EFFECT OF OXIDIZED MULTIWALLED CARBON NANOTUBES AND HYPERTHERMIA TREATMENT ON EMT6 TUMOR MICROENVIRONMENT

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DEDICATION

This thesis is dedicated to my father, who taught me that the best kind of knowledge to have is that which is learned for its own sake. It is also dedicated to my mother, who taught me that even the largest task can be accomplished if it is done one step at a time.

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ABSTRACT

Tumor microenvironment has been identified as a crucial player in influencing anti-tumor immune response in breast cancer. Alternative treatment using combination of carbon nanotubes (CNTs) with hyperthermia (HT) has been reported to reduce tumor burden. However, its immunological mechanisms in tumor microenvironment is not yet fully understood. Hence, this study was conducted to investigate the effect of combination of ox-MWCNT and hyperthermia treatment on immune responses in tumor microenvironment. In this study, EMT6 breast cancer cells were inoculated subcutaneously into right flank of female Balb/c mice. At day 7 post-inoculation, ox-MWCNT were injected intratumorally in CNT and combined groups. Then, mice in combined and HT were subjected to local HT for three consecutive days. Mice for control group was left untreated. Mice were euthanized 10 days post-inoculation and their lymph nodes were harvested for flow cytometric (FACS) analysis while the tumors were subjected for immunohistochemistry (IHC) analysis. In another experiment, mice were euthanized 21 days post-inoculation and tumors were harvested for FACS analysis. Results from this study demonstrated that the infiltration and maturation of DCs in lymph nodes increased in combined treated groups. In addition, FACS analysis of tumor cells showed that combined treatment significantly increased the infiltration of $CD8^+$ and $CD4^+$ T cells, natural killer (NK) cells and macrophages. Furthermore, combined and HT alone has shown to drastically decrease Tregs population in tumor. On the other hand, data from the IHC studies indicated that angiogenesis activity increased in combination therapy but had less influence on inducing tumor progression. Data presented in this study highlights the potential therapeutic use of ox-MWCNT with HT treatment against breast cancer. Information gained from this research may contribute to understand on the effect of combined treatment on tumor microenvironment and may have implications in breast cancer management in clinical setting.

ABSTRAK

Persekitaran mikro tumor telah dikenal pasti sebagai pemain utama dalam mempengaruhi tindak balas imun anti-tumor dalam kanser payudara. Rawatan alternatif menggunakan gabungan nanotiub karbon (CNTs) bersama hipertermia (HT) telah dilaporkan dapat mengurangkan beban tumor. Walaubagaimanapun, mekanisme imunologi dalam persekitaran mikro tumor masih belum difahami sepenuhnya. Justeru, kajian ini dijalankan untuk mengkaji kesan rawatan hipertermia dimediasi oleh ox-MWCNT terhadap tindak balas imun dalam persekitaran mikro tumor. Dalam kajian ini, sel kanser payudara EMT6 telah diinokulasi secara subkutaneus di sebelah kanan badan mencit Balb/c betina. Pada hari ketujuh inokulasi, ox-MWCNT disuntik dalam tumor mencit kumpulan CNT dan gabungan. HT setempat dijalankan pada mencit kumpulan gabungan dan HT untuk tiga hari berturut-turut. Mencit untuk kumpulan kawalan dibiarkan tanpa rawatan. Mencit dikorbankan 10 hari selepas inokulasi dan nod limfa diambil untuk analisis sitometri aliran (FACS) manakala tumor diambil untuk analisis imunohistokimia (IHC). Dalam kajian lain, mencit dikorbankan 21 hari selepas inokulasi dan tumor diambil untuk analisis FACS. Keputusan dari kajian ini menunjukkan bahawa penyusupan masuk dan kematangan DC di nod limfa meningkat dalam kumpulan rawatan gabungan. Tambahan pula, analisis FC untuk sel tumor menunjukkan rawatan gabungan meningkatkan signifikan penyusupan masuk sel $CD8^+$ dan $CD4^+$ T, sel pembunuh semula jadi (NK) dan makrofaj. Di samping itu, gabungan dan HT sahaja menunjukkan pengurangan ketara populasi Tregs dalam tumor. Selain itu, data daripada analisis IHC menunjukkan bahawa aktiviti angiogenesis meningkat dalam terapi gabungan tetapi kurang berpengaruh dalam mendorong perkembangan tumor. Data yang dikemukakan dalam kajian ini menengahkan potensi terapeutik penggunaan ox-MWCNT dalam rawatan HT sebagai pendekatan terapeutik terhadap kanser payudara. Informasi yang didapati daripada kajian ini mungkin menyumbang kepada pemahaman tentang kesan rawatan menggunakan ox-MWCNT dan HT dalam mikro persekitaran mikro tumor dan mungkin mempunyai implikasi dalam pengurusan kanser payudara dalam persekitaran klinikal.

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LIST OF ABBREVIATIONS

HT	-	Hyperthermia
WHO	-	World Health Organization
IARC	-	International Agency for Research on Cancer
Hsp	-	Heat shock protein
CNT	-	Carbon nanotubes
SWCNT	-	Single-walled carbon nanotubes
MWCNT	-	Multiwalled carbon nanotubes
Ox-MWCNT	-	Oxidized MWCNT
UTM	-	Universiti Teknologi Malaysia
NK	-	Natural Killer
NIR	-	Near-infrared radiation
VEGF	-	Vascular endothelial growth factor
MMP-9	-	Matrix metalloproteinase 9
UKM	-	Universiti Kebangsaan Malaysia
AEC	-	Animal Ethics Committee
ICD	-	International Classification of Diseases
BRCA	-	Breast cancer gene
MRI	-	Magnetic Resonance Imaging
SPION	-	Super-paramagnetic iron oxide nanoparticles
FDA	-	Food and Drug Administration
ROS	-	Reactive oxygen species
ECM	-	Extracellular matrix
TGF-β1	-	Transforming growth factor beta 1
MHC	-	Major histocompatibility complex
IL	-	Interleukin
IFN	-	Interferon
TNF	-	Tumor necrosis factor
TIL	-	Tumor infiltrating lymphocytes
DC	-	Dendritic cell
Treg	-	Regulatory T cell

CD	-	Cluster of Differentiation
FoxP3	-	Forkhead/flanking helix nuclear transcription factor
LN	-	Lymph nodes
dLN	-	Draining lymph nodes
CCR7	-	CC-chemokine receptor 7
CO_2	-	Carbon dioxide
FBS	-	Fetal bovine serum
PBS	-	Phosphate-buffered saline
NaCl	-	Sodium chloride
i.p.	-	Intraperitoneal
s.c.	-	Subcutaneous
i.t.	-	Intratumoral
rpm	-	Rotation per minute
^oC	-	Degree Celsius
hr	-	Hour
min	-	Minute
g	-	Gram
mg	-	Milligram
µL	-	Microlitre
nm	-	Nanometre
mL	-	Millilitre
M	-	Molar
mg/mL	-	Milligram per millilitre
mm^2	-	Square millimetre
PI	-	Propidium iodide
NEDB	-	Non-enzymatic dissociation buffer
AF	-	Alexa Fluor
PE	-	Phycoerythrin
APC	-	Allophycocyanin
FSC	-	Forward scatter
SSC	-	Side scatter
KX	-	Ketamine/xylazine
HCl	-	Hydrochloric acid

FFPE	-	Formalin-fixed paraffin embedded
TBS	-	Tris-buffered saline
DAB	-	3,3-diaminobenzidine
APC	-	Antigen presenting cells
CAFs	-	Carcinoma-associated fibroblasts

LIST OF SYMBOLS

n	-	number
P	-	p-value
ns	-	Non-significant

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CHAPTER 1

INTRODUCTION

1.1 Research Background

Breast cancer is responsible for the leading cause of death among females. According to the International Agency for Research on Cancer (IARC), breast cancer is ranked fifth as the global cause of death which involved 627,000 death in 2018 (World Health Organisation, 2018). Current therapeutic approaches include surgery, chemotherapy, and radiotherapy are however not necessarily effective in treating cancer. The incompetence of current breast cancer treatment has become the major factor for these unfavorable progressions. The difficulties in treating cancer are due to its distinctive immune evasion, metastases, and resistance to cancer therapies (Yagawa et al., 2017). The therapeutic effects of current cancer treatment are also affected by post-treatment infections, inefficient drug delivery to tumor site, therapeutic drug resistance and tumor relapse (Lee et al., 2018). Collectively, these negative drawbacks have increased the urgency to search for effective treatment with minimal risk. Hence, researchers are interested on finding cancer therapies that can promote therapeutic effect and reduce adverse side effects. These can be achieved by hyperthermia treatment which possessed promising potentials in enhancing therapeutic efficacy without causing detrimental side effects.

Hyperthermia or thermotherapy is considered as minimally invasive procedure in treating cancer through heat induction. This treatment increases body temperature from normal temperature to fever range between 40^{o}C to 44^{o}C which could be applied either regionally or whole body (Behrouzkia et al., 2016; Evans et al., 2016). In addition, hyperthermia is also reported for wound healing application (Ibelli et al., 2018) and osteoarthritis treatment (Jeziorski, 2018). Surprisingly, hyperthermia treatment also has the potential in reducing tumor burden by targeting the tumor tissue and activates the immune system simultaneously (Evans et al., 2016; Cheng et al.,

2019). Furthermore, heat induction may upregulate anti-tumor response by inducing the expression of heat shock protein (Hsp), necrosis in the tissue, and reducing tumor vascularization (Xie et al., 2011; Kargozar et al., 2020).

The integration of CNTs in hyperthermia treatment is known to be beneficial in cancer therapy due to its versatile physiochemical properties. Due to its intrinsic properties such as good thermal conductor, minimally invasive, harmless, and highly effective, CNT has been exploited to be integrated in thermal therapy application for example HT treatment. Despite its effect in reducing tumour burden, hyperthermia is reported to have an uneven heat distribution, which reduces treatment efficiency. There is evidence of nanoparticles-mediated hyperthermia which improves heat uniformity. CNTs emerged as a promising nanoparticle used in improving hyperthermia efficiency due to its versatile characteristics. This includes its thermal and electrical conductivities (Sanginario et al., 2017). Combination of carbon nanotubes in hyperthermia treatment facilitates and maintains uniform thermal distribution on the targeted tumor tissue. Studies also reported that HT treatment combined with CNTs prolonged the survival rate and inhibit the tumour progression in tumor-bearing mice (Sanginario et al., 2017; Radzi, 2019).

It has been established in our laboratory that CNT-mediated hyperthermia treatment eliminates tumor burden in EMT6 breast tumor (Radzi, 2019). This promising outcome prompted us to further investigate the influence of immune cells and microvasculature within the tumor microenvironment thus contributing to the anti-tumor effect. Therefore, the aim of this study is to investigate the effect of hyperthermia treatment combined with CNTs on the tumor microenvironment in promoting anti-tumor immune responses.

1.2 Problem Statement

Tumor microenvironment consists extracellular matrix and different types of cells such as endothelial cells composing tumor vasculature, immune and inflammatory cells (Belli et al., 2018). Tumor microenvironment is recognized as an

important contributor for tumor development. It is based on a complex series of biological events which result in uncontrolled growth and resistance to tumor cell eradication. As tumor cells continue to proliferate, the tumor size increases with an associated change of the tumor microenvironment (Roma-Rodrigues et al., 2013). The interaction between cancer and immune cells results in further alteration of cellular components, extracellular matrix restructuration and chaotic vascularization formation in the tumor microenvironment (Fang and DeClerck, 2013). Understanding the composition of tumor microenvironments which changes during tumor growth allows for the development of effective cancer treatments.

Hyperthermia modifies the tumor microenvironment to control tumor growth and relapse. Studies indicated that physiological responses to heat affected the tumor microenvironment by upregulating the recruitment of immune cells such as T cells, NK cells and macrophages into the tumor microenvironment (Baronzio et al., 2014) while suppressing the expression of vascular endothelial growth factor (VEGF) (Sawaji et al., 2002). Despite these encouraging findings, consistent heat distribution inside tumors is difficult to accomplish because of inadequate heat localization in tumor and short heat retention time. The efficiency of heat delivery throughout the tumor cell can also be affected by the absorption of heat energy by the normal tissue that unintentionally caused a non-specific damage to the healthy neighboring tissue (Cheng et al., 2019). Hence, CNT is suggested as a potential candidate to address the limitations encountered in this treatment.

Despite numerous studies which proved that CNT-mediated hyperthermia reduces tumor burden, understanding of tumor microenvironment following the treatment is inadequate. There is a need for further investigation to elucidate the immune response mechanisms. It is of interest to see how CNT-mediated hyperthermia treatment alters the tumor microenvironment which contributes to anti-tumor response.

1.3 Research Objectives

The followings are the proposed objectives for this study:

(a) To investigate the effect of HT and CNTs treatment on dendritic cells activation of immune cells in the lymph nodes by flow cytometric analysis.

(b) To assess the infiltration of immune cells in the tumour microenvironment following treatment with hyperthermia and CNTs using flow cytometric analysis.

(c) To determine the expression of angiogenesis markers in the tumour microenvironment following the treatment of hyperthermia and CNTs using immunohistochemical analysis.

1.4 Scope of the Study

This study focused on the therapeutic effect of hyperthermia treatment in combination with carbon nanotubes (CNTs) for the treatment of breast cancer. This research involved inoculating EMT6 breast cancer cells into the animal model (murine) followed by administration of carbon nanotubes into the tissue and treatment with a near-infrared radiation (NIR). The study includes the flow cytometric analysis of lymph nodes and tumor. In addition, immunohistochemical analysis of the tissue of the tumor were also conducted. The protocols involved are approved protocol by the Universiti Kebangsaan Malaysia Animal Ethics Committee (UKM AEC; code 65/2019).

1.5 Significance of the Study

The goal of this study is to explore CNT-mediated hyperthermia treatment as alternative breast cancer treatment. This study highlights benefits of CNT-mediated

hyperthermia in improving anti-tumor response which lead to inhibition of tumor growth, enhances anti-tumor response, and subsequently reduces the mortality rate. Fundamental knowledge from this project may have a direct implication for breast cancer treatment in clinical settings. This research highlights the benefit of non-invasive nanomaterials-mediated treatment, which will reduce economic burden in breast cancer cases.

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