UNIVERSITI PUTRA MALAYSIA

EFFECT OF RECOMBINANT HUMAN ERYTHROPOIETIN AND ITS COMBINATION WITH TAMOXIFEN ON VIABILITY OF MCF-7 CELLS IN THREE-DIMENSIONAL CULTURE

HARETH YAHYA AHMED SHUJAAEDIN

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By

HARETH YAHYA AHMED SHUJAAEDIN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

May 2019

DEDICATION

To my mother, Taqiah, my father, Yahya, my wife, Asma'a, my sons, Mohammed, Akram, and Hamzah, and my daughter, Noran

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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Chairman : Professor Rasedee bin Abdullah, PhD
Faculty : Institute of Bioscience

Anemia is often a side-effect of cancers. Erythropoietin (EPO) is the erythropoiesis-stimulating hormone produced by the kidneys that is used in the treatment of anemias. This hormone is being used concurrently with chemotherapeutic drugs in the treatment of cancers. From previous studies, EPO alone or combined with cancer therapeutics showed inconsistent effects on cancer cells viability in the two-dimensional (2D) cell cultures. Unlike in 2D cell cultures, the effects of drugs on three-dimensional (3D) cell cultures mimic their effects on live tissues. Currently, there is no study that determined the effect of EPO alone or combined with tamoxifens on MCF-7 cell in 3D cultures (spheroids). Thus, the general objective of the study was to determine the effect of recombinant human EPO (rHuEPO) alone and in combination with tamoxifen (rHuEPO-tamoxifen combination) on the viability of MCF-7 breast cancer cell spheroids. The specific objectives of the study were to generate stable *in vitro* MCF-7 spheroids, determine the effects of rHuEPO and rHuEPO-tamoxifen combination treatments on the viability and MCF-7 cell cycle phase of the spheroids. MCF-7 cells were grown in monolayer culture until 85 % confluency was reached. Then, the MCF-7 spheroids were generated by the conventional hanging drop (CHD) combined ultra-low adhesive plate (ULAT). The MCF-7 spheroids were then treated with 0.1, 3.12, 6.25, 10, 12.5, 25, 50, 100, and 200 IU/mL rHuEPO, 10 µg/mL (IC_{50}) tamoxifen, or 10 µg/mL tamoxifen in combination with 10, 100 or 200 IU/mL rHuEPO for 24, 48 or 72 h. The effects of rHuEPO and combination rHuEPO-tamoxifen treatments on MCF-7 spheroids were determined using the (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay, neutral red retention, trypan blue exclusion assay, DNA fragmentation, acridine orange/propidium iodide staining, caspases activation assays, and cell cycle assay and flow cytometry analyses after staining with propidium iodide. Treatment with rHuEPO, at high concentrations of 10, 50, 100, and 200 IU/mL decreased cell viability and ($p<0.05$) caspase-3, -8, or -9 activities in the MCF-7 spheroids in dose-dependent manner. Treatment with rHuEPO alone increased the population of MCF-7 cells in the subG1/G0 phase also in dose- and time-dependent

manner. Based on the annexin-V and cell cycle assays, tamoxifen and the rHuEPO-tamoxifen combination treatments both caused the MCF-7 cells in the spheroids to undergo apoptosis. However, the cytotoxic effect of rHuEPO-tamoxifen combination was more intense than the effect of tamoxifen alone. Although treated cells showed morphological characteristics of apoptosis, the caspase activities were downregulated after rHuEPO-tamoxifen combination treatments, suggesting that the MCF-7 cell death was not associated with caspase activities. In conclusion, the study showed that the antiproliferative effects of rHuEPO toward the MCF-7 cells is via cytostasis. This study also suggests that rHuEPO is not only safe for use with chemotherapeutic drugs to treat cancer-related anemias, but also potentiate the toxic effects of tamoxifen toward breast cancer cells.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

KESAN ERITROPOIETIN MANUSIA REKOMBINAN DAN GABUNGANNYA DENGAN TAMOKSIFEN TERHADAP KEBOLEHHIDUPAN SEL MCF-7 DALAM KULTUR TIGA-DIMENSI

Oleh

HARETH YAHYA AHMED SHUJAAEDIN

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Anemia kerap merupakan kesan sampingan kanser. Eritropoietin (EPO) adalah hormon perangsang eritropoiesis yang dihasilkan oleh ginjal, diguna merawat anemia. Hormon ini di guna bersama drug kemoterapi dalam rawatan kanser. Daripada kajian yang lepas, EPO secara bersendirian atau bergabung dengan terapeutik kanser menunjukkan kesan yang tidak konsisten terhadap kebolehhidupan sel kanser dalam kultur sel dua-dimensi (2D). Bukan seperti dalam kulture sel 2D, kesan drug terhadap kultur sel tiga-dimensi (3D) menyerupai kesannya pada tisu hidup. Kini, tiada kajian yang menentukan kesan EPO bersendirian atau tergabung dengan tamoxifen terhadap sel MCF-7 dalam kultur 3D (sferoid). Justeru, objektif umum kajian ini ialah untuk menentukan kesan EPO manusia recombinan (rHuEPO) secara bersendirian dan bergabung dengan tamoksifen (rHuEPO-tamoksifen penggapungan) terhadap kebolehhidupan sferoid sel kanser payudara MCF-7. Objektif khusus kajian ialah untuk menjanakan sferoid MCF-7 *in vitro* yang stabil, menentukkan kesan perlakuan rHuEPO dan rHuEPO-tamoksifen terhadap kebolehhidupan, dan fasa kitaran sel MCF-7 dalam sferoid. Sel MCF-7 ditumbuhkan dalam kultur monolapisan hingga mencapai konfluensi 85 %. Kemudian, sferoid MCF-7 dijanakan mengguna kaedah titis tergantung konvensional (CHD), gabungan plat lekatan ultra-rendah (ULAT). Sferoid MCF-7 ini diperlakukan pula dengan 0.1, 3.12, 6.25, 10, 12.5, 25, 50, 100, and 200 IU/mL rHuEPO, 10 µg/mL (IC_{50}) tamoksifen, atau 10 µg/mL tamoksifen secara gabungan dengan 10, 100 atau 200 IU/mL rHuEPO selama 24, 48 atau 72 h. Kesan perlakuan rHuEPO dan gabungan rHuEPO-tamoksifen terhadap sferoid MCF-7 ditentukan menguna assai (4,5-dimetiltiazol-2-il)-2,5-difenil tetrazolium bromida, pengekalan merah neutral, penyisihan biru tripan, fragmentasi DNA, pewarnaan oren akridina/propidium iodida, assai pengaktifan kaspase, dan assai kitaran sel, dan analisis sitometri aliran selepas pewarnaan propidum iodida. Perlakuan dengan rHuEPO pada kepekatan tinggi 10, 50, 100, dan 200 IU/mL mengurangkan kebolehhidupan sel dan aktiviti kaspase-3, -8, -9 dalam sferoid MCF-7 secara bersandarkan dos. Perlakuan dengan rHuEPO sahaja meningkatkan populasi sel MCF-7 pada fasa subG1/G0 secara

bersandarkan dos and masa. Berasaskan assai annexin-V dan kitaran sel, perlakuan tamoksifen dan gabungan rHuEPO-tamoksifen menyebabkan sel MCF-7 dalam sferoid untuk mengalami apoptosis. Bagaimanapun, kesan sitototoksik gabungan rHuEPO-tamoksifen lebih kuat daripada kesan tamoksifen secara bersendirian. Walaupun sel terperlaku menunjukkan ciri morfologi apoptosis, aktiviti kaspase diturun pengawalaturannya selepas perlakuan gabungan rHuEPO-tamoxifen, menyarankan kematian sel MCF-7 ini tiada kaitan dengan aktiviti kaspase. Kesimpulannya, kajian ini menunjukkan kesan antipemproliferatan rHuEPO terhadap sel MCF-7 ialah melalui sitostasis. Kajian ini juga menyarankan rHuEPO bukan sahaja selamat untuk diguna secara gabungan dengan drug kemoterapeutik dalam rawatan anemia berkaitan kanser, tapi juga memperkuatkan kesan toksik tamoksifen terhadap sel kanser payudara.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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AIF	Apoptotis-inducing factor
Akt	Protein kinase B (PKB)
Apaf-1	Apoptosis protease-activating factor-1
Bax	bcl-2-like protein 4
Bcl-xL,	B-cell lymphoma-extra large
℃	Degrees centigrade
CC	Correlation coefficient
CD34	Human stem cells
CD8+	Cytotoxic T cells
CEPO	Carbamylated Erythropoietin
CFU-E	Colony forming unit erythroid
CFU-Es	Colony forming unit erythroids
CHD	Conventional hanging drop
c-IAP2	Baculoviral IAP repeat-containing protein3
CICD	Caspase-independent cell death
CKD	*Chronic kidney disease*
CUT	The resultant technique from CHD and ULAT techniques combination in this thesis
CV	Coefficient of variance
Cys161	Cysteine 161
Cys29	Cysteine 29
Cys33	Cysteine 33
Cys7	Cysteine 7
DOL	Developed overlay
2D	Two dimensional

3D	Three dimensional
ECM	Extracellular matrix
ECRB	Eppendorf A-4-62 centrifuge rotor 1 MTP buckets
EPO	Erythropoietin
EPOR	Erythropoietin receptor
EPORs	Erythropoietin receptors
EPO-TAMNLC	Nanostructured lipid carrier coated EPO
ER	Estrogen receptor
ERK	Extracellular signal–regulated kinase
ESBR	Eppendorf swing-bucket rotor with tubes rack
FBS	Fetal bovine serum
FGF	Fibroplast growth factor
Fig	Figure
Flt-1	Vascular endothelial growth factor receptor 1 gene
g	Gravitational force
GMD	Geometric mean diameter
h	Hour
HDFa	Human dermal fibroblasts, adult
HIF1-α	Hypoxia inducible factor alfa
HIV	Human immunodeficiency viruses
HPC	Hematopoietic precursor cells
Hsp70	70 kilo Dalton heat shock proteins
IC_{50}	Half maximal inhibitory concentration
iFBS	Inactivated fetal bovine serum
IκB	*kappa*-B *kinase*
IL-8	Interleukin 8

JAK2	Janus kinase 2
Kd	kilo Dalton
LFM-13	Bruton′s tyrosine inhibitor
MAPK	mitogen-activated protein kinase
MCF-7	Michigan Cancer Foundation-7
MDA-MB-231	M.D. Anderson metastasis Breast cancer
min	Minutes
mL	Millimeter
µg	Microgram
µL	microliter
µm	micrometer
mm^3	Milliliter cube
MMP-2	Matrix metalloproteinase-2
mRNA	Messenger Ribonucleic Acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NFκB	Nuclear factor-kappa B
NSCLC	Non-small cell lung cancer
OD	Optical density
PBS	Phosphate buffer saline
PI3K	Phosphoinositide 3-kinases
PKB	Protein kinase B
Poly-HEMA	Poly(2-hydroxyethyl methacrylate
R	radius
Ras	Small G protein family
rHuEPO	Recombinant Human Erythropoietin
RPMI1640	Roswell Park Memorial Institute medium

S	surface area
SCa-1	Stem cells antigen-1
SD	Standard deviation
SERM	Selective estrogen receptor modulators
STAT5	Signal transducer and activator of transcription
Tam	tamoxifen
TF	Transcription factor
ULAT	Ultra-low adhesion technique
V	volume
VEGF	vascular endothelial growth factor
XIAP	X-linked inhibitor of apoptosis protein

CHAPTER 1

INTRODUCTION

Breast cancer is the highest prevalence cancer among females. It affects approximately 2.1 million women annually. It is the major cause of cancer-related deaths in women. While the women in the developed country are most affected, the prevalence rate of breast cancer are continuously increasing worldwide. The incidence rate of breast cancer in females is about 18% of all women cancer (Akram *et al*., 2017); ("WHO | Breast cancer," n.d.).

Unlike normal cells, cancer cells defeat growth suppressor genes and acquire the ability for prolonged uncontrolled proliferation. As in any type of cancer, such neoplastic change leads up to lose the normal function and structure of the breast tissue. Breast cancer might develope either from myoepithelial, epithelial or a stem cell generating myoepithelial or epithelial cancer cells. Numerous reasons and predisposing factors contribute in breast cancer occurrence (Gusterson *et al*., 1982); (Graña and Reddy, 1995); (Evan and Vousden, 2001).

Estrogen has a central role in breast cancer incidence. Over exposure of cells to estrogen always associated with breast cancer that characterized by DNA damage and gene alteration. Estrogen exerts this effect by binding estrogen receptor in breast cells. Blockade of estrogen receptor (ER) with an estrogen antagonist agent will abolish estrogen effect. Selective estrogen receptor modulators (SERM) are exogenous molecules that have the ability to bind estrogen receptors in breast cells. They can antagonize estrogen and eliminate its effect. Tamoxifen is the most common drug among SERM which is widely used in treatment of ER-positive breast cancer (Riggs and Hartmann, 2009).

Breast cancer patients affected by anemia as a result of tamoxifen treatment or the disease itself (Leonard et al., 2005).

The glycoprotein hormone, erythropoietin, is one of the most important cytokines in the maintenance of homeostasis. The primary function of EPO is in the regulation and stimulation bone marrow production of erythrocytes, the oxygen carrier in blood circulation. Erythropoietin is secreted mainly by the kidneys as a consequence of hypoxia, as in anemia. The recombinant form of EPO, Recombinant EPO (rHuEPO) is primarily used for the correction and prevention of anemia. This drug is also commonly used to alleviate cancer- and chemotherapy-related anemias.

The effect of EPO is partially governed by oxygen level in the cellular environment. In hypoxia, the hypoxia-inducible factor-1α (HIF-1α) become upregulated, which in turn triggers EPO action (Wang and Semenza, 1993). On cells, EPO acts through the EPO receptors (EPOR). The expression of EPO receptor (EPOR) is higher in cells under hypoxic than normoxic condition (Ribatti, 2010). Therefore, the EPO-EPOR binding is high in hypoxic state, a condition that occurs at the core of tumor tissues.

Although, erythroid precursors are rich in EPOR, these receptors are not confined to hematopoietic cells only. Other normal tissues, such as endothelial, smooth muscle, and the nervous system cells also express EPORs (Ogunshola and Bogdanova, 2013). The receptors are also found in numerous tumors; thus, it was suggested that EPO treatment may post risks to cancer patients (Chan *et al*., 2017). Although, some researches had claimed that EPO may be pro-proliferative for cancer cells (Acs *et al*., 2001), there are other studies suggested that EPO or its derivatives may not adversely affect cancer growth (Gewirtz *et al*., 2006; Belda-Iniesta *et al*., 2007; Sairah *et al*., 2009) but may in fact show anticancer effects (Våtsveen *et al*., 2016; Beh *et al*., 2017).

It is premature at this time to conclude that rHuEPO can safely be used together with chemotherapeutics in the treatment of cancers. However, earlier studies in our laboratory seemed to suggest that rHuEPO is not only safe but also may potentiate the antiproliferative effects of anticancer drugs. Erythropoietin elevated the anticancer effects of doxorubicin by increasing the activities of caspase-3/7 and -9 in breast cancer cell lines (Radwan *et al*., 2016). When EPO was incorporated in tamoxifen-loaded nanostructured lipid carriers, the formulation enhanced the cytotoxic effect of tamoxifen on MCF-7 cells (Beh *et al*., 2017). The EPOR are not only located on the cell membrane also around the nucleus (Beh *et al*., 2019), indicating EPO can bypass the surface receptor mediated action and directly exert effects on the nucleus. These are evidences of the usefulness of EPO in the treatment of cancers that only requires further clinical trial verifications using appropriate treatment conditions.

The benefits of using EPO in the treatment of anemia due to cancers still outweighs the debatable claim that it is risky to cancer patients (Littlewood, 2001; Lundholm *et al*., 2004; Gupta *et al.*, 2010), although these studies recommended caution when using to treat cancer patients (Belenkov *et al*., 2004; Henke *et al*., 2006; Phillips *et al*., 2007). However, erythropoietin enhanced treatment outcome in combined immunodeficient mice bearing small subcutaneous tumors (Silver and Piver, 1999). The study showed greater improvement in tumor regression with combination EPO-cisplatin treatment than with cisplatin alone. In fact, mice treated with EPO-cisplatin group appeared healthier than those treated with cisplatin only. The synergic effects between EPO and chemotherapeutic was also shown by Sigounas *et al*., (2004). In their study, it was shown that using EPO in combination with cisplatin, mitomycin C or cyclophosphamide to treat animals bearing Lewis lung carcinoma produced synergistic effects between EPO and anticancer agents in the suppression of tumor growth.

The differences of effect shown with EPO treatment seem to be due to various factors such as disease stage, patient history, tumor origin or cell line, anticancer agent used, and experimental design. The type of erythropoietin derivatives used and the cellular expression of EPO receptors may also count for the difference in the effects of EPO (Trincavelli *et al*., 2013). In addition, the dose of EPO or derivatives used may contribute to the variability of results produced by various studies. For example, on the rodent mammary adenocarcinoma, treatment with rHuEPO in the presence of Taxol (paclitaxel) for 24 h showed variable results that was dependent on concentration of rHuEPO, with the number of apoptotic cells decreasing with increase in rHuEPO (Hardee et al., 2006).

The three-dimensional (3D) cancer cell cultures, because it closely approximates *in vivo* tumors are now gaining popularity as an *in vitro* model in cancer studies. The 3D cancer cell or spheroid cultures has the mass and some complexity of the *in vivo* tumors that is not present in the 2D cultures. The 3D microenvironment construction also mimics *in vivo* tumor mainly by the presence of the hypoxic core, thus, the cancer spheroid is a compatible model as model in the investigation the effect EPO on the MCF-7 cancer cells.

1.1 Problem statement

As aforementioned above, the 3D cell culture model closely reflects the *in vivo* tumors and it is a compatible model to investigate EPO effect on the MCF-7 cancer cells, though, until the moment there is no study that showed the effect of rHuEPO treatment on cell viability, cell cycle and sensitivity to tamoxifen of MCF-7 cells grown in this model.

1.2 Hypothesis

Treatment of MCF-7 spheroids with rHuEPO or rHuEPO-tamoxifen combination will significantly reduce the cell viability comparing to untreated or tamoxifen alone-treated cells respectively.

1.3 General objectives of the study

The general objective of the study was to determine the effects of rHuEPO and rHuEPO-tamoxifen combination treatment on 3D MCF-7 cell culture viability.

1.4 Specific objectives of the study

The specific objectives are to:

1. generate a stable *in vitro* 3D MCF-7 cell culture.
2. determine the effects of rHuEPO treatments on viability and cell cycle phase of MCF-7 cells from 3D cultures.
3. determine the effect of rHuEPO on the sensitivity of MCF-7 cells from 3D cultures to tamoxifen.

1.5 Expected results

The three-dimensional (3D) cancer cell cultures create a central hypoxic mass. Under hypoxic condition, hypoxia-inducible factor (HIF1-α) is activated which followed by overexpression of the vascular endothelial growth factor (VEGF). The simultaneous overexpression of both factors brings tumors aggressiveness phenotype, angiogenesis, metastasis, and resistance to apoptotic activity (Semenza *et al*., 2003; Hamdan and Zihlif, 2014; Zuazo-Gaztelu and Casanovas, 2018). Furthermore, VEGF produced by MCF-7 breast cancer cells stimulates cell growth by an autocrine pathway *in vitro* (Guo *et al*., 2003; Jun *et al*., 2017). Targeting HIF1-α by inhibitory agents will reduce the cancer aggressiveness. Erythropoietin was evident to inhibit HIF1-α and VEGF transcription in both MCF-7 breast and ovarian cancer cells *in vitro* (Hale *et al*., 2006). Thus, in the study, rHuEPO is expected to inhibit HIF1-α and subsequently bridle its effects which, in part, will translated to cell viability reduction and sensitivity increase to tamoxifen treatments.

1.6 Conclusion

The use of EPO in the treatment of cancers-associated and chemotherapy-induced anemia has raised concerns. Although, some studies suggested that EPO promotes cancer cell proliferation and tumor growth, there are others that did not show these effects. In fact, the results from the study conduct in our own laboratory suggest that, with the right condition and appropriate time in application, EPO is not only innocuous to cancer cells but may be may potentiate the therapeutic effects of anticancer agents.

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LIST OF PUBLICATIONS

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