ASCORBIC ACID LOADED PLGA NANOPARTICLES GEL AS POTENTIAL ORAL SQUAMOUS CELL CARCINOMA SITE-SPECIFIC TREATMENT

BY

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ABSTRACT

Oral squamous cell carcinoma (OSCC) represents the majority of oral cancer. Chemotherapy is commonly used to treat OSCC especially as the disease advanced. However, conventional chemotherapy is associated with terrible adverse effects and the occurrence of chemoresistance which causes treatment failure. Therefore, the quest for a more effective and safer alternative has intensified. High dose ascorbic acid has been evidenced to confer anticancer effects via generation of reactive oxygen species (ROS) and through epigenetic mechanisms. Poly lactic-co-glycolic acid (PLGA) was used as the encapsulating polymer for the delivery of ascorbic acid to the cancer cells. A rapid UV-visible spectrophotometric method was validated as per ICH guideline and applied throughout this study for the quantification of ascorbic acid. Double emulsion solvent evaporation method was used to fabricate ascorbic acid loaded PLGA (AA-PLGA) nanoparticles. Optimisation of formulation was carried out by multilevel categoric full factorial design based on different surfactant concentrations and surfactant types used in the primary emulsion. The particle size of the optimised formulation was found to be 252 ± 2.98 nm, polydispersity index (PDI) of 0.151 ± 0.02, zeta potential of -20.93 ± 0.87 mV and encapsulation efficiency of 69.73 ± 1.07%. Scanning electron microscope images revealed the spherical shape of nanoparticles. The drug release behaviour exhibited a biphasic pattern namely initial burst release followed by slower release. The optimised nanoparticles formulation was further incorporated into different concentrations of Carbopol® gel. The pH of the prepared formulations was well within the pH range of the oral cavity which is between 6.2 – 7.6. Statistical analysis indicated that Carbopol® concentration significantly (p-value < 0.05) affected viscosity, spreadability and mucoadhesion of the gels. The properties of the prepared formulations were compared with a Carbopol® based commercial product (Kin Care) as a reference. Carbopol® polymer with a strength of 1% was chosen as the optimum gelling agent for AA-PLGA nanoparticles gel. The viscosity of the formulation with 1% Carbopol® was slightly higher (p-value < 0.05) than the commercial product, yet the spreadability and adhesion results were comparable (p-value > 0.05). The rheological study showed that all the gels exhibited a pseudoplastic behaviour which is desirable as it facilitates the flow of gel out of the tube and can form a viscous gel at the application site. Besides, the optimum gel formulation exhibited a zero-order kinetic release of ascorbic acid nanoparticles for 6 hours duration. Hence, it is a good candidate for topical application on the oral mucosa. The optimised AA-PLGA nanoparticles were subjected to cytotoxic assay against the OSCC SCC-25 cell line. Through in vitro cytotoxicity study, AA-PLGA nanoparticles mediated a significant (p-value < 0.05) reduction of cancer cell viability in a dose-dependent manner with an IC_{50} value of 2420 µg/mL. Severe cellular morphological changes were examined with an inverted microscope after 24 hours of incubation with AA-PLGA nanoparticles evidenced the cancer cell death in the SCC-25 cell line. The results of the present study support the feasibility of AA-PLGA nanoparticles gel to treat OSCC and hope the formulation can open a new avenue for OSCC therapy.

ملخص البحث

سرطان الخلايا الحرشفية الفموي (OSCC) يمثل غالبية سرطان الفم. العلاج الكيميائي التقليدي يرتبط بآثار ضارة رهيبة وظاهرة التشبع الكيميائي في سرطان الفم التي تسبب قصورا في العلاج. ولذلك، فإن الجهود قد تكثفت سعيا إلى إيجاد بديل أكثر فعالية وأمان. حمض الأسكوربيك يضفي تأثيرات مضادات للسرطان من خلال توليد أنواع الأكسجين المتفاعل (ROS) ومن خلال آليات epigenetic. استخدام حمض الجليكوليك متعدد حمض اللاكتيك (PLGA) باعتباره البوليمر المغلف للحمض الأسكوربيك لإيصاله إلى الخلايا السرطانية. وقد تم تطبيق طريقة قياس الأشعة فوق البنفسجية طوال هذه الدراسة على كمية حمض الأسكوربيك. وقد استخدمت طريقة تبخر المذيبات المزدوجة لصنع أحماض الأسكوربيك المحملة بالمواد النانوية المشحونة (AA-PLGA). تم إجراء تحسين الصياغة من خلال تصميم عاملي شامل متعدد المستويات يعتمد على تركيزات معاملات التوتر السطحي المختلفة وأنواعها المستخدمة في المستحلب الأولي. حجم الجسيمات في الصيغة المثلى هو 252 ± 2.98 نانومتر، ومؤشر تعدد الجسيمات (PDI) من 0.151 ± 0.02، وإمكانية زيتا من -20.93 ±0.87 ميغا إلكترون فولت، وكفاءة التغليفر 69.73 ± 1.07%. كشفت الصور المجهرية الإلكترونية عن الشكل الكروي للجسيمات النانوية. أظهر سلوك إطلاق الدواء نمطًا ثنائي الطور. وأُدمجت الصيغة المثلى للجسيمات النانوية في تركيزات مختلفة من هلام الكربوبول. وكان الأس الهيدروجيني للتركيبات المعدة في نطاق الأس الهيدروجيني لتجويف الفم الذي يتراوح بين 6.2 – 7.6. أشار التحليل الإحصائي إلى أن تركيز الكاربوبول أثر بشكل كبير على اللزوجة والقدرة على التوزيع والالتصاق. وتمت مقارنة خصائص التركيبات المعدة بمنتج تجاري قائم على كن كير (Kin Care) كمرجع. تم اختيار كربوبول بوليمر بقوة 1% كعامل أمثل للهلام النانوي AA-PLGA. أظهرت جميع المواد الهلامية سلوكًا زائفًا مرغوبًا لأنها تسهل تدفق الهلام من الأنبوب وقادرة على تكوين هلام لزج في موقع التطبيق. ومن ثم، فهو مرشح جيد للتطبيق الموضعي على المخاط الشفوي. تم اختبار تركيبة الجسيمات النانوية المحسنة للتأثير السام للخلايا على خط خلايا OSCC. ساهمت الجزيئات النانوية AA-PLGA في تخفيض كبير في قدرة الخلايا السرطانية على البقاء بطريقة تعتمد على الجرعة وتبلغ قيمتها IC_{50} من 2420ميكروغرام/مل. وكشفت نتائج هذه الدراسة عن إمكانية وجود هلام النانو AA-PLGA لعلاج OSCC وفتح طريق جديد لعلاج OSCC.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Science in Pharmacy.

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LIST OF ABBREVIATIONS

AA-PLGA	Ascorbic acid loaded PLGA
OSCC	Oral squamous cell carcinoma
HPV	Human papillomavirus
SVCT	Sodium-dependent vitamin C transporters
5-hmC	5-hydroxymethylcytosine
TET	Ten-eleven-translocation
PLGA	Poly lactic-co-glycolic acid
PDI	Polydispersity index
UV	Ultraviolet
SD	Standard deviation
LOD	Limit of detection
LOQ	Limit of quantification
PVA	Polyvinyl alcohol
PBS	Phosphate buffer solution
ATR-FTIR	Attenuated total reflectance-fourier transform infrared spectroscopy
ANOVA	Analysis of variance
CGM	Complete growth media
ROS	Reactive oxygen species
DMSO	Dimethyl sulfoxide
TBEA	Trypan blue exclusion assay
GSH	Reduced glutathione
GSSG	Oxidised glutathione

MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Oral cancer accounts for 377, 713 new cases and 177, 757 death in 2020, making it the seventeenth most common cancer worldwide (Sung et al., 2021). Meanwhile, 742 new cases and 403 deaths were reported in Malaysia in 2020 (World Health Organization, 2021). It is associated with significant mortality, especially if the patient is diagnosed at an advanced stage (Carolina et al., 2017). Though oral cancer was not among the top 10 cancers in Malaysia, it is still a major concern because two-third of the cases reported are diagnosed at a late stage (Ghani, Razak, et al., 2019). According to Yaaqob et al., (2019), the oral cancer stage was statistically important, with patients in the late stage (stage III and stage IV) having a five times greater chance of dying compared to those in early-stage (stage I and stage II). Furthermore, Malaysians had a poorer 5-year survival rate of 40.9% compared to the world 5-year survival rate of roughly 50%. (Ghani, Razak, et al., 2019). Often, chemotherapy is required in the late-stage as well as in the metastatic oral cancer (Hartner, 2018). Cisplatin and fluorouracil, both available in intravenous injection, are known as the most commonly used anticancer agents in oral cancer (Ketabat et al., 2019). Various detrimental adverse effects due to systemic conventional chemotherapy approach have been reported and remain the major concern to the patients (Marcazzan et al., 2018). For example, cisplatin is associated with three distinct adverse effects, which are renal toxicity, neurotoxicity, and gastrointestinal toxicity (Aldossary, 2019). Besides, chemoresistance developed worldwide in various cancer, including OSCC and was recognised as the primary source

of treatment failure (S. Li et al., 2018). Cisplatin resistance has been reported where more than 30% of patients acquired resistance to cisplatin during early treatment administration. Meanwhile, the others slowly develop resistance after several rounds of chemotherapy (Zhuang et al., 2017). Hence, a new innovative and safer alternative for oral cancer therapy is highly in need.

The pharmacological ascorbic acid dose, which is administered intravenously, has been proven as a promising anticancer agent in both in vivo and in vitro studies (Vissers & Das, 2018). Its cytotoxicity effect against cancer cells has been exhibited in various cell lines, including oral cancer cell lines (Baek et al., 2017; Ohwada et al., 2017; Zhou et al., 2020). However, the instability of ascorbic acid in a bulk aqueous system was regarded as a challenge for its formulation. In certain conditions such as exposure to air and light, ascorbic acid undergoes reversible oxidative degradation to dehydroascorbic acid. Dehydroascorbic acid can further be hydrolysed irreversibly to an inactive compound called 2,3-diketogulonic acid (Sheraz et al., 2015). Nevertheless, the application of nanotechnology particularly encapsulating ascorbic acid in nanoparticles has been shown to preserve its stability and enhance the delivery in a sustained release manner (Duarah et al., 2017). Topical, localised, and non-invasive approach are favourable strategies for oral cancer treatment (Ferreira et al., 2020). Apart from that, easy access to the affected area has made local intraoral drug delivery an attractive route for the delivery of chemotherapeutic agents (Matos et al., 2020). Nonetheless, environmental factor particularly continuous saliva secretion should come into consideration in developing a suitable formulation for oral cavity application. This normal physiologic phenomenon would dilute and remove the drug from the target site (Desai, 2018). Thus, the incorporation of the mucoadhesive polymer is necessary to

overcome this issue by interacting with the mucous glycoprotein of the oral mucosa (Netsomboon & Bernkop-Schnürch, 2016; Sarma et al., 2019).

Therefore, this study aims to formulate optimised ascorbic acid loaded polymeric nanoparticles intended to treat oral cancer. The formulated nanoparticles were further incorporated into a mucoadhesive polymer, specifically Carbopol® as an oral gel for topical and controlled release delivery of ascorbic acid to the tumour site with better residence time. The formulation was tested for the cytotoxicity activity against an OSCC cell line by using an MTT assay.

1.2 PROBLEM STATEMENT

As far as this is concerned, conventional chemotherapy approach is associated with several disadvantages particularly terrible adverse effects to the patient and occurrence of chemoresistance towards commonly used chemotherapy which accounts for the treatment failure, disease recurrence and metastasis (S. Li et al., 2018; Marcazzan et al., 2018; Zhuang et al., 2017). Thus, identifying a new potential anticancer agent and developing a safe, effective and non-invasive drug delivery is necessary for complementary or alternative treatment of OSCC. A high dose of ascorbic acid has been shown to be cytotoxic against a variety of cancer lines, including OSCC (Baek et al., 2017; Ohwada et al., 2017; Zhou et al., 2020). Topical administration of high dose ascorbic acid directly to cancerous site is favourable due to the non-invasive and straightforward approach. However, formulating ascorbic acid is difficult due to its instability in the bulk aqueous system (Sheraz et al., 2015). Several approaches have been adopted to preserve its stability and enhance its delivery to the target site such as formulation of multiple emulsion, microparticles including nanoparticles (Duarah et al., 2017; Kheynoor et al., 2018; Sheraz et al., 2015). Besides, salivary washout also is the

challenge to retain the potential anticancer agent longer to the targeted site (Desai, 2018). However, the application of the mucoadhesive agent reported prolonging drug residence time (Kassab et al., 2017). Table 1.1 summarises problems and solutions of this study.

Table 1.1 Problems and solutions of this study

Problems	**Solutions**
Dreadful adverse effects and chemoresistance reported in oral cancer due to the conventional chemotherapy approach impair the effectiveness of cancer treatment (Li et al., 2018).	Identify a new potential anticancer agent namely ascorbic acid (Baek et al., 2017; Ohwada et al., 2017; Zhou et al., 2020).
Formulating ascorbic acid is difficult due to its instability in the bulk aqueous system (Sheraz et al., 2015).	Formulation of nanoparticles (Duarah et al., 2017; Kheynoor et al., 2018).
Salivary washout and low drug retention are the major hindrance associated with topical delivery of the drug directly in the oral cavity (Desai, 2018).	Application of mucoadhesive polymer namely Carbopol® as oral gel (Desai, 2018).

1.3 LITERATURE REVIEW

1.3.1 Oral Cancer

Oral cancer is a malignant tumour that develops on the lips or in the oral cavity such as the tongue, cheeks, floor of the mouth, hard and soft palate (Hinaz & Geetha, 2018). The term oral cancer is frequently used interchangeably with oral squamous cell carcinoma (OSCC) since more than 90% of malignant tumour developed in the oral cavity represented by OSCC (Gunjal et al., 2020). OSCC is explained as an invasive abnormal growth of epithelial cells with variable levels of squamous differentiation derived from stratified squamous epithelium of the oral mucosa (Suciu et al., 2014). The clinical presentation of oral cancer is broad (Wong & Wiesenfeld, 2018). Non-healing ulcer, white lesion, red patches, uncontrolled growth of oral mucosa, lymph node enlargement, mobile teeth, tooth pain, orofacial pain and mouth bleeding are the distinct signs and symptoms of oral cancer (Muthu et al., 2018). Lip and oral cavity cancer account for 377, 713 new cases and 177, 757 death in 2020, putting it the seventeenth most common cancer worldwide (Sung et al., 2021). The prevalence of oral cancer differs greatly depending on the geographical region where it is being diagnosed. Globally, the majority of the highest rate of oral cancer incidence is concentrated in South Asia, Central Asia and Oceania. South Asian countries such as India and Sri Lanka possess the highest incidence of oral cancer cases (Miranda-Filho & Bray, 2020).

Oral cancer creates a massive burden on public health. It is associated with a dismal prognosis especially if the patient is diagnosed at a late stage (Parkinson, 2018). Almost 86% of oral cancers are diagnosed at an advanced stage (Haron et al., 2020). Though oral cancer was not among the top 10 cancer in Malaysia, it is still a major concern since two-third of the cases reported are diagnosed at a late stage (Chou et al., 2019). Limited access to specialists and lack of awareness of oral cancer and its

alarming signs and symptoms are the contributing factors related to late diagnosis (Ghani, Razak, et al., 2019; Haron et al., 2020). Moreover, the 5-year survival rate of oral cancer of the Malaysian population is 40.9% lower than the global survival rate which is around 50% (Ghani, Ramanathan, et al., 2019). The recurrence and metastasis of oral cancer continue to happen after the 5-year survival yet with a reduced rate (Chou et al., 2019).

The occurrence of oral cancer is strongly related to exposure to the risk factors such as tobacco smoking, alcohol consumption, betel nut chewing and human papillomavirus (HPV). Indeed, frequent exposure to multiple risk factors produces a higher probability of developing oral cancer. For instance, practising multiple risks concurrently such as smoking, drinking alcohol and chewing betel nuts exhibited the highest risk for developing oral cancer (Ghani, Razak, et al., 2019). Besides, habit of risks practised in Malaysia significantly correlated with the ethnicity. Smoking alone is the most common habit practised by Malays. Smoking and drinking alcohol are common in Chinese meanwhile quid chewing is common in Indians (Ghani, Razak, et al., 2019).

Interestingly, the anatomical location and clinical characteristic of oral cancer are correlated to the risk factors practised. Those smoking and drinking alcohol reported more endophytic tumour and frequently developed on the lateral border of the tongue and mouth floor. Meanwhile, those practising chewing betel quid which is usually held in the buccal sulcus would develop an exophytic tumour on the gingiva, buccal sulcus and buccal mucosa (Warnakulasuriya, 2009). This finding is consistent with a study conducted on the Malaysian population. Tongue and floor of the mouth is the most common tumour site in Malays and Chinese. Meanwhile, buccal mucosa and gingiva are the common locations for Indians and Indigenous people (Ghani, Razak, et al., 2019)