

Basic Medical Research

Effect of hypoxia-inducible factor-1 α induction by CoCl₂ on breast cancer cells survival: influence of cytochrome-c and survivin

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Abstrak

Latar belakang: Jaringan tumor biasanya mengalami hipoksia karena terganggunya suplai oksigen ke daerah tersebut. Respons adaptasi terhadap keadaan hipoksia jaringan kanker diperantarai oleh (HIF-1 α). HIF-1 α meningkatkan ekspresi protein pro-apoptosis, sitokrom-c dan meningkatkan ekspresi anti-apoptosis survivin. Dalam penelitian ini akan dipelajari peran HIF-1 α terhadap ketahanan hidup sel kanker payudara melalui pengaturan pro-apoptosis sitokrom-c dan anti-apoptosis survivin.

Metode: Cell line kanker payudara T47D diinduksi dengan CoCl₂, kemudian dipanen dan dilakukan analisis terhadap ekspresi HIF-1 α , sitokrom-c, survivin dan viabilitas sel.

Hasil: Induksi HIF-1 α dengan CoCl₂ menyebabkan peningkatan protein dan mRNA HIF-1 α , protein sitokrom-c dan mRNA survivin, namun tidak menyebabkan perubahan viabilitas sel.

Kesimpulan: Induksi HIF-1 α tidak menyebabkan perubahan pada viabilitas sel kanker payudara T47D akibat pengaturan keseimbangan antara ekspresi pro-apoptosis sitokrom-c dan anti-apoptosis survivin.

Abstract

Background: Tumor tissue usually became hypoxic due to disruption of oxygen supply. Adaptation response to hypoxia is mediated by transcription factor, hypoxia-inducible factor-1 α (HIF-1 α). HIF-1 α signaling is known to increase the expression of pro-apoptotic protein cytochrome-c, and anti-apoptotic survivin. In this study we wanted to analyze the role of HIF-1 α on breast cancer cells survival through pro-apoptosis cytochrome-c and anti-apoptosis survivin regulation.

Methods: Breast cancer cell lines T47D were induced by CoCl₂ then harvested to analyze the expression of HIF-1 α , protein cytochrome-c, mRNA survivin and cell viabilities.

Results: HIF-1 α induction by CoCl₂ causes the increase of protein and mRNA of HIF-1 α , cytochrome-c protein, and survivin mRNA, but does not cause the changes in cell viability.

Conclusion: HIF-1 α induction have no effects on breast cancer cell line T47D viabilities due to the balance regulation between pro-apoptosis expression cytochrome-c and anti-apoptosis survivin.

Keywords: apoptosis, cell viability, cytochrome-c, hypoxia-inducible factor-1 α , survivin

pISSN: 0853-1773 • eISSN: 2252-8083 • <http://dx.doi.org/10.13181/mji.v23i3.933> • Med J Indones. 2014;23:139-46

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The incidence of breast cancer was ranked the highest of cancer in Indonesia in 2012.¹ Cancer develops if apoptosis is disturbed and cell growth is faster and live longer than normal. In other words, tumor growth is supported by the ability to avoid apoptosis.² The tumor blood vessels are structurally and functionally abnormal causing temporarily irregular of blood flow which leads to hypoxic environment in the tumor tissue, and finally induce the increase of hypoxia-inducible factor-1 α (HIF-1 α) protein stability in various tumor tissues.³

HIF-1 α is a transcription factor of many target genes that functions in hypoxia adaptation response. Protein HIF-1 has two sub-units, HIF- α and β . HIF-1 α domain contains basic-Helix-Loop-Helix (bHLH) at the N-terminal, two domains Per-ARNT-Sim (PAS), an oxygen-dependent degradation domain (ODD) which mediates the stability of HIF-1 dependent oxygen, and a transactivation domain (TAD) at the C-terminal (C-TAD).⁴

The presence of oxygen causes prolyl hydroxylase (PHD) enzyme to catalyze the hydroxylation of proline residues 402 and 564 in the ODD domain of HIF-1 α protein in cytoplasm.⁵ The hydroxylated HIF- α is recognized by von Hippel Lindau (VHL) protein thus bind to it. The HIF- α protein that binds to vHL can be degraded through ubiquitination.⁴ During hypoxic condition, the hydroxylation of HIF-1 α protein does not occur due to the lack of oxygen as a substrate for PHD thus increasing the stability of HIF-1 α in the cytoplasm.⁶ The stabilized form of HIF-1 α protein will translocate from the cytoplasm to the nucleus, where it heterodimerizes with HIF-1 β . The HIF- α /HIF- β complex binds to hypoxic responsive elements (HREs) sequence located in the upstream of the target genes of HIF- α protein leading to induction of the expression of target genes which play some roles in tumor growth, such as erythropoietin (EPO), vascular endothelial growth factor (VEGF), glucose transporter-1, 3 (GLU1, 3) and glucose transporter 1 (GLUT-1), carbonic anhydrase-9 (CA IX) and lactate dehydrogenase-A (LDHA).⁷

Besides increasing the survival of tumor cells in hypoxic conditions, HIF-1 α is also involved in apoptosis of tumor cells. Ardyanto found that the induction of HIF-1 α by CoCl₂ for 6 hours in gastric carcinoma cell line increased the HIF-1 α protein expression leading to decreased and increased in apoptosis and proliferation, respectively.⁸ HIF-1 α

protein can affect the release of cytochrome-c through the p53 pathway and BNIP3.⁹ Various signals that induce apoptosis will converge in mitochondrial as a releaser of cytochrome-c and then will activate the caspase cascade that leads to apoptosis.

Several studies have shown that HIF-1 α can inhibit apoptosis pathway by increasing the expression of inhibitors of apoptosis proteins such as survivin.¹⁰ Peng, et al¹¹ found that HIF-1 α protein can activate the transcription of survivin gene in breast cancer cells by binding directly to the promoters of genes survivin. Survivin inhibits the conversion of pro-caspase 9 to the active form. It has been demonstrated that survivin has become a target for cancer treatment, in which, inhibition of its expression was shown to increase the effectiveness of chemotherapy and chemoradiation.¹²

Induction of HIF-1 α by CoCl₂ with various concentrations has been performed on variety of cells, including tumor cells. Cobalt can inactivate PHD by binding to the iron binding site of PHD because of its structure similar to the iron (Fe²⁺). The result is that HIF-1 α does not undergo hydroxylation by PHD and is not degraded by ubiquitin, which resulted in increased stability of HIF-1 α .¹³ The aim of this study is to analyze the role of HIF-1 α on breast cancer cells survival through pro-apoptosis cytochrome-c and anti-apoptosis regulation.

METHODS

Cell culture and treatments

Breast cancer cell line T47D was used as a sample because it represents most of the breast cancer characteristics, i.e. derived from ductal carcinoma breast cancer cells, estrogen and progesteron receptor positive, and p53 mutan. T47D cells were induced by CoCl₂ then we examined the expression of HIF-1 α , cell viability, cytochrome-c and survivin.

HIF induction

T47D cells were grown in 24-well plate with a number of 2.5 X 10⁴ cells in each well and maintained in 1 mL of medium Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 ug/mL streptomycin, and 292 ug/mL L-glutamine at 37°C, 5% CO₂ for 24-48 hours. After reaching

80% confluent, the medium was discarded and then the cells were induced with CoCl_2 250 μM or 500 μM dissolved in DMEM without FBS for 2, 6, 24 or 48 hours. T47D cells with the treatment but without CoCl_2 induction was used as a control. After induction by CoCl_2 , cells were harvested and were analyzed for HIF expression, cell viability and cytochrome-c.

ELISA HIF-1 α

Cells that have been induced by CoCl_2 were examined for HIF-1 α protein by ELISA using Human/Mouse HIF-1 α Total Immunoassay (R&D Systems) reagent kit.

HIF- α mRNA and survivin mRNA RT-PCR

RNA samples were isolated and amplified using SYBR-Green kit (BioRad). The HIF-1 α primer sequences used were 5'-CCA GCA ACT TGA TGA TGA GG-3' (forward) and 5'-TTG ATT GAG TGC AGG GTC AG-3' (reverse) (designed by Dr.dr. Novi Silvia Hardiany, MBIomed) whereas Survivin primer sequences used were 5'-GCC AGA CAT TGA AGA CGA CCC GGA-3' (forward) and 5'-CGC CGG TCG ATG GCA ACT TT-3' (reverse).¹⁴

Cytochrome-c analysis

Cytochrome-c concentration was analyzed using sandwich ELISA technique by Human Cytochrome-c Immunoassay kit (Quantikine ®).

Viability assay by MTS method

The number of living cells after induction with CoCl_2 was measured by MTS method using CellTiter 96R Aqueous Non-Radioactive Cell Proliferation Assay (a) (Promega). Reagent used is a solution of a substance containing tetrazolium [3-(4,5-dimethylthiazol-2-yl)-5-(3carboxymethoxyphenyl)-2 - (4-sulfophenyl)-2H-tetrazolium, inner salt; MTS(a)] and a metabolic binding reagent (phenazines methosulfate; PMS).

Statistical analysis

The results of each measurement were analyzed using ANOVA method for significance and with Pearson correlation test for correlation analysis. Both of them were analyzed using statistical program SPSS 11.5. Statistical significance was set at $p < 0.05$.

RESULTS

T47D cultures

T47D cells obtained from Dr.rer.nat. Marselina Irasonia Tan, MS (School of Life Sciences, ITB) was grown and propagated in DMEM medium with 10% FBS (Figure 1).

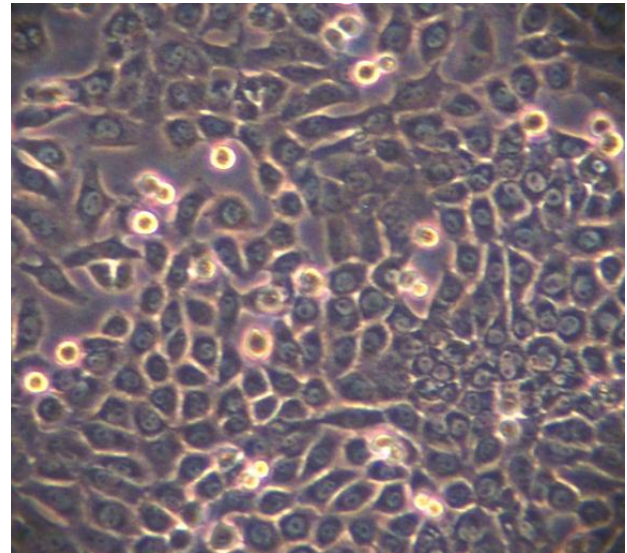


Figure 1. T47D cells were cultured in DMEM medium

Optimization of CoCl_2 incubation

Optimization of CoCl_2 induction was performed on T47D cells. T47D cells were induced by 500 μM CoCl_2 for 2, 6, 24, and 48 hours to determine the most optimal time for incubation. After induction with various period of time, it was concluded that the concentration of HIF-1 α protein were highest in six hours after incubation. CoCl_2 incubation more than six hours showed a decreased in HIF-1 α protein concentrations (Figure 2). Based on this result, the induction of HIF-1 α were conducted by six hour incubation of 500 μM CoCl_2 .

Protein HIF-1 α analysis

HIF-1 α protein concentration increased in line with increased concentrations of CoCl_2 given. However there is no significant difference between the ratio of the concentration of HIF-1 α induced by 250 μM and 500 μM CoCl_2 with the control sample (Figure 3). Strong positive correlation was found between the increase in the concentration of CoCl_2 and the concentration of HIF-1 α although the difference did not reach statistically significant (Pearson, $r = 0.971$, $p = 0.154$).

HIF-1 α
(ng/g protein)

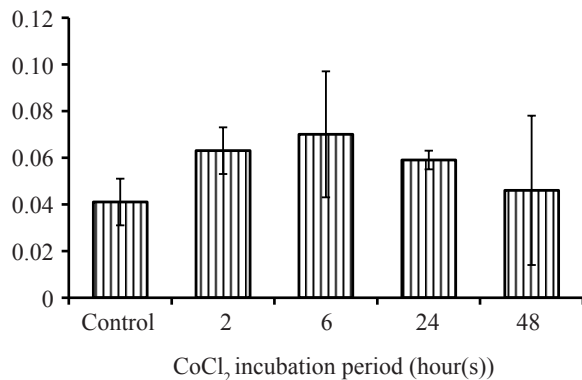


Figure 2. Optimization of CoCl₂ incubation time. T47D cells were induced by CoCl₂ 500 μ M for 2, 6, 24, and 48 hours to determine the most optimal incubation time. It was concluded that the increase in HIF-1 α protein concentrations were highest after induction for six hours

Ratio of HIF-1 α
concentration against control

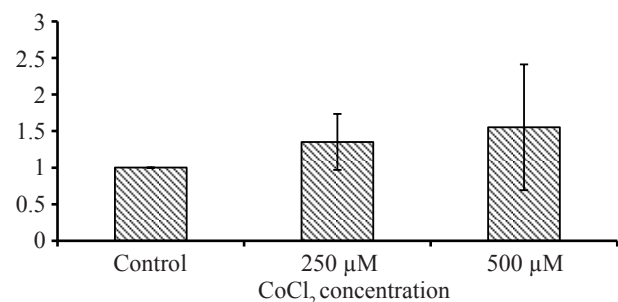


Figure 3. Effect of CoCl₂ induction on HIF-1 α protein concentration. CoCl₂ induction led to an increase in HIF-1 α protein concentration in T47D cells though it was not significant ($p > 0.05$)

Analysis of HIF-1 α mRNA

mRNA expression of HIF-1 α of T47D cells were increased in line with the increased concentrations of CoCl₂ (Figure 4). However, the mRNA expression of HIF-1 α was shown no significant difference between control and CoCl₂-induced cells. Strong positive significant correlations (Pearson, $r = 0.998$) was found between the increase of HIF-1 α protein concentrations and mRNA expression of HIF-1 α . Strong positive correlation was also found between HIF-1 α protein concentrations expression although it was not significant (Pearson, $r = 0,976$).

Cytochrome-c analysis

Cytochrome-c concentration increased in line with

increased concentrations of CoCl₂. However, the concentration of cytochrome-c T47D were not reach statistically difference between control cells and T47D cells-induced by 250 and 500 μ M CoCl₂ (t-test, $p > 0.1$) (Figure 5). There was strong positive correlation between the concentration of CoCl₂ and cytochrome-c (Pearson, $r = 0.998$).

Survivin expression analysis

Survivin mRNA expression was increased with increased concentrations of CoCl₂. Significant difference was found between the expression of survivin mRNA of control cells and cells induced by 250 μ M CoCl₂ (t-test, $p = 0.005$) and 500 μ M CoCl₂, respectively (t-test, $p = 0.024$) (Figure 6).

Ratio of normalized
HIF-1 α mRNA
expression

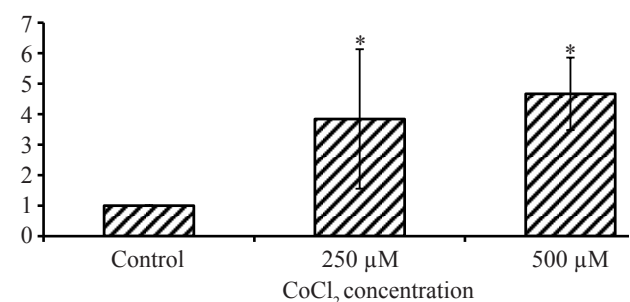


Figure 4. Effect of CoCl₂ induction on HIF-1 α mRNA and protein expression. CoCl₂ induction led to an increase in HIF-1 α mRNA expression of T47D cells. There is significant difference of HIF-1 α mRNA expression between control and cells induced by 500 μ M CoCl₂ (t-test, $p = 0.004$). Symbol ‘*’ shows statistically significant vs control

Ratio of cytochrome-c con-
centration against
control

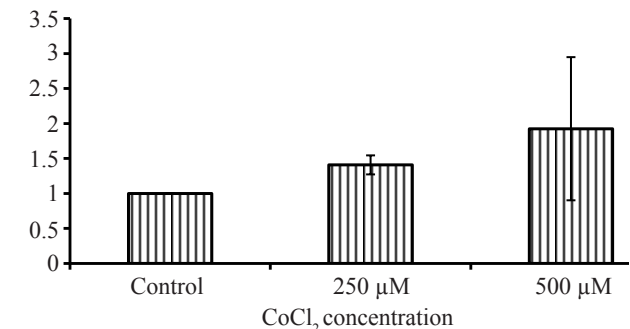


Figure 5. The influence of the CoCl₂ induction on cytochrome-c. CoCl₂ induction led to an increase in cytochrome-c concentration of T47D cells. No significant difference of cytochrome-c concentration between control (without induction) and T47D cells-induced by CoCl₂ (t-test, $p > 0.1$)

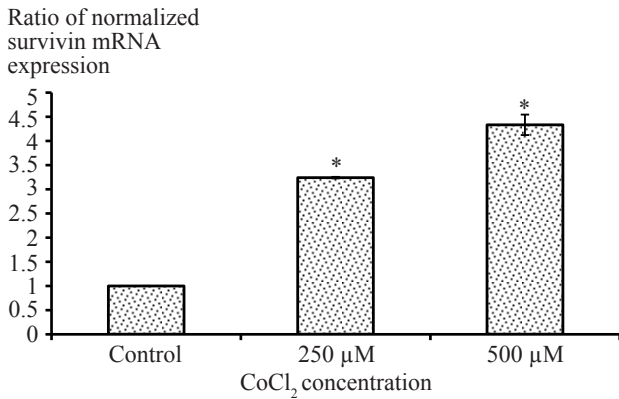


Figure 6. Effect of CoCl₂ induction on survivin mRNA expression. CoCl₂ induction leads to increased expression of survivin mRNA in T47D cells. Significant difference was found between the expression of survivin mRNA of T47D control cells (without induction) and T47D cells induced by CoCl₂ 250 μM. Symbol ‘*’ shows statistically significant vs control

Analysis of cell viability

T47D cells’ viability after induction with CoCl₂ did not show any differences. There is no significant difference of viability ratios between control cells and cells induced by CoCl₂ (t-test, $p > 0.05$) (Figure 7).

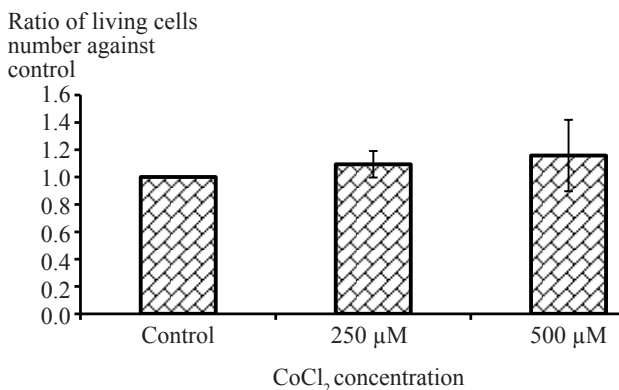


Figure 7. CoCl₂ induction effect on cell viability. There was no significant difference between viability ratios of control cells (without induction) and T47D cells induced by CoCl₂ 250 and 500 μM (t-test, $p > 0.05$)

Correlation between HIF and cytochrome-c, survivin and cell viability

There was strong positive correlation between HIF-1α protein and the cytochrome-c (Pearson, $r = 0.985$) in T47D cells induced by CoCl₂. Strong positive correlation was obtained between survivin mRNA expression and HIF-1α protein (Pearson, $r = 0.999$).

In addition, there was a strong positive correlation between survivin mRNA expression and HIF-1α mRNA expression (Pearson, $r = 0.994$). There was no significant correlation between HIF-1α protein concentration and cell viability.

DISCUSSION

CoCl₂ induction, HIF-1α, and cytochrome-c

From the present study, we found a strong positive correlations between the increase of CoCl₂ concentration and HIF-1α protein as well as mRNA expression in T47D cells. These results were in consistent with the study conducted by Hendrawan, et al¹⁵ which found that the gene expression of HIF-1α myocardial cells increased gradually in line with the duration of hypoxia and reached a peak at day 21. We also found a strong positive relationship between the concentration of HIF-1α protein and of cytochrome-c in the cytoplasm of T47D cells induced by CoCl₂ and between the expression of HIF-1α mRNA and the concentration of cytochrome-c. These results are consistent with the study of Ardyanto which show that the apoptotic index of myocardial cells increases with duration of hypoxia, although there is no significant association between increased expression of HIF-1α with the increase in the apoptotic index.¹⁶

HIF-1α protein can induce cell apoptotic through two pathways, the p53 pathway and Bcl-2/adenovirus E1B 19 kDa-interacting protein 3 (BNIP3) pathway. P53 protein is a tumor suppressor protein and a transcription factor of various pro-apoptotic genes. P53 protein can be induced by many toxic substances, including gamma radiation, ultraviolet, genotoxic drugs and oxidative stress. Meanwhile, BNIP3 is a pro-apoptotic protein of Bcl-2 proteins family which have only BH3 domain.⁹

HIF-1α protein can increase the stability of p53 protein. The increase of p53 protein stability thus inhibit the anti-apoptotic proteins Bcl-2 and Bcl-XL so that the pro-apoptotic proteins Bax and Bak can be freely increase the permeability of mitochondrial cell membrane. The increased of mitochondrial cell membrane permeability will increase the cytochrome-c removal from mitochondrial intermembrane space to cytoplasm.⁹

Although p53 was mutated in most cancers, HIF-1 α protein can induce apoptosis through BNIP3 pathway. HIF-1 α protein can bind to hypoxia response element (HRE) sequence in the promoter region of BNIP3 gene to increase BNIP3 gene expression.¹⁷ The increase of BNIP3 expression would inhibit anti-apoptosis Bcl-2 and Bcl-XL so that the pro-apoptotic protein Bax and Bak can induce the removal of cytochrome-c from mitochondrial intermembrane space to cytoplasm. The cytochrome-c in the cytoplasm will bind to Apaf-1 protein to form apoptosome body. Apoptosome body will then activate caspase-9, and the active caspase-9 will activate the apoptotic executor caspase-3. Once activated, the apoptotic executor caspase-3 will induce apoptosis.⁹

Despite causing an increase in pro-apoptotic protein cytochrome-c in cytoplasm, the excessive expression of HIF-1 α in breast carcinoma also strongly associated with poor clinical prognosis. The increased expression of HIF-1 α in breast cancer also associated with increased in tumor progression which characterized by excessive vascularization, tumor invasion, tumor size, tumor metastasis, tumor stage and degree of histopatologic.¹⁸

HIF-1 α and survivin

There is strong positive correlation between HIF-1 α protein concentration and survivin mRNA expression in this study. We also found strong positive correlation between HIF-1 α mRNA expression and survivin mRNA expression.

Strong positive correlation between the expression of HIF-1 α and mRNA survivin expression showed that HIF-1 α play a role in increasing the expression of survivin which can cause the inhibition of apoptosis leading to the increase of tumor growth. Peng, et al¹¹ found that HIF-1 α protein can activate the transcription of survivin gene in breast cancer cell line MCF-7 by binding directly to the promoters of survivin genes. The increase of HIF-1 α gene expression can increase the promoter activity of survivin in gene in breast cancer cell line MCF-7, but not in normal breast cell line MCF-10A. The results of this study indicate that the increased expression of HIF-1 α gene is associated with increased expression of survivin in breast cancer cell line T47D.

Expression of survivin appears to have a very large correlation with the expression of Bcl-2 (anti-apoptotic protein) and the decreased apoptotic index in breast cancer.¹⁹ Increased survivin expression is also associated with an increased risk of recurrences, locoregional lymph node invasion and metastasis.¹⁰ Survivin can inhibit caspase-9 activation by cytochrome-c. The consequence of this inhibition on caspase-9 activation is that the apoptotic executor caspase-3 will be stopped so that the apoptosis will be inhibited.¹⁰

The inhibition of HIF-1 α mRNA in lung adenocarcinoma cell line and colorectal carcinoma cell line downregulates the expression of survivin mRNA.²⁰ It is also found that inhibition of survivin *in vitro* and *in vivo* suppressed the potential growth tumor and increased the sensitivity of tumor cells to chemotherapy agents and also inhibited the occurrence of angiogenesis. Inhibition of survivin mRNA expression also halted the growth of mice lymphoma tumor cells.²¹

It has been known that the increased stability of HIF affects mainly the increase in survivin gene expression compared to the release of cytochrome-c into the cytoplasm. Inhibition of HIF is expected to suppress anti-apoptotic protein survivin so that apoptotic will continue. Inhibition of HIF-1 α would also suppress the release of cytochrome-c into the cytoplasm which causes apoptosis does not occur so that cancer cells will continue to proliferate. This cancer cells proliferation can be inhibited by administration of cytotoxic chemotherapy drugs.²² Sekarutami²³ have shown that patients with cervical cancer who received combination of chemoradiotherapy plus hypoxia response modifier therapy (KON) showed the highest response (90.9%) compared to patients who only received chemoradiotherapy (69.2%) or radiotherapy plus KON (63.6%).

HIF and cell viability

Induction of HIF-1 α in this study did not affect the viability of T47D cells. Analysis about the role of HIF-1 α towards the pro-apoptotic protein cytochrome-c and anti-apoptotic survivin in previous studies were conducted in separate studies. In addition, the role of HIF-1 α toward pro and anti-apoptotic proteins has never been studied on T47D breast cancer cells line, but rather on the breast cancer cell line MCF-7. This

study integrates the two factors: the role of HIF-1 α towards pro-apoptotic cytochrome-c and anti-apoptosis in the same cells, ie breast cancer cells line T47D. Thus, the role of HIF-1 α protein with respect to the balance regulation between pro-apoptotic and anti-apoptotic was directly visible from no differences in cells viabilities before and after HIF-1 α induction.

The increasing of pro-apoptotic protein cytochrome-c after induction of HIF-1 α by CoCl₂ administration in this study succeeded in inhibiting the increase of T47D breast cancer cells viabilities. Meanwhile, the increasing of survivin after induction of HIF-1 α by CoCl₂ was succeeded to inhibit T47D cells apoptosis. The end result of pro-apoptotic protein cytochrome-c and anti-apoptotic survivin expression regulation by HIF-1 α is the balance of apoptotic pathway, as shown by the absence of differences in the T47D cells viabilities before and after HIF-1 α induction by CoCl₂.

Based on the above results, it can be concluded that CoCl₂ induction caused an increase of HIF-1 α that were correlated with the increased of pro-apoptotic cytochrome-c protein as well as anti-apoptotic survivin mRNA level. Therefore, the induction of HIF-1 α by CoCl₂ did not cause changes in breast cancer cell viability.

In conclusion, this study shown that HIF-1 α induction have no effects on breast cancer cell line T47D viabilities because of the balance regulation between pro-apoptosis expression cytochrome-c and anti-apoptosis survivin. Therefore, our results motivate careful assessment of the possibility of HIF-1 α as a target therapy in cancer.

Conflicts of interest

All authors have nothing to disclose.

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