Original Article

The impact of rotenone-modulated oxidative stress on the survival of human breast cancer stem cells (CD24- /CD44+)

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Running Title: Rotenone-modulated oxidative stress in human BCSCs
Abstract

Background
Cancer stem cells (CSCs) have been proven to be tumorigenic and may be responsible for the resistance to chemo-radiation therapy, disease recurrence and metastasis. Chemo-radiation therapy modulates oxidative stress in cancer cells, leading to cellular adaption response including modulation of cell survival and antioxidant defense mechanisms. However, the redox status alteration of breast CSCs is not yet clearly understood. The aim of this study was to elaborate the impact of rotenone-modulated oxidative stress on the survival of human breast CSCs (CD24-/CD44+) which might be beneficial to understand the underlying mechanism of chemo-radiation therapy resistance.

Methods
Human breast CSCs (CD24-/CD44+) and non-CSCs (CD24-/CD44-) were treated with rotenone and DMSO (vehicle) for 6 hours, respectively. The effects of rotenone on oxidative stress were assessed by analysing intracellular reactive oxygen species (ROS) level using dihydroethidium assay, as well as mRNA expression and specific activity of MnSOD antioxidant. Finally, cell survival was determined using MTS assay, as well as through analysis of intracellular cytochrome-c level and survivin mRNA expression.

Results
Our results showed that rotenone could not modulate the superoxide level of human breast CSCs (CD24-/CD44+), in contrast to that of non-CSCs (CD24-/CD44-). Albeit MnSOD synthesis in human breast CSCs has been excessively enhanced following rotenone treatment, the enzyme activity was still lower than in non-CSCs. Moreover,
the cell viability of CSCs was higher than that of non-CSCs, which could be related to the increase of survivin.

**Conclusions**
We conclude that human breast CSCs (CD24-/CD44+) could survive better than their counterpart non-CSCs (CD24-/CD44-) when treated with rotenone. This impact might be associated with the increase of antioxidant MnSOD and survivin mRNA expression.

**Keywords**
Human breast cancer stem cells, CD24-/CD44+, CD24-/CD44-, rotenone, ROS, superoxide radicals, oxidative stress, MnSOD, survivin
Introduction
Breast cancer has the highest incidence, 5-year prevalence and mortality among other cancers in Indonesian population [1]. It has been reported that recurrence of breast cancer after conventional therapy is initiated by a side population of residual cancer cells [2]. Cancer stem cells (CSCs) have been proven to be tumorigenic side population of cancer cells which have stemness characters similar to normal stem cells, such as self-renewal, pluripotency and a high survival rate [3,4]. Breast CSCs could be identified based on the presence of several surface antigen markers, such as CD44+, CD24−, ESA+ and CD133+ of different breast CSCs subpopulation [5,6]. As to other CSCs, breast CSCs are considered responsible for resistance to chemoradiation therapy, disease recurrence and metastasis [2,7,8]. Consequently, the development of effective breast cancer therapy requires more attention on targeting the eradication of breast CSCs [7].

One of the general strategies of breast cancer therapy is to treat cancer cells, excessively with free radicals [8]. As a consequence, the homeostasis between free radicals and endogenous antioxidant defense mechanisms in cancer cells will be disturbed, known as oxidative stress, leading to various type of cell death such as apoptosis and autophagy [9,10]. Chemo-radiation therapy modulates oxidative stress in cancer cells which induces cellular adaption responses including modulation of cell survival and antioxidant defense mechanisms. Previous studies have reported that the level of ROS-scavenging enzymes such as superoxide dismutase (SOD), glutathione peroxidase and catalase have been variously altered in cancer [11-13], leading to distinct responses towards oxidative stress-based therapy. However, little is currently known about the redox status alteration of breast CSCs, particularly conferring their ability to regulate cell survival and, therefore resist oxidative stress-based therapy.
The aim of this study was to analyze the impact of rotenone-modulated oxidative stress on the cell survival of human breast CSCs (CD24+/CD44+) compared with their counterpart non-CSCs (CD24-/CD44-). Rotenone blocks electrons flow from complex I to co-enzyme Q of electron transport chain in mitochondria. Thus, rotenone increases the production of superoxide anion ($O_2^-$) radicals, one of the major endogenous reactive oxygen species (ROS) [14,15]. We hypothesized that breast CSCs could survive from oxidative stress, possibly due to up-regulation of antioxidant enzyme expression mainly MnSOD. Our preliminary study has demonstrated that MnSOD is highly expressed in human breast CSCs (unpublished data). These findings indicated that human breast CSCs have a lower intracellular ROS level following rotenone treatment, hence could survive better than the non-CSCs. We also found that this impact is strongly associated with the increase of antioxidant MnSOD and survivin mRNA expression. This study may contribute to the understanding of underlying mechanism of chemo-radiation therapy resistance in breast cancer and provide insights into the development of a new therapeutic strategy by specifically targeting breast CSCs.

**Materials and methods**
The present study was conducted in accordance with the Declaration of Helsinki (1964). The ethical clearance has been approved by the Ethical Committee in Health Research, Faculty of Medicine, Universitas Indonesia. Clinical specimens were collected from five Indonesian breast cancer patients underwent surgery at Cipto Mangunkusumo General Hospital, Jakarta.
Isolation and Sorting of Human Breast CSCs (CD24-/CD44+)

Breast cancer specimens (~1 gram) were obtained within approximately 1 hour after surgical removal of the tumor. The tumor was then soaked in a sterile DMEM medium, mechanically dissociated and homogenated with a sterile scalpel blade. To obtain a single cell suspension, homogenate was mixed with 0.14% collagenase IV in PBS buffer and incubated at 37°C for 3-4 hours. Every 15-20 minutes, cell suspension was mixed. After incubation, collagenase was inactivated by adding 10% FBS in DMEM into cell suspension. Subsequently, cell suspension were washed in PBS and centrifuged at 300 g for 10 min. After re-suspension of cell pellets, single cell suspension was filtered using 40-μm cell strainer, washed with PBS and finally cultured with DMEM/10% FBS.

Sorting procedures were performed using MACS technology, as described in manufacturer’s protocol. Briefly, single cell suspension (1 x 10^6 cells) of breast cancer tissue was first selected using human CD24 microbeads and sorted using MS columns and Mini MACS® (Miltenyi Biotec) separator to obtain CD24 + and CD24- cell fractions. CD24- cells that were not bound to the magnetic beads were grown as monolayer culture in high glucose DMEM (Gibco®), supplemented with 3.7 g/L of sodium bicarbonate, 1% Penicillin-Streptomycin and 10% heat-inactivated FBS. About 1 x 10^6 CD24- cells were harvested and selected using human CD44 microbeads (Miltenyi Biotec) and sorted using MS columns and Mini MACS® (Miltenyi Biotec) separator to obtain CD24-/CD44+ and CD24-/CD44- cell fractions. Each sorting procedure was performed twice.

Cell Culture
Human breast CSCs (CD24-/CD44+) were grown in high glucose Dulbecco’s modified Eagle’s medium/F12 (DMEM/F12 Gibco®) without fetal bovine serum (FBS) supplemented with 1% Penicillin-Streptomycin, whereas non-CSCs (CD24-/CD44-) were grown in high glucose DMEM (Gibco®) supplemented with 3.7 g/L of sodium bicarbonate, 1% Penicillin-Streptomycin and 10% heat-inactivated FBS. The medium was refreshed every 2-3 days. The condition for cell culture was 5% CO₂ and 19% O₂ at 37°C. The cells were subcultured with 0.25% Trypsin and 1% EDTA whenever the cultures reached 80-90% of confluence.

**Rotenone Treatment**

Human breast CSCs (CD24-/CD44+) or non-CSCs (CD24-/CD44-) (10⁵ cells) were treated with DMSO-diluted rotenone (Sigma Aldrich) in serum-free DMEM medium at concentration of 0.5 µM, 5 µM and 50 µM, respectively. Control was cells treated with DMSO (vehicle) at equivalent concentrations used to dilute rotenone. After 6 hours of incubation under condition of 5% CO₂ at 37°C, cells were finally harvested with 0.25% Trypsin and 1% EDTA, counted and extracted for further analysis.

**Intracellular Superoxide Anion (O₂•⁻) Measurement using Dihydro-Ethidium (DHE)**

Measurement of intracellular O₂•⁻ production was performed using superoxide sensitive probe dihydroethidium (DHE) as described previously [16]. Fresh stock of DHE (Invitrogen) was prepared by dissolving DHE stock solution into PBS to get final concentration of DHE was 20 µM. DHE label is light sensitive, so that it was protected before, during and after the experiments. Briefly, 2x10⁴ cells were collected by trypsinization and washed twice with sterile PBS 1x. Cells were suspended in 1 ml
of PBS and loaded with DHE label (20 μM) for 30 min at 37°C. The cells were rinsed with PBS twice and fluorescence intensity was measured immediately with spectrofluorometer (Perkin Elmer®) with excitation λ at 488nm and emission λ at 585nm.

**Analysis of MnSOD and Survivin mRNA Expression using Real Time RT-PCR**

Total RNA was isolated from harvested cell culture of breast CSCs (CD24-/CD44+) and non-CSCs (CD24-/CD44-) using TriPure Isolation kit (Roche®). The procedures followed the protocols provided in the kit. Total RNA was determined using spectrophotometry at λ 260 nm.

cDNA of MnSOD and survivin were synthesized from 200 ng of total RNA samples and amplified using iScript One Step RT-PCR Kit with SYBR Green (BioRad®), according to the manufacturer’s protocol. The reactions were cDNA synthesis for 10 minutes at 50°C; inactivation of iScript reverse transcriptase for 5 minutes at 95°C; PCR cycles (40 cycles) for 10 seconds at 95°C; 30 seconds at 59°C (for MnSOD and 18Sr RNA) or at 61°C (for survivin); 30 seconds at 72°C.

Primers used for MnSOD and 18SrRNA mRNA amplification were as described in our previous report [17]. Primers used for survivin cDNA amplification were designed using Primer-Blast program and NCBI Gene Bank [NM_001168.2]. Primers for survivin mRNA were 5’-GCCAGATGACGACCCCATAGAGGA-3’ (forward) and 5’-TCGATGGCACGGCGCACTTTT-3’ (reverse) with amplicon size of 204bp. 18S rRNA was used as reference gene (external standard). Aquabidest were used as a non-template control (NTC) to reduce the false positive result. Level of mRNA
expression in rotenone-treated cells was relatively determined using Livak formula and normalized to DMSO-treated cells as a control.

**Analysis of MnSOD specific activity**

Total protein was isolated from harvested cell culture of breast CSCs and non-CSCs using Tripure Isolation kit (Roche®) according to manufacturer’s protocol. MnSOD enzyme activity was measured using xanthine oxidase inhibition assay (RanSOD® kit), as previously described [17]. To inhibit the Cu/ZnSOD, natrium cyanide (5 mM) was firstly added into each sample and the mixture was incubated for 5 minutes at room temperature. Xanthine oxidase was then added to the mixture and the color change was measured by spectrophotometer at λ 505 nm after 30 seconds and 3 minutes. The enzyme activity was calculated as a percentage inhibition of the samples plotted to the standard curve. The specific activity of MnSOD enzyme was calculated as enzyme activity (in Unit) per mg protein. Protein concentration was measured using spectrophotometer at 280 nm and plotted to the BSA (Bovine Serum Albumin) standard curve.

**Cell viability assay (MTS Assay)**

Cell viability after rotenone treatment was determined using tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) as a substrate provided in CellTiter 96 Non-radioactive Cell Proliferation Assay kit® (Promega, USA). MTS assay depends on bioreduction of tetrazolium by the mitochondria in live cells into insoluble formazan product in the cell culture medium. MTS is a non-radioactive calorimetric assay that quantifies proliferating cells in a population of cells. It is composed of solutions of a tetrazolium
compound (MTS) and an electron-coupling reagent (phenazine ethosulfate). Briefly, 10^4 cells were pretreated with rotenone at a gradient concentration 0.5 µM, 5 µM, 50 µM and 500 µM for up to 6 hours and proceed by addition of 20µl of MTS/PMS mixture (MTS:PMS = 20:1) to each well and incubated for 1-4 hours under standard conditions of 5% CO₂ at 37°C. The purple formazan product (indicative of reduction of MTS) became visible and the absorbance was measured using BioRad® microplate reader at 490 nm. The 490 nm absorbance is directly proportional to the number of living cells in the culture.

Analysis of cytochrome-c level
Analysis of cytochrome-c level was performed using Human Cytochrome-c Immunoassay Quantikine kit ® (R&D Systems). After rotenone treatment, 1 x 10^5 cells were washed, trypsinized and centrifuged to obtain pellet of cells. Cells pellet were then diluted in 300µl cell lysis buffer, incubated for 1 hour at room temperature. After centrifugation at 1000xg for 15 minutes, the supernatants were obtained and used for cytochrome-c measurement. The procedure was performed according to the manufacturer’s protocol. The cytochrome-c level was measured using microplate reader at 450 nm.

Statistical analysis
All values of rotenone-treated cells were compared with those of control cells and presented as mean ± standard error (SE). Statistical evaluation of significant differences was performed using the Student's t-test.
Results
In this present study, we have succeeded to isolate CD24-/CD44+ and CD24-/CD44- cells from human breast cancer tissue. Both cells were grown as monolayer cultures (Figure 1A and B). In order to maintain stemness property and prevent differentiation, we particularly treated CD24-/CD44+, a putative breast CSCs, with DMEM/F12 without FBS, in comparison with the medium used to culture CD24-/CD44- (DMEM supplemented with FBS). As demonstrated in Figure 1, human breast cancer CD24-/CD44+ cells tended to stick together forming mammospheres (Figure 1A), whereas the morphology of CD24-/CD44- cells was dominated by fibroblast-like cells (Figure 1B). In addition, we observed that the growth of CD24-/CD44+ cells was slower than their counterpart cells (CD24-/CD44-). In a parallel study, we could confirm that the isolated human breast cancer CD24-/CD44+ have considerably higher expression of Oct-4, a major pluripotent gene, as well as higher mammosphere forming unit, as a proof of tumorigenic capability, compared with their counterpart CD24-/CD44- cells (data not shown), hence implying the CD24-/CD44+ cells as human breast CSCs while the CD24-/CD44- cells as non-CSCs.

Superoxide level of human breast CSCs (CD24-/CD44+)
Modulation of oxidative stress in human breast CSCs (CD24-/CD44+) and non-CSCs (CD24-/CD44-) was performed using rotenone - a complex I electron transport chain inhibitor – in order to simulate chemo-radiation therapy based on ROS generation particularly superoxide radicals. Rotenone concentrations used in this study were 0.5 μM, 5 μM and 50 μM based on our previous study that performed rotenone treatment for T98G glioblastoma cell line (in preparation manuscript). Another recent study using human breast adenocarcinoma (MCF-7) cell line observed a significant decrease of cell viability following treatment of 50 μM rotenone [18]. In addition to those
results, incubation period of rotenone within 4 to 8 hours significantly increased the
generation of ROS in human neuronal stem cells [15].

We also treated the cells with DMSO in order to control the effects arising from the
solvent DMSO on the modulation of oxidative superoxide. DMSO is generally used as a
cryoprotectant for the preservation of cells and also used as an organic solvent [19].
Although DMSO has been established to have a low level of toxicity at low
concentration [20], however recent study has revealed that DMSO may modulate
oxidative stress in the cell cultures when given at high concentration [21]. Our results
demonstrated that DMSO did not affect the superoxide level of human breast CSCs
(CD24-/CD44+) as well as of their counterpart non-CSCs (CD24-/CD44-).

To specifically monitor the level of superoxide radicals (O$_2^-$) in the cells produced
upon rotenone treatment, we performed dihydroethidium (DHE) assay. DHE is able to
freely permeate through cell membranes and reacts with superoxide anions to form
fluorescent product 2-hydroxyethidium which can be detected by spectrofluorometer
[16]. In determining the effect of rotenone on superoxide level in human breast CSCs
(CD24-/CD44+) and non-CSCs (CD24-/CD44-), DHE intensity of rotenone-treated
cells was normalized to DMSO-treated cells as a control. The results demonstrated
that there were no significant differences of superoxide level between human breast
CSCs (CD24-/CD44+) treated with rotenone concentration of 0.5, 5 and 50 µM
compared with their respective control (Figure 2). In contrast to that, superoxide level
in the non-CSCs (CD24-/CD44-) could obviously be induced by rotenone in a dose-
dependent manner, which shown significantly higher at concentration of 5 µM (± 1.3-
fold higher) and 50 µM (±1.7-fold higher) than in their CSCs counterpart (p < 0.05).
The fact that rotenone treatment did not affect the superoxide level of human breast CSCs (CD24-/CD44+) compared with the non-CSCs (CD24-/CD44-) could indicate that human breast CSCs (CD24-/CD44+) might be more resistant to oxidative stress. It appears that breast CSCs possibly have a mechanism to overcome the formation of ROS levels caused by the rotenone treatment. Therefore, the antioxidant system in breast CSCs also needs to be analysed to determine the redox status in breast CSCs.

MnSOD expression of human breast CSCs (CD24-/CD44+)

To examine the effect of rotenone treatment on the antioxidant system, we analyzed the MnSOD gene expression and activity. MnSOD is a major endogenous antioxidant enzyme that eliminates superoxide radicals (O$_2^•-$) generated by the mitochondrial electron transport chain [22]. MnSOD mRNA expression level and specific activity of MnSOD may be altered under several physiological and pathophysiological conditions including environmental factors such as availability of manganese ions [22,23]. The suppression of MnSOD expression in ovarian cancer cells enhanced superoxide level which further induced cell proliferation in vitro and tumor growth in vivo [13]. It is also crucial to analyze its specific activity since MnSOD is synthesized in the cytoplasm as a precursor and post-translationally transported into the mitochondrial matrix via an amino-terminal targeting sequence.

Analysis of MnSOD mRNA expression level in breast CSCs (CD24-/CD44+) and non-breast CSCs (CD24-/CD44-) were performed based on the relative expression to the control cells merely treated with DMSO. In this study, we found that mRNA MnSOD expression level in CD24-/CD44+ cells following rotenone treatment of 0.5, 5 and 50 µM was predominantly enhanced about 3-, 5-, and 66-fold compared with
those in CD24-/CD44- cells (Figure 3A, B). Statistical analysis using Student’s t-test revealed significant differences between those two cells at all concentrations of rotenone. In contrast to its mRNA expression level, no significant differences in MnSOD specific activity could be found between human breast CSCs (CD24-/CD44+) and non-CSCs (CD24-/CD44-) following the rotenone treatment at any concentrations (Figure 4). Nonetheless, the specific activity of MnSOD in both cells tended to increase in a dose-dependent manner.

**Cell survival of human breast CSCs (CD24-/CD44+)**

**Cell viability**

To determine the ability of cells in maintaining or recovering their viability after rotenone treatment, we performed MTS assay which depends on bioreduction of tetrazolium by mitochondria in live cells into insoluble formazan product in the cell culture medium. Comparative analysis of viability between breast CSCs (CD24-/CD44+) and non-CSCs (CD24-/CD44-) was performed based on the ratio level of rotenone-treated cells to control (DMSO-treated cells). The results revealed that rotenone treatment could decrease cell viability in both human breast CSCs and non-CSCs (Figure 5). However, the viability of human breast CSCs was significantly higher than those of the non-CSCs, particularly at rotenone concentration of 50 µM (~6-fold, p<0.01).

**Cell apoptosis**

Rotenone provokes overproduction of superoxide radicals in mitochondria, leading to stimulation of cell apoptosis through intrinsic pathway. Cytochrome-c is one of pro-apoptotic molecules that involves in the initial activation of other cytosolic pro-apoptotic proteins to cause morphological and biochemical damage with eventual cell
death. Increase level of intracellular cytochrome-c signifies the occurrence of cell apoptosis through mitochondrial (intrinsic) pathway [24]. Surprisingly, the level of intracellular cytochrome-c in all samples (either treated and untreated of CSCs or non-CSCs) were similar to those of blanks which merely consisted of sample diluents (Figure 6A), indicating cytochrome-c protein level could not be detected in these samples. Here, we have verified that the negative results obtained from this analysis was not due to technical errors, since the positive control using human glioblastoma T98G cells treated with rotenone (Figure 6), as well as various concentrations of standard provided in the kit (data not shown) showed significantly higher absorbances at 450 nm compared with the blanks. Therefore, we suggested that rotenone treatment did not increase cytochrome-c level in both CSCs and non-CSCs, indicating no impact of rotenone treatment on the intrinsic pathway of apoptosis.

**Expression of survivin mRNA**

We further analysed the involvement of survivin as anti-apoptotic factor on the survival of human breast CSCs after rotenone treatment. Survivin plays a definite role in inhibiting the activation of many pro-apoptotic proteins such as caspase-9 and caspase activators [25]. To compare the survivin mRNA expression level between human breast CSCs (CD24-/CD44+) and non-CSCs (CD24-/CD44-), the expression level in rotenone-treated cells was firstly normalized to DMSO-only treated cells. In this study, we showed that the expression level of survivin mRNA in the breast CSCs following rotenone treatment was drastically increased (~15-fold at rotenone 0.5 µM and ~2.5-fold at 5 µM, ~1.9-fold at 50 µM) than that in the non-CSCs (Figure 7A, B), indicating the role of survivin on the cell viability of human breast CSCs.
Discussion
Several earlier studies have proposed that cancer recurrence after chemo-radiation is initiated by a subpopulation of residual malignant cells which are highly resistant to drug treatment and are believed to be cancer stem cells (CSCs) [2,26,27]. Although it has been considered that the CSC subpopulations might use redox regulatory mechanisms to promote cell survival and resistance to anticancer therapy [8,28], no direct evidence has been reported. In this study, human breast cancer cells from clinical specimens of Indonesian patients have been isolated and sorted to obtain the CSC (CD24-/CD44+) subpopulation based on its predominantly higher expression level of Oct-4 pluripotent gene compared with its counterpart non-CSCs (CD24-/CD44-) (data not shown). Furthermore, we deliberately performed rotenone induction to elucidate the impact of oxidative stress modulation on the survival of human breast CSCs and non-CSCs.

The inability of rotenone treatment to modulate oxidative stress status in human breast CSCs (CD24-/CD44+)
Rotenone is a natural toxin derived from plant extracts of the Leguminosae family from South America. In everyday life, rotenone is used as an insecticide. Since this substance blocks electron flow from NADH to co-enzyme Q at complex I of respiratory chain in mitochondria, it is able to increase the production of superoxide radicals, and further induced cytotoxicity [14,15,29]. Moreover, rotenone has been suggested to act as an anti cancer agent [14].

In fact, our study revealed that ROS level in human breast CSCs (CD24-/CD44+), could not be enhanced by rotenone although treated with high doses, in comparison with their counterpart (CD24-/CD44-) cells. We presumed that human breast CSCs
may have a mechanism that could overcome the accumulation of ROS after rotenone induction, therefore enabling avoidance of cell death as well as maintenance of cell viability. Previous studies using normal hematopoietic stem cells and mammary epithelial stem cells have reported that maintaining ROS level at a lower state than their mature progeny is required in order to prevent cellular differentiation and to maintain long term self-renewal [8,28]. Rather more comparable result was the presence of breast CSC subset in MMTV-Wnt1 mouse tumors (Thy^+CD24^+Lin^- cells) containing lower ROS level than the corresponding non-tumorigenic cells [28].

Oxidative stress is defined as a disturbance in the equilibrium between free radicals / ROS and endogenous antioxidant defense mechanisms [9]. In response to the overproduction of ROS (especially superoxide radicals) induced by rotenone, the first cellular antioxidant activated will be MnSOD, an isoform of the superoxide dismutase (SOD) located in inner membrane of mitochondria. MnSOD eliminates anion superoxide (O_2^-) by converting it into hydrogen peroxide (H_2O_2) [22]. In the present study, we demonstrated the inability of rotenone to modulate the redox status of human breast CSCs, as shown by their superoxide levels compared with DMSO-treated cells. On the other hand, the superoxide level of human breast non-CSCs investigated in this study could be modulated by rotenone treatment in a dose-dependent manner. We strongly implied that the differential response on rotenone treatment between CSCs and non-CSCs associated with the differential expression level of MnSOD gene between those cells. Following rotenone treatment, MnSOD gene expression in human breast CSCs was highly induced, in contrast to the non-CSCs. Several studies have previously reported that after ionizing radiation exposure, cancer cells up-regulated the expression of genes involved in antioxidant synthesis. It
has also been suggested that high expression of antioxidant in CSCs contributes to therapy resistance [2,7,22].

Interestingly, the result of this study revealed that although the synthesis of MnSOD mRNA in human breast CSCs has been excessively increased, its enzyme specific activity remained relatively unaffected compared with that of their control and the non-CSCs. A similar result was also shown by our study using human glioblastoma multiforme T98G cell lines treated by rotenone [in preparation manuscript]. Therefore, we assumed that rotenone-treated cells responded to accumulated free radicals by increasing the MnSOD mRNA synthesis, in order to fulfill the availability of MnSOD protein. Nevertheless, this response still could not enhance the enzyme activity. The plausible explanation of this phenomenon might be due to the overconsumption of MnSOD activity to scavange overaccumulation of superoxide radicals produced by rotenone. Taken together, we indicated that MnSOD enzyme plays a remarkable role on the maintaining of low ROS level in human breast CSCs (CD24-/CD44+).

**The higher survival of human breast CSCs (CD24-/CD44+) following rotenone treatment compared with non-CSCs (CD24-/CD44-)**

The success of cancer therapy is basically assessed by its ability to affect the survival of cancer cells. When cancer cells are sensitized to any conventional cancer therapy, such as chemotherapy, radiation as well as hormonal therapy, it is expected that the cancer cell survival is decreased leading to eradication of the cancer cells. Conventional regimen of anti-cancer therapy is generally based on the assumption that all cancer cells have the same potential of malignancy, without considering the
presence of breast CSCs [2]. A number of recent scientific reviews and studies have enthusiastically described CSCs as a minor population of breast cancer cells that play roles on high survival rate and resistance of cancer cells to anti-cancer therapies [7,8]. Therefore, the development of an anti-cancer therapy targeted to breast CSCs is urgently required so that cancer can be eradicated from the root.

It should be noted that most of chemo-radiation therapy acts by modulating oxidative stress of cancer cells. In this study, rotenone is used to simulate the effect of free radical-based anti-cancer therapies. The impact of rotenone on cell survival can be examined by analysing cell proliferation, cell viability and apoptosis. Even though viability of both breast CSCs (CD24-/CD44+) and non-CSCs (CD24-/CD44-) investigated in the present study were reduced after rotenone treatment compared with untreated cells, we should remark that breast CSCs were more viable than non-CSCs when treated with increasing concentrations of rotenone (Figure 5). Combined with the result of survivin mRNA expression (Figure 7), substantially higher expression of survivin in breast CSCs (CD24-/CD44+) is thought to precede the higher viability of breast CSCs compared with those in non-CSCs (CD24-/CD44-). Thus, we presumed that the increase of survivin mRNA expression level might be another mechanism owned by breast CSCs (CD24-/CD44+) to survive from the impact of oxidative stress modulation. In addition, we found that rotenone treatment could not increase the intracellular cytochrome-c in both breast CSCs (CD24-/CD44+) and non-CSCs (CD24-/CD44-), indicating that the decreased of cell viability after rotenone treatment was not caused by intrinsic apoptosis. As previously reported, survivin could inhibit the release of mitochondrial cytochrome-c. Therefore, they have suggested that survivin may involve in inhibiting the intrinsic pathway of cell apoptosis [30].
Additional studies should be performed to elaborate the mechanism of cell death targeted by oxidative stress modulator, in particular to those generating superoxide radicals.

Comparing with other previous reports [2,8,13], our findings confirmed that human breast CSCs (CD24-/CD44+) have conserved low level of ROS characteristic, which probably helps to protect their genomes from endogenous and exogenous ROS-mediated damages and hence maintaining their stemness towards oxidative stress. The present study revealed that the mechanism maintaining cell viability in breast CSCs after rotenone treatment may be partially due to the increased production of free radical scavengers including, but not limited to, MnSOD and the up-regulation of expression of anti-apoptotic molecules such as survivin.

Heterogeneity of cancer cells may influence the resistance to existing conventional cancer therapies such as ionizing radiation and chemotherapy [31]. As a consequence, studies on further investigation of oxidative stress modulation in human breast CSCs is crucial for the development of a more effective regimen of breast cancer therapy [8]. This should include a novel strategy to suppress MnSOD as well as survivin expression in order to enhance cell apoptosis and simultaneously increase sensitivity to chemoradiation therapy.

**Conclusions**
This is the first study providing clear evidence that human breast CSCs (CD24-/CD44+) could survive better than their counterpart non-CSCs (CD24-/CD44-) when treated with rotenone, an oxidative stress modulator. The underlying mechanism for
this impact can be ascribed to the remarkably increase of antioxidant MnSOD and survivin expression. The present study provides insights towards identification of CSC-resistance mechanisms against oxidative stress modulating therapy. Further studies are required to develop novel therapeutic strategies to overcome the low ROS level state and hence increasing cell death targeted to human breast CSCs.

Competing interests
The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' contributions
SIW conceived, designed and coordinated the whole study, interpreted the results and was responsible for the writing of the manuscript. GA carried out the whole experiments, participated in the data analysis and helped to draft the manuscript. NSH participated in the CSCs sorting procedures and rotenone induction as well as performed the statistical analysis. AB carried out the human breast cancer cells isolation and culture. SWAJ contributed in the study design, data analysis and interpretation of the results. All authors have read and approved the final manuscript.

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Purnamawati, M. Biomed in the laboratory works, as well as the contribution of dr. Erwin Danil Y, SpBO in providing the breast cancer specimens.

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Figures

Figure 1. Human breast cancer stem cells (CD24-/CD44+) formed mammospheres. (A) Human breast CSCs (CD24-/CD44+) were grown in high glucose Dulbecco’s modified Eagle’s medium/F12 (DMEM/F12, Gibco®) without fetal bovine serum (FBS), supplemented with 1% Penicillin-Streptomycin. (B) Human breast non-CSCs (CD24-/CD44-) were grown in high glucose DMEM (Gibco®), supplemented with 3.7g/L of sodium bicarbonate, 1% Penicillin-Streptomycin and 10% heat-inactivated FBS.

Figure 2. Superoxide anion ($O_2^-$) level of human breast CSCs (CD24-/CD44+) treated with rotenone was unaffected. About $2 \times 10^4$ human breast CSCs (CD24-/CD44+) and non-CSCs (CD24-/CD44-) were treated with various concentrations of rotenone and DMSO, respectively. Intracellular superoxide anion level was determined using DHE assay, as described under “Material and Methods”. Fluorescence intensity was measured using spectrofluorometer with excitation $\lambda$ at 485 nm and emission $\lambda$ at 585 nm, and normalized to that in control group (DMSO-treated cells). All values are means ± SE, $n=6$. Significant differences at * ($p<0.05$) were determined using two-tailed Student’s t-test.

Figure 3. Overexpression of MnSOD mRNA in human breast CSCs (CD24-/CD44+) treated with rotenone. About $2 \times 10^4$ human breast CSCs (CD24-/CD44+) and non-CSCs (CD24-/CD44-) were treated with various concentrations of rotenone and DMSO as vehicle, respectively. Total RNA was extracted from the cells and cDNA product was amplified using Real-Time RT-PCR methods, as described under “Material and Methods”. (A) Ratio of MnSOD mRNA expression level in breast
CSCs compared with that in non-CSCs. Expression level was relatively calculated and normalized to those from control (DMSO-treated cells) and reference gene (18S rRNA). All values are means ± SE, n = 6. Significant differences (*: p < 0.05 and **: p < 0.01) was determined using two-tailed Student’s t-test. (B) 2% agarose gel electrophoresis of RT-PCR products of MnSOD and 18S rRNA in human breast CSCs and non-CSCs samples treated with 5µM rotenone (R) and DMSO at equivalent concentration used to dilute 5µM rotenone (C). Product size of MnSOD cDNA amplicon was 216 bp and 18S rRNA was 155 bp. Lane M was a 100bp DNA ladder marker. NTC was referred as non-template control for MnSOD primers.

**Figure 4. MnSOD specific activity in human breast CSCs (CD24-/CD44+) treated with rotenone was unaffected.** About 2x10^4 human breast CSCs (CD24-/CD44+) and non-CSCs (CD24-/CD44-) were treated with various concentrations of rotenone and DMSO (control), respectively. Total protein was extracted from the cells as described under “Material and Methods”. MnSOD specific activity was measured using xanthine oxidase inhibition method (RanSOD kit ®). Cu/ZnSOD activity was inhibited by adding NaCN 5mM into each sample. The optical density was measured by spectrophotometer at λ 505 nm after 30 seconds and 3 minutes. Level of MnSOD specific activity was normalized to that in DMSO-treated cells. All values are means ± SE, n = 6. No significant difference (p > 0.05) was determined using Student’s t-test.

**Figure 5. Rotenone treatment increased the viability of human breast CSCs (CD24-/CD44+) compared with the non-CSCs (CD24-/CD44-).** Cell viability was determined using MTS assay (CellTiter 96 Non-radioactive Cell Proliferation Assay
kit®, Promega). About $2 \times 10^4$ human breast CSCs (CD24-/CD44+) and non-CSCs (CD24-/CD44-) were treated with various concentrations of rotenone and DMSO (vehicle), respectively, then followed by addition of 20µl of MTS/PMS mixture (MTS:PMS = 20:1) to each well and incubated for 1-4 hours under standard conditions of 5% CO$_2$ and 37°C. The absorbance at 490 nm was directly proportional to the number of living cells in the culture. Cell viability level was normalized to that in DMSO-treated cells. All values are means ± SE, n = 6. Significant differences (*: p < 0.05; **: p < 0.01) was determined using two-tailed Student’s t-test.

**Figure 6. Rotenone treatment did not affect the cytochrome-c level of human breast CSCs (CD24-/CD44+) and non-CSCs (CD24-/CD44-).** (A) About $2 \times 10^4$ human breast CSCs (CD24-/CD44+) and non-CSCs (CD24-/CD44-) were treated with various concentrations of rotenone and DMSO (vehicle), respectively. Analysis of cytochrome-c level was performed using Human Cytochrome-c Immunoassay Quantikine kit® (R&D Systems). Blank consisted of calibrator diluents only. Positive control used was T98G cells (human glioblastoma multiforme) treated with 50 µM rotenone. Control was cells (CD24-/CD44+ or CD24-/CD44-) without any treatment. The absorbance of samples was measured using ELISA reader at λ 450 nm. Level of cytochrome-c was performed based on the absorbance of rotenone-treated cells normalized to that of DMSO-treated cells. All values are means ± SE, n = 6. No significant difference was found (p > 0.05) between treated groups and control (two-tailed Student’s t-test).

**Figure 7. Overexpression of survivin mRNA in human breast CSCs (CD24-/CD44+) treated with rotenone.** About $2 \times 10^4$ human breast CSCs (CD24-/CD44+)
and non-CSCs (CD24-/CD44-) were treated with various concentrations of rotenone and DMSO (vehicle), respectively. Total RNA was extracted from the cells and cDNA product was amplified using Real-Time RT-PCR methods, as described under “Material and Methods”. (A) Ratio of survivin mRNA expression level in breast CSCs compared with that in non-CSCs. Expression level was relatively calculated and normalized to those from control (DMSO-treated cells) and reference gene (18S rRNA). All values are means ± SE, n = 6. Significant differences (*: p < 0.05 and **: p < 0.01) was determined using two-tailed Student’s t-test. (B) 2% agarose gel electrophoresis of RT-PCR products of survivin and 18S rRNA in human breast CSCs and non-CSCs treated with 0.5 µM rotenone (R) and DMSO at equivalent concentration used to dilute 0.5 µM rotenone (C). Product size of survivin amplicon was 204 bp and 18S rRNA was 155 bp. Lane M was a 100bp DNA ladder marker. NTC was referred as non-template control for survivin primers.
Figure 4

Figure 5
Figure 6

Figure 7