

Establishment and characterisation of a herpes simplex virus type 2 murine challenge model for the evaluation of a novel microbicide

T Kgoe



orcid.org/ 0000-0003-4771-8282

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Supervisor: Prof RK Hayeshi

Co-supervisor: Dr L Damelin

Assistant-supervisor: Dr A Okem

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PREFACE

This dissertation is presented in article format and consists of one manuscript peer reviewed for publication. The dissertation was prepared as per the North-West University (NWU) guidelines for postgraduate studies and the requirements of the publishing journal.

All in vivo biological studies were carried out by myself after successful completion of short courses on basic cell culturing techniques and animal handling and the principles of research on animals. Prof. Rose Hayeshi, Dr. Ambrose Okem and Dr. Leonard Damelin prepared the study design and assisted with data analysis and interpretation. A part of the virus plaque assay was carried out at the Cell biology unit at the Centre for HIV and STIs, National Institute for Communicable Diseases, National Health Laboratory Service, with the assistance of Dr. Leonard Damelin. Flow cytometry analysis was conducted at the Center of Excellence for Pharmaceutical Sciences (Pharmacén™), NWU. The analysis was performed by myself with the assistance of Prof. Lissinda Du Plessis, who also conducted the data analysis.

The animal studies were carried out in accordance with the NWU code of conduct for researchers (ethics number NWU-00170-18-A5) in the AAALAC accredited animal facility (Department of Science and Technology/North-West University Preclinical Drug Development Platform Vivarium; PCDDP). HSV-2 viral infection was performed by Kobus Venter and Jacob Mabena. I was involved mainly in monitoring the mice every day and recording their mass and clinical scores.

Histological analysis of the vaginal and rectal tissues was conducted by myself with the training of Prof. Che Weldon at the department of zoology, NWU. The images were captured by myself with the help of Dr. Courtney Cook from the unit for Environmental Sciences and Management.

Part of my study was presented at the Drug Safety Africa meeting held in Potchefstroom, South Africa (November, 2018); and Academy of Pharmaceutical Sciences South Africa held in Pretoria, South Africa (October, 2019) in which I received an award for best oral presenter.

DECLARATION

I, Tumelo Olebogeng Kgoe (the student), hereby declare that this dissertation is a record of my own work (except where citations or acknowledgements indicate otherwise) and that this research study has not been submitted to any other university.

A handwritten signature in black ink, appearing to read 'Tumelo Olebogeng Kgoe', with a long horizontal stroke extending to the right.

Date: 21/11/2019

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I would like to dedicate this dissertation to me. One day I will eventually read through this body of work and it will remind me of my time here at the PCDDP. Had I not believed in myself, I would not have been in this position that I am in today. To my parents, the people that always support me with every decision that I make in my life, this one is for you. To my home girls and home boys that have always picked me up when I was in need of support, this one is for you. Although this dissertation will have my name on it, I could not have completed it without the following people that contributed

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ABSTRACT

Introduction

Herpes simplex virus type 2 (HSV-2) has been a global epidemic for many years. This disease is prevalent in Europe, North America, and Africa. HSV-2 is one of the most common sexually transmitted infections (STIs) in low-income countries with high incident rates of HIV/AIDS. With more than 20 million cases reported annually, it has become an issue of great concern. As an incurable STI, preventative alternatives are preferred over the development of treatments. There is a need to develop preventative strategies to effectively reduce HSV infection. Animal models are the preferred biological systems for the testing of safety and efficacy of the newly developed products, including vaccines and novel microbicides. In our study, we developed and characterised a murine model which can be applied in the evaluation of Bi-SP, a novel bismuth (III) complex which has previously been shown to display anti-microbial activity *in vitro* against HSV-2 and other STI causing agents.

Methods

For model establishment, six to eight-week old fasted male (n = 8) and medroxyprogesterone pre-treated female (n = 8) BALB/c mice were administered 10 µl of sterile phosphate buffered saline (PBS) rectally and vaginally respectively. Ten minutes after PBS administration, the mice were infected with 16.6 µl of HSV-2 strain G at a viral titer of 1×10^6 plaque forming units. The mice were monitored for infection twice a day for the duration of the study. Vaginal and rectal swab samples were collected from all animals 2 days post infection, immediately before euthanasia and after euthanasia. The swab samples were assayed for viable virus by virus plaque assay and CD4⁺ and CD8⁺ analysis using flow cytometry. Rectal and vaginal tissue samples from male and female mice respectively were collected from euthanised animals for histology. The tissue samples were fixed in formalin and were processed through a series of dehydration and elucidation steps and embedded in histoplast wax. The tissues were transversely sectioned with a microtome at 5 – 7 µm sections. Sectioned bands were mounted on glass microscopic slides and were stained with hematoxylin & eosin. The stained tissues were evaluated for epithelial cell disruption and evidence of inflammation under a compound microscope.

Safety assessment of Bi-SP was conducted in male (n = 5) and female BALB/c mice (n = 5) at 5 µg/animal. Administration of the novel microbicide was done rectally and vaginally using a 2 – 20 µl pipette. Rectal and vaginal tissue samples were collected 2 hours after Bi-SP administration for histopathology.

In determining the efficacy of the novel microbicide, female mice were administered Bi-SP at 5 µg/animal in PBS (n = 10), 5 µg/animal in a hypo-osmotic 2.7% HEC gel (n = 10) and 2.7% HEC gel only as control (n = 10), vaginally. The male mice were administered Bi-SP at 5 µg/animal (n = 20) and 10 µg/animal (n = 10) in PBS, rectally. Ten minutes after microbicide administration, the mice were infected with HSV-2 strain G at a titer of 1×10^6 PFU. The male (n = 1) and female (n = 1) control mice were administered with sterile PBS. The experiments were conducted in a biosafety level (BSL)-3 laboratory and the mice were monitored for infection twice a day over a period of 19 days. During the course of the study, the animals were monitored and clinical scores were assigned. Once the animals reached the humane endpoint clinical score, they were euthanised. The rectal and vaginal swab samples were collected 2 days post-infection and immediately before euthanasia. For virus plaque assay, only swabs collected 2 days post infection were used while CD4⁺ and CD8⁺ analysis was done using swabs from 2 days post infection and immediately before euthanasia. Vaginal and rectal tissues were excised from the female and male mice respectively, after euthanasia. The tissues were sliced open longitudinally and stored in formalin for histological analysis.

Results

The survival rate of male and female BALB/c mice infected with HSV-2 was reduced to 0%, 9 days post infection. All the animals reached severe clinical symptoms between days seven and nine post infection. The symptoms included severe inflammation perianally and perivaginally in male and female mice respectively. Severe swelling of the bladder and constipation in the digestive tract were observed. Virus plaque assays confirmed the presence of viable virus in the collected swab samples. Histological evaluation revealed elevated levels of inflammatory infiltrate in the submucosa and severe disruption on the mucosa, more specifically the glands and the glandular crypts in rectal tissues collected from male mice. A thinned squamous epithelium and an infiltration of inflammatory cells was observed in vaginal tissue collected from female mice, which was indicative of tissue damage. The presence of the virus from swab samples and the effect HSV-2 had on the vaginal and rectal mucosa is confirmation of the susceptibility of BALB/c mice to HSV-2 strain G.

Bi-SP at 5 µg/animal did not cause tissue damage. The microbicide demonstrated significant activity ($p < 0.05$) against HSV-2, subsequently increasing the survival rates from 0% in the PBS administered groups of both male and female mice, to 10% in female mice administered with Bi-SP at 5 µg/animal in PBS and 60% in female mice administered with Bi-SP at 5 µg/animal in 2.7% HEC gel. Female mice administered with 2.7% HEC gel only also had a survival rate of 60%. The survival rate in male mice increased to 40% in mice administered with 5 µg/animal and 30% in mice administered with 10 µg/animal in PBS. The presence of viable virus in swab samples was

confirmed using plaque assay and CD4⁺ and CD8⁺ cells were detected in vaginal swabs but not in rectal swabs.

Conclusion

Both female and male BALB/c mice were highly susceptible to HSV-2, whether infected vaginally or rectally, making them a model for use in microbicide safety and efficacy studies. The susceptibility of BALB/c mice allowed for the study on the effects of the novel microbicide Bi-SP, on HSV-2 in vaginal and rectal tissues. Bi-SP was proven to be safe to use at 5 µg/animal as it did not cause any tissue damage in the vaginal and rectal mucosa. The microbicide improved the survival of HSV-2 infected mice, an indication of the antiviral effects against HSV-2

Therefore, we can conclude that BALB/c mice were highly susceptible to HSV-2 strain G at 1 x 10⁶ pfu and are a suitable model for rectal and vaginal HSV-2 challenge studies. Although Bi-SP displayed significant antiviral activity against HSV-2, the microbicide will be need to be optimised to yield better survival rates than it has for the current study.

Keywords

BALB/c, Bi-SP, CD4⁺, CD8⁺, Herpes simplex virus, HSV-2, sexually transmitted infections, STI

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List of abbreviations

AIDS	Acquired Immune Deficiency Syndrome
ARV	antiretroviral
BSL	biosafety level
BLT	bone marrow/liver/thymic
CD4⁺	Helper T cells
CD8⁺	Cytotoxic T cells
CO₂	carbon dioxide
DNA	Deoxyribonucleic acid
DMEM	Dulbucco's modified eagle's medium
EDTA	ethylenediamtetracetic acid
FBS	foetal bovine serum
GUD	Genital ulcer disease
HPV	human papillomavirus
HEC	hydroxyethylene cellulose
H&E	Hematoxylin & eosin
HSV	Herpes Simplex Virus
HIV	Human Immunodeficiency Virus
HSV-1	Herpes simplex virus type 1
HSV-2	Herpes simplex virus type 2
IFN	Interferons
IFN-α	Interferon alpha

IFN-γ	Interferon gamma
IVC	individually ventilated cages
KZN	KwaZulu Natal
ml	millilitre
MPA	medroxyprogesterone acetate
MTT	Thiazolyl blue tetrazolium bromide
NHP	Nonhuman primate
NICD	National institute of clinical diseases
NK	Natural Killer cells
NOD	Nucleotide-binding oligomerization domain
NLR	Nucleotide-binding oligomerization domain like receptors
nm	Nanometer
N-9	nonoxynol-9
NWU	North-West University
pDC	Plasmacytoid dendritic cells
PenStrep	penicillin/streptomycin
PFU	plaque forming units
PBS	Phosphate Buffered Saline
RIG-1	Retinoic acid-inducible gene I
RLR	Retinoic acid-inducible gene I like receptors
SCID	severe combined immune deficiency
STI	Sexually transmitted infection

TLR	Toll-like receptors
TLR9	Toll like receptor 9
USA	United States of America
μl	microliter
μg	microgram

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CHAPTER 1 INTRODUCTION, PROBLEM STATEMENT AND AIMS

1.1 Introduction

Herpes simplex virus (HSV) is an infection that is prevalent in the world, and common in developing countries experiencing severe HIV/AIDS epidemics (Brown *et al.*, 2007). There are two types of herpes simplex viruses, HSV type 1 (HSV-1), which is known to cause cold sores, and HSV type 2 (HSV-2), the sexually transmitted subtype that occurs in the genital and rectal area (Looker *et al.*, 2017a). HSV-2 infection causes ulcers and blisters around the genital and rectal region and is the leading cause of genital ulcer disease (Johnston & Corey, 2016).

According to Daniels *et al.* (2016), an estimated 400 million people are living with HSV-2 and about 20 million cases are reported annually. The global prevalence among 15 to 49-year olds is estimated to be approximately 11.3% (Looker *et al.*, 2017b; Schiffer & Gottlieb, 2017). In South Africa, a 31% prevalence of HSV-2 in females aged 15 - 29 years old has been recorded, with incidents documented in KwaZulu-Natal (KZN) as high as 80% among commercial female sex workers (Daniels *et al.*, 2016). Although there is no cure for HSV-2, antiviral therapy can reduce transmission and the duration of symptoms.

Microbicides are topically applied anti-infective compounds, which were initially developed to combat and reduce the global prevalence of HIV-1 transmission (Catalone *et al.*, 2005a), however they also offer a broad-spectrum activity against other STIs, namely HSV, human papillomavirus, *Neisseria gonorrhoeae* and *Chlamydia trachomatis*, amongst others (Achilles *et al.*, 2002).

Due to low toxicity exhibited by bismuth, it is considered a safe metal to use in biological systems (Yang *et al.*, 2015). Bismuth complexes, as active ingredients in therapeutic agents, have been used in the treatment of gastrointestinal diseases (diarrhoea, indigestion, and nausea), and for antimicrobial and anticancer therapy (Andrews *et al.*, 2006; Keogan & Griffith, 2014; Marcus *et al.*, 2015b; Sampognaro *et al.*, 2017; Yang *et al.*, 2015). With work on non-toxic microbicides continually increasing, a bismuth (III) complex, Bi-SP, a novel metal-based microbicide that has recently been developed, has proven to be an active compound in inhibiting common STIs, including HIV and HSV-2 *in vitro*.

In drug development, the use of animal models for pre-clinical testing is a crucial step before any drug can be enrolled for clinical evaluation (Barre-Sinoussi & Montagutelli, 2015a; Veazey *et al.*, 2012). Animal models, more specifically rodent models, are cost-effective as they are easy to breed and can be genetically manipulated for any study. BALB/c mice are inbred

strains of mice that share a similar genetic structure. These are ideal models of choice in studies involving HSV-2 infection (Sartori *et al.*, 2016b; Varese *et al.*, 2018). Not only are they susceptible to HSV-2, but they can also be used to study immune responses associated with the compounds administered to them (Galdeano C, 2015).

1.2 Problem Statement

Due to the high prevalence and incidence rates of HIV and HSV-2 co-infection in Africa, particularly in Sub-Saharan Africa, microbicide research is more valuable than ever. With the development of new microbicides, there is a need for animal models to test novel microbicides for safety and efficacy. *In vitro* tests provide valuable information about the activity of the microbicide against pathogens, however, *in vivo* studies can provide a more in-depth understanding of how the microbicide might affect the mucosal areas as well as immune responses. In response to this problem, our study proposes to establish and characterise a mouse model for microbicide testing and to evaluate the safety and efficacy of the novel bismuth (III) complex (Bi-SP), which has a lack of *in vivo* information.

1.3 Aims and objectives

1.3.1 Aims

The first aim of this study was to establish a mouse model of HSV-2 rectal and vaginal mucosal infection in BALB/c mice. The second aim is to apply the model to evaluate efficacy of the novel microbicide for the prevention of HSV-2.

1.3.2 Objectives

- To establish and characterise an HSV-2 challenge mouse model using male and female BALB/c mice aged 6 to 8 weeks old
- To evaluate Bi-SP for safety and efficacy for the prevention of HSV-2 using male and female BALB/c mice
- To evaluate the vaginal and rectal tissue for epithelial integrity/disruption and leukocyte infiltration using histology
- To confirm the viral infection using virus plaque assay
- To detect CD4⁺ and CD8⁺ T cell counts with the use of flow cytometry

1.4 Dissertation layout

Chapter 1 is an introductory chapter, providing a brief background on HSV-2, microbicides and animal models. This chapter also states the problem statement and provides aims and objectives in order to address the problems.

Chapter 2 is a thorough literature review of HSV-2, discussing the sexually transmitted infection's epidemiology and the effect it has on the immune response. Also discussed in this chapter is information on microbicides about the different types and what and who they are intended for. The importance of animal models and the different types in vaccine and microbicide research is discussed in this chapter as well.

Chapter 3 is the chapter that describes how the HSV-2 challenge BALB/c male and female mice model was established and characterised. This chapter evaluated the usefulness of BALB/c mice in HSV-2 studies by testing their susceptibility to the virus when infected vaginally and rectally with 1×10^6 pfu of HSV-2 strain G. Clinical scores of the mice were recorded throughout the study to monitor the onset of infection in vaginally and rectally infected female and male mice. Immune responses, more specifically CD4⁺ and CD8⁺ were also evaluated to monitor the effects of HSV-2 on the innate and adaptive immunity of BALB/c mice

Chapter 4 is presented as a manuscript for publication according to the instruction for authors' guideline of the *Journal of Virology*® (JVI). In this chapter, the novel microbicide Bi-SP was evaluated for safety at 5 µg/animal in female and male mice via vaginal and rectal administration. Once the microbicide was cleared for being safe to use, the BALB/c mice that were characterised in Chapter 3 were used as HSV-2 challenge models to evaluate the efficacy of Bi-SP *in vivo*. The effect of the microbicide in inhibiting viral replication was observed with the increased survival of the mice.

Chapter 5 is the last chapter that highlights the main conclusion from this study and addresses the limitations that were encountered throughout the study and how future work can address them.

References

- Achilles, S. L., Shete, P. B., Whaley, K. J., Moench, T. R. & Cone, R. A. 2002. Microbicide efficacy and toxicity tests in a mouse model for vaginal transmission of *Chlamydia trachomatis*. *Sexually Transmitted Diseases*, 29(11):655-664.
- Andrews, P. C., Deacon, G. B., Forsyth, C. M., Junk, P. C., Kumar, I. & Maguire, M. 2006. Towards a structural understanding of the anti-ulcer and anti-gastritis drug bismuth subsalicylate. *Angewandte Chemie International Edition English*, 45(34):5638-5642.
- Barre-Sinoussi, F. & Montagutelli, X. 2015. Animal models are essential to biological research: issues and perspectives. *Future Science OA*, 1(4):FSO63.
- Brown, J. M., Wald, A., Hubbard, A., Rungrueangthanakit, K., Chipato, T., Rugpao, S., Mmiro, F., Celentano, D. D., Salata, R. S., Morrison, C. S., Richardson, B. A. & Padian, N. S. 2007. Incident and prevalent herpes simplex virus type 2 infection increases risk of HIV acquisition among women in Uganda and Zimbabwe. *AIDS*, 21(12):1515-1523.
- Catalone, B. J., Kish-Catalone, T. M., Neely, E. B., Budgeon, L. R., Ferguson, M. L., Stiller, C., Miller, S. R., Malamud, D., Krebs, F. C., Howett, M. K. & Wigdahl, B. 2005. Comparative safety evaluation of the candidate vaginal microbicide C31G. *Antimicrobial Agents and Chemotherapy*, 49(4):1509-1520.
- Daniels, B., Wand, H., Ramjee, G. & Team, M. D. P. 2016. Prevalence of Herpes Simplex Virus 2 (HSV-2) infection and associated risk factors in a cohort of HIV negative women in Durban, South Africa. *BMC Research Notes*, 9(1):510.
- Galdeano C, M. 2015. Stimulation of Innate Immune Cells Induced by Probiotics: Participation of Toll-Like Receptors. *Journal of Clinical & Cellular Immunology*, 06(01):2.
- Johnston, C. & Corey, L. 2016. Current Concepts for Genital Herpes Simplex Virus Infection: Diagnostics and Pathogenesis of Genital Tract Shedding. *Clinical Microbiology Reviews*, 29(1):149-161.
- Keogan, D. M. & Griffith, D. M. 2014. Current and potential applications of bismuth-based drugs. *Molecules*, 19(9):15258-15297.
- Looker, K. J., Elmes, J. A. R., Gottlieb, S. L., Schiffer, J. T., Vickerman, P., Turner, K. M. E. & Boily, M. C. 2017a. Effect of HSV-2 infection on subsequent HIV acquisition: an updated systematic review and meta-analysis. *Lancet Infectious Diseases*, 17(12):1303-1316.
- Looker, K. J., Magaret, A. S., May, M. T., Turner, K. M. E., Vickerman, P., Newman, L. M. & Gottlieb, S. L. 2017b. First estimates of the global and regional incidence of neonatal herpes infection. *Lancet Global Health*, 5(3):e300-e309.
- Marcus, E. A., Sachs, G. & Scott, D. R. 2015. Colloidal bismuth subcitrate impedes proton entry into *Helicobacter pylori* and increases the efficacy of growth-dependent antibiotics. *Alimentary Pharmacology & Therapeutics*, 42(7):922-933.

Sampognaro, P., Vo, K. T., Richie, M., Blanc, P. D. & Keenan, K. 2017. Bismuth Subgallate Toxicity in the Age of Online Supplement Use. *Neurologist*, 22(6):237-240.

Sartori, G., Jardim, N. S., Marcondes Sari, M. H., Dobrachinski, F., Pesarico, A. P., Rodrigues, L. C., Jr., Cargnelutti, J., Flores, E. F., Prigol, M. & Nogueira, C. W. 2016. Antiviral Action of Diphenyl Diselenide on Herpes Simplex Virus 2 Infection in Female BALB/c Mice. *Journal of Cellular Biochemistry*, 117(7):1638-1648.

Schiffer, J. T. & Gottlieb, S. L. 2017. Biologic interactions between HSV-2 and HIV-1 and possible implications for HSV vaccine development. *Vaccine*.

Varese, A., Remes Lenicov, F., Gonzalez Prinz, M., Paletta, A., Ernst, G., Maeto, C., Merlotti, A., Sabatte, J., Simula, S., Holgado, M. P., Dantas, E., Geffner, J. & Ceballos, A. 2018. Seminal vesicle fluid increases the efficacy of intravaginal HSV-2 vaccination. *Mucosal Immunology*, 11(2):536-548.

Veazey, R. S., Shattock, R. J., Klasse, P. J. & Moore, J. P. 2012. Animal models for microbicide studies. *Current HIV Research*, 10(1):79-87.

Yang, Y., Ouyang, R., Xu, L., Guo, N., Li, W., Feng, K., Ouyang, L., Yang, Z., Zhou, S. & Miao, Y. 2015. Review: Bismuth complexes: synthesis and applications in biomedicine. *Journal of Coordination Chemistry*, 68(3):379-397.

CHAPTER 2 LITERATURE REVIEW

2.1 Herpes simplex virus

Herpes simplex virus (HSV) is one of the most common sexually transmitted infections (STIs) that has one of the highest global rates of incidents. Herpes is a linear, double-stranded DNA virus (150 – 200 nm in diameter) belonging to the *alphaherpesviridae* family (Ali *et al.*, 2018; Dropulic & Cohen, 2012; Lee *et al.*, 2019). Two types of HSV exist, type 1 which is a non-genital infection normally associated with orofacial lesions due to infection in the mouth or eye, and type 2 is normally associated with genital herpes (Whitley & Baines, 2018). This lifelong and infectious disease can only be contracted through physical contact with the infected area, whether it is HSV-1 through kissing, or HSV-2 through sexual interaction (Looker & Garnett, 2005). HSV is considered a neurotropic and neuroinvasive virus, indicating it is a virus capable of infecting nerve cells. While HSV-1 can be contracted from as early as childhood, HSV-2 is more likely to be contracted around the age of sexual debut (Lee *et al.*, 2019). Regardless of the type, herpes is a mucocutaneous infection, targeting epithelial mucosa, where the virus infects somatic and autonomic nerve endings (Fatahzadeh & Schwartz, 2007).

2.1.1 Epidemiology and pathogenesis of HSV-2

Genital herpes is one of the most common STIs globally, with most infected people in the age group 14 to 49 years old (McQuillan *et al.*, 2018), and more than two thirds of the World's population infected with either type 1 and/or 2 (Schmid, 2019). It is estimated that over 400 million cases of HSV-2 have been reported (Daniels *et al.*, 2016; Keller *et al.*, 2019; Pieknik *et al.*, 2019; Ramchandani *et al.*, 2018), with 19 to 20 million infections reported on a yearly basis (Agyemang *et al.*, 2018; Keller *et al.*, 2019).

The rate of HSV-2 prevalence varies in different regions, for instance Sub-Saharan Africa experiences over 50% infection for males and over 80% for females, with an overall 90% prevalence in Africa (Daniels *et al.*, 2016). With Africa having the highest prevalence rates of both types of HSV, 87% for HSV-1 and 14.4% for HSV-2, it is clear that HSV-2 is more prevalent in low-income regions (Francis, 2018). In South Africa, studies have shown a 31% prevalence of HSV-2 in women aged 15 to 26, while an 84% prevalence was shown among female commercial sex workers in the province of KwaZulu-Natal (Daniels *et al.*, 2016).

Risk factors for genital HSV infection include older age, female gender, black race, poor socio-economic status, low-level education, prior sexually transmitted disease, early age at first sexual intercourse, and a high number of lifetime sexual partners (Fatahzadeh & Schwartz,

2007). Previous analysis of HSV-2 risk factors showed that women whose sexual debut was at an age younger than 16 years old were at higher risk of being HSV-2 positive. Women who had not attended high school were at greater risk of having acquired HSV-2 (Abbai *et al.*, 2015).

HSV-2 is transmitted through sexual intercourse, and is caused by the introduction of the virus onto the mucosal surface or through the lesions of the skin during sexual contact (Brugha *et al.*, 1997). At primary infection, the virus infects epithelial cells and starts replicating at the site of infection (Fatahzadeh & Schwartz, 2007), then continues to spread to nerve endings, followed by retrograde axonal transport to establish persistent infection in the sacral ganglia, where lifetime latency is established in the infected host (Johnston & Corey, 2016; Schiffer & Gottlieb, 2017). HSV-2 reactivates within ganglia almost constantly and travels back to the genital tract via axons, where it again spreads amongst keratinocytes (Schiffer & Gottlieb, 2017).

2.1.2 Immune responses associated with HSV-2

The innate immunity is essential for the initial detection of invading pathogens and subsequent activation of the adaptive immune response. It is the first line of defense in preventing viral invasion and replication before signalling the adaptive immune response (Chan *et al.*, 2011; Koyama *et al.*, 2008).

The adaptive immune responses to HSV-2 includes the cellular response mediated by CD4⁺ and CD8⁺ T cells, and the humoral response mediated by B-cells and antibodies. The presence of CD4⁺ and CD8⁺ T cells after infection are important for both genital epithelial immunity and for the clearing of the virus (Aoshi *et al.*, 2011; Chan *et al.*, 2011). However, it has been observed in patients with an impaired immune system that the amount of CD8⁺ T cells is lower in the case of high HSV-2 shedding at the site of infection (Sandgren *et al.*, 2016).

The presence of HSV-2 increases the population of active CD4⁺ T cells, which are directed to the epithelial layer to provide the necessary protection (Sandgren *et al.*, 2016). When the virus is active, high levels of CD4⁺ T cells have been detected at the sites of local inflammation. CD4⁺ T cells are usually the first to the site of infection, and they have been reported as the principle recruiters of CD8⁺ T cells during recurrent HSV-2 (Sandgren *et al.*, 2016). After infection, CD8⁺ T cells are recruited and take on the role of clearing the virus (Dropulic & Cohen, 2012), and controlling HSV-2 shedding from the nerve cells to prevent future reactivation and the formation of new lesions (Schiffer *et al.*, 2011).

2.2 Microbicides

Microbicides are topically applied anti-infective compounds that prevent HIV and other STIs, including HSV. They are self-administered agents that are applied directly to the genital tract and rectum before sexual intercourse (Bourne *et al.*, 2000; Lederman *et al.*, 2006; Stone, 2002). Microbicides were developed to provide women controlled protection against sexually transmitted diseases (Achilles *et al.*, 2002). The need for such agents was based on the discovery that women were eight times more susceptible to STIs than men (Omar & Bergeron, 2011). Therefore, microbicides empower women, as male condoms have always been the main contraceptive for the prevention of infections, viruses and pregnancy. However, microbicides should not be confused with contraceptives, and are not a substitute for condoms. Microbicides were initially developed to combat and reduce the global prevalence of HIV-1 transmission (Catalone *et al.*, 2005a), but being discrete female-controlled products, they offer a broad-spectrum activity against other common STIs, namely HSV, human papillomavirus (HPV), *Neisseria gonorrhoeae*, and *Chlamydia trachomatis* (Achilles *et al.*, 2002).

Microbicides can be administered in various topical application forms, including creams and gels. These products should be safe for human use, have a similar vaginal acidic environment and should be affordable (Omar & Bergeron, 2011). An effective microbicide will not only be required to provide adequate protection within moments after use, it should also maintain its activity against pathogens in the genital tract for a considerable amount of time thereafter (Bourne *et al.*, 2000). In order for microbicides to be efficient, they must inactivate viral transmission while the virus is still in the vaginal lumen, prevent the virus from attaching to its target host, and prevent viral replication after infection (Stone, 2002). By deactivating the virus, preventing viral shedding and maintaining the vaginal pH, microbicides are able to provide protection from unwanted infections (Abdel-Aleem, 2011).

There are two types of microbicides, broad-spectrum microbicides and antiretroviral (ARV) mediated microbicides. The most common broad-spectrum microbicides, which are referred to as the first generation microbicides are nonoxynol-9 (N-9), a commercially available spermicide that has been used for over 40 years (Abdel-Aleem, 2011), SAVVY (C31G), a potent antiviral agent with activity against HSV and HIV-1 (Catalone *et al.*, 2005a), acyclovir, which reduces and shortens the clinical severity and duration of the HSV episode (Brugha *et al.*, 1997), famciclovir, a synthetic antiviral drug that is used to reduce the frequency of HSV outbreaks (Perry & Wagstaff, 1995) and valacyclovir, which reduces viral shedding and recurrences (Brugha *et al.*, 1997; Schiffer *et al.*, 2011). Acyclovir, valacyclovir and famciclovir are the microbicides in use today, which serve their specific purpose from the treatment of

cold sores, growth inhibition properties towards HSV-1 and -2, to the treatment of repeat outbreaks from both types of HSV, respectively (Johnston & Corey, 2016). Carraguard, Buffergel and PRO 2000, also broad-spectrum microbicides, were shown to be safe for preventative and therapeutic usage for a wide range of STIs, but failed in clinical trials and were deemed ineffective (Abdel-Aleem, 2011), which in turn led to the research of new microbicides referred to as the second generation microbicides.

The second-generation microbicides, which are all based on antiretroviral formulations include tenofovir, which is sometimes called PMPA gel and dapivirine and formerly called TMC 120 (McMahon *et al.*, 2011). These microbicides show substantial protection against infection with HIV. The advantage of second-generation microbicides is that they are formulated from drugs designed to attack HIV, but there is also a possibility that they might not be as protective towards other STIs unlike the first generation microbicides (Abdel-Aleem, 2011). The ideal microbicide should prevent both the transmission and acquisition of HIV and HSV-2 (Brown *et al.*, 2007).

Microbicides, like any other drugs, are composed of different chemical formulations designed for their specific purpose. Even if the drug works *in vitro*, it has to be safe enough to be used *in vivo*. Microbicides such as N-9 and SAVVY are part of a list of microbicides which have successfully undergone preclinical testing and show good activity against HSV-2, but unfortunately cause damage to the epithelial layer, increasing the susceptibility to HIV. Furthermore, when evaluating a novel microbicide, safety assessment is of most importance to determine if this drug can be marked safe to use. It is important to note that microbicides are mainly applied topically intravaginally and intrarectally, and thus the safety markers can be determined by examination of the cervicovaginal tissue.

According to Abdel-Aleem (2011), in order to receive permission to test a drug candidate in humans, the developers have to show that the drug is unlikely to be harmful and that it may be beneficial to humans. Some examples of safety marker tests include, but are not limited to, determining the impact of microbicides on vaginal cytokines and chemokines, leukocyte infiltration, and effects of microbicide treatment on vaginal epithelium to name a few (Galen *et al.*, 2007).

Bismuth is a post-transitional metal, which is naturally found in minerals such as bismuthinite (Bi_2S_3) and bismite (Bi_2O_3) (Sanderson, 2019). This element can be found on the periodic table with an atomic number of 83 and an atomic weight of 208.980. The name bismuth is speculated to have originated from the German word “weismuth,” which literally translates to “white substance” (Salvador *et al.*, 2012). As a post-transitional metal, the characteristics of

bismuth differ from transitional metals. It is a soft metal with a high electrical resistance compared to transitional metals, which are known for their excellent conductivity and hard structure. Bismuth has been widely used in industry. Due to its characteristics, such as its photoconductivity and refractive index, bismuth oxide (Bi_2O_3) thin films have been applied in a number of applications including their use in sensors and optical coatings (Gujar *et al.*, 2006; Gujar *et al.*, 2005). Other well-known bismuth complexes include bismuth oxychloride (BiOCl), which has been implemented in cosmetics as a white pigment used in nail, cleansing and hair colouring products (Kulikov *et al.*, 2012), and bismuth subcarbonate ($(\text{BiO})_2\text{CO}_3$), which has been used in the medicinal world as an antibacterial agent (Chen *et al.*, 2010).

The most commonly known product containing bismuth as an active pharmaceutical ingredient in medicinal chemistry is Pepto-Bismol, a stomach ache medication, which is used to treat diarrhoea and other gastrointestinal related issues (Bowen *et al.*, 2019). Other bismuth containing products used in medicinal chemistry include colloidal bismuth substrate, which has a direct inhibitory effect on *Campylobacter pylori*, a bacterium which causes gastritis and is involved in duodenal ulcer development (Buck, 1990; Wagstaff *et al.*, 1988) and ranitidine bismuth citrate (Vondracek, 1998) and colloidal bismuth pectin (Nie *et al.*, 1999), which are both used for the treatment of *Helicobacter pylori*, a bacterium known to cause stomach ulcers. The use of the mentioned complexes are some of many bismuth containing compounds that are efficient in the treatment of gastrointestinal complications, including diarrhoea and peptic ulcer diseases (Yang *et al.*, 2015). The therapeutic record of accomplishment of bismuth complexes has attracted a lot of attention in vaccine and microbicide research. Bi-SP, a metal-based broad-spectrum novel bismuth (III) complex, has already been investigated *in vitro* for the rapid inactivation of HIV-1 as well as viral and bacterial/protozoan STIs, namely HSV-2, HPV-16 and *Chlamydia trachomatis*.

2.3 Animal models

An animal model is a simple representation of a complex system (Denayer *et al.*, 2014). With the use of animals, certain procedures and tests, which could be unethical to conduct on humans, can be performed such as the development and design of surgical techniques before being conducted on humans (Barre-Sinoussi & Montagutelli, 2015b). Animal models are valuable for drug development, for example in testing prevention and therapeutic strategies against infection by HIV-1 and other sexually transmitted pathogens. They can also be used to acquire valuable information on the pharmacokinetics of vaginally- or rectally-delivered compounds, including the longevity of protective effects.

The ideal animal model should replicate, to a major extent, both a human disease phenotype and its underlying causality and be based on the study that is to be conducted (McGonigle & Ruggeri, 2014). It is important that researchers work with animals that have similar systems to those of humans (Vandamme, 2015). Anatomical, genetical and physiological similarities between animals and humans should allow the model of choice to be able to mimic the pathological responses of humans as closely as possible, making the animal model more efficient for pre-clinical testing before moving on to clinical tests (Barre-Sinoussi & Montagutelli, 2015b; McGonigle & Ruggeri, 2014).

2.3.1 Types of animal models in microbicide research

Nonhuman primate (NHP) models are considered the ideal animal model for studying the pathogenesis of viruses such as HIV (Barouch *et al.*, 2012b) and HSV-2 (Crostarosa *et al.*, 2009). The most commonly used NHP models are macaques, which include the rhesus, pigtail and cynomolgus subtypes.

Rhesus macaques are presently still the model of choice for microbicide safety and efficacy testing, where they can be used for vaginal and rectal viral challenges (Crostarosa *et al.*, 2009; Yu *et al.*, 2009). Pigtail macaques have been widely used for vaginal and rectal microbicide safety and efficacy testing (Patton *et al.*, 2009). Some investigators prefer them over rhesus monkeys for vaginal challenge studies because like humans, they give birth all year round whereas rhesus macaques have breeding seasons. The cynomolgus macaques have been used in HIV research and show applicability for future microbicide studies (Veazey *et al.*, 2012). NHP models are however more expensive to maintain (VandeBerg & Williams-Blangero, 1997b) and require highly trained personnel to handle them (Daadi *et al.*, 2014).

Rodent models are the most commonly used models due to their simplicity in experiments and their ability to be genetically modified. Rodent models have been used to study HSV induced immune responses and pathogenesis and the most commonly used species include mice, rats, rabbits and guinea pigs (Kollias *et al.*, 2015; Vandamme, 2015). Murine models are more cost-efficient than other models, easy to breed and can be genetically manipulated for different types of studies (Vandamme, 2015). These models have been used to study latent and recurrent infections of HSV (Stanberry, 1994) and mucosal immunity to HSV-2 in the genital mucosa (Kollias *et al.*, 2015).

Murine models that are currently in use include the humanised bone marrow/liver/thymic (BLT) mouse model (Lavender *et al.*, 2018), the severe combined immune deficiency (SCID) mice (Miyoshi *et al.*, 1999a), and BALB/c mice (Sartori *et al.*, 2016a). The BLT mice are generally

considered more relevant for mucosal challenge models, and for microbicide efficacy screening since this model has human mucosal target cells. The BLT mice are used more frequently in HIV related studies than in HSV-2 studies (Denton *et al.*, 2011). The SCID mice have always been used in HIV-1 research (Miyoshi *et al.*, 1999b), but the limited reconstitution and distribution of relevant viral target cells into their mucosal tissues has limited their value in the field of microbicide research (Veazey *et al.*, 2012).

BALB/c mice are inbred strains of mice that have been well characterised and used widely in immunology studies (Dinkel *et al.*, 1999). These inbred mice are free of genetic variations that could increase variation in experimental results, making them useful in vaccine development and studies of infectious disease. BALB/c mice are frequently used in microbicide studies as they have been established for HSV-2 genital infection (Da Costa *et al.*, 2001; Diaz & Knipe, 2016; Sanjuan & Zimberlin, 2001; Wilson *et al.*, 2009). One of the reasons for the use of BALB/c mice is that they are susceptible to HSV-2 (compared to the C57BL/6 strain) (Chew *et al.*, 2009), allowing for the study of immune responses caused by the virus, and the activity of novel microbicides and vaccines against these viruses.

2.3.2 Immune responses in HSV-2 infected BALB/c mice

In-depth studies on the release of CD4⁺ and CD8⁺ T cells in response to HSV-2 have been studied more in mice than in humans. In mice, the expression of CD4⁺ and CD8⁺ T cells in the presence of HSV-2 has been studied in detail (Kwant-Mitchell *et al.*, 2009; Parr & Parr, 1997; Parr & Parr, 2003). Although it is believed that CD8⁺ T cells are responsible for viral clearance, studies conducted in mice suggest that CD4⁺ T cells might be important for acute HSV-2 viral clearance (Chan *et al.*, 2011). Interferon gamma (IFN- γ), which is produced early after infection by natural killer (NK) cells and later by CD4⁺ T cells, has been shown to be a crucial cytokine for the control of HSV in mouse models (Kwant-Mitchell *et al.*, 2009). CD4⁺ T cells are recruited to the site of infection and release IFN- γ , which stimulates the recruitment of CD8⁺ T cells that are responsible for viral clearance and by suppressing reactivation of HSV-2 by inhibiting viral replication (Chentoufi & Benmohamed, 2012). These cells are recruited after infection in order to be active in the early stages of infection and contribute to the early immune control (Chew *et al.*, 2009). As previously demonstrated by Sandgren *et al.* (2016), Dropulic and Cohen (2012), and Schiffer *et al.* (2011), the roles of CD4⁺ and CD8⁺ in human HSV-2 clearance also have the same function in mice. This relationship in T cell roles highlights a common biomarker for HSV-2 immunity in both human and mice models.

2.3.3 Establishing an HSV-2 model

2.3.3.1 HSV-2 strains used in animal models of infection

Herpes simplex virus has a variety of strains, which differ based on their genetic diversity across different regions of the world (Johnston *et al.*, 2017). The most commonly used strains of HSV-2 in research are the 333, HG52 and G strains (Da Costa *et al.*, 2001; Johnston *et al.*, 2017; Kwant-Mitchell *et al.*, 2009; Marshak *et al.*, 2014). Strain G is a popular strain used in microbicide research and has been shown to cause infection in *ex vivo* (Calenda *et al.*, 2017a) and in *in vivo* studies (Marshak *et al.*, 2014).

2.3.3.2 Mode of infection

In previous studies conducted by Iversen *et al.* (2017b) and Marshak *et al.* (2014), HSV-2 was delivered locally in the vaginal and rectal mucosa using a pipette. A viral titer of 1×10^6 PFU for HSV-2 strain G was adapted from a study conducted by Calenda *et al.* (2017b). This is a non-invasive way of infecting female and male mice with the virus. The hormone medroxyprogesterone (MPA) is administered in female mice prior to infection, in order to induce the thinning of the vaginal epithelium, which in turn will increase the susceptibility of the animal model to the virus infection (Kaushic *et al.*, 2003; Veazey, 2013). A progesterone derivative mostly used in HSV-2 challenge studies is notably Depo-Provera, which is a brand name for medroxyprogesterone acetate (MPA) (Iversen *et al.*, 2017a; Marshak *et al.*, 2014; Wilson *et al.*, 2009). This is the most frequently used hormone in HSV-2 microbicide studies and is administered subcutaneously.

2.3.3.3 Assays used to characterise mouse models

2.3.3.3.1 Virus plaque assay

Virus plaque assays are considered one of the most accurate methods for the direct quantification of infectious virions, and are used to determine the number of infectious viruses present in a given sample (Baer & Kehn-Hall, 2014). Plaque assays usually involve growing Vero cells infected with a serially diluted virus, HSV-2 in this case, of an unknown concentration in 6 or 24 well culture plates. Typically, semi-solid overlays, such as agar, are used to restrict viral spread, preventing the spread of the virus to other cells in order to form “plaques.” After an incubation period, the agar overlay is removed and a dye, such as the commonly used crystal violet, is added; trypan blue can also be used to detect dead and dying cells in cytotoxicity assays and for routine assessment of cell viability. This dye provides a

counterstain that allows for the identification of the formed plaques, which will appear white on a background of the blue dye (Iversen *et al.*, 2017a).

2.3.3.3.2 Histology

Histology is the study of the microscopic structure of biological material using various stains including masons stain, Golgi stain, toluidine blue and the most commonly used haematoxylin and eosin (H&E) stain. After the application of the desired stain, the biological material is sectioned and examined under a microscope (electron or light microscope) (Alturkistani *et al.*, 2016). The process of histological staining takes five key stages, which involve fixation, processing, embedding, sectioning and staining (Alturkistani *et al.*, 2016). Histological evaluation of vaginal and rectal cavities collected from BALB/c mice has been used to detect epithelial cell disruption, macrophages and the release of inflammatory chemokines from mice administered with various treatments before being infected with HSV-2 (Galen *et al.*, 2007). Due to the invasiveness of HSV-2, histological evaluations of the mucosal surface and epithelial layer allow the observation of the epithelial disruption or inflammation caused by the virus (Gillgrass *et al.*, 2005).

2.3.3.3.3 Flow cytometry

Flow cytometry is a type of methodology that allows real-time analysis of cellular composition, cell signalling and other relevant immunological pathways. It has many applications and can be used on virtually any cellular source, blood, body fluid, tissue and bone marrow (Abraham & Aubert, 2016). Flow cytometric assays range from qualitative to quantitative (relative and absolute) and phenotyping to functional, besides being useful for assessing specific protein expression, cell viability, apoptosis and death, cellular interactions and cell enrichment (Abraham & Aubert, 2016; Brussaard *et al.*, 2000). Flow cytometry in HSV-2 model characterisation can be used to detect the presence of CD4⁺ and CD8⁺ T cells (Posavad *et al.*, 2017).

2.4 Conclusion

HSV-2 is a major concern globally and in developing countries, specifically regions presenting with a high HIV/AIDS epidemic. With the ongoing research on preventative measures for reducing the spread of this STI, microbicides are the products of choice to decrease the ever-increasing cases of STI's. Although there have been microbicides dedicated for the prevention of HIV and HSV-2, broad-spectrum microbicides are in high demand and Bi-SP aims to fill that gap in microbicide development. BALB/c mouse models are ideal candidates due to their high susceptibility to the virus and their ability to be used to assess biomarkers of HSV-2 infection.

References

- Abbai, N.S., Wand, H. & Ramjee, G. 2015. Socio-demographic and behavioural characteristics associated with HSV-2 sero-prevalence in high risk women in KwaZulu-Natal. *BMC Research Notes*, 8:185.
- Abdel-Aleem, H. 2011. Vaginal microbicides: An overview. *Middle East Fertility Society Journal*, 16(1):14-18.
- Abraham, R.S. & Aubert, G. 2016. Flow Cytometry, a Versatile Tool for Diagnosis and Monitoring of Primary Immunodeficiencies. *Clinical Vaccine Immunology*, 23(4):254-271.
- Achilles, S.L., Shete, P.B., Whaley, K.J., Moench, T.R. & Cone, R.A. 2002. Microbicide efficacy and toxicity tests in a mouse model for vaginal transmission of Chlamydia trachomatis. *Sexually Transmitted Diseases*, 29(11):655-664.
- Agyemang, E., Magaret, A.S., Selke, S., Johnston, C., Corey, L. & Wald, A. 2018. Herpes Simplex Virus Shedding Rate: Surrogate Outcome for Genital Herpes Recurrence Frequency and Lesion Rates, and Phase 2 Clinical Trials End Point for Evaluating Efficacy of Antivirals. *The Journal of Infectious Diseases*, 218(11):1691-1699.
- Ali, M.K., Hathal, H.D. & Almoayed, H.A. 2018. Prevalence and Diagnosis of Genital Herpes by Immunological and Molecular Study. *Iraqi Journal of Medical Sciences*, 16(1).
- Alturkistani, H.A., Tashkandi, F.M. & Mohammedsaleh, Z.M. 2016. Histological stains: a literature review and case study. *Global Journal of Health Science*, 8(3):72.
- Aoshi, T., Koyama, S., Kobiyama, K., Akira, S. & Ishii, K.J. 2011. Innate and adaptive immune responses to viral infection and vaccination. *Current Opinion in Virology*, 1(4):226-232.
- Baer, A. & Kehn-Hall, K. 2014. Viral concentration determination through plaque assays: using traditional and novel overlay systems. *Journal of Visualized Experiments*(93):e52065.
- Barouch, D.H., Klasse, P.J., Dufour, J., Veazey, R.S. & Moore, J.P. 2012. Macaque studies of vaccine and microbicide combinations for preventing HIV-1 sexual transmission. *Proceedings of the National Academy of Sciences of the United States of America*, 109(22):8694-8698.
- Barre-Sinoussi, F. & Montagutelli, X. 2015. Animal models are essential to biological research: issues and perspectives. *Future Science OA*, 1(4):FSO63.
- Bourne, N., Stanberry, L.R., Kern, E.R., Holan, G., Matthews, B. & Bernstein, D.I. 2000. Dendrimers, a new class of candidate topical microbicides with activity against herpes simplex virus infection. *Antimicrobial Agents and Chemotherapy*, 44(9):2471-2474.
- Bowen, A., Agboatwalla, M., Pitz, A., Salahuddin, S., Brum, J. & Plikaytis, B. 2019. Effect of Bismuth Subsalicylate vs Placebo on Use of Antibiotics Among Adult Outpatients With Diarrhea in Pakistan: A Randomized Clinical Trial. *JAMA Network Open*, 2(8):e199441.
- Brown, J.M., Wald, A., Hubbard, A., Rungruengthanakit, K., Chipato, T., Rugpao, S., Mmiro, F., Celentano, D.D., Salata, R.S., Morrison, C.S., Richardson, B.A. & Padian, N.S. 2007. Incident and prevalent herpes simplex virus type 2 infection increases risk of HIV acquisition among women in Uganda and Zimbabwe. *AIDS*, 21(12):1515-1523.

- Brugha, R., Keersmaekers, K., Renton, A. & Meheus, A. 1997. Genital herpes infection: a review. *International Journal of Epidemiology*, 26(4):698-709.
- Brussaard, C.P., Marie, D. & Bratbak, G. 2000. Flow cytometric detection of viruses. *Journal of Virological Methods*, 85(1-2):175-182.
- Buck, G.E. 1990. Campylobacter pylori and gastroduodenal disease. *Clinical Microbiology Reviews*, 3(1):1-12.
- Calenda, G., Villegas, G., Barnable, P., Litterst, C., Levendosky, K., Gettie, A., Cooney, M.L., Blanchard, J., Fernandez-Romero, J.A., Zydowsky, T.M. & Teleshova, N. 2017. MZC Gel Inhibits SHIV-RT and HSV-2 in Macaque Vaginal Mucosa and SHIV-RT in Rectal Mucosa. *Journal of Acquired Immune Deficiency Syndrome*, 74(3):e67-e74.
- Catalone, B.J., Kish-Catalone, T.M., Neely, E.B., Budgeon, L.R., Ferguson, M.L., Stiller, C., Miller, S.R., Malamud, D., Krebs, F.C., Howett, M.K. & Wigdahl, B. 2005. Comparative safety evaluation of the candidate vaginal microbicide C31G. *Antimicrobial Agents and Chemotherapy*, 49(4):1509-1520.
- Chan, T., Barra, N.G., Lee, A.J. & Ashkar, A.A. 2011. Innate and adaptive immunity against herpes simplex virus type 2 in the genital mucosa. *Journal of Reproductive Immunology*, 88(2):210-218.
- Chen, R., Cheng, G., So, M.H., Wu, J.L., Lu, Z., Che, C.M. & Sun, H.Z. 2010. Bismuth subcarbonate nanoparticles fabricated by water-in-oil microemulsion-assisted hydrothermal process exhibit anti-Helicobacter pylori properties. *Materials Research Bulletin*, 45(5):654-658.
- Chentoufi, A.A. & Benmohamed, L. 2012. Mucosal herpes immunity and immunopathology to ocular and genital herpes simplex virus infections. *Clinical and Developmental Immunology*, 2012:149135.
- Chew, T., Taylor, K.E. & Mossman, K.L. 2009. Innate and adaptive immune responses to herpes simplex virus. *Viruses*, 1(3):979-1002.
- Crostarosa, F., Aravantinou, M., Akpogheneta, O.J., Jasny, E., Shaw, A., Kenney, J., Piatak, M., Lifson, J.D., Teitelbaum, A., Hu, L., Chudolij, A., Zydowsky, T.M., Blanchard, J., Gettie, A. & Robbiani, M. 2009. A macaque model to study vaginal HSV-2/immunodeficiency virus co-infection and the impact of HSV-2 on microbicide efficacy. *PLoS ONE*, 4(11):e8060.
- Da Costa, X.J., Morrison, L.A. & Knipe, D.M. 2001. Comparison of different forms of herpes simplex replication-defective mutant viruses as vaccines in a mouse model of HSV-2 genital infection. *Virology*, 288(2):256-263.
- Daadi, M.M., Barberi, T., Shi, Q. & Lanford, R.E. 2014. Nonhuman primate models in translational regenerative medicine. *Stem Cells and Development*, 23 Suppl 1:83-87.
- Daniels, B., Wand, H., Ramjee, G. & Team, M.D.P. 2016. Prevalence of Herpes Simplex Virus 2 (HSV-2) infection and associated risk factors in a cohort of HIV negative women in Durban, South Africa. *BMC Research Notes*, 9(1):510.
- Denayer, T., Stöhr, T. & Roy, M.V. 2014. Animal models in translational medicine: Validation and prediction. *European Journal of Molecular & Clinical Medicine*, 2(1).

- Denton, P.W., Othieno, F., Martinez-Torres, F., Zou, W., Krisko, J.F., Fleming, E., Zein, S., Powell, D.A., Wahl, A., Kwak, Y.T., Welch, B.D., Kay, M.S., Payne, D.A., Galloway, P., Appella, E., Estes, J.D., Lu, M. & Garcia, J.V. 2011. One percent tenofovir applied topically to humanized BLT mice and used according to the CAPRISA 004 experimental design demonstrates partial protection from vaginal HIV infection, validating the BLT model for evaluation of new microbicide candidates. *Journal of Virology*, 85(15):7582-7593.
- Diaz, F.M. & Knipe, D.M. 2016. Protection from genital herpes disease, seroconversion and latent infection in a non-lethal murine genital infection model by immunization with an HSV-2 replication-defective mutant virus. *Virology*, 488:61-67.
- Dinkel, A., Aicher, W.K., Warnatz, K., Burki, K., Eibel, H. & Ledermann, B. 1999. Efficient generation of transgenic BALB/c mice using BALB/c embryonic stem cells. *Journal of Immunological Methods*, 223(2):255-260.
- Dropulic, L.K. & Cohen, J.I. 2012. The challenge of developing a herpes simplex virus 2 vaccine. *Expert Review of Vaccines*, 11(12):1429-1440.
- Fatahzadeh, M. & Schwartz, R.A. 2007. Human herpes simplex virus infections: epidemiology, pathogenesis, symptomatology, diagnosis, and management. *Journal of the American Academy of Dermatology*, 57(5):737-763; quiz 764-736.
- Francis, A.-Y. 2018. Co-Infection Prevalence of Herpes Simplex Virus Types 1 and 2 with Human Papillomavirus and Associated Risk Factors among Asymptomatic Women in Ghana. *International Journal of Infectious Diseases and Therapy*, 3(3).
- Galen, B.T., Martin, A.P., Hazrati, E., Garin, A., Guzman, E., Wilson, S.S., Porter, D.D., Lira, S.A., Keller, M.J. & Herold, B.C. 2007. A comprehensive murine model to evaluate topical vaginal microbicides: mucosal inflammation and susceptibility to genital herpes as surrogate markers of safety. *Journal of Infectious Diseases*, 195(9):1332-1339.
- Gillgrass, A.E., Tang, V.A., Towarnicki, K.M., Rosenthal, K.L. & Kaushic, C. 2005. Protection against genital herpes infection in mice immunized under different hormonal conditions correlates with induction of vagina-associated lymphoid tissue. *Journal of Virology*, 79(5):3117-3126.
- Gujar, T.P., Shinde, V.R., Lokhande, C.D. & Han, S.H. 2006. Electrosynthesis of Bi₂O₃ thin films and their use in electrochemical supercapacitors. *Journal of Power Sources*, 161(2):1479-1485.
- Gujar, T.P., Shinde, V.R., Lokhande, C.D., Mane, R.S. & Han, S.H. 2005. Bismuth oxide thin films prepared by chemical bath deposition (CBD) method: annealing effect. *Applied Surface Science*, 250(1-4):161-167.
- Iversen, M., Paludan, S. & Holm, C. 2017a. Vaginal HSV-2 Infection and Tissue Analysis. *Bio-Protocol*, 7(13).
- Iversen, M.B., Paludan, S.R. & Holm, C.K. 2017b. Vaginal HSV-2 Infection and Tissue Analysis. *Bio-Protocol*, 7(13).
- Johnston, C. & Corey, L. 2016. Current Concepts for Genital Herpes Simplex Virus Infection: Diagnostics and Pathogenesis of Genital Tract Shedding. *Clinical Microbiology Reviews*, 29(1):149-161.

- Johnston, C., Magaret, A., Roychoudhury, P., Greninger, A.L., Reeves, D., Schiffer, J., Jerome, K.R., Sather, C., Diem, K., Lingappa, J.R., Celum, C., Koelle, D.M. & Wald, A. 2017. Dual-strain genital herpes simplex virus type 2 (HSV-2) infection in the US, Peru, and 8 countries in sub-Saharan Africa: A nested cross-sectional viral genotyping study. *PLoS Medicine*, 14(12):e1002475.
- Kaushic, C., Ashkar, A.A., Reid, L.A. & Rosenthal, K.L. 2003. Progesterone increases susceptibility and decreases immune responses to genital herpes infection. *Journal of Virology*, 77(8):4558-4565.
- Keller, M.J., Huber, A., Espinoza, L., Serrano, M.G., Parikh, H.I., Buck, G.A., Gold, J.A., Wu, Y., Wang, T. & Herold, B.C. 2019. Impact of Herpes Simplex Virus Type 2 and Human Immunodeficiency Virus Dual Infection on Female Genital Tract Mucosal Immunity and the Vaginal Microbiome. *Journal of Infectious Diseases*, 220(5):852-861.
- Kollias, C.M., Huneke, R.B., Wigdahl, B. & Jennings, S.R. 2015. Animal models of herpes simplex virus immunity and pathogenesis. *Journal of Neurovirology*, 21(1):8-23.
- Koyama, S., Ishii, K.J., Coban, C. & Akira, S. 2008. Innate immune response to viral infection. *Cytokine*, 43(3):336-341.
- Kulikov, E., Latham, K. & Adams, M.J. 2012. Classification and discrimination of some cosmetic face powders using XRF spectrometry with chemometric data analysis. *X-Ray Spectrometry*, 41(6):410-415.
- Kwant-Mitchell, A., Ashkar, A.A. & Rosenthal, K.L. 2009. Mucosal innate and adaptive immune responses against herpes simplex virus type 2 in a humanized mouse model. *Journal of Virology*, 83(20):10664-10676.
- Lavender, K.J., Pace, C., Sutter, K., Messer, R.J., Pouncey, D.L., Cummins, N.W., Natesampillai, S., Zheng, J., Goldsmith, J., Widera, M., Van Dis, E.S., Phillips, K., Race, B., Dittmer, U., Kukolj, G. & Hasenkrug, K.J. 2018. An advanced BLT-humanized mouse model for extended HIV-1 cure studies. *AIDS*, 32(1):1-10.
- Lederman, M.M., Offord, R.E. & Hartley, O. 2006. Microbicides and other topical strategies to prevent vaginal transmission of HIV. *Nature Reviews Immunology*, 6(5):371-382.
- Lee, D.H., Zuckerman, R.A. & Practice, A.S.T.I.D.C.o. 2019. Herpes simplex virus infections in solid organ transplantation: Guidelines from the American Society of Transplantation Infectious Diseases Community of Practice. *Clinical Transplantation* :e13526.
- Looker, K.J. & Garnett, G.P. 2005. A systematic review of the epidemiology and interaction of herpes simplex virus types 1 and 2. *Sexually Transmitted Infections*, 81(2):103-107.
- Marshak, J.O., Dong, L. & Koelle, D.M. 2014. The murine intravaginal HSV-2 challenge model for investigation of DNA vaccines. *Methods in Molecular Biology*, 1144:305-327.
- McGonigle, P. & Ruggeri, B. 2014. Animal models of human disease: challenges in enabling translation. *Biochemical Pharmacology*, 87(1):162-171.
- McMahon, J.M., Morrow, K.M., Weeks, M., Morrison-Beedy, D. & Coyle, A. 2011. Potential impact of vaginal microbicides on HIV risk among women with primary heterosexual partners. *Journal of the Association Nurses in AIDS Care*, 22(1):9-16.

McQuillan, G., Kruszon-Moran, D., Flagg, E.W. & Paulose-Ram, R. 2018. Prevalence of Herpes Simplex Virus Type 1 and Type 2 in Persons Aged 14-49: United States, 2015-2016. *NCHS Data Brief*(304):1-8.

Miyoshi, H., Smith, K.A., Mosier, D.E., Verma, I.M. & Torbett, B.E. 1999a. Transduction of human CD34(+) cells that mediate long-term engraftment of NOD/SCID mice by HIV vectors. *Science*, 283(5402):682-686.

Miyoshi, H., Smith, K.A., Mosier, D.E., Verma, I.M. & Torbett, B.E. 1999b. Transduction of human CD34+ cells that mediate long-term engraftment of NOD/SCID mice by HIV vectors. *Science*, 283(5402):682-686.

Nie, Y., Li, Y., Wu, H., Sha, W., Du, H., Dai, S., Wang, H. & Li, Q. 1999. Colloidal bismuth pectin: an alternative to bismuth subcitrate for the treatment of *Helicobacter pylori*-positive duodenal ulcer. *Helicobacter*, 4(2):128-134.

Omar, R.F. & Bergeron, M.G. 2011. The future of microbicides. *International Journal of Infectious Diseases*, 15(10):e656-660.

Parr, M.B. & Parr, E.L. 1997. Protective immunity against HSV-2 in the mouse vagina. *Journal of Reproductive Immunology*, 36(1-2):77-92.

Parr, M.B. & Parr, E.L. 2003. Vaginal immunity in the HSV-2 mouse model. *International Reviews of Immunology*, 22(1):43-63.

Patton, D.L., Sweeney, Y.T. & Paul, K.J. 2009. A summary of preclinical topical microbicide rectal safety and efficacy evaluations in a pigtailed macaque model. *Sexually Transmitted Diseases*, 36(6):350-356.

Perry, C.M. & Wagstaff, A.J. 1995. Famciclovir. A review of its pharmacological properties and therapeutic efficacy in herpesvirus infections. *Drugs*, 50(2):396-415.

Pieknik, J.R., Bertke, A.S. & Krause, P.R. 2019. Herpes Simplex Virus 2 in Autonomic Ganglia: Evidence for Spontaneous Reactivation. *Journal of Virology*, 93(11).

Posavad, C.M., Zhao, L., Dong, L., Jin, L., Stevens, C.E., Magaret, A.S., Johnston, C., Wald, A., Zhu, J., Corey, L. & Koelle, D.M. 2017. Enrichment of herpes simplex virus type 2 (HSV-2) reactive mucosal T cells in the human female genital tract. *Mucosal Immunology*, 10(5):1259-1269.

Ramchandani, M., Selke, S., Magaret, A., Barnum, G., Huang, M.W., Corey, L. & Wald, A. 2018. Prospective cohort study showing persistent HSV-2 shedding in women with genital herpes 2 years after acquisition. *Sexually Transmitted Infections*, 94(8):568-570.

Salvador, J.A., Figueiredo, S.A., Pinto, R.M. & Silvestre, S.M. 2012. Bismuth compounds in medicinal chemistry. *Future Medicinal Chemistry*, 4(11):1495-1523.

Sanderson, R.T. 2019. Bismuth. <https://www.britannica.com/science/bismuth> Date of access: October 16 2019.

Sandgren, K.J., Bertram, K. & Cunningham, A.L. 2016. Understanding natural herpes simplex virus immunity to inform next-generation vaccine design. *Clinical and Translational Immunology*, 5(7):e94.

- Sanjuan, N.A. & Zimmerlin, M.N. 2001. Pathogenesis of herpes simplex virus type 2 experimental genital infection in pregnant mice. *FEMS Immunology and Medical Microbiology*, 30(3):197-202.
- Sartori, G., Jardim, N.S., Marcondes Sari, M.H., Dobrachinski, F., Pesarico, A.P., Rodrigues, L.C., Jr., Cargnelutti, J., Flores, E.F., Prigol, M. & Nogueira, C.W. 2016. Antiviral Action of Diphenyl Diselenide on Herpes Simplex Virus 2 Infection in Female BALB/c Mice. *Journal of Cellular Biochemistry*, 117(7):1638-1648.
- Schiffer, J.T. & Gottlieb, S.L. 2017. Biologic interactions between HSV-2 and HIV-1 and possible implications for HSV vaccine development. *Vaccine*.
- Schiffer, J.T., Magaret, A., Selke, S., Corey, L. & Wald, A. 2011. Detailed analysis of mucosal herpes simplex virus-2 replication kinetics with and without antiviral therapy. *Journal of Antimicrobial Chemotherapy*, 66(11):2593-2600.
- Schmid, D.S. 2019. Mixing It Up: New Insights Into Interspecies Recombination Between Herpes Simplex Virus Type 1 and 2. *Journal of Infectious Diseases*.
- Stanberry, L.R. 1994. Animal models and HSV latency. *Seminars in Virology*, 5(3):213-219.
- Stone, A. 2002. Microbicides: a new approach to preventing HIV and other sexually transmitted infections. *Nature Reviews Drug Discovery*, 1(12):977-985.
- Vandamme, T.F. 2015. Rodent models for human diseases. *European Journal of Pharmacology*, 759:84-89.
- VandeBerg, J.L. & Williams-Blangero, S. 1997. Advantages and limitations of nonhuman primates as animal models in genetic research on complex diseases. *Journal of Medical Primatology*, 26(3):113-119.
- Veazey, R.S. 2013. Animal models for microbicide safety and efficacy testing. *Current Opinion in HIV and AIDS*, 8(4):295-303.
- Veazey, R.S., Shattock, R.J., Klasse, P.J. & Moore, J.P. 2012. Animal models for microbicide studies. *Current HIV Research*, 10(1):79-87.
- Vondracek, T.G. 1998. Ranitidine bismuth citrate in the treatment of Helicobacter pylori infection and duodenal ulcer. *Annals of Pharmacotherapy*, 32(6):672-679.
- Wagstaff, A.J., Benfield, P. & Monk, J.P. 1988. Colloidal bismuth subcitrate. A review of its pharmacodynamic and pharmacokinetic properties, and its therapeutic use in peptic ulcer disease. *Drugs*, 36(2):132-157.
- Whitley, R. & Baines, J. 2018. Clinical management of herpes simplex virus infections: past, present, and future. *F1000Res*, 7.
- Wilson, S.S., Cheshenko, N., Fakioglu, E., Mesquita, P.M., Keller, M.J. & Herold, B.C. 2009. Susceptibility to genital herpes as a biomarker predictive of increased HIV risk: expansion of a murine model of microbicide safety. *Antiviral Therapy*, 14(8):1113-1124.
- Yang, Y., Ouyang, R., Xu, L., Guo, N., Li, W., Feng, K., Ouyang, L., Yang, Z., Zhou, S. & Miao, Y. 2015. Review: Bismuth complexes: synthesis and applications in biomedicine. *Journal of Coordination Chemistry*, 68(3):379-397.

Yu, R.R., Cheng, A.T., Lagenaur, L.A., Huang, W., Weiss, D.E., Treece, J., Sanders-Beer, B.E., Hamer, D.H., Lee, P.P., Xu, Q. & Liu, Y. 2009. A Chinese rhesus macaque (*Macaca mulatta*) model for vaginal *Lactobacillus* colonization and live microbicide development. *Journal of Medical Primatology*, 38(2):125-136.

CHAPTER 3 ESTABLISHMENT AND CHARACTERISATION OF A MOUSE MODEL OF HSV-2

3.1 Introduction

HSV-2, commonly known as genital herpes is a sexually transmitted infection (STI) that can cause serious complications in immunocompromised patients (Wald *et al.*, 1997). It is an incurable STI which, sheds frequently from genital surfaces even in the absence of signs and symptoms of the disease (Posavad *et al.*, 2017). HSV-2 is a double-stranded DNA virus belonging to the *alphaherpesviridae* family (Bayliss & Piguet, 2018; Kumar *et al.*, 2016), which affects more than 400 million people in the age group 15-49-years old and is the main cause of genital ulcer disease (Looker *et al.*, 2015). The prevalence of HSV-2 in Europe and the USA is in the range of 15-20% and as high as 80% in Sub-Saharan Africa (SSA) (Abbai *et al.*, 2015; Dudek *et al.*, 2011). Paz-Bailey *et al.* (2007) reported that in SSA regions, the prevalence of HSV-2 is as high as 80% among women compared to 50% among men.

The immune response to HSV-2 comprises of the innate and adaptive immunity being triggered in order to clear the viral infection. Helper T (CD4⁺) and cytotoxic T (CD8⁺) cells are some of the many immune responses associated with a response to a foreign invasion (Zhang *et al.*, 2018b). CD4⁺ T cells are critically important in genital epithelial immunity during primary HSV infection, whereas CD8⁺ T cells mostly have a role in clearing infection in neurons. After HSV infection, CD8⁺ T cells accumulate in the skin near the sensory nerve endings (Chentoufi & Benmohamed, 2012). These cells rapidly control the shedding of HSV from the nerve endings and the infection of epithelial cells in order to prevent the formation of new lesions (Chew *et al.*, 2009).

Animal models have for decades been used for biomedical research (Fujiwara, 2018). Nonhuman primates (NHP) have successfully been the models of choice in vaccine and microbicide safety and efficacy studies for many years (Barouch *et al.*, 2012a; Daadi *et al.*, 2014; Veazey, 2013; Veazey *et al.*, 2012). Although they share similar genetic and physiologic mechanisms with humans, they are quite expensive to maintain, especially for high volume studies (VandeBerg & Williams-Blangero, 1997a). Due to the costs associated with primates, many studies are being conducted in rodent models. Rodents such as guinea pigs, rabbits, rats and mice have been at the forefront of pre-clinical drug development studies. This is due to their low cost and high reproducibility (Diaz & Knipe, 2016; Marshak *et al.*, 2014; Wilson *et al.*, 2009).

The ability to study latent and recurrent HSV infections and the advantage of genetic modification make the rodent models ideal for infectious challenge studies. BALB/c mice were the models of choice in this study as they are inbred strains and are genetically identical, which is beneficial in studying the immune responses caused by the virus. They have been used in several genital HSV-2 studies (Da Costa *et al.*, 2001; Wilson *et al.*, 2009; Zhang *et al.*, 2018a). BALB/c mouse models have shown to be more susceptible to HSV-2 compared to other commonly used mouse strains (Halford *et al.*, 2004), including humanised bone marrow liver thymus (BLT) and severe combined immunodeficient (SCID), which have been previously utilised for microbicide and HIV-1 vaccine studies (Denton *et al.*, 2008; Denton *et al.*, 2011; Lavender *et al.*, 2018; Miyoshi *et al.*, 1999b; Princiotta *et al.*, 2018). In this study, we have established and characterised a BALB/c mouse model by infecting the mice with HSV-2 strain G.

3.2 Materials and methods

3.2.1 Materials

Thiazolyl blue tetrazolium bromide (MTT) and molecular biology grade agarose tablets were purchased from Melford (Ipswich, United Kingdom). African green monkey kidney (vero) cells and HSV-2 strain G were obtained from the National Institute of Clinical Diseases (NICD) (Sandringham, Johannesburg, South Africa). Formalin was obtained from PathCare (Goodwood, South Africa). Histoplast wax was purchased from Thermofisher Scientific (Waltham, Massachusetts, US). Dulbecco's modified eagle's medium: nutrient mixture F-12 (DMEM/F12), foetal bovine serum (FBS), penicillin-streptomycin-amphotericin B mixture and trypsin/EDTA were purchased from Lonza (Basel, Switzerland). Medroxyprogesterone acetate (MPA), phosphate-buffered saline (PBS) tablets and D-(+)-Glucose were obtained from Sigma-Aldrich (Missouri, United States). Dental brushes (0.8 mm) were purchased from Dis-Chem. Super-X Plex® Cytokine Kit was purchased from Antigenix America (Melville, United States).

3.3 Methods

3.3.1 Genital herpes mouse model establishment

Six to eight-week-old male and female BALB/c mice were bred at the DST/NWU Preclinical Drug Development Platform vivarium located at the Potchefstroom campus of the North-West University. The initial weight of the male mice ranged between 18.84 – 22.61 g and for the female mice between 14.56 – 19.43 g. The female mice (n = 8) were subcutaneously pre-treated with MPA (20 mg/animal) suspended in PBS, six days prior to the infection. The male

mice ($n = 8$) were fasted for twelve hours to prevent interruption from faeces during administration in the rectal area before the start of the experiment, water was provided *ad libitum*. The female and male mice received PBS (10 μ l) 10 minutes before the animals were infected with 1×10^6 PFU of HSV-2 strain G (16.6 μ l). PBS was administered to replicate the method for when microbicides and vaccines are given before viral infection. The control group was administered PBS without HSV-2. PBS and the virus were delivered vaginally in female mice and rectally in male mice using a 2 - 20 μ l pipette (Figure 1). Mice were monitored twice a day for 10 days. The scoring was as follows: 0). Normal, 1). Perianal/genital erythema, 2). Perianal swelling and erythema (may have slightly ataxic gait and/or slightly ruffled coat), 3). Purulent lesions, partial or complete paralysis in one or both hind limbs, visible weight loss or dehydration, very poor grooming, perianal urine staining due to loss of bladder control and, 4). Immobile, complete hind limb paralysis, severe dehydration, little or no grooming (Marshak et al., 2014). A clinical score of 3 and above constituted immediate euthanasia as this score represented the humane endpoint. Vaginal and rectal swabs were collected using 0.8 mm dental brushes two days post-infection and immediately before the animals were euthanised. The swab samples were suspended in 250 μ l of PBS, then flash-frozen in liquid nitrogen and stored at -80°C . Aliquots of the swab samples collected two days post infection were used for virus plaque assay. For the flow cytometry analysis, samples collected two days post infection and immediately before euthanasia were used. Vaginal and rectal tissues were excised from the female and male mice respectively. The tissues were sliced open longitudinally and stored in formalin for histological analysis. Representative tissue sections obtained from control mice not infected with HSV-2 were used to provide examples of intact tissue structures of the rectal and vaginal cavity. All the experiments were approved by the NWU-AnimCareREC ethics committee with the ethics number NWU-00170-18-A5 and were conducted in the environmentally controlled biosafety level (BSL) 3 laboratory. The animals were housed (five mice per cage) in individually ventilated cages (IVC) under normal conditions (room temperature $22 \pm 1^{\circ}\text{C}$, internal cage temperature $24 \pm 2^{\circ}\text{C}$, room humidity 40 – 72.7 %, cage humidity 40 – 57 % and pressure in cages at 60 air changes/hour).

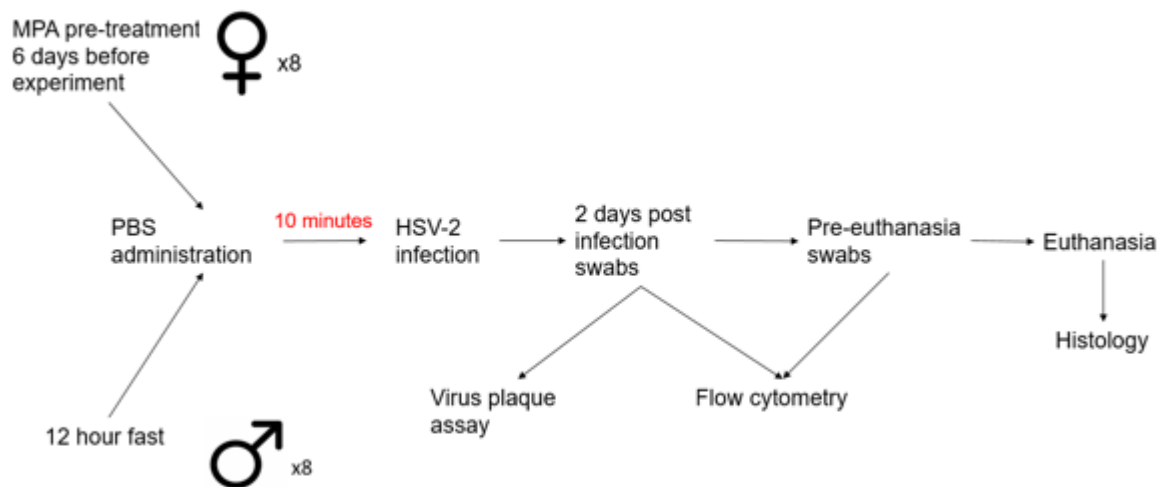


Figure 1: Schematic representation of the process for establishing and characterising an HSV-2 murine model

3.3.2 Assessment of epithelial integrity/disruption and leukocyte infiltration in rectal and vaginal tissues

For two hours, the excised formalin-fixed vaginal and rectal tissues were washed under running water and underwent a process of dehydration, elucidation and fixation, as shown by the schematic representation (Figure 2A). The tissues were transversely fixed in histoplast wax and sectioned with a microtome at a thickness of 5 - 7 μm . The sectioned bands were placed in a water bath set at 40°C, mounted on glass microscope slides and left to dry overnight in an oven set at 35°C. The tissues were stained with hematoxylin-eosin (H&E) and the coverslips were mounted on slides using Entellan™ mounting media (Figure 2B). The stained sections were evaluated for epithelial cell disruption and evidence of inflammation under a compound microscope (Nikon Eclipse E800 biological research microscope).

mixture (cDMEM). The cells were incubated until a confluence of 80% was reached. After the vero cells reached the desirable confluence, the growth medium was aseptically removed and the cells were washed with 15 ml of PBS before being chemically detached with the use of trypsin-EDTA for 3 minutes. Trypsin was deactivated with cDMEM and the cells were transferred to a 15 ml falcon centrifuge tube and centrifuged for 3 minutes at 1136 x g. The cell supernatant was discarded and the cells were suspended in cDMEM and counted using a hemocytometer. A total of 1×10^5 cells were seeded per well in a 12 well plate and were grown overnight in a cell culture incubator. Aliquots of 100 μ l of rectal and vaginal swab samples collected from male and female mice infected with HSV-2 were incubated on the vero monolayers once a confluence of 70% was reached in the 12-well plate. The samples were incubated for 4 hours on the vero cell monolayers, gently swirling the flask every hour to ensure equal sample distribution. The medium was removed after 4 hours of incubation and replaced with assay medium, which was prepared as follows: 5 ml of cDMEM, 1 ml of cDMEM enriched with 0.42 g of glucose and 24 ml of plain DMEM-F12 without FBS containing 1% of antibiotics to make a total volume of 30 ml. In 27 ml of the assay media, 3 ml of 4% agarose solution was added, the plates were left for 10 minutes for the agarose to solidify before being placed in an incubator set at 37°C, 5% CO₂ for a further two to three days to allow plaque formation. After incubation, the wells were stained with MTT dye and left for 2 – 3 hours before the plaques were counted.

3.3.4 Measurement of CD4⁺ and CD8⁺ cell count using flow cytometry

Rectal and vaginal swab samples were thawed and 5 μ l of each was taken for analysis. The samples were serially diluted threefold (1:10, 1:100, 1:1000) for a total volume of 100 μ l in reading buffer (Super-X Plex© Cytokine Kit). Samples were deactivated under UV light for 6 minutes on ice. Thereafter, the swab samples were incubated with FITC-conjugated anti-CD4 or anti-CD8 antibodies (5 μ l each) for 2 hours at 4°C in the dark. To obtain the baseline for normal T cells, swab samples collected from uninfected mice were used. For quality control purposes, the inclusion of an unstained sample from an infected mouse was used. The acquisition was performed on the BD Accuri™ C6 (BD Biosciences) and analysis was performed on the CSampler™ software: 100 000 cells/sample were acquired with the fluidics set at a slow flow rate with the FSC threshold set at 80 000. The cells were gated using forward scatter (FSC-A) versus side scatter area (SSC-A) (Figure 3).

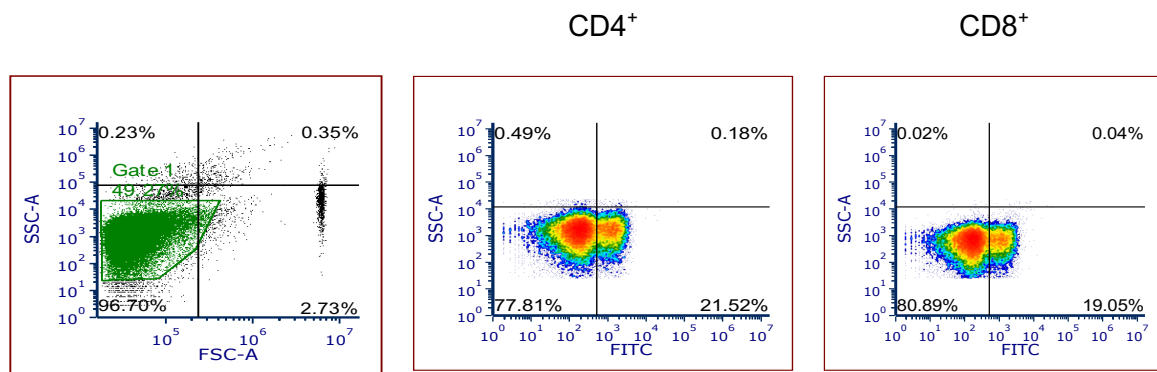


Figure 3: The gating of cells in the swab samples. Plot A (SSC vs FSC) is used to gate the population of interest based on size (FSC; forward scatter proportional to the square diameter of the cell) and complexity (SSC; side scatter) of the cells. Plot B (SSC vs FITC) is a density plot that uses the number of events (cells) to help distinguish the population of interest that is the florescent body (FITC/FL1-H). The red colour indicates the largest number of cells.

3.3.5 Statistical analysis

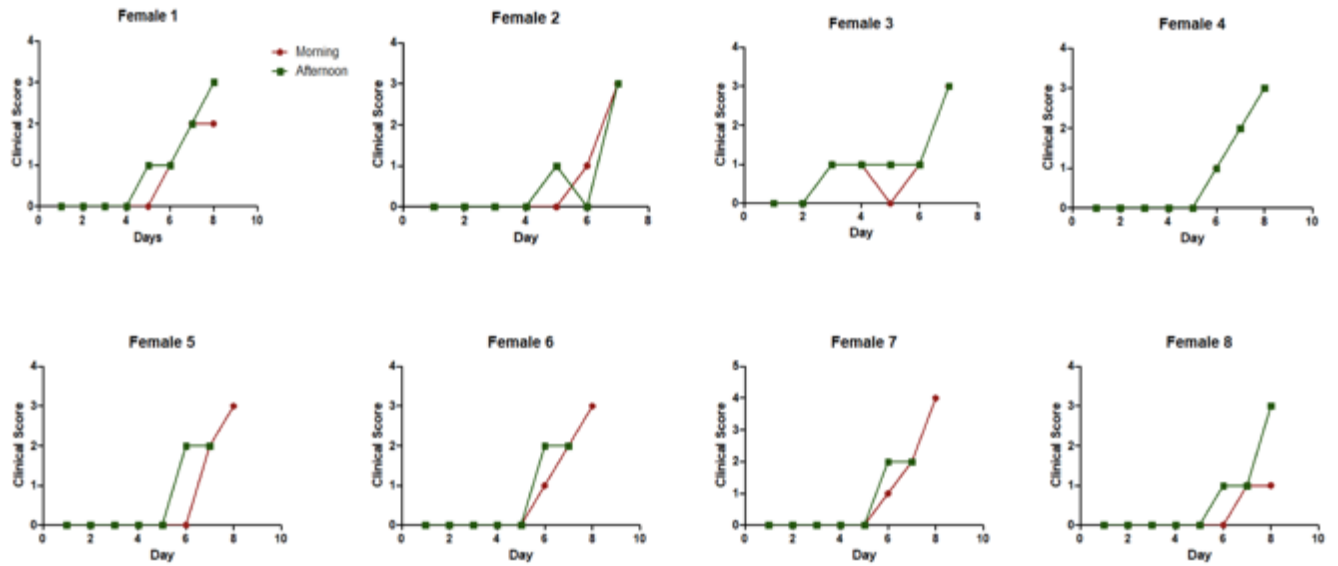
Kaplan-Meier survival curves were assessed with the log-rank test using GraphPad Prism v5 (GraphPad Software, Inc.). P values < 0.05 were considered statistically significant.

3.4 Results

3.4.1 Rectal and vaginal HSV-2 challenge

With the onset of infection, it was observed that the female mice presented with earlier clinical signs of infection compared to the male mice (Figure 4A and B). The first signs of infection were from the visible swelling on the surface of the vaginal and anal skin of the female and male mice respectively. In female mice (n = 8), early clinical signs of infection were observed from day three in mouse 3 in the morning (Figure 4A) and signs of infection in male mice (n = 8) were observed from day three in mouse 2 in the morning (Figure 4B). The majority of the female mice started presenting signs of infection on days five and six (Figure 4A) and the male mice on days six and seven (Figure 4B).

A.



B.

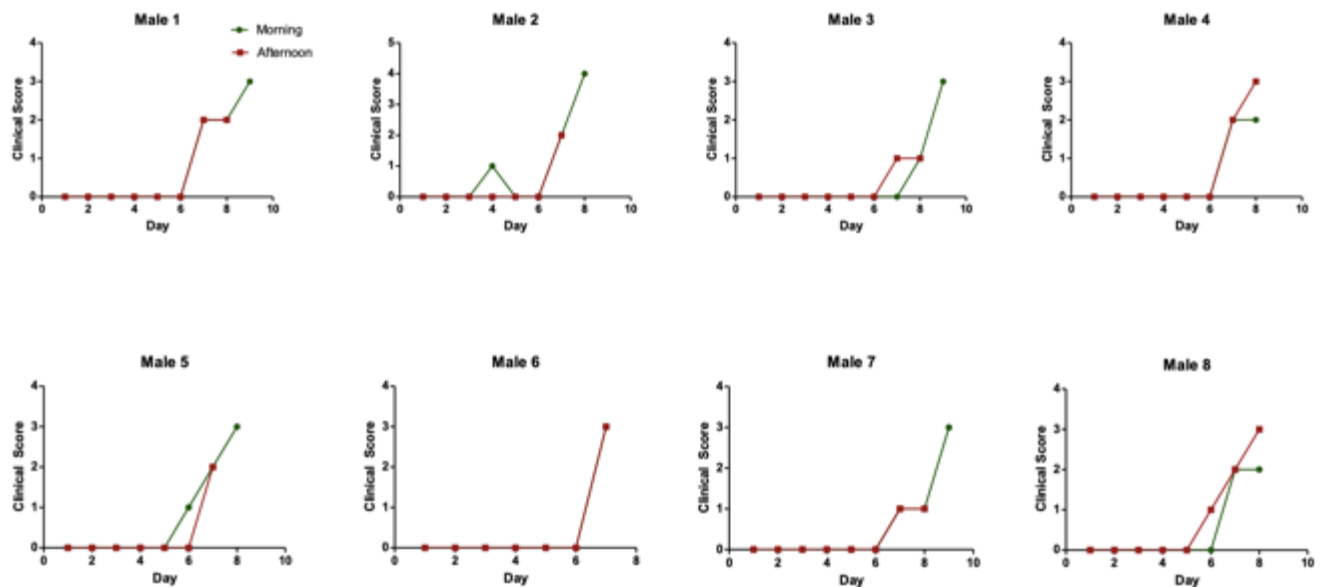


Figure 4: Clinical scores of HSV-2 infected **A.** female and **B.** male mice recorded twice a day over nine days in the morning and in the afternoon

The survival curves indicate the rate of survival (reaching of humane endpoint) of both the female and male mice after infection with HSV-2. The rectal and vaginal cavity vary in structure and environment; therefore, the male and female mice reached the humane endpoint at different times. Although the male mice survived a day longer than the female mice, there was

no significant difference ($p = 0.0946$) in the rate of survival between the two groups (Figure 5).

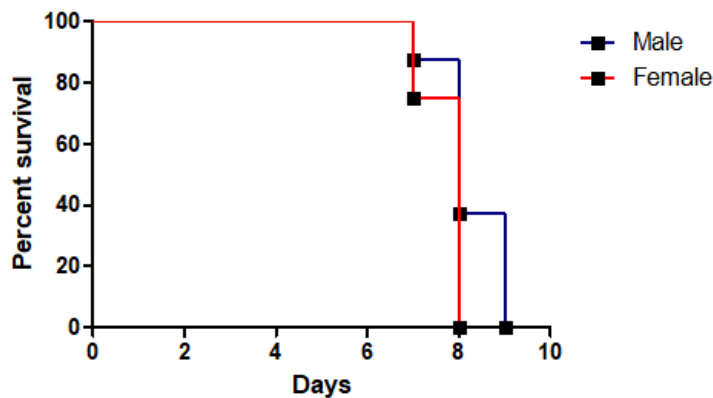


Figure 5: The survival rate of female and male mice ($p = 0.0946$) post-infection with HSV-2 strain G. Vaginal and rectal inoculation of HSV-2 lead to complete mortality (humane endpoint reached) under 10 days post-infection.

3.4.2 Effect of HSV-2 on the epithelial surface of rectal and vaginal tissues excised from male and female mice respectively

Representative tissue sections obtained from animals after euthanasia eight to nine days post-viral infection are presented in Figure 6. The uninfected rectal tissue from a male mouse control (Figure 6A) is observed with intact glands, muscularis mucosae and visible glandular crypts, indicating healthy tissue. Rectal tissue collected from a male mouse euthanised on day 8 had elevated levels of inflammatory infiltrate in the submucosa: severe disruption on the mucosa, more specifically on the glands and the glandular crypts were observed with small regions of the focused cell infiltrate (Figure 6B). The infection had a negative influence on the digestive tract resulting in constipation of the mice. Upon dissection, all eight male mice and five of the eight female mice had full bladder and dried faeces inside the colon compared to the uninfected mice, which were not constipated and had a normal bladder as seen in dissected healthy mice.

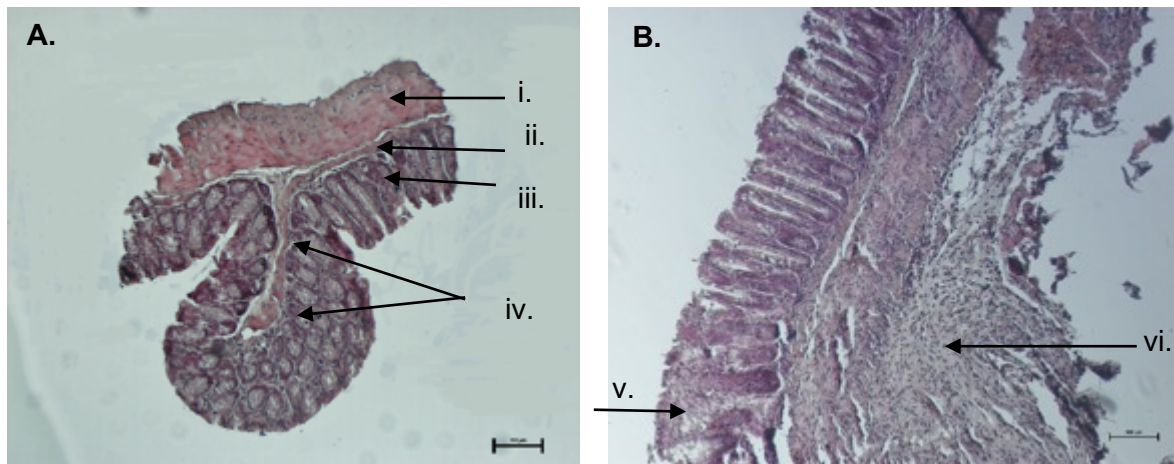


Figure 6: Hematoxylin-eosin stained rectal tissue sections for signs of inflammation and epithelial cell integrity. **A.** rectal tissue collected from an uninfected male mouse. **i.** submucosa, **ii.** muscularis mucosae, **iii.** glands, **iv.** glandular crypts **B.** rectal tissue section collected from a mouse infected with HSV-2 which was euthanised 8 days post-infection. **v.** structural damage to the glands on the mucosa, and **vi.** submucosa with an increased leukocyte infiltration. All images were captured at 10X magnification.

Figure 7A is representative of vaginal tissue excised from an uninfected female control mouse presenting with an intact squamous epithelium and a smooth muscle layer. The structural features are indicated on the images by black arrows. The representative vaginal tissue from an infected female mouse features a thinned squamous epithelium and an infiltration of inflammatory cells on the surface of the smooth muscle layer (Figure 7B), a visual indication of tissue disruption.

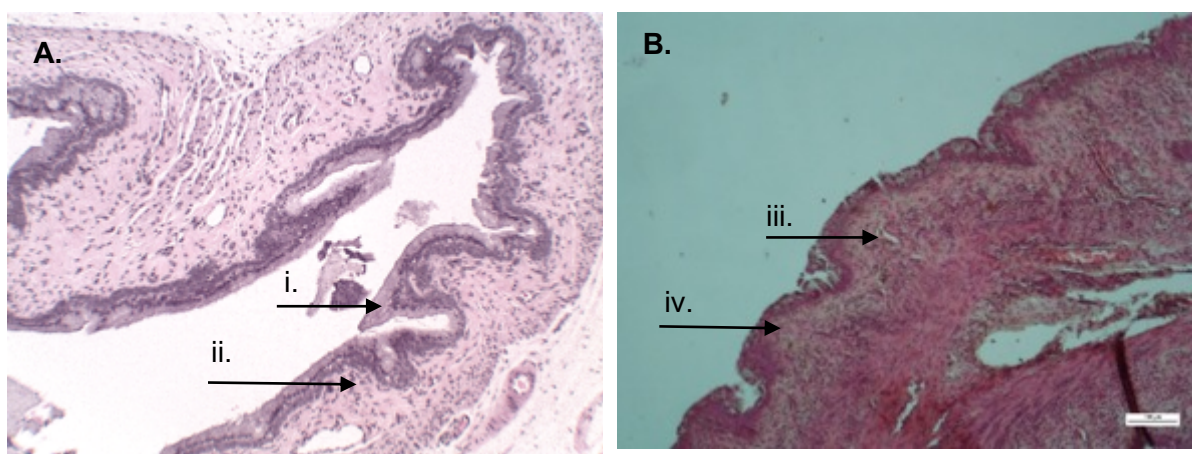


Figure 7: Hematoxylin-eosin stained vaginal sections for signs of inflammation and epithelial cell integrity. **A.** vaginal tissue collected from an uninfected female mouse. **i.** squamous epithelium, **ii.** smooth muscle layer. **B.** Vaginal tissue collected from a female mouse infected

with HSV-2 and euthanised 8 days post-infection. iii. increased leukocyte infiltration and iv. thinning of squamous epithelium. All images were captured at 10X magnification.

3.4.3 HSV-2 viability in rectal and vaginal swab samples from challenged male and female BALB/c mice

Samples collected 2 days post-HSV-2 infection developed plaques, which was an indication of successful viral inoculation in both male and female mice. It can be seen in Figure 8 that there was plaque formation in all the samples.

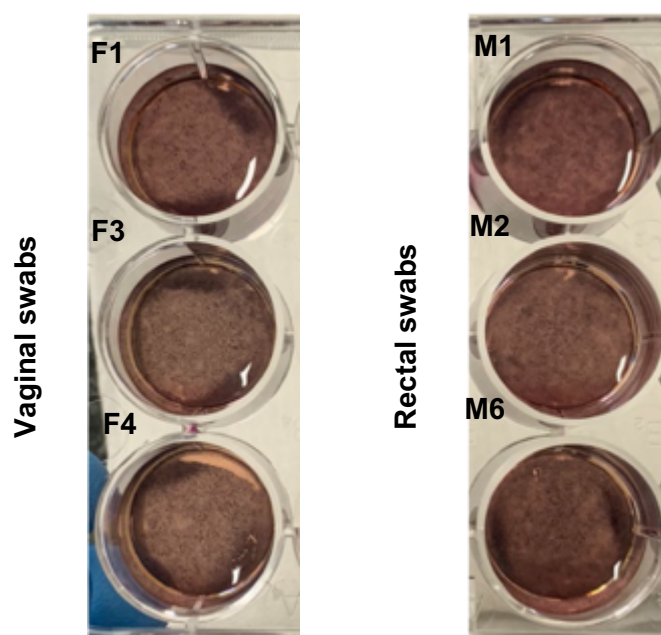


Figure 8: HSV-2 plaque overlay utilising 12 well plates. Vero cells were plated at 1×10^5 cells in 12 well plates and infected with 100 μ l of vaginal and rectal swab samples collected 2 days post infection.

3.4.4 CD4⁺ and CD8⁺ population in genital swab samples

Swab samples from uninfected mice were used to obtain a control of the normal T cell count present in healthy female (Figure 9A) and male (Figure 9B) mice. CD4⁺ and CD8⁺ cells were detected in vaginal swab samples from infected female mice (Figure 10) and were not detected in rectal swabs from infected male mice (Figure 11). Positive staining and detection of CD4⁺ and CD8⁺ are presented with the percentages indicated in the lower right quadrant of the pseudo colour plots. The percentages represent the total number of positively stained cells that were fluorescently detected using the BD Accuri™ C6 flow cytometer.

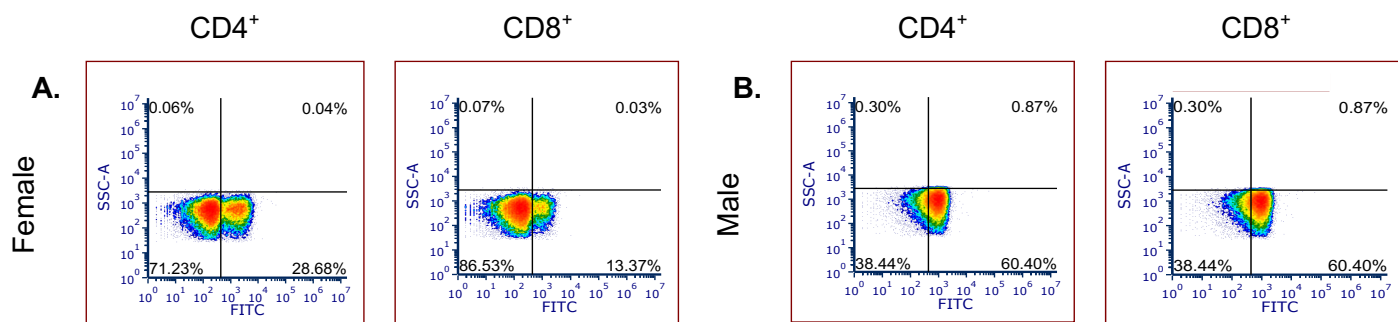


Figure 9: Percentages of CD4⁺ and CD8⁺ T cells in vaginal and rectal swab samples stained with anti-CD4 and anti-CD8 FITC-conjugated antibodies, respectively.

Randomly selected vaginal swab samples ($n = 3$) collected 2 days post infection had CD4⁺ in two of the three mice, and CD8⁺ in 2 of the 3 mice. Samples collected immediately before euthanasia had CD4⁺ in three out of three mice, and CD8⁺ in one of the three mice (Figure 10). The rectal swabs did not contain either CD4⁺ or CD8⁺ cells (Figure 11).

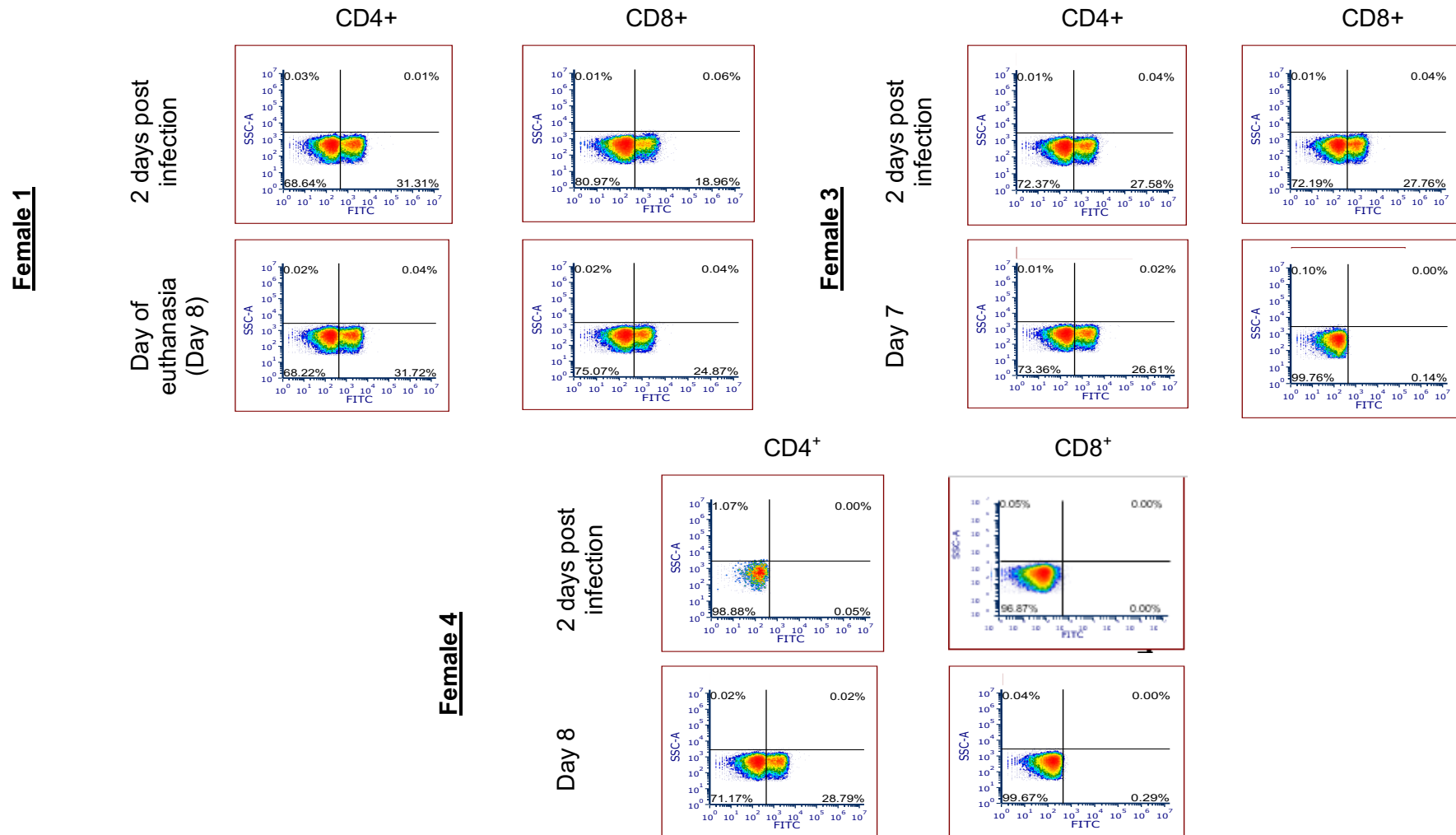


Figure 10: Percentages of CD4⁺ and CD8⁺ T cells from vaginal swab samples collected from infected female mice.

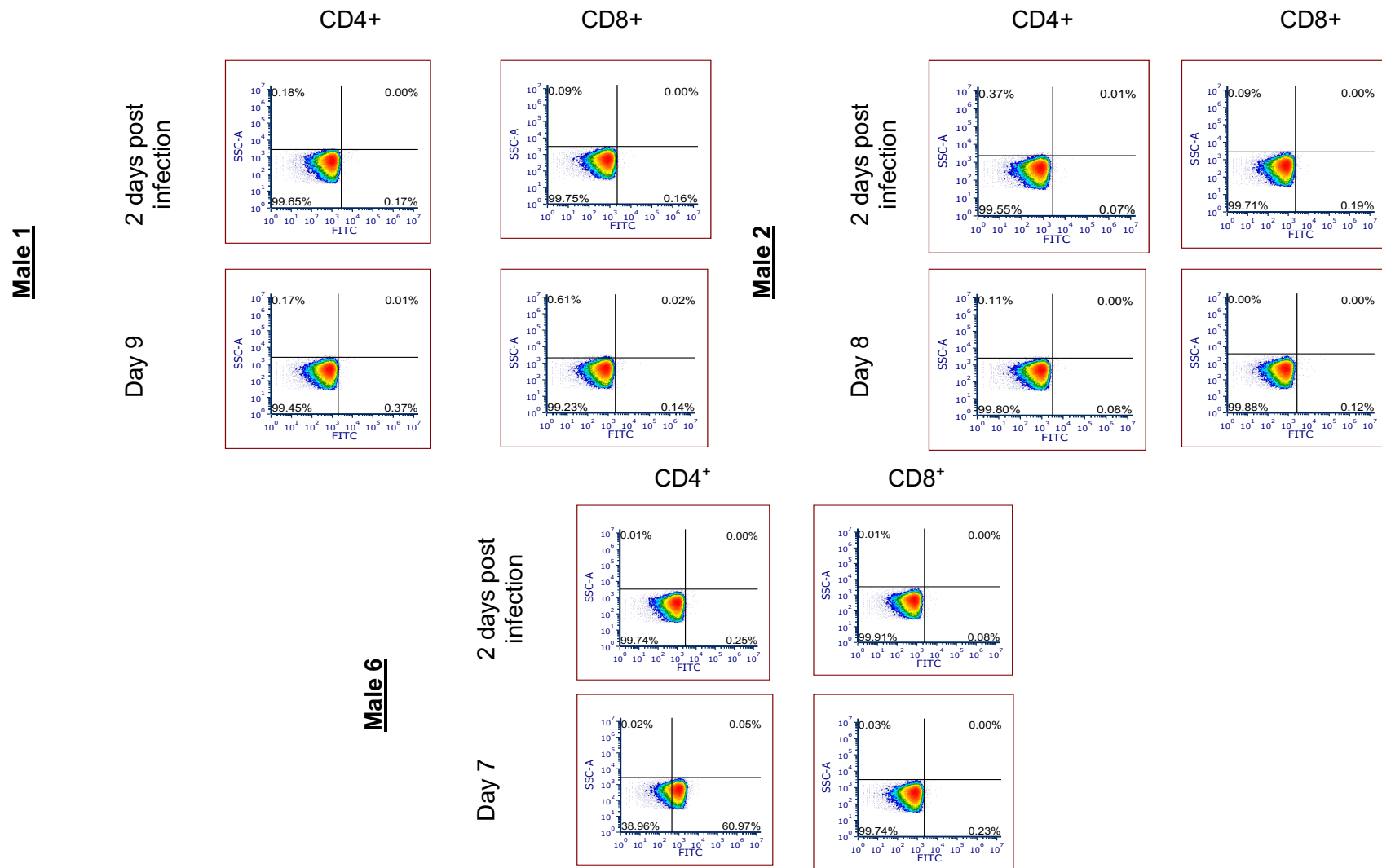


Figure 11: Percentages of CD4⁺ and CD8⁺ T cells from rectal swab samples collected from infected male mice.

3.5 Discussion

Animal models have been used for many years in vaccine and microbicide studies and the most popular models in recent years are rodents, more specifically murine models. In our study, we characterised a genital herpes model that can be applied for future studies in vaccine and microbicide testing. We chose the BALB/c models as they are cost efficient and easy to breed.

In this study, female and male BALB/c mice, eight each, were infected vaginally and rectally with HSV-2 strain G. With the observed survival rates obtained, HSV-2 was seen to be a fatal virus in mouse models compared to humans, as previously demonstrated by the findings of Marshak et al. (2014). The active virus degraded the health of the mice to the point where all the mice were euthanised only seven days after the infection in female mice, and eight days in male mice. Using virus plaque assay, infection and active viral shedding was confirmed in the vaginal and rectal swab samples collected two days post infection from each animal. A dilution series is usually a standard in plaque assays in order to obtain the ideal dilution that will allow for the counting of the plaques (Baer & Kehn-Hall, 2014). In the present study, the focus was to determine if there was viable virus in the samples. It was observed that all the randomly selected vaginal and rectal swab samples that were analysed contained viable virus.

The vaginal tissue consists essentially of a multilayer of squamous epithelia, and a significant structural difference in the thickness of the squamous epithelium was found when comparing vaginal tissues of healthy and infected female mice. The thickness of the vaginal epithelium is known to be influenced by hormonal influences (Anderson *et al.*, 2014) and defence against viruses or pathogens (Vincent *et al.*, 2013). The population of leukocytes as confirmed by a veterinarian, was significantly increased in the smooth muscle of the excised tissues.

Although, genital herpes has a higher prevalence in females in heterosexual relationships, HSV-2 is also a major concern among men in homosexual relationships (Russell et al., 2001). There is paucity of information on the HSV-2 infectious studies conducted in the rectal area. Although the environment of the rectum is not as complex as the vagina, we aim to emphasise the importance of conducting studies involving the use of this site to generate information that would be valuable in the future of microbicides.

While factors including the presence of medroxyprogesterone to increase susceptibility in female mice exist (Kaushic et al., 2003), in male mice, rectal administration is known to have a fast absorption rate due to the blood and lymph supply in the rectal mucosa for the increase in systemic absorption, thus possibly deeming the rectal mucosa easily susceptible to the virus

as well (Lowry, 2016). The structural damages to the rectal tissues were easily identified as the glands and the glandular crypts were severely disrupted. These structural features are usually well defined as also seen in our experiment on the epithelium tissues collected from the healthy mice.

The immune response caused by viruses has been explored in previous studies where chemokines and cytokines were the immune responses of interest (Cummins & Doncel, 2009). Many of these studies were conducted in female mouse models (Thapa & Carr, 2009; Thapa *et al.*, 2008; Zhang *et al.*, 2018a). These types of studies have rarely been conducted in male mice. Studies conducted by Chan *et al.* (2011) and Chew *et al.* (2009) have confirmed that the CD4⁺ and CD8⁺ T cell expression has major roles in the immunity against HSV-2 and were therefore the main focus, in this study. Past studies conducted in humans and mice confirmed that it was possible to detect CD4⁺ and CD8⁺ T cells in blood (Chentoufi & Benmohamed, 2012; Dropulic & Cohen, 2012). However, in this study, swab samples were used instead of blood samples and both cell types were detected in the vaginal swab samples: the presence of CD4⁺ and CD8⁺ in the rectal swab specimens was not detected. There is limited research that has been conducted in profiling the immune cell phenotypes in the rectal mucosa from rectal swabs in HSV-2 infected BALB/c mice. In a *Chlamydia*-related study in mice, effective detection of immune responses from rectal swabs was performed (Pais *et al.*, 2017). Immune responses from rectal swab samples could also be detected in healthy bovine (McNeilly *et al.*, 2007) and in Simian Immunodeficiency Virus infected macaques (Tuero *et al.*, 2015) to name a few. Furthermore, previous studies conducted by Pérez-Ruiz and Berraondo (2016) and Yasuda *et al.* (2011), showed that CD4⁺ and CD8⁺ cells were detected from rectal samples through immunohistochemistry in rectal tissues and rectum cancer tumours from humans. Therefore, BALB/c mice may not be an appropriate mouse model for the prediction of CD4⁺ and CD8⁺ responses in rectal mucosa.

3.6 Conclusion

There was evidence of inflammation at the site of infection, with visible swelling on the perianal and perivaginal skin. Through histological examination, an increase in leukocytes were identified, suggesting successful viral invasion. Biomarker analysis was conducted using flow cytometry to investigate CD4⁺ and CD8⁺ T cell immune response to HSV-2 infection further. The CD4⁺ and CD8⁺ cells were detected in vaginal swab samples but not in rectal swab samples. The study has shown that BALB/c mice are indeed susceptible to HSV-2, as previously stated in the literature. Furthermore, the model can be used for genital herpes challenge studies and for the analysis of CD4⁺ and CD8⁺ in the vaginal mucosa rather than

the rectal mucosa. This particular model can be used for future studies for evaluating the efficacy of novel microbicides and vaccines.

References

- Abbai, N. S., Wand, H. & Ramjee, G. 2015. Socio-demographic and behavioural characteristics associated with HSV-2 sero-prevalence in high risk women in KwaZulu-Natal. *BMC Research Notes*, 8:185.
- Anderson, D. J., Marathe, J. & Pudney, J. 2014. The structure of the human vaginal stratum corneum and its role in immune defense. *American Journal of Reproductive Immunology*, 71(6):618-623.
- Baer, A. & Kehn-Hall, K. 2014. Viral concentration determination through plaque assays: using traditional and novel overlay systems. *Journal of Visualized Experiments* (93):e52065.
- Barouch, D. H., Klasse, P. J., Dufour, J., Veazey, R. S. & Moore, J. P. 2012. Macaque studies of vaccine and microbicide combinations for preventing HIV-1 sexual transmission. *Proceedings of the National Academy of Sciences of the United States of America*, 109(22):8694-8698.
- Bayliss, R. J. & Piguet, V. 2018. The Ticking CLOCK of HSV-2 Pathology. *Journal of Investigative Dermatology*, 138(1):17-19.
- Chan, T., Barra, N. G., Lee, A. J. & Ashkar, A. A. 2011. Innate and adaptive immunity against herpes simplex virus type 2 in the genital mucosa. *Journal of Reproductive Immunology*, 88(2):210-218.
- Chentoufi, A. A. & Benmohamed, L. 2012. Mucosal herpes immunity and immunopathology to ocular and genital herpes simplex virus infections. *Clinical and Developmental Immunology*, 2012:149135.
- Chew, T., Taylor, K. E. & Mossman, K. L. 2009. Innate and adaptive immune responses to herpes simplex virus. *Viruses*, 1(3):979-1002.
- Cummins, J. E., Jr. & Doncel, G. F. 2009. Biomarkers of cervicovaginal inflammation for the assessment of microbicide safety. *Sexually Transmitted Diseases*, 36(3 Suppl):S84-91.
- Da Costa, X. J., Morrison, L. A. & Knipe, D. M. 2001. Comparison of different forms of herpes simplex replication-defective mutant viruses as vaccines in a mouse model of HSV-2 genital infection. *Virology*, 288(2):256-263.
- Daadi, M. M., Barberi, T., Shi, Q. & Lanford, R. E. 2014. Nonhuman primate models in translational regenerative medicine. *Stem Cells and Development*, 23 Suppl 1:83-87.
- Denton, P. W., Estes, J. D., Sun, Z., Othieno, F. A., Wei, B. L., Wege, A. K., Powell, D. A., Payne, D., Haase, A. T. & Garcia, J. V. 2008. Antiretroviral pre-exposure prophylaxis prevents vaginal transmission of HIV-1 in humanized BLT mice. *PLoS Medicine*, 5(1):e16.
- Denton, P. W., Othieno, F., Martinez-Torres, F., Zou, W., Krisko, J. F., Fleming, E., Zein, S., Powell, D. A., Wahl, A., Kwak, Y. T., Welch, B. D., Kay, M. S., Payne, D. A., Gallay, P., Appella, E., Estes, J. D., Lu, M. & Garcia, J. V. 2011. One percent tenofovir applied topically to

humanized BLT mice and used according to the CAPRISA 004 experimental design demonstrates partial protection from vaginal HIV infection, validating the BLT model for evaluation of new microbicide candidates. *Journal of Virology*, 85(15):7582-7593.

Diaz, F. M. & Knipe, D. M. 2016. Protection from genital herpes disease, seroconversion and latent infection in a non-lethal murine genital infection model by immunization with an HSV-2 replication-defective mutant virus. *Virology*, 488:61-67.

Dropulic, L. K. & Cohen, J. I. 2012. The challenge of developing a herpes simplex virus 2 vaccine. *Expert Review of Vaccines*, 11(12):1429-1440.

Dudek, T. E., Torres-Lopez, E., Crumpacker, C. & Knipe, D. M. 2011. Evidence for differences in immunologic and pathogenesis properties of herpes simplex virus 2 strains from the United States and South Africa. *Journal of Infectious Diseases*, 203(10):1434-1441.

Fujiwara, S. 2018. Humanized mice: A brief overview on their diverse applications in biomedical research. *Journal of Cellular Physiology*, 233(4):2889-2901.

Halford, W. P., Balliet, J. W. & Gebhardt, B. M. 2004. Re-evaluating natural resistance to herpes simplex virus type 1. *Journal of Virology*, 78(18):10086-10095.

Kaushic, C., Ashkar, A. A., Reid, L. A. & Rosenthal, K. L. 2003. Progesterone increases susceptibility and decreases immune responses to genital herpes infection. *Journal of Virology*, 77(8):4558-4565.

Kumar, S. P., Chandy, M. L., Shanavas, M., Khan, S. & Suresh, K. V. 2016. Pathogenesis and life cycle of herpes simplex virus infection-stages of primary, latency and recurrence. *Journal of Oral and Maxillofacial Surgery Medicine and Pathology*, 28(4):350-353.

Lavender, K. J., Pace, C., Sutter, K., Messer, R. J., Pouncey, D. L., Cummins, N. W., Natesampillai, S., Zheng, J., Goldsmith, J., Widera, M., Van Dis, E. S., Phillips, K., Race, B., Dittmer, U., Kukolj, G. & Hasenkrug, K. J. 2018. An advanced BLT-humanized mouse model for extended HIV-1 cure studies. *AIDS*, 32(1):1-10.

Looker, K. J., Magaret, A. S., Turner, K. M., Vickerman, P., Gottlieb, S. L. & Newman, L. M. 2015. Global estimates of prevalent and incident herpes simplex virus type 2 infections in 2012. *PloS ONE*, 10(1):e114989.

Lowry, M. 2016. Rectal drug administration in adults: how, when, why. *Nursing times*, 112(8):12-14.

Marshak, J. O., Dong, L. & Koelle, D. M. 2014. The murine intravaginal HSV-2 challenge model for investigation of DNA vaccines. *Methods in Molecular Biology*, 1144:305-327.

McNeilly, T. N., Naylor, S. W., Mitchell, M. C., McAteer, S., Mahajan, A., Smith, D. G., Gally, D. L., Low, J. C. & Huntley, J. F. 2007. Simple methods for measurement of bovine mucosal antibody responses in vivo. *Veterinary Immunology and Immunopathology*, 118(1-2):160-167.

Miyoshi, H., Smith, K. A., Mosier, D. E., Verma, I. M. & Torbett, B. E. 1999. Transduction of human CD34+ cells that mediate long-term engraftment of NOD/SCID mice by HIV vectors. *Science*, 283(5402):682-686.

Pais, R., Omosun, Y., He, Q., Blas-Machado, U., Black, C., Igietseme, J. U., Fujihashi, K. & Eko, F. O. 2017. Rectal administration of a chlamydial subunit vaccine protects against genital infection and upper reproductive tract pathology in mice. *PLoS ONE*, 12(6):e0178537.

Paz-Bailey, G., Ramaswamy, M., Hawkes, S. J. & Geretti, A. M. 2007. Herpes simplex virus type 2: epidemiology and management options in developing countries. *Sexually Transmitted Infections*, 83(1):16-22.

Perez-Ruiz, E. & Berraondo, P. 2016. Immunological Landscape and Clinical Management of Rectal Cancer. *Frontiers in Immunology*, 7:61.

Posavad, C. M., Zhao, L., Dong, L., Jin, L., Stevens, C. E., Magaret, A. S., Johnston, C., Wald, A., Zhu, J., Corey, L. & Koelle, D. M. 2017. Enrichment of herpes simplex virus type 2 (HSV-2) reactive mucosal T cells in the human female genital tract. *Mucosal Immunology*, 10(5):1259-1269.

Princiotta, A. M., Vrbanc, V. D., Melillo, B., Park, J., Tager, A. M., Smith, A. B., 3rd, Sodroski, J. & Madani, N. 2018. A Small-Molecule CD4-Mimetic Compound Protects Bone Marrow-Liver-Thymus Humanized Mice From HIV-1 Infection. *Journal of Infectious Diseases*, 218(3):471-475.

Russell, D. B., Tabrizi, S. N., Russell, J. M. & Garland, S. M. 2001. Seroprevalence of herpes simplex virus types 1 and 2 in HIV-infected and uninfected homosexual men in a primary care setting. *Journal of Clinical Virology*, 22(3):305-313.

Thapa, M. & Carr, D. J. 2009. CXCR3 deficiency increases susceptibility to genital herpes simplex virus type 2 infection: Uncoupling of CD8+ T-cell effector function but not migration. *Journal of Virology*, 83(18):9486-9501.

Thapa, M., Welner, R. S., Pelayo, R. & Carr, D. J. 2008. CXCL9 and CXCL10 expression are critical for control of genital herpes simplex virus type 2 infection through mobilization of HSV-specific CTL and NK cells to the nervous system. *The Journal of Immunology*, 180(2):1098-1106.

Tuero, I., Mohanram, V., Musich, T., Miller, L., Vargas-Inchaustegui, D. A., Demberg, T., Venzon, D., Kalisz, I., Kalyanaraman, V. & Pal, R. 2015. Mucosal B cells are associated with delayed SIV acquisition in vaccinated female but not male rhesus macaques following SIVmac251 rectal challenge. *PLoS pathogens*, 11(8):e1005101.

VandeBerg, J. L. & Williams-Blangero, S. 1997. Advantages and limitations of nonhuman primates as animal models in genetic research on complex diseases. *Journal of Medical Primatology*, 26(3):113-119.

Veazey, R. S. 2013. Animal models for microbicide safety and efficacy testing. *Current Opinion in HIV and AIDS*, 8(4):295-303.

Veazey, R. S., Shattock, R. J., Klasse, P. J. & Moore, J. P. 2012. Animal models for microbicide studies. *Current HIV Research*, 10(1):79-87.

Vincent, K. L., Vargas, G., Wei, J. N., Bourne, N. & Motamedi, M. 2013. Monitoring vaginal epithelial thickness changes noninvasively in sheep using optical coherence tomography. *American Journal of Obstetrics and Gynecology*, 208(4):282. e281-282. e287.

Wald, A., Corey, L., Cone, R., Hobson, A., Davis, G. & Zeh, J. 1997. Frequent genital herpes simplex virus 2 shedding in immunocompetent women. Effect of acyclovir treatment. *Journal of Clinical Investigation*, 99(5):1092-1097.

Wilson, S. S., Cheshenko, N., Fakioglu, E., Mesquita, P. M., Keller, M. J. & Herold, B. C. 2009. Susceptibility to genital herpes as a biomarker predictive of increased HIV risk: expansion of a murine model of microbicide safety. *Antiviral Therapy*, 14(8):1113-1124.

Yasuda, K., Nirei, T., Sunami, E., Nagawa, H. & Kitayama, J. 2011. Density of CD4(+) and CD8(+) T lymphocytes in biopsy samples can be a predictor of pathological response to chemoradiotherapy (CRT) for rectal cancer. *Radiation Oncology*, 6(1):49.

Zhang, M., Deng, X., Guan, X., Geng, L., Fu, M., Zhang, B., Chen, R., Hu, H., Hu, K., Zhang, D., Li, M., Liu, Y., Gong, S. & Hu, Q. 2018. Herpes Simplex Virus Type 2 Infection-Induced Expression of CXCR3 Ligands Promotes CD4(+) T Cell Migration and Is Regulated by the Viral Immediate-Early Protein ICP4. *Frontiers in Immunology*, 9:2932.

CHAPTER 4 SAFETY, EFFICACY, AND BIOMARKER ASSESSMENTS OF A BROAD-SPECTRUM BISMUTH (III) COMPLEX MICROBICIDE IN A GENITAL HERPES MOUSE MODEL

The paper was written in accordance to the guidelines of the Journal of Virology

Chapter 4: Safety, efficacy, and biomarker assessments of a broad-spectrum bismuth (III) complex microbicide in a genital herpes mouse model

Tumelo Kgoe¹, Rose Hayeshi*¹, Ambrose Okem¹, Leonard Damelin²

¹ Department of Science and Technology/North West University Preclinical Drug Development Platform, North-West University, Potchefstroom, South Africa

² National Institute for Communicable Diseases, Johannesburg, South Africa

*Corresponding author

ABSTRACT

Background

Herpes simplex virus - 2 (HSV-2) is a serious global epidemic with an estimated 400 million people infected and about 20 million cases reported annually. There is an urgent need for broad-spectrum microbicides to inhibit HSV-2, particularly in countries with high HSV-2/HIV-1 co-infection.

Methods

Female mice were vaginally administered Bi-SP in PBS at 5 µg/animal (n = 10), Bi-SP in 2.7% HEC gel at 5 µg Bi-SP/animal (n = 10) and 2.7% HEC gel only (n = 10). Male mice were rectally administered Bi-SP in PBS at 5 µg/animal (n = 20) and 10 µg/animal (n = 10). Administrations were conducted 10 minutes before vaginal and rectal viral infection with 1 x 10⁶ PFU HSV-2. Vaginal and rectal swab samples were collected two days post-infection for virus plaque assay and flow cytometry and immediately before euthanasia to determine CD4⁺ and CD8⁺ T cell counts. The rectal and vaginal tissues collected were evaluated for tissue integrity.

Results

Bi-SP demonstrated significant ($p < 0.05$) antiviral activity against HSV-2, subsequently increasing the survival rates to 10 - 60% in female groups and 40% in the male groups at a dose of 5 $\mu\text{g}/\text{animal}$ in PBS. Virus plaque assay confirmed the presence of viable virus in vaginal and rectal swab samples. CD4^+ and CD8^+ cells were detected in vaginal swab samples and none was detected in rectal samples. Histological analysis confirmed the safety of the microbicide at a dose of 5 $\mu\text{g}/\text{animal}$ as there were no signs of tissue damage.

Conclusion

Bi-SP has antiviral activity against HSV-2 but its delivery requires further optimization to increase the survival rates of both vaginally and rectally infected BALB/c mice. Bi-SP did not fully protect the vaginal and rectal mucosa from virus penetration and the influence it had on the immune responses was inconclusive and will need to be further investigated.

Keywords

Antiviral, BALB/c, Bi-SP, CD4^+ , CD8^+ , HSV-2

4.1 Introduction

Herpes simplex virus (HSV) is an incurable, sexually transmitted infection (STI), which is the leading cause of genital ulcer disease (Johnston & Corey, 2016). There are two types of HSV, HSV type 1 (HSV-1) and HSV type 2 (HSV-2), also referred to as oral and genital herpes respectively. HSV is spread through sexual contact, including vaginal, anal and oral sex. HSV-2 is more prevalent in women than in men, with an overall prevalence of 53.3% and 17% respectively in South Africa (Abbai et al., 2015; Kenyon et al., 2013). Men are more likely to spread the infection because of their multiple sexual partners compared to women, hence the higher rates of infection in females than in males (Fatahzadeh & Schwartz, 2007).

One way to manage and prevent HSV-2 is with the use of topical microbicides which can be applied locally to the vaginal and rectal mucosa. The most commonly used microbicides with potent antiviral activities against HSV include acyclovir and valacyclovir. Acyclovir is a product used to treat cold sores caused by HSV-1 (Ikawa et al., 2019), and valacyclovir is an antiviral drug used in both the prevention and treatment of HSV-2 (Zuckerman et al., 2007). Although these drugs have proven to have antiviral activity against both types of HSV-2, broad-spectrum microbicides are still needed to combat the spread of sexually transmitted infections.

The use of bismuth complexes in medicine has been traced to as early as the 1900s, with the main commercial product being Pepto-Bismol, a medication for stomach aches with bismuth as its active pharmaceutical ingredient (API) (Keogan & Griffith, 2014). Other products that contain bismuth as an API, include but are not limited to, colloidal bismuth subcitrate, which is used to treat peptic ulcer disease (Wagstaff et al., 1988), ranitidine bismuth citrate, an antimicrobial against *Helicobacter pylori* (Vondracek, 1998) and colloidal bismuth pectin, used for the treatment of *Helicobacter pylori* (Marcus et al., 2015a) as an alternative to bismuth subcitrate (Nie et al., 1999).

Bismuth complexes are mainly used for the treatment of gastrointestinal complications (Keogan & Griffith, 2014). Bismuth salts form insoluble complexes when placed in an aqueous solution (Udalova et al., 2008). For example, colloidal bismuth subcitrate forms a barrier that protects stomach lesions from the ulcer causing bacteria (Wagstaff et al., 1988), and bismuth subsalicylate forms a layer on the gastric mucosa, protecting it from the stomach acid (Andrews et al., 2006). The anti-inflammatory and antibacterial activity demonstrated by the mentioned compounds highlight the potential of using bismuth complexes in vaccines and microbicides for the prevention of STIs.

Bi-SP, is a novel bismuth (III) complex, being investigated as a broad-spectrum microbicide. In *in vitro* tests against a number of common STIs, including; HIV-1, HPV-16, *Chlamydia trachomatis* and HSV-2, Bi-SP has shown antiviral activity and inhibition of growth for the mentioned STIs. The microbicide exhibited extremely low toxicity towards mammalian cells and was shown to have potent activity against HIV-1, was highly active against HPV-16 and very active against HSV-2 in the inhibition of viral replication *in vitro*. Therefore, Bi-SP shows promise as a microbicide.

Hydroxyethyl cellulose (HEC), a non-ionic polymer, is used as a thickening agent and as a vehicle in various microbicide studies (Abdool Karim *et al.*, 2015; Bax *et al.*, 2002; Stoddart *et al.*, 2011). Due to its structure and characteristics, HEC can serve as a protective barrier against microorganisms (Boddupalli *et al.*, 2010), and be used as a carrier to deliver drugs through topical administration (Ribeiro *et al.*, 2018). The inclusion of HEC in 70% phosphate buffered saline as a drug delivery vehicle will enable fluid absorption in the vaginal tract, enabling better colloid distribution and coating of the vaginal mucous surface in the absence of any adverse effects (Ensign *et al.*, 2017).

In this study, Bi-SP, a water-soluble bismuth (III) complex that has potent broad-spectrum activity against HSV-2 and HIV-1 *in vitro*, was evaluated for safety and antiviral efficacy *in vivo* using a BALB/c genital herpes mouse model.

4.2 Materials and methods

4.2.1 Materials

African green monkey kidney (vero) cells, HSV-2 strain G, bismuth (III) complex (Bi-SP) and 2.7% hydroxyethyl cellulose (HEC) gel in 70% PBS (200 – 220mOsm/Kg) were all obtained from the National Institute of Communicable Diseases (NICD) (Johannesburg, South Africa). Anti-Mouse CD4-FITC and anti-Mouse CD8-FITC and Super-X Plex[®] Cytokine Kit were all purchased from Antigenix America (Melville, United States). Dulbecco's modified eagle's medium: nutrient mixture F-12 (DMEM/F12), foetal bovine serum (FBS), penicillin-streptomycin-amphotericin B mixture and trypsin/EDTA were purchased from Lonza (Basel, Switzerland). Thiazolyl blue tetrazolium bromide (MTT) and molecular biology grade agarose tablets were purchased from Melford (Ipswich, United Kingdom). Formalin was acquired from PathCare (Goodwood, South Africa). Histoplast wax was obtained from Thermofisher Scientific (Waltham, Massachusetts, US). Medroxyprogesterone acetate (MPA), phosphate-buffered saline (PBS) tablets and D-(+)-Glucose were obtained from Sigma-Aldrich (Johannesburg, South Africa). Dental brushes (0.8 mm) were purchased from Dis-Chem (Potchefstroom, South Africa).

4.2.2 Methods

4.2.2.1 Safety assessment of Bi-SP in BALB/c mice

Male and female BALB/c mice were used to evaluate the safety of Bi-SP as a topical application for the prevention of HSV-2. Female BALB/c mice aged 6-8-weeks-old (n = 5) were hormonally pre-treated six days prior to the start of the experiment with a subcutaneous injection of MPA suspended in sterile PBS solution to a final dose of 20 mg/mouse. The male mice (n = 5), aged 6-8 weeks-old were fasted 12 hours before the start of the experiment with water provided *ad libitum* to prevent the interruption of faeces during administrations in the rectal area. Rectal and vaginal administration of Bi-SP was delivered at 5 µg/animal in PBS with the use of a 2 – 20 µl pipette. The mice were euthanised by cervical dislocation two hours after receiving Bi-SP and the vaginal and rectal tissues were surgically excised and stored in formalin. Excised vaginal and rectal tissues were sent to a qualified pathologist at PathCare veterinary laboratories for pathological examinations. All experiments were approved by the NWU-AnimCareREC ethics committee (ethics number NWU-00170-18-A5) and were conducted in an environmentally controlled biosafety level (BSL) 3 laboratory. The animals were housed (five mice per cage) in individually ventilated cages (IVC) under normal

conditions (room temperature $22 \pm 1^{\circ}\text{C}$, internal cage temperature $24 \pm 2^{\circ}\text{C}$, room humidity 40 – 72.7 %, cage humidity 40 – 57 % and pressure in cages at 60 air changes/hour).

4.2.2.2 Efficacy evaluation of Bi-SP against HSV-2 in vaginally and rectally infected female and male mice respectively

Female (n = 30) and male (n = 30) BALB/c mice aged 6-8 weeks-old were used to evaluate the antiviral activity of Bi-SP against HSV-2. Male mice were fasted twelve hours before the experiment day with water provided *ad libitum* to prevent faecal interruption during Bi-SP administration and HSV-2 infection, and female mice were administered with MPA six days before the experiment day. The female mice were administered with 5 µg/animal Bi-SP in a PBS vehicle (n = 10) (Group 2), 5 µg/animal Bi-SP in a hypo osmotic 2.7% HEC gel vehicle (n = 10) (Group 3) and 2.7% HEC gel vehicle only (n = 10) (Group 4). The male mice were administered with 5 µg/animal Bi-SP, Group 2 (n = 10) and Group 3 (n = 10), and 10 µg/animal Bi-SP (n = 10) (Group 4) using PBS as the delivery vehicle. The control mice (Group 1), one male and one female, were not administered the virus. Both male and female mice were infected with HSV-2 strain G at 1×10^6 pfu 10 minutes after microbicide and vehicle administration. The efficacy of Bi-SP was assessed using Kaplan-Meier curves to monitor the survival rates.

The animals from each group were marked 1-10. All the animals were monitored twice a day for 19 days using a range of clinical scores. The scoring was as follows: 0). Normal, 1). Perianal/genital erythema, 2). Perianal swelling and erythema (may have slightly ataxic gait and/or slightly ruffled coat, the normal mouse coat is glossy and shiny), 3). Purulent lesions, partial or complete paralysis in one or both hind limbs, visible weight loss detected by daily mass measurements or dehydration, very poor grooming, perianal urine staining due to loss of bladder control and, 4). Immobile, complete hind limb paralysis, severe dehydration, little or no grooming (Marshak et al., 2014). The humane endpoint was set at a clinical score of 3 and 4 and constituted immediate euthanasia. Vaginal and rectal swabs were collected using 0.8 mm dental brushes two days post-infection and immediately prior to euthanasia. The swab samples were suspended in 250 µl of PBS and were flash-frozen in liquid nitrogen before being stored at -80°C . Aliquots of the swab samples collected two days post-infection were used for virus plaque assay, and samples collected two days post infection and immediately after euthanasia were used for flow cytometry. Vaginal and rectal tissue samples were excised from euthanised animals and were stored in formalin until use for histological analysis for the evaluation of tissue integrity and morphology.

4.2.2.2.1 Histology

The excised vaginal and rectal tissues fixed in formalin were washed under running water for two hours and underwent a process of dehydration. The tissues were transversely fixed in histoplast wax and were sectioned with a microtome (Reicket-Junn 2050, Leica microsystems GmbH, Germany) at a thickness of 5 – 7 μm . The sectioned bands were placed in a water bath set at 40°C, mounted on glass microscope slides and left to dry overnight in an oven set at 35°C. The tissues were stained with H&E and the coverslips were mounted on the slides using Entellan™ mounting media. The stained sections were evaluated for epithelial cell disruption and evidence of inflammation under a compound microscope (Nikon's Eclipse E800 biological research microscope).

4.2.2.2.2 HSV-2 plaque assay

Vaginal and rectal swab samples collected from three representative samples from infected female and male mice respectively were added on vero cell monolayers, seeded in a 12 well plate and grown to a confluence of 60 – 80% in DMEM-F12 supplemented with 10% FBS and 1% antibiotics (penicillin-streptomycin-amphotericin B mixture). The growth medium containing the swab samples was removed after three hours incubation and fresh assay medium containing 4% agarose was added to the wells and allowed to solidify at room temperature. The cells were incubated for three days in a cell culture incubator set at 37°C with 90% humidity and an inflow of CO₂ at 5% to allow the plaques to form before the addition of MTT (10 mg/ml in PBS) to visualise the plaques.

4.2.2.2.3 Measurement of CD4⁺ and CD8⁺ cell counts

Samples of vaginal (n = 3) and rectal (n = 3) swabs (two days post-infection and before euthanasia) at 5 μl were diluted to a ratio of 1:1000 in reading buffer (Super-X Plex © Cytokine Kit) to make up a volume of 100 μl in a 96 well plate. The samples were placed on ice and exposed to UV light for six minutes to deactivate infectious viral particles of HSV-2. Anti-Mouse CD4 and anti-Mouse CD8 (5 μl each) were added to the samples and incubated for two hours in the dark at 4°C. An unstained sample from an infected mouse was used for quality control purposes. The acquisition was performed on a BD Accuri™ C6 flow cytometer (BD Biosciences) using the BD CSampler™ Software at 100 000 cells/sample with the fluidics set at a slow flow rate and threshold set at 80 000. For data analysis, the population of cells were gated based on size (FSC, forward scatter proportional to the square diameter of the cell) and complexity (SSC, side scatter) of the cells. The gate used was SSC-A vs. FSC-A to detect the cell population of interest

4.2.2.2.4 Statistical analysis

Kaplan-Meier survival curves were constructed using GraphPad Prism v5 (GraphPad Software, Inc.). The log rank-test was used to compare the different test groups statistically. P values < 0.05 were considered significant.

4.3 Results

4.3.1 Bi-SP has anti-viral effects against HSV-2 and improves the survival of the infected mice

HSV-2 is an invasive virus in female and male mice, resulting in a 0% survival rate in both the male and female mice. The Kaplan-Meier curves, Figures 2 and 3, indicate the improvement in the survival rate of mice administered with Bi-SP before viral infection compared to control mice administered with PBS only. The vaginal and rectal swab samples collected from infected mice two days post infection presented with viable virus, which served as confirmation of infection.

4.3.1.1 Effect of Bi-SP on the survival rate of female mice and the inhibition of HSV-2

The female mice that were administered with 5 μ g/animal of Bi-SP in PBS presented with an increase in survival rate by 10% from 0% (Figure 2). Furthermore, after two days post infection, the vaginal swabs presented with positive plaques in the representative samples (Table 1).

Higher survival rates were observed when the female mice received Bi-SP at 5 μ g/animal using a hypo-osmotic 2.7% HEC gel as the delivery vehicle, increasing the survival rate to 60% (Figure 1). Out of 10 vaginal swab samples that were analysed, six did not present with any viable virus two days post-infection (Table 1).

The female mice that were administered with 2.7% HEC gel also only had a significant survival rate of 60% (Figure 1). In the 10 vaginal swab samples that were analysed, only two samples tested negative for viable virus while the rest presented with viable virus (Table 1).

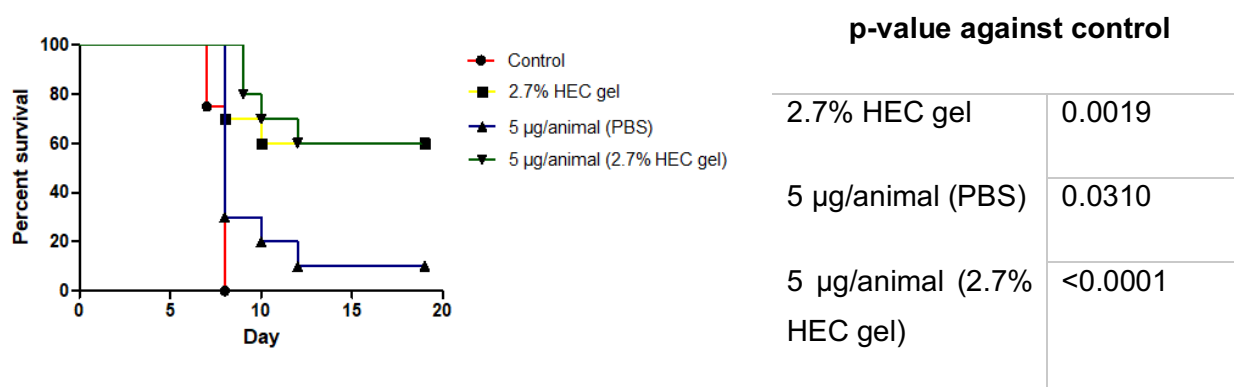


Figure 1: Kaplan-Meier survival curves of female mice in different test groups

Table 1: Viable HSV-2 in vaginal swab samples collected 2 days post infection representative of the different test groups

Female control	
Mouse ID	Plaques formed
1.1	+ ^a
1.3	+
1.4	+
5 µg/animal (PBS)	
Mouse ID	Plaques formed
2.1	+(5)
2.5	+
2.7	+
5 µg/animal (2.7% HEC gel)	
Mouse ID	Plaques formed
3.1	- ^b
3.2	-
3.3	-
3.4	-
3.5	-
3.6	+(11)
3.7	+(11)
3.8	-
3.9	+(8)
3.10	+
2.7% HEC gel	
Mouse ID	Plaques formed
4.1	+(11)
4.2	+(1)
4.3	+(2)
4.4	+
4.5	-
4.6	+
4.7	+
4.8	+(1)
4.9	-
4.10	+

^a positive for plaque formation but too many to count, ^bnegative for plaque formation, (-) number of plaques counted

4.3.1.2 Effect of Bi-SP on the survival rate of male mice and the inhibition of HSV-2

The male mice administered with 5 µg/animal in PBS had a better survival rate compared to the group that received 10 µg/animal and the control (Figure 2). The control group had more plaques compared to the micrbicide treated groups (Table 2). There was viable virus in samples collected two days post infection in all the male groups administered with 5 µg/animal and 10 µg/animal (Table 2).

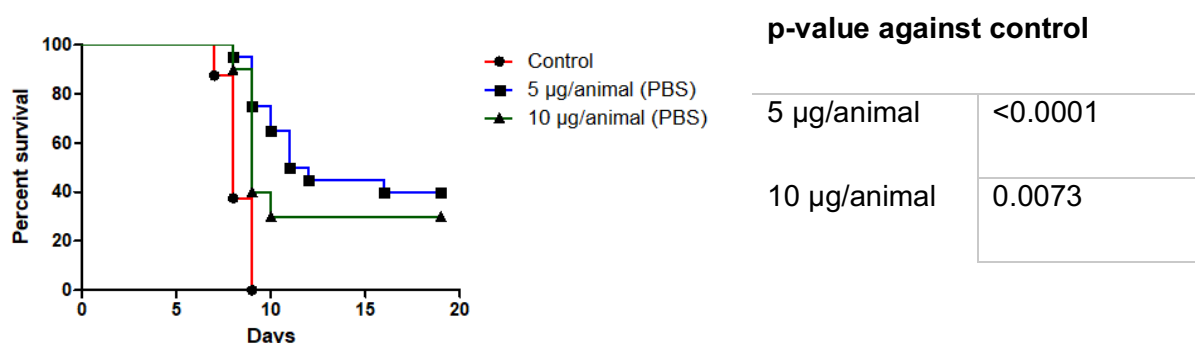


Figure 2: Kaplan-Meier survival curves of male mice at different test groups

Table 2: Rectal swab samples representative of each test group (5 µg/animal and 10 µg/animal) were assayed for plaque formation

Male control	
Mouse ID	Plaques formed
1.1	+ ^a
1.2	+
1.6	+
5 µg/animal (PBS)	
Mouse ID	Plaques formed
2.1	+(9)
2.3	+(9)
2.6	+(15)
3.3	+(11)
3.6	+(15)
3.7	+(4)
10 µg/animal (PBS)	
Mouse ID	Plaques formed
4.4	+(3)
4.7	+(2)

4.1	+(1)
-----	------

^a positive for plaque formation but too many to count

4.3.1.3 Comparison of female and male groups administered with 5 µg/animal of Bi-SP in PBS

The administration of Bi-SP at 5 µg/animal affected the survival rates of female and male mice differently. The humane end point was reached faster in vaginally challenged mice compared to the rectally challenged male mice. There was a significant difference ($p < 0.05$) in the rate of survival between the genders.

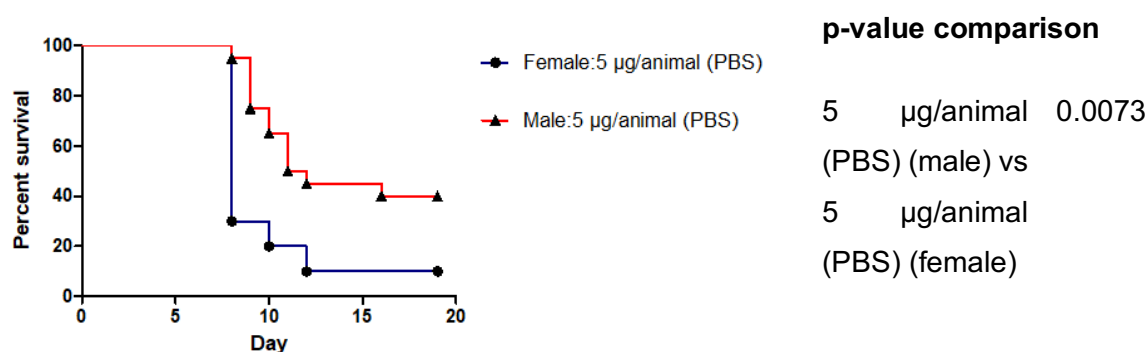


Figure 3: Comparison of female and male test groups administered with Bi-SP at 5 µg/animal

4.3.2 Bi-SP does not cause damage to the vaginal and rectal mucosa at 5 µg/animal

Histopathology reports indicated no epithelial tissue damage rectally and vaginally after administration of Bi-SP at 5 µg/animal to the male and female mice. Figure 4A is a representative tissue section that displays the structure of an intact rectum structure with visible mucosa, comprising of intact glands, glandular crypts, muscularis mucosa and submucosa. Figure 4B is a representative section of an intact vaginal tissue structure with an undamaged squamous epithelium and smooth muscle tissue, these structural features are indicated by the black arrows.

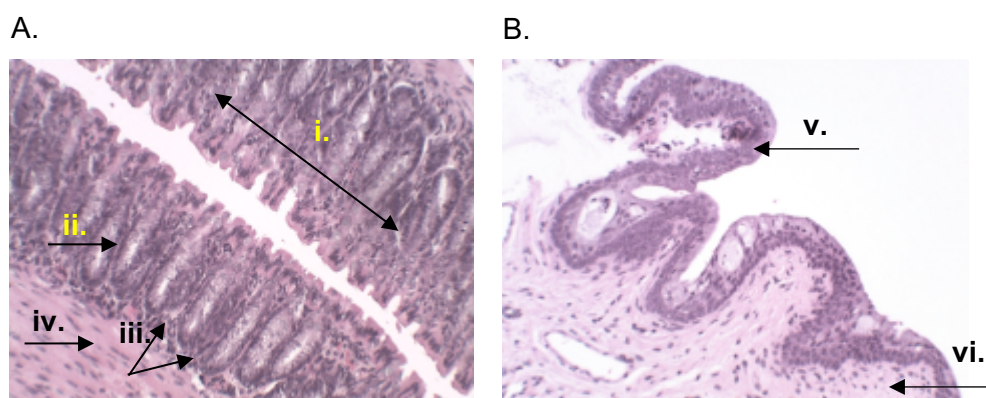


Figure 4: Hematoxylin and Eosin stained rectal and vaginal tissues of male and female mice administered with 5 µg/animal of Bi-SP. **A.** Rectal tissue with intact **i.** mucosa, **ii.** glands, **iii.** glandular crypts, and **iv.** submucosa. **B.** Vaginal tissues with intact **v.** squamous epithelium and **vi.** smooth muscle layer. The images were captured at 10X magnification.

4.3.3 Bi-SP did not prevent HSV-2 from causing structural damage to the epithelium of vaginal and rectal tissues

The vaginal and rectal tissues collected from the infected female and male mice presented with structural damages on the squamous epithelium of the vaginal mucosa (Figure 5) and on the glands and glandular crypts epithelial layer of the rectal mucosa (Figure 6). Although, the microbicide prolonged the survival of the mice, it did not prevent the virus from reaching the tissues, thereby causing an inflammatory response as indicated with the infiltration of leukocytes on the smooth muscle layer of the vaginal tissues (Figure 5) and on the submucosa of the rectal tissue (Figure 6).

It was observed that the squamous epithelial layer was relatively thin on tissues collected from female mice administered with Bi-SP using PBS as a delivery vehicle (Figure 5B). Representative rectal tissue sections (Figures 6A and C) presented with an epithelial layer in which the glands and glandular crypts were not structurally present. This was observed in the majority of the rectal tissues excised from the infected male mice. Tissues collected from mice administered with 10 µg/animal (Figure 6B and D) presented with better tissue integrity compared to the tissues collected from mice administered with 5 µg/animal. Although structural damage to the glands and glandular crypts was observed in both groups, many of the tissues presented with a separation of the muscularis mucosa and the submucosa, which is probably caused by an error in the tissue preparation process (Figure 6B and D).

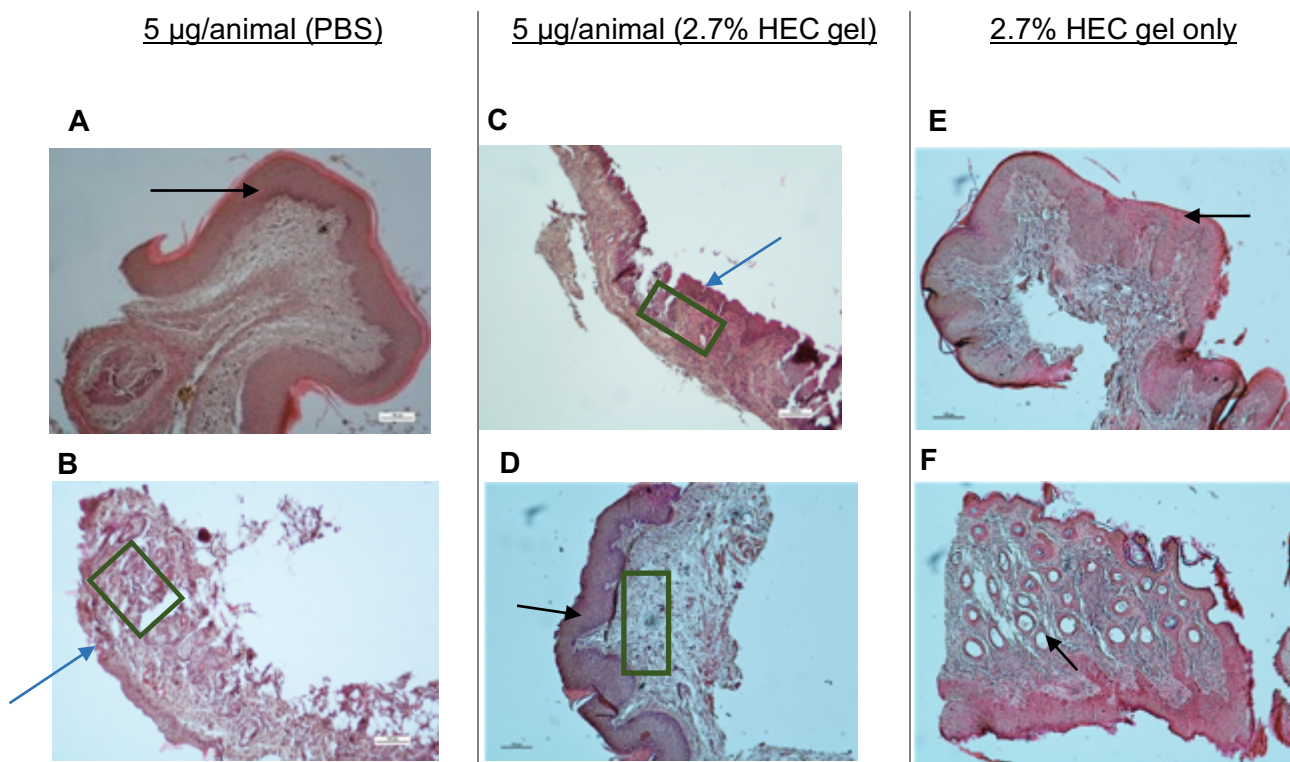
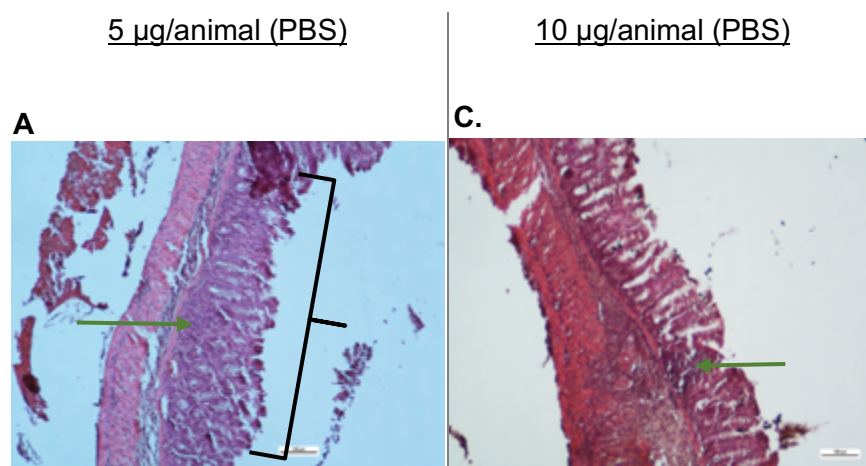


Figure 5: Vaginal tissues from 3 female groups administered with 5 μ g/animal of Bi-SP in PBS vehicle **A.** (day 8) and **B.** (day 19), 2.7% HEC gel only **C.** (Day 7) and **D.** (Day 19), and 5 μ g/animal of Bi-SP in 2.7% HEC gel vehicle **E.** (Day 9) and **F.** (Day 19). All images were captured at 10X magnification. Arrows indicate thickened epithelium (black), the thinned epithelium (blue) and inflammatory cell infiltration (green block).



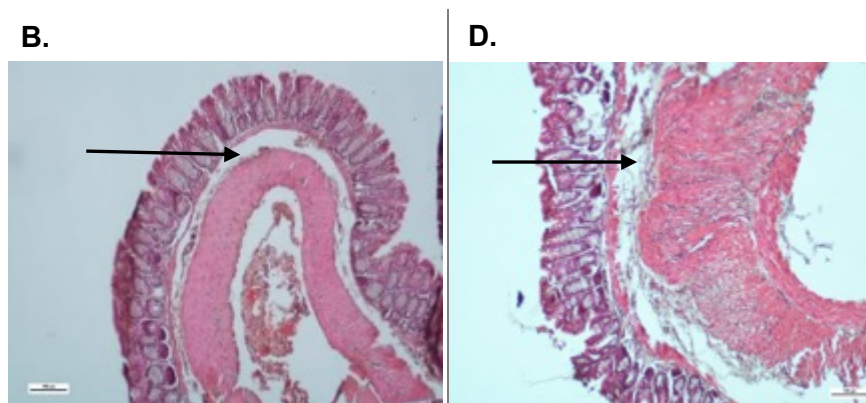


Figure 6: Rectal tissues collected from male mice administered with 5 µg/animal and 10 µg/animal of Bi-SP in PBS vehicle. **A.** Tissue collected on day 8 and **B.** day 19 from mice administered with 5 µg/animal of Bi-SP. **C.** tissue collected on day 8 and, **D.** collected on day 19 from mice administered with 10 µg/animal of Bi-SP. The Bi-SP was reconstituted in PBS and all images were captured at 10X magnification. Arrows indicate damaged glandular crypts and glands (green), separation of the connective tissue and submucosa (black) and damage to the structural activity of the rectal mucosa (square brackets).

4.3.4 Effect of Bi-SP on the immune responses of HSV-2 infected-mice

The CD4⁺ and CD8⁺ T cells were detected in vaginal swab samples in all the test groups. Figure 7 is a representative figure of CD4⁺ and CD8⁺ percentages from healthy female (Figure 7A) and male (Figure 7B) mice. Of the three representative vaginal swab samples collected two days post-infection (Figure 8), two had CD4⁺, and none had CD8⁺. In samples collected immediately before euthanasia, one of the three had CD4⁺, and two had CD8⁺. In the group administered with 5 µg/animal in HEC gel (Figure 9), three out of three mice presented with CD4⁺, and two of the three had CD8⁺ in samples collected 2 days post infection. For samples collected immediately before euthanasia, three out of three had CD4⁺, and two of the three had CD8⁺. For 2.7% HEC gel only (Figure 10), one of the three mice had CD4⁺, and one of the three had CD8⁺ in 2 days post infection samples. One of the three had CD4⁺, and two had CD8⁺ in samples collected immediately before euthanasia. No CD4⁺ and CD8⁺ T cells were detected in male mice administered in doses of 5 µg/animal and 10 µg/animal (Figure 11). The percentages of CD4⁺ in infected female mice remained unchanged and CD8⁺ percentages varied from animal to animal. In the male mice, no CD4⁺ or CD8⁺ T cells were detected in both healthy (Figure 7B) and infected mice.

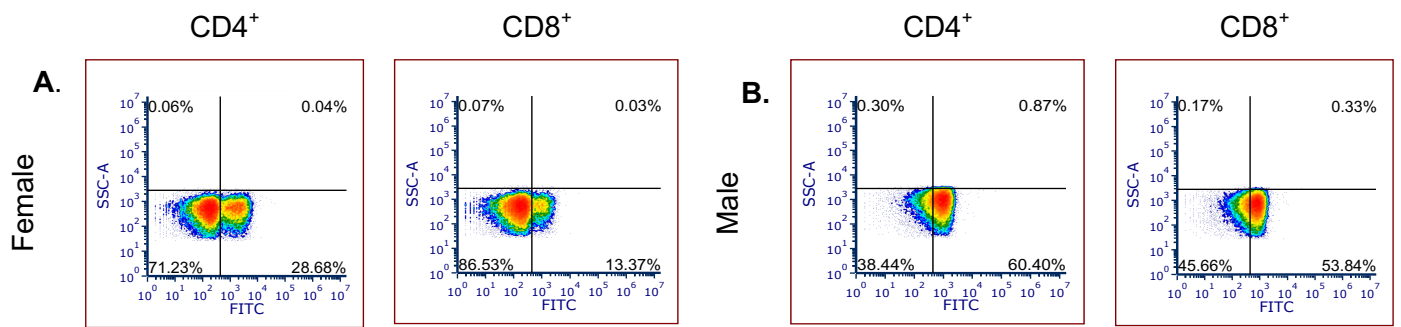


Figure 7: CD4⁺ and CD8⁺ percentages from vaginal and rectal swabs collected from healthy female and male mice.

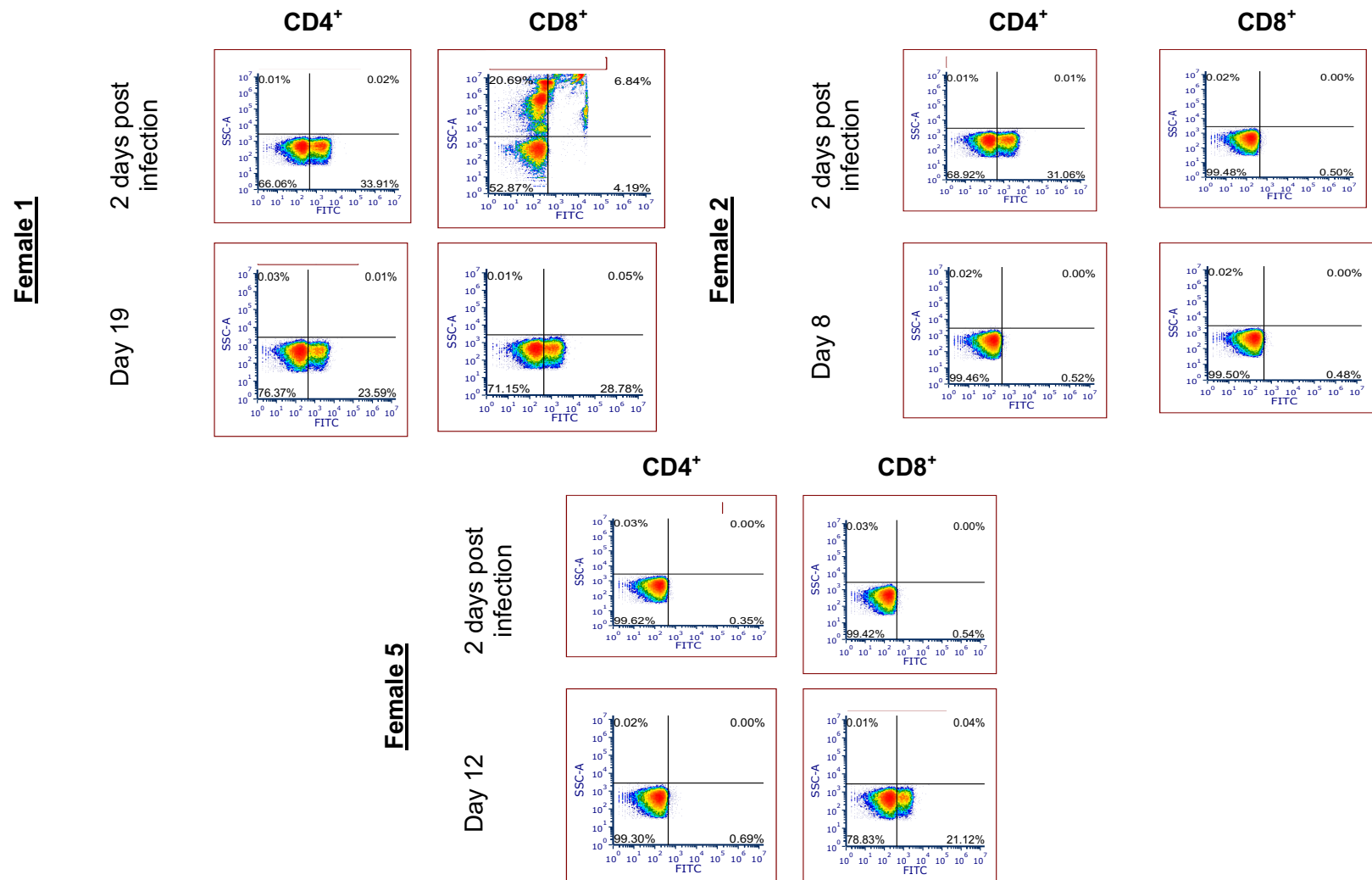


Figure 8: CD4⁺ and CD8⁺ expression from vaginal swab samples of female mice that received Bi-SP at 5 µg/animal in PBS

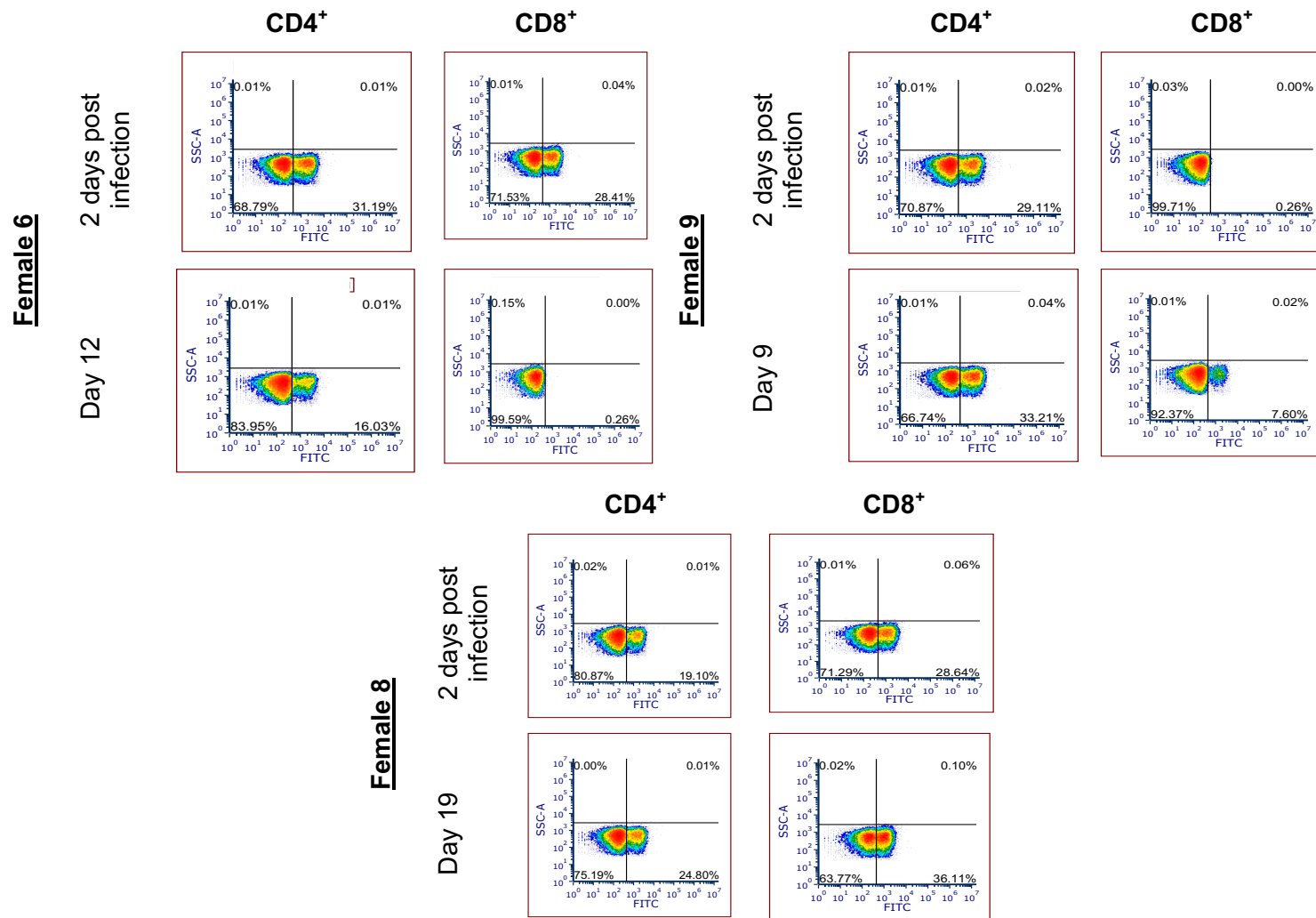


Figure 9: CD4⁺ and CD8⁺ expression from female mice administered with Bi-SP in 2.7% HEC gel

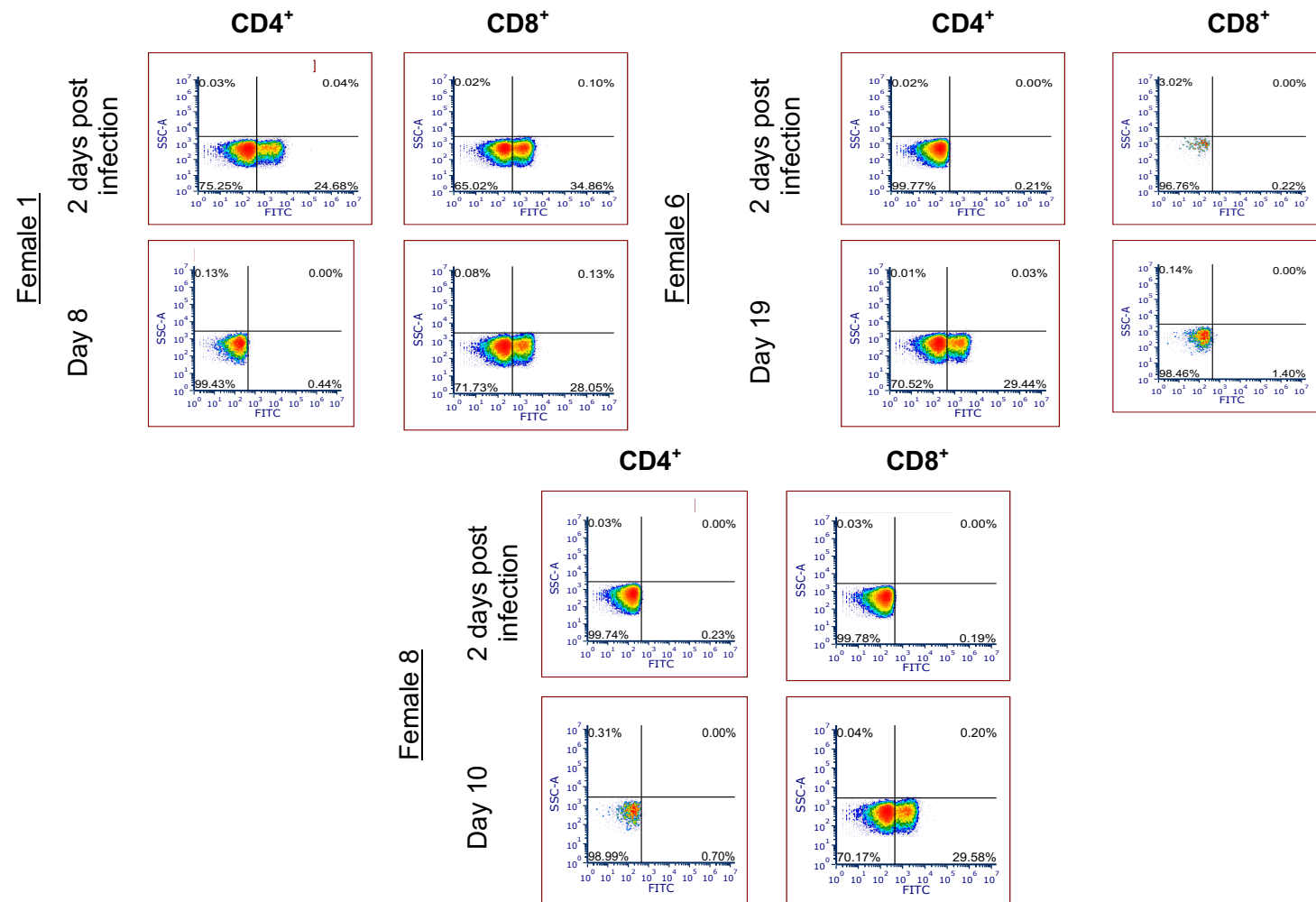


Figure 10: CD4⁺ and CD8⁺ expression in vaginal swab samples from HSV-2 infected female mice administered with 2.7% HEC gel only

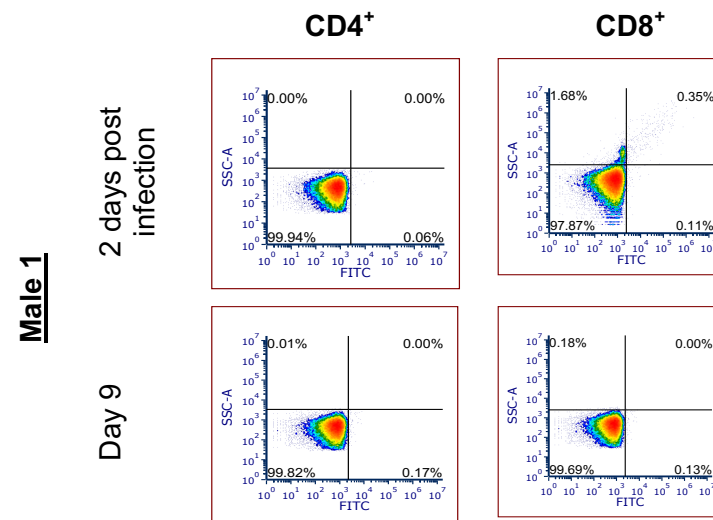


Figure 11: CD4⁺ and CD8⁺ expression from rectal swab samples of HSV-2 infected male mice administered with 5 µg/animal in PBS

4.4 Discussion

HSV-2 has been a major concern in countries experiencing severe HIV/AIDS. An HSV-2 epidemic contributes directly to the increase in HIV incidence due to increased susceptibility of HIV in HSV-2 positive individuals (Freeman *et al.*, 2006). One of the major gaps in the development of broad-spectrum microbicides is the lack of animal models that can be used to analyse the safety and efficacy of novel microbicides. The understanding of the vaginal and rectal mucosal immunity in the protection of foreign pathogens is essential to understanding the susceptibility of the mice to sexually transmitted infections. Bi-SP has shown broad-spectrum activity *in vitro* against HIV and many STIs including HSV-2 (LH Damelin, personal communication). The *in vitro* antiviral activity of Bi-SP is well-known but the microbicide has not been evaluated *in vivo*. The safety assessment and efficacy evaluation of Bi-SP will provide information on the effectiveness of the microbicide as a prophylactic against HSV-2.

In this study, the safety of the microbicide was assessed in female and male mice which were administered with Bi-SP vaginally and rectally. The prophylactic capability of Bi-SP was also evaluated in HSV-2 infected mice. Through histological evaluation, it was confirmed that Bi-SP did not cause any tissue damage when tested for safety at 5 µg/animal. In the present study, single administration of Bi-SP was used, whereas in many studies repeated exposure of microbicide was routinely used (Catalone *et al.*, 2005b; Galen *et al.*, 2007; Wilson *et al.*, 2009). However, in this study, we aimed to highlight the prophylactic effects of Bi-SP from a single administration as initial proof of concept.

The administration of Bi-SP increased the survival in female mice to 10% (Bi-SP in PBS vehicle) and 60% (Bi-SP in 2.7% HEC gel vehicle). For the male mice, survival rate was increased to 30% (5 µg/animal in PBS) and 40% (10 µg/animal in PBS). There was a significant difference in the rate of survival when comparing male and female mice that were administered with 5 µg/animal of Bi-SP in PBS. The rectally administered male mice had a better survival rate than the vaginally administered female mice. The reason for the better survival rate in male mice administered the microbicide by the rectal route is believed to be caused by the high absorption from the rectal cavity (van Hoogdalem *et al.*, 1991). Although the number of plaques in male mice administered with 10 µg/animal was lower than any other group, a higher dose of Bi-SP did not significantly increase the survival rate of the males compared to mice administered with 5 µg/animal. Furthermore, the results of the plaque assays are not quantitative and only three representative samples per group were used. The groups of female mice administered with Bi-SP at 5 µg/animal in 2.7% HEC gel and those administered with 2.7% HEC gel only were the only groups in which samples from all the animals were analysed.

It was observed that the 2.7% HEC gel protected mice against HSV-2 when administered as the vehicle for the microbicide and alone in the control group. HEC gels in isotonic saline (Tien *et al.* 2005), have been previously shown to have no protective effect against HSV-2 (Fernandez-Romero *et al.*, 2012). However, the 2.7% HEC gel used in this study was prepared with 70% PBS, and was moderately hypo-osmotic, as it had been previously established that hypo-osmotic gels induce fluid absorption in the vaginal tract, facilitating better drug absorption and distribution (Ensign *et al.*, 2017). It is likely that this hypo-osmotic formulation contributed to the protective effect of the gel as upon dissection of the vaginal tissues of mice that received hypo-osmotic HEC gel, a visible coating of HEC was clearly evident on the vaginal mucosa indicating that fluid absorption within the vaginal tract induced the formation of a viscous HEC layer over the vaginal surface and inhibited viral infection. Importantly though, the hypo-osmotic HEC formulation was not able to inactivate HSV-2 since 80% of mice that received gel alone had viable virus, two days post viral challenge compared with 40% for animals that received gel and Bi-SP.

Bi-SP however, did not protect the vaginal and rectal mucosa from viral invasion. Histological evaluation of all the vaginal tissues from the female mice presented with a relatively thin squamous epithelium and an increase in leukocyte infiltration. Tissues excised between day eight and twelve presented with thinned squamous epithelium and visible tissue inflammation. These structural features were indicated by the increased inflammatory cell infiltration into the smooth muscle layer, similar to the findings of Quispe Calla *et al.* (2016). The majority of the rectal tissues from the male mice presented with severe damage on the glands and the glandular crypts. The virus affected multiple sites on the tissues despite the mice being treated with the microbicide prior to infection. Upon dissection, the majority of the mice that received the prophylaxis and those that were administered with the 2.7% HEC gel had a full bladder and faecal matter in the intestines, suggesting they were constipated. According to Assi *et al.* (2014), these symptoms are common with herpes infection around the anal region.

It was observed through flow cytometry that the CD4⁺ and CD8⁺ cell counts in the selected samples representing the different test groups varied across all the female mice. There was no difference in the percentages of CD4⁺ and CD8⁺ expression between mice that were euthanised earlier and those that survived to the end of the study. There have been documented results of CD4⁺ and CD8⁺ cells in female mice infected vaginally with HSV-2 with decreased amounts of T cells after viral infection, which reduced immunity to the vaginal epithelium as demonstrated by Parr and Parr (1997). The CD4⁺ and CD8⁺ percentages in infected females across the different test groups did not present with higher or lower percentages when compared to the cell count of the cells in the healthy mouse. Therefore, the presence of the virus had no influence on the percentages of CD4⁺ and CD8⁺ cells in the female mice that were analysed for the study. CD4⁺

and CD8⁺ cells were not detected in rectal swab samples collected from both the healthy mice and microbicide administered male mice infected with HSV-2. These results confirm the findings of the previous chapter for BALB/c model establishment, which showed that CD4⁺ and CD8⁺ could not be detected in rectal swabs. Therefore, the model is considered potentially unsuitable for detection of CD4⁺ and CD8⁺ from rectal mucosa. Although Bi-SP was safe to use at 5 µg/animal and did not have any adverse effects on the vaginal and rectal mucosa, the information gathered from the CD4⁺ and CD8⁺ immune response is unclear and further testing will need to be conducted with more animals for clear and conclusive data.

4.5 Conclusion

Bi-SP did not bring about any irritation to the mouse vaginal and rectal mucosae after a two-hour exposure at 5 µg/animal and did not cause any adverse effects as proven by histological analysis. The increase in concentration of the microbicide did not significantly improve the survival rates in the male mice either. Bi-SP administered using PBS and 2.7% HEC as delivery vehicles did not fully protect the vaginal and rectal mucosa from HSV-2 invasion.

Bi-SP's antiviral activity did not affect the expression of CD4⁺ and CD8⁺ T cells in terms of increase or decrease in both HSV-2 infected female and male mice. In female mice, the percentages of CD4⁺ did not vary as much as the CD8⁺ percentages in vaginal swab sample collected from the one female mouse control. CD4⁺ and CD8⁺ cells were however not detected in rectal swabs from infected male mice or the control mice.

There is some evidence of antiviral effects of Bi-SP for the prevention of HSV-2 in the BALB/c mouse model, but further work in optimising the delivery of the drug is necessary.

4.6 Abbreviations

API: Active pharmaceutical ingredient; CO₂: Carbon dioxide; DMEM/F12: Dulbecco's Modified Eagle Medium; EDTA: Ethylenediaminetetraacetic acid; FBS: Foetal bovine serum; HEC: Hydroxyethylcellulose; HIV: Human immunodeficiency virus; HSV: Herpes simplex virus; H&E: Hematoxylin and eosin; pfu: plaque forming units; PBS: Phosphate Buffered Saline; MTT: Thiazolyl blue tetrazolium bromide; STI: Sexually transmitted infection; UV: Ultraviolet; °C: degrees celcius

4.7 Acknowledgements

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References

- Abbai, N.S., Wand, H. & Ramjee, G. 2015. Socio-demographic and behavioural characteristics associated with HSV-2 sero-prevalence in high risk women in KwaZulu-Natal. *BMC Research Notes*, 8:185.
- Abdool Karim, S.S., Abdool Karim, Q., Kharsany, A.B., Baxter, C., Grobler, A.C., Werner, L., Kashuba, A., Mansoor, L.E., Samsunder, N., Mindel, A., Gengiah, T.N. & Group, C.T. 2015. Tenofovir Gel for the Prevention of Herpes Simplex Virus Type 2 Infection. *New England Journal of Medicine*, 373(6):530-539.
- Andrews, P.C., Deacon, G.B., Forsyth, C.M., Junk, P.C., Kumar, I. & Maguire, M. 2006. Towards a structural understanding of the anti-ulcer and anti-gastritis drug bismuth subsalicylate. *Angewandte Chemie International Edition English*, 45(34):5638-5642.
- Assi, R., Hashim, P.W., Reddy, V.B., Einarsdottir, H. & Longo, W.E. 2014. Sexually transmitted infections of the anus and rectum. *World Journal of Gastroenterology*, 20(41):15262-15268.
- Bax, R., Douville, K., McCormick, D., Rosenberg, M., Higgins, J. & Bowden, M. 2002. Microbicides--evaluating multiple formulations of C31G. *Contraception*, 66(5):365-368.
- Boddupalli, B.M., Mohammed, Z.N., Nath, R.A. & Banji, D. 2010. Mucoadhesive drug delivery system: An overview. *Journal of Advanced Pharmaceutical Technology & Research*, 1(4):381-387.
- Catalone, B.J., Kish-Catalone, T.M., Neely, E.B., Budgeon, L.R., Ferguson, M.L., Stiller, C., Miller, S.R., Malamud, D., Krebs, F.C., Howett, M.K. & Wigdahl, B. 2005. Comparative safety evaluation of the candidate vaginal microbicide C31G. *Antimicrobial Agents and Chemotherapy*, 49(4):1509-1520.
- Ensign, L., Cone, R. & Hanes, J. 2017. Nanoparticle formulations with enhanced mucosal penetration: Google Patents.
- Fatahzadeh, M. & Schwartz, R.A. 2007. Human herpes simplex virus infections: epidemiology, pathogenesis, symptomatology, diagnosis, and management. *Journal of the American Academy of Dermatology*, 57(5):737-763; quiz 764-736.
- Fernandez-Romero, J.A., Abraham, C.J., Rodriguez, A., Kizima, L., Jean-Pierre, N., Menon, R., Begay, O., Sedor, S., Ford, B.E., Gil, P.I., Peters, J., Katz, D., Robbiani, M. & Zydowsky, T.M. 2012. Zinc acetate/carrageenan gels exhibit potent activity in vivo against high-dose herpes simplex virus 2 vaginal and rectal challenge. *Antimicrobial Agents and Chemotherapy*, 56(1):358-368.
- Freeman, E.E., Weiss, H.A., Glynn, J.R., Cross, P.L., Whitworth, J.A. & Hayes, R.J. 2006. Herpes simplex virus 2 infection increases HIV acquisition in men and women: systematic review and meta-analysis of longitudinal studies. *AIDS*, 20(1):73-83.
- Galen, B.T., Martin, A.P., Hazrati, E., Garin, A., Guzman, E., Wilson, S.S., Porter, D.D., Lira, S.A., Keller, M.J. & Herold, B.C. 2007. A comprehensive murine model to evaluate topical vaginal microbicides: mucosal inflammation and susceptibility to genital herpes as surrogate markers of safety. *Journal of Infectious Diseases*, 195(9):1332-1339.
- Ikawa, Y., Fujiki, T., Nishimura, R., Noguchi, K., Koshino, E., Fujiki, A., Fukuda, M., Kuroda, R., Mase, S., Araki, R., Maeba, H., Shiraki, K. & Yachie, A. 2019. Improvement of refractory

acyclovir-resistant herpes simplex virus type 1 infection by continuous acyclovir administration. *Journal of Infection and Chemotherapy*, 25(1):65-67.

Johnston, C. & Corey, L. 2016. Current Concepts for Genital Herpes Simplex Virus Infection: Diagnostics and Pathogenesis of Genital Tract Shedding. *Clinical Microbiology Reviews*, 29(1):149-161.

Kenyon, C., Colebunders, R., Buve, A. & Hens, N. 2013. Partner-concurrency associated with herpes simplex virus 2 infection in young South Africans. *International journal of STD & AIDS*, 24(10):804-812.

Keogan, D.M. & Griffith, D.M. 2014. Current and potential applications of bismuth-based drugs. *Molecules*, 19(9):15258-15297.

Marcus, E.A., Sachs, G. & Scott, D.R. 2015. Colloidal bismuth subcitrate impedes proton entry into *Helicobacter pylori* and increases the efficacy of growth-dependent antibiotics. *Alimentary Pharmacology and Therapy*, 42(7):922-933.

Nie, Y., Li, Y., Wu, H., Sha, W., Du, H., Dai, S., Wang, H. & Li, Q. 1999. Colloidal bismuth pectin: an alternative to bismuth subcitrate for the treatment of *Helicobacter pylori*-positive duodenal ulcer. *Helicobacter*, 4(2):128-134.

Quispe Calla, N.E., Vicetti Miguel, R.D., Boyaka, P.N., Hall-Stoodley, L., Kaur, B., Trout, W., Pavelko, S.D. & Cherpes, T.L. 2016. Medroxyprogesterone acetate and levonorgestrel increase genital mucosal permeability and enhance susceptibility to genital herpes simplex virus type 2 infection. *Mucosal Immunology*, 9(6):1571-1583.

Ribeiro, A.M., Magalhães, M., Veiga, F. & Figueiras, A. 2018. Chapter 41-1. Cellulose-Based Superabsorbent Hydrogels. p. 1-29).

Stoddart, C.A., Neff, C.P., Kurisu, T., Ndolo, T., Fox, K. & Akkina, R. 2011. A Topical Microbicide Gel Formulation of CCR5 Antagonist Maraviroc Prevents HIV-1 Vaginal Transmission in Humanized RAG-hu Mice. *PLoS ONE*, 6(6).

Udalova, T., Logutenko, O., Timakova, E., Afonina, L., Naydenko, E. & Yukhin, Y.M. 2008. Bismuth compounds in medicine. (In. 2008 Third International Forum on Strategic Technologies organised by: IEEE. p. 137-140).

van Hoogdalem, E.J., de Boer, A.G. & Breimer, D.D. 1991. Pharmacokinetics of rectal drug administration, part I. *Clinical pharmacokinetics*, 21(1):11-26.

Vondracek, T.G. 1998. Ranitidine bismuth citrate in the treatment of *Helicobacter pylori* infection and duodenal ulcer. *Annals of Pharmacotherapy*, 32(6):672-679.

Wagstaff, A.J., Benfield, P. & Monk, J.P. 1988. Colloidal bismuth subcitrate. A review of its pharmacodynamic and pharmacokinetic properties, and its therapeutic use in peptic ulcer disease. *Drugs*, 36(2):132-157.

Wilson, S.S., Cheshenko, N., Fakioglu, E., Mesquita, P.M., Keller, M.J. & Herold, B.C. 2009. Susceptibility to genital herpes as a biomarker predictive of increased HIV risk: expansion of a murine model of microbicide safety. *Antiviral Therapy*, 14(8):1113-1124.

Zuckerman, R.A., Lucchetti, A., Whittington, W.L., Sanchez, J., Coombs, R.W., Zuniga, R., Magaret, A.S., Wald, A., Corey, L. & Celum, C. 2007. Herpes simplex virus (HSV) suppression with valacyclovir reduces rectal and blood plasma HIV-1 levels in HIV-1/HSV-2-seropositive men:

a randomized, double-blind, placebo-controlled crossover trial. *Journal of Infectious Diseases*, 196(10):1500-1508.

CHAPTER 5 CONCLUSIONS AND FUTURE RECOMMENDATIONS

5.1 Conclusion

- The BALB/c genital herpes mouse model was successfully established. The mice were infected rectally and vaginally with HSV-2 at a titre of 1.0×10^6 pfu. The virus proved to be very invasive in both male and female mice as clinical signs could be observed three days post infection.
- With supporting evidence from the 0% survival rate, the confirmation of infection by the use of plaque assays, structural tissue damage and visible leukocyte infiltration from histological analysis, it can be confirmed that BALB/c mice are indeed susceptible to HSV-2 vaginally and rectally and exhibit the effects caused by the virus.
- Flow cytometry analysis confirmed that CD4⁺ and CD8⁺ cells were only detected in vaginal swabs and not in rectal swabs. However, this data is not sufficient to make a valid conclusion as only three samples per group were analysed.
- The novel microbicide Bi-SP was shown to be safe for use in the animal models at 5 µg/animal.
- The microbicide increased the survival rate of female mice to 10%, and when delivered using a 2.7% HEC gel as a delivery vehicle, the survival rate of the female mice increased from 10% to 60%. In male mice, the survival rate was increased to 30 and 40% when administered with Bi-SP at 5 µg/animal and 10 µg/animal respectively. This proves that the microbicide is effective in the prevention of viral replication in the vaginal and rectal mucosa.

5.2 Research limitations

- Only one male and one female mouse control (uninfected mice) were used for histology, virus plaque assay, and flow cytometry.
- For the virus plaque assay and flow cytometry, only three samples per test group were used. It would have been ideal to use all the samples ($n = 10$) to provide more definitive results, but this could not be achieved due to time constraints.
- No antigen specific T cell responses were measured
- No viability marker was used for CD4⁺ and CD8⁺ analysis
- HEC alone appeared to block infection

5.3 Future recommendations

- Increasing the number of control mice to at least 3 mice per group will be beneficial to have conclusive results that can be used for comparison. Virus plaque assay and analysis of CD4⁺ and CD8⁺ should be performed on all the vaginal and rectal swab samples instead of randomly selected samples.
- Do further research such as immunohistochemistry analysis on excised tissues on the rectal mucosa of BALB/c mice.
- Conduct further analysis of more HSV-2-related immune responses such as cytokines and chemokines in particular CCL2, CCL5, CXCL9 and IL-6 which can be beneficial in translating from *in vivo* to clinical studies.
- Quantify HSV-2 in vaginal and rectal swab samples by the use of reverse transcription polymerase chain reaction (RT-PCR)
- Serial dilutions can be performed with rectal and vaginal swab samples in order to quantify the virus using virus plaque assay
- Bi-SP should be delivered in a less hypo-osmotic HEC gel to avoid gel formation.

APPENDIX 1: HISTOPATHOLOGY REPORTS FOR BI-SP SAFETY EVALUATION

** Pathologists **		PathCare Veterinary Laboratories
Drs Dietrich, Voigt & Mia		Neels Bothma Str, N1 City
		Tel : +27 21 596 3400
		Vet Facility No: PR16/14260

PracNum: 5200539	FINAL REPORT	Page: 1
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Doctor
NORTH WEST UNIVERSITY VIVARIUM
ATT: COR BESTER
11 HOFFMAN STREET
2531 POTCHEFSTROOM

Patient
RAT NO 6 BESTER OTHER

Age : Sex : DOB: 150:M:		
ID Number	NOT AVAILABLE	Tel No (H) NOT AVAILABLE
Other Number	NONE	Tel No (C) NONE
Specimen	18:ZH765	Lab Ref
Collection Date	2018-03-08 :	617159412
Received Date	2018-03-29 12:21	
Report Date	2018-05-09 15:44	File No. NWU-00290-17-AS/

----- Histopathology -----

CLINICAL HISTORY:
Reference NWU-00290-17-AS/samples 6 to 10 (mouse vaginal tracts).
Five female mice received 10 micro litre of a microbicide test compound intravaginally.

They were euthanised 2 hours after administration and vaginal tracts were collected in formalin for histopathology.

MACROSCOPY:
Five mouse vaginal samples were received in buffered formalin for evaluation by light microscopy.

Pathcare reference	PCDDP ID
18:765	Female 1
18:766	Female 2
18:767	Female 3
18:768	Female 4
18:769	Female 5

MICROSCOPY:
Longitudinal sections from the vaginal tracts were prepared for histopathology.
On histopathology, there was no evidence of epithelial cell disruption nor

		Lab Ref	Page : 2
NORTH WEST UNIVERSITY VIVARIUM	617159412	RAT NO 6 BESTER OTHER	
Specimen	Collection Date	Received Date	Report Date
18:ZH765	2018-03-08 :	2018-03-29 12:21	2018-05-09 15:44

MICROSCOPY

(Continued)

inflammation in any of the vaginal tissue sections evaluated.

COMMENTS:

A single intravaginal administration in female mice of a microbicidal test compound was not associated with any microscopic changes on histopathology.

ICD-10 : Z01.7

Signed Out by DR SOPHETTE GERS on 2018-03-29 16:20:00
For Consultation Call : 021 - 5963516
END OF REPORT : Total Number of Pages : 2

**** Pathologists ****
Drs Dietrich, Voigt & Mia

PathCare Veterinary Laboratories
Neels Bothma Str, W1 City
Tel : +27 21 596 3400
Vet Facility No: FR16/14260

PracNum: 5200539

FINAL REPORT

Page: 1

Doctor
NORTH WEST UNIVERSITY VIVARIUM
ATT: COR BESTER
11 HOFFMAN STREET
2531 POTCHEPSTROOM

Patient
RAT 1 BESTER OTHER

Age : Sex :	DOB :	150:M:	
ID Number		NOT AVAILABLE	Tel No (H) NOT AVAILABLE
Other Number		NONE	Tel No (C) NONE
Specimen	:	18:ZH760	Lab Ref
Collection Date	:	2018-03-08 :	617159418
Received Date	:	2018-03-29 13:09	
Report Date	:	2018-05-09 15:44	File No. NWU-00290-17-A5/

----- Histopathology -----

CLINICAL HISTORY:

Reference NWU-00290-17-A5/samples 1 to 5 (mouse rectums) .

Five male mice received 10 microliter of a microbicide test compound intrarectally.

Mice were euthanized two hours after administration and rectums were collected in formalin for histopathology (Samples 1 to 5) .

MACROSCOPY:

Five mouse rectal samples were received in buffered formalin for evaluation by light microscopy.

Pathcare reference	PCDDP ID
18:760	Male 1
18:761	Male 2
18:762	Male 3
18:763	Male 4
18:764	Male 5

		Lab Ref	Page : 2
NORTH WEST UNIVERSITY VIVARIUM		617159418	RAT 1 BESTER OTHER
Specimen	Collection Date	Received Date	Report Date
18:ZH760	2018-03-08 :	2018-03-29 13:09	2018-05-09 15:44

MICROSCOPY:

Longitudinal sections of rectum were prepared for histopathological evaluation.

On histopathology there was no evidence of epithelial cell disruption nor inflammation in any of the rectal tissue sections.

COMMENTS:

A single intrarectal administration in male mice of a microbicidal test compound was not associated with any microscopic changes on histopathology.

DIAGNOSIS:

ICD-10 : Z01.7

Signed Out by DR SOPHETTE GERS on 2018-03-29 16:20:00
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APPENDIX 2: AUTHOR GUIDELINES FOR VIROLOGY

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number of supplemental material files is limited to 10. No supplemental material should be included in the main manuscript. Supplemental files should be submitted in the following standard formats.

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Research Articles should include the elements described in this section.

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2. Bina XR, Taylor DL, Vikram A, Ante VM, Bina JE. 2013. *Vibrio cholerae* ToxR downregulates virulence factor production in response to cyclo(Phe-Pro). *mBio* 4:e00366-13.
3. Winnick S, Lucas DO, Hartman AL, Toll D. 2005. How do you improve compliance? *Pediatrics* 115:e718–e724.
4. Falagas ME, Kasiakou SK. 2006. Use of international units when dosing colistin will help decrease confusion related to various formulations of the drug around the world. *Antimicrob Agents Chemother* 50:2274–2275. (Letter.) [“Letter” or “Letter to the editor” is allowed but not required at the end of such an entry.]
5. Cox CS, Brown BR, Smith JC. *J Gen Genet*, in press.* [Article title is optional; journal title is mandatory.]
6. Forman MS, Valsamakis A. 2011. Specimen collection, transport, and processing: virology, p 1276–1288. In Versalovic J, Carroll KC, Jorgensen JH, Funke G, Landry ML, Warnock DW (ed), *Manual of clinical microbiology*, 10th ed, vol 2. ASM Press, Washington, DC.
7. da Costa MS, Nobre MF, Rainey FA. 2001. Genus I. *Thermus* Brock and Freeze 1969, 295, ^{Al} emend. Nobre, Trtper and da Costa 1996b, 605, p 404–414. In Boone DR, Castenholz RW, Garrity GM (ed), *Bergey's manual of systematic bacteriology*, 2nd ed, vol 1. Springer, New York, NY.
8. Fitzgerald G, Shaw D. In Waters AE (ed), *Clinical microbiology*, in press. EFH Publishing Co, Boston, MA.* [Chapter title is optional.]
9. Green PN, Hood D, Dow CS. 1984. Taxonomic status of some methylotrophic bacteria, p 251–254. In Crawford RL, Hanson RS (ed), *Microbial growth on C₁ compounds*. Proceedings of the 4th International Symposium. American Society for Microbiology, Washington, DC.
10. Rotimi VO, Salako NO, Mohaddas EM, Philip LP. 2005. Abstr 45th Intersci Conf Antimicrob Agents Chemother, abstr D-1658. [Abstract title is optional.]
11. Smith D, Johnson C, Maier M, Maurer JJ. 2005. Distribution of fimbrial, phage and plasmid associated virulence genes among poultry *Salmonella enterica* serovars, abstr P-038, p 445. Abstr 105th Gen Meet Am Soc Microbiol. American Society for Microbiology, Washington, DC. [Abstract title is optional.]
12. Garcia CO, Paira S, Burgos R, Molina J, Molina JF, Calvo C, Vega L, Jara LJ, Garcia-Kutzbach A, Cuellar ML, Espinoza LR. 1996. Detection of *Salmonella* DNA in synovial membrane and synovial fluid from Latin American patients using the polymerase chain reaction. *Arthritis Rheum* 39(Suppl 9):S185. [Meeting abstract published in journal supplement.]
13. O'Malley DR. 1998. PhD thesis. University of California, Los Angeles, CA. [Title is optional.]
14. Stratagene. 2006. Yeast DNA isolation system: instruction manual. Stratagene, La Jolla, CA. [Use the company name as the author if none is provided for a company publication.]
15. Odell JC. April 1970. Process for batch culturing. US patent 484,363,770. [Include the name of the patented item/process if possible; the patent number is mandatory.]
16. Harrison F, Roberts AEL, Gabriliska R, Rumbaugh KP, Lee C, Diggle SP. 2015. A 1,000-year-old antimicrobial remedy with antistaphylococcal activity. *mBio* 6:e01129-15. [Original article that describes how data submitted to a database were generated.]
17. Harrison F, Roberts AEL, Gabriliska R, Rumbaugh KP, Lee C, Diggle SP. 2015. Data from “A 1,000-year-old antimicrobial remedy with antistaphylococcal activity.” Dryad Digital Repository <https://doi.org/10.5061/dryad.mn17p>. [Citation for the database where the data in the previous reference were deposited; the URL is necessary.]
18. Wang Y, Rozen D. 2016. Colonization and transmission of the gut microbiota of the burying beetle, *Nicrophorus vespilloides*, through development. *bioRxiv* <https://doi.org/10.1101/091702>.

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Magalon A, Mendel RR. 15 June 2015, posting date. Biosynthesis and insertion of the molybdenum cofactor. *EcoSal Plus* 2015 <https://doi.org/10.1128/ecosalplus.ESP-0006-2013>.

APPENDIX 3: ETHICS TRAINING



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YUNIBESITHI YA BOKONE-BOPHIRIMA
NOORDWES-UNIVERSITEIT
POTCHEFSTROOM CAMPUS

Private Bag X6001, Potchefstroom
South Africa 2620

Tel: 018 299-1111/2222
Web: <http://www.nwu.ac.za>

Health Sciences Ethics Office for Research,
Training and Support
Tel: 018 299 2082
Fax:
Email: minnie.greeff@nwu.ac.za

18 May 2017

Dear Mr Tumelo Kgoe

PROOF OF ATTENDANCE

This letter certifies that you have attended the 2 day ethics training, entitled:

The Basics of Health Research Ethics

presented by Prof Minnie Greeff (Head of the Health Sciences Ethics Office for Research, Training and Support) on 2 and 3 May 2017.

This proof of attendance, as recognised by HREC and the Ethics Office, NWU, is valid for 3 years and expires on the 3rd of May 2020.

Yours sincerely

A handwritten signature in black ink, appearing to be 'M. Greeff'.

Prof Minnie Greeff
Head of Health Sciences Ethics
Office for Research, Training and Support

A handwritten signature in black ink, appearing to be 'A. Kotzé'.

Prof Awie Kotzé
Dean of Faculty of Health Sciences



Private Bag X6001, Potchefstroom
South Africa 2520

Tel: +2718 299-1111/2222
Web: <http://www.nwu.ac.za>

**Health Sciences Ethics Office for Research,
Training and Support**
Tel: +2718 299 2089
Email: minie.greeff@nwu.ac.za

7 March 2018

Dear Mr Tumele Kgoe

PROOF OF ATTENDANCE

This letter certifies that you have attended the half-day ethics training, entitled:

The SANS document: As regulation for research with animals

presented by Prof Christiaan B Brink (Chairperson of AnimCare) on 24 January 2018.

This proof of attendance, as recognised by AnimCare and the Ethics Office, Faculty of Health Sciences, NWU, is valid for 3 years and expires on 24 January 2021.

Yours sincerely,

Prof Minie Greeff
Head of Health Sciences Ethics Office
for Research, Training and Support

Prof Awie Kotzé
Executive Dean: Faculty of Health
Sciences

Original details: (10167306) C:\Users\NWU\USER\Google Drive\9. Research and Postgrad Education\9.1.5.7 Training\Templates\9.1.5.7.5_CA_SANSDOC_1st2018.docm
7 March 2018

File reference: 9.1.5.7.5



Faculty of Health Sciences

Vivarium

This is to certify that

TUMELO KGOE

ID 9401025110082

has attended the Introductory Short Course on

Animal Handling and the Principles of Research on Animals

11 – 13 April 2018

Dean:
Faculty of Health Sciences

Head:
Vivarium (PCDDP)

APPENDIX 4: CONFERENCE PRESENTATIONS

POSTER PRESENTATION

Tumelo Kgoe¹, Rose Hayeshi¹, Ambrose Okem¹, Leonard Damelin²

¹ DST/NWU Preclinical Drug Development Platform (PCDDP), North West University, Potchefstroom, South Africa

² National Institute for Communicable Diseases, Sandringham, Johannesburg

Establishment and characterisation of a murine HSV-2 model for the evaluation of a novel microbicide. Drug Safety Africa Conference. North-West University, Potchefstroom, South Africa: 20 – 22 November 2018

Tumelo Kgoe, Rose Hayeshi, Ambrose Okem, Leonard Damelin

Establishment and characterisation of a murine herpes simplex virus type 2 (HSV-2) model for the evaluation of novel microbicides. The Academy of Pharmaceutical Sciences of the Pharmaceutical Society of South Africa (APSSA) conference. Kleinkaap Boutique Hotel, Centurion. 9 – 11 October 2019

*The Academy of Pharmaceutical Sciences of the
Pharmaceutical Society of South Africa*



congratulates

Tumelo Kgoe

*as the winner of the best oral presentation award
at the APSSA conference 2019*

Chairman

10 October 2019

Date

APPENDIX 5: LANGUAGE EDITING CERTIFICATE

Gill Smithies

Proofreading & Language Editing Services

59, Lewis Drive, Amanzimtoti, 4126, Kwazulu Natal

Cell: 071 352 5410 E-mail: moramist@vodamail.co.za

Work Certificate

To	Ms. Tumelo Kgoe
Address	DST/NWU Preclinical Drug Development Platform (PCDDP) Centre of Excellence for Pharmaceutical Sciences, North West University, Private Bag X6001, Potchefstroom.
Date	24/11/2019
Subject	Dissertation: Establishment and characterisation of a herpes simplex virus type 2 murine challenge model for the evaluation of a novel microbicide
Ref	GS/TK/01

I, Gill Smithies, certify that I have proofed the following for language, grammar and style,

Chapters 1 – 5: Establishment and characterisation of a herpes simplex virus type 2 murine challenge model for the evaluation of a novel microbicide, by Tumelo Kgoe,

to the standard as required by NWU, Potchefstroom Campus.

Gill Smithies