CHARACTERIZATION OF LIPOSOME-ENCAPSULATED TYLOSIN, *IN VITRO* CYTOTOXICITY AND ITS ANTIBACTERIAL ACTIVITY AGAINST *Corynebacterium pseudotuberculosis* ISOLATED FROM GOATS

By

MOHAMMAD EHSAN SADDIQI

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Veterinary Science

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Chairman : Associate Professor Arifah Abdul Kadir, PhD
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Corynebacterium pseudotuberculosis is the causative agent of caseous lymphadenitis in goats and it is highly resistant to many conventional antibiotics. Delivery of antibiotics through liposomal encapsulation provides a promising strategy for the effective treatment of resistant bacterial pathogens. The objectives of the study are to characterize, evaluate cytotoxicity and efficacy of liposomal tylosin against *C. pseudotuberculosis* isolated from goats. Liposomes-encapsulated tylosin was produced by the conventional thin-film hydration method. The prepared liposomal dispersions particle size, polydispersity index (PDI), and zeta potential were measured by dynamic light scattering. Cytotoxicity effect of liposome-encapsulated tylosin and free tylosin was evaluated against the normal mouse fibroblast and normal human dermal fibroblast primary cells using MTT assay. The antibacterial activity of free and liposomal tylosin against *C. pseudotuberculosis* was performed using microbroth dilution and resazurin methods and the antibiofilm activity was evaluated using minimum biofilm inhibitory concentration and minimum biofilm eradication concentration (MBEC) by crystal violet and resazurin-based colourimetric methods. The mean diameter of 171.6 ± 3.22 nm was obtained for liposomal tylosin with 0.236 ± 0.002 and -4.75 ± 1.6 mV of PDI and zeta potential, respectively. The encapsulation efficiency of liposomal tylosin was 47.7 ± 2.8%. The release behaviour of tylosin from the conventional liposomes is based on initial fast release followed by sustained and slow-released as compared to free tylosin. Cytotoxicity findings revealed higher cell viability (above 70%) for liposomal tylosin and below 70% for free tylosin at a high concentration of 1024 µg/mL. The MIC of conventional, cationic, fusogenic and fuso_cationic tylosin was 32 µg/mL which is much higher than free tylosin with the MIC of 1 µg/mL. In contrast, the MBEC was 1024 µg/mL, 256 µg/mL, 512 µg/mL and 1024 µg/mL for conventional, cationic, fusogenic, and fuso-cationic, respectively, whereas it was 2048 µg/mL for free tylosin. Cationic, fusogenic, and fuso-cationic liposomal tylosin had a mean diameter of

114.8 ± 0.115 nm, 114.4 ± 0.62 nm, and 117.7 ± 1.08 nm, respectively. The cationic liposomal tylosin revealed the average PDI and zeta potential of 0.135 ± 0.014 and 35.3 ± 1.9 mV, respectively, whereas, the mean size of 114.4 ± 0.62 nm, PDI of 0.16 ± 0.019, and zeta potential of -28.6 ± -0.115 mV was recorded for the fusogenic formulation. The fuso-cationic liposomal tylosin showed an average of 117.7 ± 1.08 nm, 0.155 ± 0.01, and 21.83 ± 0.21 mV for size, PDI, and surface charge, respectively. The encapsulation efficiency of cationic, fusogenic, and fuso-cationic liposome-encapsulated tylosin were 34.26 ± 1.15%, 46.63 ± 0.6%, and 41.36 ± 1.2%, respectively. In conclusion, the findings indicate that the liposome as a nanoparticle improved the biocompatibility of tylosin and enhanced the antibacterial activity of tylosin, therefore, it can be considered as a potential strategy to revive the routine antibiotic efficacy against resistant bacteria.

Keywords: Caseous Lymphadenitis, Biofilm, Liposomes, Cytotoxicity, Tylosin

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan ijazah Master Sains Veterinar

PENCIRIAN, SITOTOKSISITI *IN VITRO* DAN AKTIVITI ANTIBAKTERIA TILOSIN BERKAPSULASI LIPOSOM TERHADAP *Corynebacterium pseudotuberculosis* DIISOLASI DARIPADA KAMBING

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Corynebacterium pseudotuberculosis adalah agen penyebab limfadenitis kaseous pada kambing dan ianya sangat tahan terhadap banyak antibiotik konvensional. Penghantaran antibiotik melalui enkapsulasi liposomal merupakan strategi yang menjanjikan rawatan berkesan terhadap patogen bakteria tahan antibiotik. Objektif kajian adalah untuk menciri dan menilai kesan sitotoksisiti, dan keberkesanan tilosin berkapsul liposom terhadap *C. pseudotuberculosis* yang diasingkan daripada kambing. Tilosin berkapsul liposom dihasilkan melalui kaedah penghidratan filem nipis konvensional dan saiz zarah penyebaran liposom, indeks polidispersiti (PDI), dan potensi zeta diukur dengan penyerakan cahaya dinamik. Kesan sitotoksisiti tilosin berkapsul liposom konvensional dan tilosin bebas dinilai terhadap fibroblas tikus normal dan sel primer fibroblas kulit manusia normal menggunakan ujian MTT. Keberkesanan antibakteria rumusan liposomal dan tilosin bebas terhadap *C. pseudotuberculosis* ditentukan menggunakan kaedah pencairan mikrobroth dan kaedah resazurin, manakala aktiviti antibiofilem dinilai menggunakan kepekatan perencatan biofilem minimum dan kepekatan pembasmian biofilem minimum (MBEC) dengan kaedah kolorimetrik berasaskan ungu kristal dan resazurin. Keputusan menunjukkan diameter purata 171.6 ± 3.22 nm untuk tilosin berkapsul liposom dengan 0.236 ± 0.002 dan -4.75 ± 1.6 mV PDI dan potensi zeta, masing-masing. Kecekapan pengkapsulan tilosin berkapsul liposom ialah 47.7 ± 2.8%. Ciri pembebasan tilosin daripada liposom konvensional adalah berdasarkan pembebasan cepat awal diikuti dengan pembebasan berterusan dan perlahan berbanding tilosin bebas. Penemuan ujian sitotoksisiti menunjukkan viabiliti sel yang jauh lebih tinggi (melebihi 70%) bagi tilosin berkapsul liposom dan di bawah 70% ($P<0.05$) bagi formulasi bebas tilosin pada kepekatan tinggi 1024 µg/mL. Kepekatan perencatan minimum (MIC) bagi formulasi tilosin konvensional, kationik, fusogenik dan fuso-kationik ialah 32 µg/mL yang jauh lebih tinggi daripada tilosin bebas dengan MIC 1 µg/mL.

Sebaliknya, keputusan MBEC menunjukkan 1024 µg/mL, 256 µg/mL, 512 µg/mL dan 1024 µg/mL bagi konvensional, kationik, fusogenik dan fuso-kationik, manakala 2048 µg/mL bagi tilosin bebas. Formulasi tilosin berkapsul liposom kationik, fusogenik dan fuso-kationik masing-masing mempunyai diameter purata 114.8 ± 0.115 nm, 114.4 ± 0.62 nm, dan 117.7 ± 1.08 nm. Kapsul liposom kationik mendedahkan purata PDI dan potensi zeta masing-masing 0.135 ± 0.014 dan 35.3 ± 1.9 mV, manakala saiz min 114.4 ± 0.62 nm, PDI 0.16 ± 0.019, dan zeta ptential -28.6 ± -0.115 mV telah direkodkan untuk formulasi fusogenik. Tilosin terkapsul liposom fuso-kationik menunjukkan purata 117.7 ± 1.08 nm, 0.155 ± 0.01, dan 21.83 ± 0.21 mV untuk saiz, PDI dan cas permukaan, masing-masing. Kecekapan pengkapsulan tilosin berkapsul liposom kationik, fusogenik dan fuso-kationik masing-masing adalah 34.26 ± 1.15%, 46.63 ± 0.6%, dan 41.36 ± 1.2%. Kesimpulannya, dapatan kajian menunjukkan liposom sebagai nanozarah mampu meningkatkan biokompatibiliti dan aktiviti antibakteria tilosin, justeru, boleh dianggap sebagai suatu strategi yang berpotensi untuk menghidupkan semula keberkesanan antibiotik rutin terhadap bakteria tahan antibiotik.

Kata-kata kunci: Limfadenitis Kaseous, Biofilem, Liposom, Sitotoksisiti, Tilosin

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Declaration by Members of Supervisory Committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
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LIST OF ABBREVIATIONS

µL	Microlitre
µg	Microgram
ABC	ATP-binding cassette proteins
AGID	Agar gel immunodiffusion
AHLs	Acyl-homoserine lactones
APIs	Autoinducing peptides
CFU	Colony forming unit
CHEMS	Cholesteryl hemisuccinate
CLA	Caseous lymphadenitis
CO_2	Carbon dioxide
CTAB	Trimethylhexadecylammonium bromide
DDBA	Dimethyldioctadecylammonium bromide
DC-chol	Dimethylaminoethane carbamoyl cholesterol
DLS	Dynamic light scattering
DMEM	Dulbecco s modified eagle's medium
DMSO	Dimethyl sulfoxide
DMPC	Dimyristoyl phosphatidylcholine
DMPG	Dipalmitoyl phosphatidylglycerol
DOPC	Dioleoyl Phosphatidyl Choline
DOPE	1,2-dioleoylphophatidylethanolamine
DOPG	Dioleoyl Phosphatidyl Glycerol
DOTAM	2,3-dioleoyl-proyl)-trimethylamine bromide
DOTAP	1,2-dioleoyl-3-trimethylammoniopropane
DOPS	1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine
DPPA	Dipalmitoyl Phosphatidic Acid
DPPC	Dipalmitoylphosphatidylcholine
DPPG	Dipalmitoyl Phosphatidyl Glycerol
DPPS	Dipalmitoyl Phosphatidyl Serine

DSPC	Distearoylphosphatidylcholine
DSPE	Distearoyl phophoethanolamine
DNA	Deoxyribonucleic acid
eDNA	Extracellular deoxyribonucleic acid
EDTA	Ethylenediamine tetra acetic acid
EE	Encapsulation efficiency
ELISA	Enzyme-linked immunosorbent assay
EPC	Egg phosphatidylcholine
EPS	Extracellular polymeric substances
FBS	Foetal bovine serum
FDA	Food and drug administration
FITC	Fluorescein isothiocyanate
Fuso-cationic	Fusogenic-cationic
HRTEM	High-resolution electron microscopy
HPC	Hydrogenated phosphatidylcholine
HPLC	High performance liquid chromatography
LC	Loading capacity
LUVs	Large unilamellar vesicles
MBEC	Minimum biofilm eradication concentration
MHB	Mueller Hinton Broth
MIC	Minimum inhibitory concentration
mg	Milligram
mL	Milliliter
MLVs	Multilamellar vesicles
MMs	Mixed micelles
MTT bromide]	[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
MPHs	Macrolide phosphotransferases
nm	Nanometer
NIH3T3	Normal mouse embryonic fibroblast cell line

NHDF	Normal human dermal fibroblast primary cells
OD	Optical density
PBS	Phosphate buffered saline
PA	Phosphatidic acid
PC	Phosphatidyl Choline
PDI	Polydispersity index
PG	Phosphatidyl glycerol
PE	Phosphatidyl Ethanolamine
PI	Phosphatidyl Inositol
PS	Phosphatidylserine
PLD	Phospholipase D
pH	Power of hydrogen
QS	Quorum sensing
RNA	Ribonucleic acid
SA	Stearylamine
SD	Standard deviation
SUVs	Small unilamellar vesicles
TRITC	Tetramethyl rhodamine iso-thiocyanate
TSB	Tryptone soya broth
UV-Vis	Ultraviolet visible

CHAPTER 1

GENERAL INTRODUCTION

1.1 Study background

Corynebacterium pseudotuberculosis (*C. pseudotuberculosis*) is a gram positive, pleomorphic, non-spore forming, and facultative intracellular which is a family member of *Corynebacteriaceae* (Oreiby, 2015). This pathogen causes caseous lymphadenitis (CLA) also known as cheesy gland and *Morel's* disease which is a chronic and contagious bacterial disease of ruminants especially in goats (Abebe & Sisay Tessema, 2015; Oreiby, 2015). CLA is one of the economic significant diseases of small ruminants in Malaysia (Jesse *et al*., 2017). The economic consequences of the disease include reduction of meat products from the condemnation of carcass and cut-out parts, reduction of wool and leather products, reproductive efficiency reduction, removal of infected animals, and affected animal's death due to internal involvement (Abebe & Sisay Tessema, 2015; Minozzi *et al*., 2017). The disease in small ruminants is identified by abscess formation in the skin, internal and external lymph nodes, and internal organs (Corrêa *et al*., 2018). In goats, the most commonly affected lymph nodes are superficial cervical lymph nodes (Al-Gaabary *et al*., 2009). The CLA causative agent is spread almost all over the whole globe (Jeber *et al*., 2016). The first case of caseous lymphadenitis in Malaysia was recorded in 1970 at the Veterinary Research Institute, Ipoh (Osman *et al*., 2012).

Poor response of *C. pseudotuberculosis* to antimicrobial agents makes the treatment of this infection highly challengeable (Santos *et al*., 2021). There are some important factors involved in antibiotic resistance of *C. pseudotuberculosis* including 1) capsulated lesion by a thick layer (which have pus with nature of caseous within the thick capsule and 2) ability of the pathogen to survive inside the macrophage (intracellular location) (Ruiz et al., 2020). The formation of biofilm is another significant parameter that limits the efficacy of antimicrobial agents in *C. pseudotuberculosis* (Santos *et al*., 2021). Resistance to antimicrobial drugs will cause decreasing in treatment options, making the veterinarians apply more costly medicines (Scott & Menzies, 2011). Among several strategies for combating against the resistant pathogen, application of nanocarriers for delivering of antibiotic provide a promising approach to control and treat resistant bacteria (Zaidi *et al*., 2017; Lee *et al*., 2019). Nanoparticles provide a great opportunity to overcome bacterial resistance by delivery of drug in targeted site of action, increase therapeutic efficacy of conventional medicines, and reducing the adverse effects (Ruddaraju *et al*., 2020).

There are several nanoparticles that apply as drug delivery systems (Zaidi *et al*., 2017). Liposomes as one of the most popularly studied nanoparticles are

of particular significance because of their safety and specific drug targeting delivery (Zaidi *et al*., 2017; *Rukavina et al*., 2018). The incorporation of antibiotics into liposomes reveals increasing drug carrying inside bacterial cells and biofilms (Abed & Couvreur, 2014). In addition, modification of physicochemical properties of liposome including their size, coating and surface charge, and composition of bilayer and rigidity/elasticity of membrane enables researchers to tailor of liposome with desired pharmacokinetics and pharmacodynamics drug profile (Vanić *et al*., 2019). Moreover, liposomes as a delivery system for antibiotics reduce the toxicity of the drugs, increase the activity of antibiotics against the intracellular and extracellular pathogen (Drulis-Kawa & Dorotkiewicz-Jach, 2010). Liposomes are able to interact with mammalian cells through several mechanisms such as absorption, endocytosis, fusion, and lipid transfer (Pagano & Weinstein, 1978). As a result, a high local concentration of antibiotics is achievable to the cell membrane or within the bacterial cells via encapsulation of antibiotics into the liposomes (Rukavina *et al*., 2018).

Liposome as a nanocarrier has been proven that possess the ideal characteristics of a nanodevice for delivering antibiotics (Gonzalez Gomez & Hosseinidoust, 2020). Previous studies reported that many antibiotics showed high efficacy and less toxicity when encapsulated into liposome such as streptomycin (Gangadharam *et al*., 1991), ampicillin (Schumacher & Margalit, 1997), gentamicin (Vitas *et al*., 1997), amikacin (Xiong *et al*., 1999), tetracycline (Sangare *et al*., 1999), ciprofloxacin (Wong *et al*., 2003), benzylpenicillin (H. J. Kim & Jones, 2004), levofloxacin (Zhang et al., 2009), vancomycin (Sande *et al*., 2012), colistin (Wallace *et al*., 2012), clarithromycin (Alhajlan *et al*., 2013), tobramycin (Messiaen *et al*., 2013), doxycycline (Franklin *et al*., 2015), gentamicin (Alhariri *et al*., 2017), azithromycin (Vanić *et al*., 2019). With respect to the unique features of liposomes as novel drug delivery in enhancing the efficacy and reducing the side effects of antibiotics, liposome was selected as the nanocarrier for tylosin against *C. pseudotuberculosis* in this research.

Tylosin is a broad-spectrum antibiotic (Poźniak *et al*., 2020) and is commonly applied in veterinary medicine as a member of macrolides antibiotics, specifically against gram positive bacteria, mycoplasma, and anaerobic bacteria (Atef *et al*., 2009). Tylosin is applicable in many animals spp. such as cattle, sheep, goats, swine, poultry, dogs, and cats (Poźniak *et al*., 2020). Tylosin is a bacteriostatic antibiotic that prohibits bacterial growth through inhibition of protein synthesis via binding to the ribosomal 50s subunit of bacteria (Ji *et al*., 2014; Poźniak *et al*., 2020).

1.2 Problem statement

Caseous lymphadenitis is a chronic and infectious disease of goats caused by *C. pseudotuberculosis* (Corrêa *et al*., 2018). The disease induces huge

financial losses in the goats industry (Ruiz et al., 2020; Santos et al., 2021). Since the CLA causative organism is resistant to many antibiotics, conventional antimicrobial treatment of the disease is a big challenge (de Pinho et al., 2021). Many factors contribute to antimicrobial resistance of *C. pseudotuberculosis* including lesions surrounded by a thick fibrous capsule filled with pus which hinders the penetration of antibiotics and intracellular location of *C. pseudotuberculosis* (Santos *et al*., 2021). Another important parameter causes *C. pseudotuberculosis* poor responsive to conventional antibiotic is the ability of biofilm formation(Santos et al., 2021). Therefore, it is necessary to look for new approaches to treat this infectious disease in goats (Stanisic *et al*., 2018). The application of nano-antibiotics has proved to be effective against resistant bacteria (Pelgrift & Friedman, 2013). Hence, incorporating antibiotics into nanoparticles will provide a promising tool not only for carrying them in the targeted site of action, but also will provide a controlled released profile (Abed & Couvreur, 2014).

1.3 Research justification

The goat rearing industry suffers severely from caseous lymphadenitis impact in terms of economic losses and financial implications annually which require more research to be conducted in this area (Ruiz *et al*., 2020). The development of new strategies and seeking new chemotherapeutics for the treatment of these diseases are highly significant. However, the development of new chemotherapeutics is a highly time-consuming and cost-effective procedure (Hochvaldová *et al*., 2022). The application of nanoparticles may give a new life for conventional antibiotics (Parisi *et al*., 2017; Hochvaldová *et al*., 2022) by improving their distribution/pharmacokinetics, efficacy, decrease toxicity, and therapeutic index against many resistant bacteria including *C. pseudotuberculosis* (Stanisic *et al*., 2018). Usage of the appropriate approach for drug carrying to circumvent the resistance mechanism of *C. pseudotuberculosis*, antibiotics could be the ideal way to treat and eliminate this pathogen.

1.4 Research hypothesis

Hypothesis 1

- Liposome-encapsulated tylosin will have the appropriate physicochemical properties for drug delivery and tylosin is able to release sustainably from it *in vitro*.

Hypothesis 2

- Liposome-encapsulated tylosin is not toxic to normal mouse fibroblast (NIH3T3) cell line and normal human dermal fibroblasts (NHDF) primary cells.

Hypothesis 3

- Conventional liposome-encapsulated tylosin will present better antibacterial and antibiofilm activity against planktonic and biofilm forms of *C. pseudotuberculosis in vitro* as compared to free tylosin.

Hypothesis 4

- Cationic, fusogenic and fuso-cationic liposome-encapsulated tylosin will possess better antibacterial and antibiofilm activity against planktonic and biofilm forms of *C. pseudotuberculosis in vitro* than conventional liposome-encapsulated tylosin and free tylosin.

Hypothesis 5

- Cationic liposomal tylosin will indicate better antibacterial activity against planktonic and biofilm form of *C. pseudotuberculosis* as compared to conventional and fusogenic liposomal tylosin.

1.5 Research objectives

The main purpose of this research is to evaluate the *in vitro* effect of liposome-encapsulated tylosin against *C. pseudotuberculosis* isolated from goat's caseous lymphadenitis cases.
The research specific objectives are to:

1. characterize liposome-encapsulated tylosin and assess the *in vitro* release profile of tylosin.
2. evaluate the *in vitro* cytotoxicity of liposomes encapsulated tylosin in normal mouse fibroblast (NIH3T3) cell line and normal human dermal fibroblasts (NHDF) primary cells using MTT assay.
3. determine the *in vitro* antibacterial and antibiofilm effect of conventional liposome-encapsulated tylosin against planktonic and biofilm forms of *C. pseudotuberculosis.*
4. determine the *in vitro* antibacterial and antibiofilm effect of cationic, fusogenic and fuso-cationic liposome-encapsulated tylosin against planktonic and biofilm forms of *C. pseudotuberculosis*.
5. compare the antibacterial efficacy of conventional, cationic, fusogenic and fuso_cationic liposome-encapsulated tylosin.

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