the administration of andrographolide could cause cell death in BCSCs. The study performed to determine the possibility of andrographolide re-sensitising doxorubicin resistance due to increased MnSOD activity.

2 MATERIALS AND METHODS

2.1 Cell culture

Breast cancer stem cells CD24-/CD44+ were obtained from the Department of Biochemistry and Molecular Biology, Faculty of Medicine Universitas Indonesia and grown in serum-free Dulbecco's Modified Eagle Medium / Nutrient Mixture F-12 (DMEM/F-12 medium) (Gibco, Thermo Fisher Scientific, Inc. Waltham, MA, USA) supplemented with 1% penicillin-streptomycin (Gibco, Thermo Fisher Scientific, Inc. Waltham, MA, USA) and amphotericin B (Gibco, Thermo Fisher Scientific, Inc. Waltham, MA, USA). BCSCs were maintained in 5% CO2 at 37°C. Cells seeded at 1 x 10^5 cells/well in 12 well plates.

2.2 Doxorubicin and Andrographolide Treatment

Doxorubicin (Sigma-Aldrich, St. Louis, Missouri, USA) was prepared in serum-free DMEM/F12 medium at a final concentration of 0,1µM. Andrographolide prepared in DMSO (Sigma-Aldrich, St. Louis, Missouri, USA) and diluted in serum-free DMEM/F-12 medium at a final concentration of 0,285 mM. Cells were treated with 0,1 µM doxorubicin every two days. After 14 days of treatment, the cells were treated with a combination of doxorubicin and andrographolide until day 22. Counting Cells used by Trypan blue exclusion assay. Cell viability was determined by dividing the number of living cells of the treatment group to the number of living cells of the control group.s

2.3 Protein Isolation

According to the manufacturer's protocol, RIPA buffer (Thermo Fisher Scientific, Illinois, USA) was

used to isolate protein from BCSC CD24-/CD44+. The supernatant containing protein stored in a new tube at -80°C.

2.4 MnSOD Activity

MnSOD activity of protein samples was determined using the RANSOD kit (Randox Lab, Crumlin, UK). Protein samples used for the assay of SOD. To inhibit Cu / ZnSOD, 5mM sodium cyanide was added. Absorbance measured at wavelengths of 505 nm on a spectrophotometer for 30 seconds. Preparation of percentage inhibition versus standard/protein sample assay and standard inhibition curve following the recommended kit manual. The SOD activity values for protein samples were read based on a curve divided by the protein concentration.

2.5 Catalase Activity

50 µL of standard/sample were added into a tube containing 950 µL of H_2O_2. The homogenised mixture read by absorbance at 210 nm. The absorbance observations were carried out at the first 30 seconds (00:30) and two minutes after that (02:30) using a stopwatch. The catalase activity was measured using the catalase standard curve made in several dilutions, divided by the protein concentration.

2.6 MDA Levels

The concentration of MDA (malondialdehyde) formed from lipid peroxidation was measured using the Wills method. The principle is that MDA will react with thiobarbituric acid (TBA) at a temperature of 90°C-100°C in an acidic atmosphere to form a pink compound that provides maximum absorbance at a wavelength of 530 nm. The concentration is calculated based on the calculations obtained from the linear regression of the MDA standard curve. The MDA concentration divided by the protein concentration.

2.7 Statistical Analysis

The data were analysed with IBM SPSS Statistics 22. The average probability analysed with a Shapiro-Wilk test. The data performed as a mean value ± standard deviation (SD). Statistical evaluation of the significant differences performed using variance (ANOVA); the LSD/Tukey test used for multiple comparisons. The significance level was at $p<0.05$.

3 RESULTS

3.1 Measurement of MnSOD Specific Activity

The activity of antioxidant enzyme MnSOD in doxorubicin treated BCSCs and a combination of doxorubicin and andrographolide treatment after day 14 to day 22 were assayed. As shown in Figure 1, BCSCs' MnSOD activity significantly decreased compared to control with no treatment on day 4 ($p < 0.05$). There was no statistical difference between the MnSOD activity of doxorubicin treated BCSCs compared to the control group on day 14. Interestingly, there was a significant increase of MnSOD activity on day 20 of doxorubicin-induced BCSCs compared to control on the same day ($p < 0.05$) and also compared to day 14 of doxorubicin-induced BCSCs ($p < 0.05$). Andrographolide was given on the 14th day and reduced the MnSOD activity of doxorubicin-induced BCSCs significantly ($p < 0.01$).

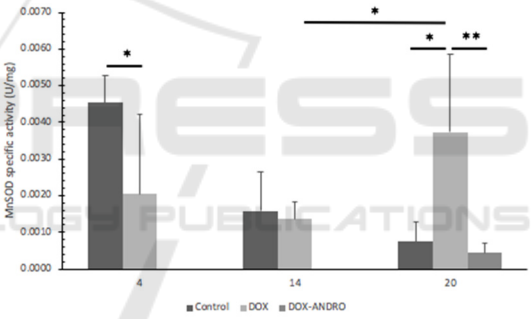

Figure 1: Effect of doxorubicin and doxorubicin-andrographolide on BCSCs MnSOD specific activity at specified times. MnSOD activity was determined using the RANSOD kit (Randox Lab, Crumlin, UK). Statistical analysis conducted as described in Materials and Methods. Significant differences from control values (*, $p < 0.05$) (**, $p < 0.01$) are indicated by the asterisks.

3.2 Measurement of Catalase Specific Activity

Catalase specific activity was measured based on a standard H_2O_2 decomposition curve by measuring the absorbance of H_2O_2 with a blank at a concentration of 1: 1000; 1: 2000; 1: 4000; 1: 8000; 1: 16000; 1: 32000 and 1: 64000. The absorbance observations at 210 nm were carried out at the first 30 seconds (00:30) and two minutes after that (02:30) using a stopwatch. Catalase activity compared in doxorubicin treated BCSCs, doxorubicin-andrographolide treated BCSCs and no treatment control. There was no difference

between the catalase activity of doxorubicin-induced and control BCSCs on day 4 and day 14. There was a significant decline of doxorubicin-induced BCSCs on day 20 compared to a control of the same day ($p < 0.05$). No significant differences observed between the catalase activity of andrographolide administration on doxorubicin-induced BCSCs' and doxorubicin-induced BCSCs.

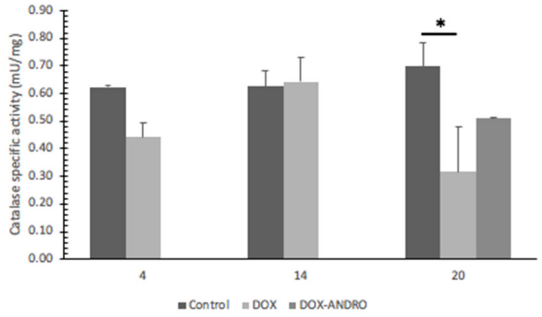

Figure 2: Effect of doxorubicin and doxorubicin-andrographolide on BCSCs catalase specific activity at specified times. Catalase activity was determined using the catalase standard curve. Statistical analysis conducted as described in Materials and Methods. Significant differences from control values (*, $p < 0.05$) (**, $p < 0.01$) are indicated by the asterisks.

3.3 MDA Levels

Oxidative stress is one of the factors that contribute to the cytotoxicity of doxorubicin. The measurement of the peroxidation product levels – malondialdehyde (MDA) was used to calculate the lipid peroxidation status in BCSCs. Lipid peroxidation was assayed by the thiobarbituric acid method. As shown in Figure 3, the MDA levels of BCSCs exposed to doxorubicin show no difference compared to control. Andrographolide administration showed a trend of increased MDA levels on doxorubicin-induced BCSCs, but the proportion was not significant.

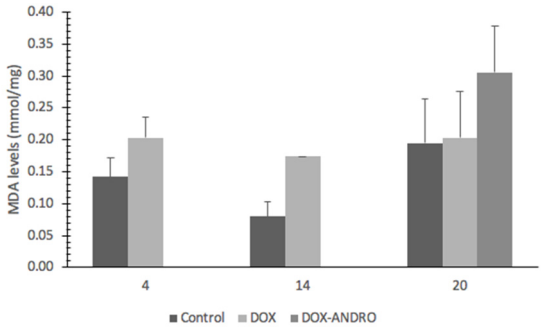

Figure 3: Effect of doxorubicin and doxorubicin-andrographolide on MDA levels at specified times. Statistical analysis conducted as described in Materials and Methods. Significant differences from control values (*, $p < 0.05$) (**, $p < 0.01$) are indicated by the asterisks.

4 DISCUSSION

Doxorubicin is one of the most effective and most used chemotherapy agents to treat breast cancer and several other cancer types. One of how doxorubicin causes cell death is through the mechanism of ROS formation, increasing oxidative stress. Increasing free radicals will cause oxidative stress. (Xu et al., 2005) Syahrani et al. demonstrated that breast cancer stem cells' sensitivity to doxorubicin decreased when given long-term exposure. Breast cancer stem cells (BCSCs) can maintain stemness and survival through increased antioxidant enzyme MnSOD activity. (Syahrani et al., 2019)

In a parallel study at the same laboratory, doxorubicin reduced CD24-/CD44+ breast cancer stem cells' viability until day ten. The viability increased on the 14th day, so it indicated the time when the BCSCs sensitivity decreases. The BCSCs in this study could not be declared resistant in a study conducted by Lukyanova et al., a doxorubicin-resistant variant of breast cancer cells was obtained by growing cells medium containing doxorubicin with stratified concentrations over a more extended period. (Lukyanova et al., 2009) The viability of CD24-/CD44+ BCSCs continued to increase up to 74.19% on the 20th day. The administration of andrographolide carried out every other day on day 14 until day 18 caused a decrease in the viability of breast cancer stem cells exposed to doxorubicin. This result indicates that andrographolide inhibits the growth of BCSCs that had reduced sensitivity to doxorubicin.

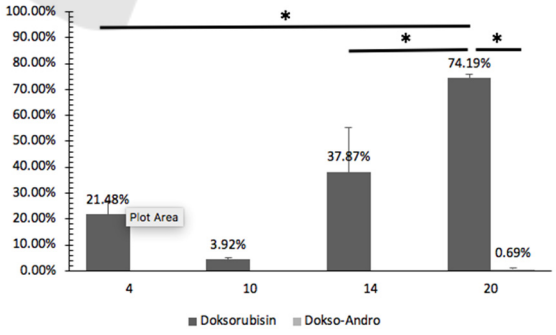

Figure 4: Effects of androgapholide on the viability of doxorubicin-induced BCSCs. BCSCs were treated with 0,1 µM doxorubicin until day 14 then, doxorubicin treated BCSCS were supplemented with 0,285 mM andrographolide.

CSCs from some types of tumours had lower ROS levels than differentiated cells. Its indicated that CSCs could maintain a low ROS level, which might help them protect themselves from damages caused by ROS. This low level of ROS was partly caused by an increase in the production of ROS scavenger enzymes. (Diehn et al., 2009) Therefore, we analysed the activity of the antioxidant enzyme. MnSOD was the most potent antioxidant enzyme because it acts as the first detoxification enzyme needed to protect cells from ROS's toxicity produced by metabolism. This enzyme catalysed the dismutation of potentially dangerous superoxide anions into hydrogen peroxide and molecular oxygen. (Oberley, 2005).

Increased expression of MnSOD protein could lead to resistance to therapy. The mechanism by which MnSOD protects cells from oxidative damages was thought by maintaining mitochondrial function and preventing the reduction in ATP synthesis caused by oxidants. (Suresh et al., 2003) In the MnSOD activity analysis using the RanSOD kit, the results of MnSOD activity from CD24-/CD44+ BCSCs exposed to doxorubicin on day four significantly lower compared to the control without treatment. It is caused by the changes in the balance of oxidative stress levels, which is one of the doxorubicin's mechanisms of action. The imbalance between high levels of oxidative stress and antioxidants could cause mutations in DNA and damage to genes that produce antioxidant proteins. (Bagchi et al., 1998) The result was that BCSCs lose their ability to fight free radicals due to disruption in antioxidant proteins' production. Also, MnSOD activity has been widely used to combat the high amount of ROS, so that the measured activity was reduced. On day 14, the MnSOD activity of CD24-/CD44+ BCSCs exposed to doxorubicin showed no significant difference compared to control. On day 20, the MnSOD activity of doxorubicin-induced BCSCs was significantly higher than the control and the doxorubicin-induced BCSCs on day 4. This research aims to clarify the role of MnSOD in causing resistance to doxorubicin in BCSCs by supplementation of androgapholide. The suppression of MnSOD by andrographolide affected the viability, sensitising BCSCs to doxorubicin treatment. Another important finding is that the MDA level after andrographolide exposure showed a higher trend compared to doxorubicin alone, although not significant, the result suggested that there was an increase in oxidative stress. We suggested that andrographolide could reduce MnSOD activity and revive the oxidative stress induced by doxorubicin, so that viability decreases.

Catalase gives protection against the deleterious effect against H_2O_2 and decomposes them to oxygen and water. Catalase is located in the peroxisomes. (Kirkman et al., 2007) On day 4 and 14, BCSCs was sensitised to doxorubicin treatment, and the catalase level was no different compared to the control without treatment. Catalase is known to have an essential role in developing tolerance to oxidative stress in cells, especially when there is limited glutathione and decreased GPx activity (Wassmann et al, 2004) or increasing H_2O_2 levels. (Yamada et al., 1991) On day 20, the catalase activity of doxorubicin-induced BCSCS was decreased significantly compared to day 14 doxorubicin-induced BCSCS and control of the same day, but the MDA level remains stable. The finding of lower catalase activity in this day might be explained with the regulation of catalase expression. The catalase core promoter is highly conserved, allowing efficient binding of transcription factors to the DNA binding sites and leading to the positive or negative regulation of catalase expression. (Nenoi et al., 2001; Glorieux et al., 2015) Epigenetic changes have been shown to regulate catalase expression in acute myelogenous leukaemia cells resistant to doxorubicin, reducing catalase protein levels compared to the parental cell lines. (Lee et al., 2012) Besides, catalase expression is also regulated at the RNA level and post-translational modifications, causing decreased catalase activity. (Glorieux et al., 2015) There was no statistical difference in the catalase activity between doxorubicin-andrographolide induced BCSCs and doxorubicin-induced BCSCs on day 20. Based on our results, we suggested an increase in MnSOD activity, but not catalase activity leading to a decrease in viability. Furthermore, increased expression of MnSOD was associated with a decrease in viability induced by oxidative stress.

Oxidative stress is one of the factors that contribute to the cytotoxicity of doxorubicin. ROS has a short life span, which is not easy to be detected. (Sanz, 2016) Measurement of lipid peroxidation end product – malondialdehyde (MDA) was used to determine lipid peroxidation as a convenient biomarker for ROS related damage. On day 4, there was no statistically significant difference between the MDA levels of BCSCs induced with doxorubicin and control. After 14 days of exposure to doxorubicin, the MDA level of BCSCs induced with doxorubicin shows a higher trend than the control group. However, when compared to day 4, MDA concentration was stable.

Similarly, the MDA level of doxorubicin-induced BCSCs on day 20 was remained stable at the same

range as day 4 and day 14. A state of redox imbalance between oxidants and antioxidants is a common hallmark of cancer resistance to treatment. High ROS exposure was given to increase oxidants within the cell and cause ROS-mediated damaged biomolecules such as DNA and protein. (Ziech et al., 2011) We suggested that the stable MDA concentration between MDA treated group showed the ability of BCSCs to maintain homeostasis and evaded cancer cell death by developing an antioxidant defence. SOD and catalase are the best enzymatic antioxidants on scavenging ROS (Banerjee et al., 2017) suggested that they were effectively scavenged free radicals and kept the amount of MDA in doxorubicin-induced BCSCs balanced. After exposure of andrographolide on day 20, there was a trend that the MDA level of doxorubicin-induced BCSCs was increasing. Andrographolide may have prooxidant and antioxidant characteristics. Previous studies have shown that andrographolide can trigger intracellular ROS formation, contributing to apoptosis in cancer cells. (Banerjee et al., 2017) In this study, andrographolide did not cause a significant increase in oxidative stress level, but it could suppress the antioxidant enzyme that scavenges ROS, MnSOD. Thus, we demonstrated that andrographolide and doxorubicin synergistically induced cell death by MnSOD suppression.

5 CONCLUSIONS

Andrographolide repeated treatment to BCSC can decrease MnSOD activity but not catalase and oxidative stress and is closely related to decreased cell viability with low sensitivity to doxorubicin.

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