CONTAMINATION, INFECTION AND INFLAMMATION CONTROL IN AN EXPERIMENTAL MUCOSAL CYST MODEL USING ATHYMIC NUDE MICE

by

Meng Wang

A thesis submitted in partial fulfillment of the requirements for the Degree of Magister Scientiae Dentium in the discipline of Oral Pathology, Faculty of Dentistry, University of the Western Cape

Supervisor: Prof JJ Hille

February 2007

Contamination, Infection and Inflammation Control in an Experimental Mucosal Cyst Model Using Athymic Nude Mice

Meng Wang

KEYWORDS

Implantation
Cyst
Experimental model
Vaginal mucosa
Nude mice
Contaminant
Infection control
Antibiotic
Disinfectant
Isolator
Non-absorbable suture

ABSTRACT

Contamination, Infection and Inflammation Control in an Experimental Mucosal Cyst Model Using Athymic Nude Mice

M. Wang

MSc (Dent) thesis, Department of Oral and Maxillofacial Pathology, Faculty of Dentistry, University of the Western Cape

Various experimental cyst models have been developed in animals to study the epithelial-mesenchymal interactions and the etiology, pathogenesis, growth and behaviour of cysts. An artificial cyst model constructed from human vaginal mucosa in athymic nude mice has been suggested as an *in vivo* biotest system to study oral mucosal diseases. However, previous investigators have rarely provided information on the identification of microbiological contaminants and infection control. This study therefore identified the common pathogens and risk factors. The effects of Terramycin in drinking water and subcutaneous injections of Gentamicin (twice daily) were examined. The silk and nylon sutures were randomly used to construct the artificial cysts and stitch the skin wounds of mice.

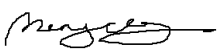
Forty- three male athymic nude mice (MFI nu nu) were implanted with human vaginal mucosal cysts under general anaesthesia with Ketamine [25mg/kg] and Medetomidine [0.5mg/kg]. Cysts in 37 mice were recovered after 9 weeks of growth. Twenty-three cyst linings had retained the original structure of the vaginal epithelium. No marked difference was present between the thickness of 9-week old cyst linings and donor vaginal epithelium. The contaminants isolated from the skin of mice before implantation were mainly normal commensals of healthy experimental animals. Coagulase negative *Staphylococci* (CNS) were the most frequently isolated contaminats (58.1%) from the implanted cyst site after 9 weeks of growth, followed by *Enterococcus faecalis* (22.6%). There was no distinct difference in the number of cases with intact cyst formation between the Terramycin/ vitamin cocktail group and the control group. The frequency of poor wound healing and/or murine epidermis ingrowth was three times higher in

animals stitched with silk sutures than in those cases where nylon sutures were used. The inflammation around silk sutures was generally more severe than that around nylon sutures. A foreign body reaction and acute inflammatory infiltrate usually surrounded the silk sutures. The loss of three mice was attributed to the high irritation of peracetic acid.

It is concluded that CNS and *Enterococci* were the most common pathogens found in the cyst model. Gentamicin proved to be more efficient against Gram-negative contaminants than Terramycin. Poor wound healing and murine epidermis ingrowth were the highest risk factors for failure of the implants. Nylon suture is an appropriate non-absorbable suture for minimising infection around experimental cysts. The prevention of microbiological contamination is compromised when the isolators are used in a substandard animal room. High irritative disinfectants and substandard animal housing must be avoided.

DECLARATION

I, Meng Wang, hereby declare that the work contained in "*Contamination, Infection and Inflammation Control in an Experimental Mucosal Cyst Model Using Athymic Nude Mice*" is my own original work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.



Meng Wang

Date: 2007-6-27

DEDICATION

This dissertation in dedicated to:

My supervisor, Prof Hille, who has given me his unselfish guidance and help

My parents, Zhensheng and Ming whose love and support are irreplaceable

My elder sister and brother-in-low, Jing and Zhangguo, who have supported me throughout my work

ACKNOWLEDGEMENTS

This research project could not have been accomplished without the continued moral, technical and financial support of many individuals, institutions and organizations. I, therefore, wish to acknowledge my sincere gratitude to all for their assistance. If someone's name has been omitted inadvertently, please accept my apologies.

Deep appreciation and sincere thanks is due to:

1. Prof JJ Hille, my mentor. HOD, Department of Oral and Maxillofacial Pathology, Faculty of Dentistry, University of the Western Cape; Chief Consulting Pathologist / Director of Oral Pathology Unit, NHLS Tygerberg Hospital laboratories. His moral support, guidance and encouragement for this project are gratefully acknowledged.

2. Mr Willem Fransman Jr, former animal technologist in the Oral and Dental Research Institute, University of the Western Cape. He provided the indispensable technical assistance and cared for the animals.

3. Dr MH Botha, Gynaecologist, Head of Unit of Gynaecological Oncology, Tygerberg Hospital, University of Stellenbosch, and his colleagues who supplied vaginal tissue specimens for this project. His continued loyal support contributed to the successful conclusion of this project.

4. Dr J van Rensburg, Head of Unit of Urogynaecology, Tygerberg Hospital, University of Stellenbosch, and his colleagues, who also provided specimens for experimental use.

5. The Department of Medical Bioscience, University of the Western Cape, for the use of their facilities.

6. Mr Llewellyn Sinclair, Animal Technologist, Animal Unit, University of the Witwatersrand, for the supply of nude mice.

7. Mr Dieter Geiger, Scientist, NHLS Anatomic Pathology, and Dept. of Anatomic Pathology, Faculty of Heath Science, University of Stellenbosch, especially for unselfishly sacrificing time and help.

8. Dr Manogari Chetty, Registrar, NHLS Oral Pathology, Tygerberg Hospital, and Department of Oral and Maxillofacial Pathology, University of the Western Cape. My deep appreciation is due to her for unselfish help and support.

9. The Oral and Dental Research Institute, University of the Western Cape, for the use of their facilities.

10. Dr David Fisher, Prof Ralf Henkel, Mr Shamieg Allie, Mr Arries, Ernest, and Semen, Department of Medical Bioscience, University of the Western Cape, for their assistance during the project.

11. Ms Pauline Hawkins, Animal Unit Manager, South Africa Vaccine Producers (Pty) Ltd, for arranging the transport of animal from JHB to CPT.

12. Mr HD van Zyl and Mr A du Toit, NHLS, for assisting with the ordering of consumables.

13. Mrs Esmeralda Voigt and Mrs Sandra Waterboer, Faculty of Dentistry, University of the Western Cape, for secretarial assistance.

14. Dr Ben Page and Dr CJF Muller, Department of Anatomy and Histology, University of Stellenbosch, for order anaesthetics agents from vet supplies.

15. Mr E Fillis and his colleagues, Assets and Insurance, University of the Western Cape, for transport of the equipments for the experiment between Tygerberg Campus and Main Campus of UWC.

16. The staff of the NHLS Anatomic Pathology and NHLS Microbiology laboratories at Tygerberg Hospital.

17. Dr Tina Roberts, Department of Oral and Maxillofacial Pathology, Faculty of Dentistry, University of the Western Cape, for her friendliness and the use of her computer.

18. The National Health Laboratory Service Research Trust and the University of the Western Cape for the generous financial support.

CONTENTS

TITLE i

KEYWORDS ii

ABSTRACT iii

DECLARATION v

DEDICATION vi

ACKNOWLEDGEMENTS vii

LIST OF TABLES xiii

LIST OF FIGURES xiv

LIST OF APPENDIX xix

CHAPTER 1: INTRODUCTION 1

CHAPTER 2: LITERATURE REVIEW 3

2.1 Evaluation and application of previous experimental cyst models in the oral environment 3

2.2 The case for human vaginal mucosa as a substitute of buccal mucosa in experimental models 5

2.3 The methodology and benefits of establishing human vaginal mucosa cysts in nude mice as an animal model to study oral mucosal properties 6

2.4 Putative causes of inflammation during the implantation cyst experiments 7

2.5 Putative microbiological contaminants of transplant models 9

2.6 General antibiotics, antimicrobials and disinfectants applied in animal research 12

CHAPTER 3: RESEARCH DESIGN AND METHODOLOGY 14

3.1 Aims and objectives 14

3.2 Nature of this study 14

3.3 Animal husbandry and care 14

3.3.1 Animal husbandry and description of the isolators: 14
3.3.2 Animal care 18
3.4 Antibiotic cover of the experimental mice 19
3.5 Management of the vaginal mucosa specimens 20
3.6 Artificial cysts creation and implantation 23
3.6.1 The construction of the artificial cysts (Fig 10) 23
3.6.2 The procedures of the implantation (Fig 11) 23
3.7 Injection of Gentamicin 27
3.8 Microbiological testing 28
3.8.1 Microbiological testing of the animals 28
3.8.2 Microbiological testing of the operation procedure 28
3.8.3 Microbiological testing of the investigators' nasal passages 28
3.8.4 Microbiological testing of the air of the animal room and isolators 28
3.8.5 Microbiological testing of the sterilized rodent food and bedding 29
3.9 Histological evalution 30
3.10 The endpoint of the animal model 31
3.11 Abbreviated research ethics statement 32

CHAPTER 4: RESULTS 33
4.1 Animal housekeeping and observations 33
4.2 Microbiological testing 37
4.2.1 The results of the skin swab before operation (Sample A) 37
4.2.2 The results of the skin swab after implantation (Sample B) 40
4.2.3 The results of the cyst swab (Sample C) 40
4.2.4 Microbiological testing of the operation procedure and investigators' nasal passages 44
4.2.5 Microbiological testing of the animal room and isolators 44
4.2.6 Microbiological testing of the food and bedding 44
4.3 The change of animals' body weight 44
4.4 Histological evaluation of the cyst wall 47
4.5 Comparison of silk suture and nylon suture 64

CHAPTER 5: DISCUSSION AND CONCLUSION 68

5.1 Identification of the contaminants and evaluation of antibiotic cover 68
5.1.1 Common pathogenic organisms 68
5.1.2 Putative source of contaminants 69
5.1.3 Inflammation and infection of the cysts 70
5.1.4 Efficiency of antibiotic coverage 71
5.2 Wound dehiscence and comparison of two non-absorbable sutures 73
5.3 Evaluation of animal model 75
5.3.1 Observation of animal housekeeping 75
5.3.2 Shortcomings of the isolator 76
5.4 Comparison of the cyst model with the Thompson studies 77
5.5 Toxicity of peracetic acid (PAA) 78
5.6 Other problems encountered in this study 79
5.7 Conclusion 80

CHAPTER 6: RECOMMENDATIONS 81

NOTE ABOUT COPYRIGHT 82
REFERENCE 83

APPENDIX I: Preparation of peracetic acid (PAA) 95
APPENDIX II: Terramycin/ Vitamin cocktail 96
APPENDIX III: Animal husbandry information form 97
APPENDIX IV:Participant information leaflet and consent form 98
APPENDIX V: Transport Medium 101
APPENDIX VI: EDTA 102
APPENDIX VII 103
1. Evaluation form of donor vaginal tissue 103
2. Evaluation form of cyst wall 103
APPENDIX VIII: Anaesthetics 104
APPENDIX IX: Other antibiotics and disinfectants 105

LIST OF TABLES

Number	Name	Page
Table 1:	Experimental groups	19
Table 2:	Evaluation of the animals and implants	34
Table 3:	The outcome details of all of the implants	36
Table 4:	Detail of the identification and antibiotic sensitivity testing of sample A	37-38
Table 5:	Summary table of the results of Sample A as identified per batch	39
Table 6:	Detail of the identification and antibiotic sensitivity testing of sample C	41-42
Table 7:	Summary table of the results of Sample C	43
Table 8:	Weight change of Control Group	45
Table 9:	Weight change of Group 2 (Terramycin/vitamin water)	45
Table 10:	Weight change of Group 3 (Gentamicin)	46
Table 11:	Weight change of Group 1 (both antibiotics)	46
Table 12:	The thickness of the control human vaginal epithelium	53
Table 13:	The thickness of the cyst epithelial lining	54
Table 14:	Evaluation of two kinds of sutures	64

LIST OF FIGURES

Figure	Page
Figure 1: Isolator	**15**
Figure 2: Materials kept in the port for sterilisation by peracetic acid	**16**
Figure 3: The food autoclave unit	**16**
Figure 4: The laminar air-flow unit for implantation procedures	**17**
Figure 5: The isolator joined with food autoclave unit by a transfer tunnel	**17**
Figure 6: The isolator joined with the laminar air-flow unit	**18**
Figure 7: The donated vaginal mucosa is immersed in transport medium	**21**
Figure 8: Two portions of donated vaginal mucosa	**21**
Figure 9A: Separating connective tissue from the epithelium by using the scalpel	**22**
Figure 9B: Completed separation of connective tissue from the epithelium	**22**
Figure 9C: Epithelium was immersed in 3mM EDTA for 2 hours treatment	**22**
Figure 10A: The instruments of the implantation	**24**
Figure 10B: The silicone tube plated on the epithelium surface	**24**
Figure10C-E: The construction of the artificial cyst	**24**
Figure 10F: The artificial cyst was immersed in transport medium	**24**
Figure11A-B: Transfer of mice from isolator to the laminar flow unit	**25**
Figure 11C, D: The applied anaesthetics before the implantation	**25**
Figure 11E: all operations were carried out in the laminar flow unit	**25**
Figure 11F-K: The implantation procedure	**26**

Figure 12: Photo of Gentamicin injection under anaesthetic condition **27**

A: Prior to the injection the animals were anaesthetized with 0.2ml Isoflurane vapourised in a 1000ml beaker inside the isolator

B: The Gentamicin was administered subcutaneously in the back (twice daily)

Figure 13: The substandard animal room **29**

Figure 14A: The implanted site with ingrowth of the epidermis and nodular scar tissue around the wound **34**

Figure 14B:Keratinized stratified squamous surface epithelium ingrowth into cyst cavity and lining scar tissue **35**

Figure 14C: Keratinized stratified squamous surface epithelium of murine epidermal origin lining the cyst cavity and scar tissue **35**

Figure 15A: Cyst necrosis and extrusion/sequestration of silastic tube due to poor wound healing **35**

Figure 15B: No implanted cyst present at the donor site **35**

Figure 16A: Photomicrographs of intact cyst formation with varying thickness of stratified squamous epithelium after 9 weeks growth **47**

Figure 16B, C: The epithelium of the cyst maintained the structure of the human vaginal mucosa **48**

Figure 17A: Photomicrograph of the vaginal mucosa processed as control tissue **49**

Figure 17B: Cyst lining derived from the same vaginal mucosa specimen (A) after 9 weeks growth **49**

Figure 18A: Photo of a larger amount of keratin in the cyst lumen **50**

Figure 18B: Photomicrograph of desquamation of the epithelial cells into the lumen **50**

Figure 19: Photomicrograph of nodular epithelial thickening at the thick region of the cyst **50**

Figure 20: Photomicrograph of 3 cysts (B-D) constructed from one donated vaginal tissue (A) **51**

Figure 21: Photomicrograph of few rete pegs had formed **51**

Figure 22A: Photomicrograph of ulcer of epithelium **52**

Figure 22B: Photomicrograph of necrotic epithelium **52**

Figure 22C: Photomicrograph of transformation between non-keratinized cyst epithelium and keratinised epidermis **52**

Figure 23: Photomicrograph of the new blood supply to implanted epithelium **55**

A) Thickened regions of epithelium where companied by hypervascular connective tissue

B) Thinner epithelium region with fewer blood vessels

Figure 24: Photomicrograph of the epidermis ingrowth and inflammation around the wound **57**

Figure 25A: Low-power photomicrograph of the inflammation in the cyst wall close to the murine dermis **57**

Figure 25B: High-power photomicrograph with details of the inflammation waround the wound suture **58**

Figure 26: Photomicrograph of the inflammation at ulcerated regions in the cyst **58**

Figure 27A: Photomicrograph of moderately dense inflammatory cell exocytosis in the cyst epithelium **59**

Figure 27B: Photomicrograph of the inflammatory cells accumulated in the cyst lumen **59**

Figure 28: Photomicrograph shows mild presence of inflammatory cells near the base of the epithelium **60**

Figure 29: Photomicrograph of the results of the Gram stains **60**

A) Gram-negative bacteria

B) Gram-positive bacteria

Figure 30: Photomicrographs of the junction between the murine host connective tissue and the human connective tissue of the artificial cyst **61**

A) Fibrosis in the implants without EDTA treatment;

B) No fibrosis at same area in the implants with EDTA treatment

Figure 31: Photomicrograph of the fibrosis around the suture **62**

Figure 32: Photomicrograph of the granulation tissue wall surrounding the fibrotic lumen contents **62**

Figure 33: Photomicrograph of the atrophic cyst **63**

Figure 34: Photomicrograph of the epithelial island in cyst wall **63**

Figure 35: Photos of wound area **65**

A) wound healing (nylon suture)

B) wound healing (silk suture)

C) epidermal ingrowth (nylon suture)

D) serious epidermal ingrowth (silk suture)

E) poor wound healing (nylon suture)

F) poor wound healing (silk suture)

Figure 36: Photomicrograph of the sutures **66**

A) Mild inflammation around nylon suture in the implant without contaminants

B) Mild inflammation around silk suture in the implant without contaminants
C) Inflammation around nylon suture in the implant with contaminants
D) Severe inflammation around silk suture in the implant with contaminants

Figure 37: High-power photomicrograph of a foreign body reaction around a silk suture. Foreign body giant cells were present **67**

LIST OF APPENDIX

Number	Name	Page
Appendix I:	Preparation of peracetic acid (PAA)	95
Appendix II:	Terramycin/vitamin cocktail	96
Appendix III:	Animal husbandry information form	97
Appendix IV:	Participant information leaflet and consent form	98
Appendix V:	Transport Medium	101
Appendix VI:	EDTA	102
Appendix VII:	1. Evaluation form of donor vaginal tissue	103
	2. Evaluation form of cyst wall	103
Appendix VIII:	Anaesthetics	104
Appendix IX:	Other antibiotics and disinfectants	105

CHAPTER 1: INTRODUCTION

There is a need for a stable experimental cyst model offering a closed environment similar to the oral cavity and which allows for controlled studies on the mucosal absorption mechanisms and local reaction patterns to introduced agents such as carcinogens, viruses, drugs and vaccines. The experimental cysts have to be constructed from fragments of non-keratinised mucosa to simulate natural oral conditions. However, the availability of disease-free fresh human oral mucosa in dimensions suitable for experimental purposes is very limited.

An implanted cyst model constructed from human vaginal mucosa has been established in athymic nude mice by Thompson and co-workers (Thompson *et al*, 2001a; b; Thompson, 2002). The cyst linings had retained the structure and permeability features of the vaginal epithelium. It has been suggested that this cyst model could be used as an *in vivo* biotest system to study oral diseases, because buccal and vaginal epithelia have similar histological characteristics, protective functions and permeability characteristics to water and chemical substances (Thompson *et al*, 2001; Van der Bijl *et al*, 1997; 1998a; b; c; 1999; 2000).

Because the susceptibility of athymic nude mice to infection is far greater than that for normal animals, the implantation sites and cysts in athymic mice have often been described to be affected by acute pyogenic infection typically occurring 10-14 days following implantation (Holmstrup *et al*, 1981d; Mackenzie *et al*, 1979). This often leads to death of the animals or unsuccessful identification of the transplant tissue. The containment or prevention of infectious disease is thus an important issue in the achievement of reliable experimental results. After Gentamicin antibiotic cover was introduced and Terramycin added to drinking water, the recovery rate of the transplant tissue raised from 47% to 87% (Holmstrup *et al*, 1981a; Mackenzie *et al*, 1979; Thompson & Van Wyk, 1996; Van Wyk & Thompson, 1992).

Other causes of infection and failure of the implants due to an inflammatory reaction can be attributed to contamination (ingrowth) of the implant site with murine cutaneous tissue and tissue reactions to absorbable suture material and other contaminants (Mackenzie *et al*, 1979; Thompson, 2002).

It obviously is mandatory to establish and maintain a strictly clean general environment; in particular a sterile working environment for the animals is required. During the project, the animals must be housed in isolators to avoid direct contact with each other or humans, and all cages and materials used including bedding, food and water must be autoclaved or sterilised before use.

The present project was initiated to: 1) identify the common pathogens and factors which could adversely affect the establishment and maintenance of this elegant *in vivo* biotest model; 2) test the efficacy of Terramycin added to drinking water and subcutaneous injections of Gentamicin (twice daily) in preventing infection of the animals and the experimental implantation sites; 3) test if the use of non-absorbable suture material around the implant and closing the incision wound reduces the inflammatory response and subsequent fibrosis.

CHAPTER 2: LITERATURE REVIEW

2.1 Evaluation and application of previous experimental cyst models in the oral environment

Cyst models in jawbones. Cysts are more common in the jaws than in any other bone because of the many rests of epithelium remaining in the tissues after dental development. There are two classes of epithelial-lined cysts of the jaws: one is derived from the odontogenic epithelium; and the second is from the non-odontogenic epithelium (Soskolne *et al*, 1976). In a number of studies, cysts produced in experimental animals were used to study the pathogenesis of jaw cysts. Some of them described the transplantation of tooth germs into soft tissue sites (Bartlett *et al*, 1973; Klein *et al*, 1971; Riviere & Sabet, 1973), while others used epithelium that was implanted into deeper connective tissue (Baker & Mitchell, 1965; Epstein & Kligman, 1957). Soskolne *et al* (1976), Ramanathan and Philipsen (1981) have described experimental models in which they implanted keratinised epithelium into the mandibles of rats in order to study the pathogenesis and growth of jaw cysts, and to determine their ultimate fate.

Cyst models developed from oral mucosa. The structure of normal human oral epithelium varies in different anatomical regions with respect to a number of characteristics including thickness, pattern of keratinisation, the epithelial connective tissue interface and the biochemical properties of the cells (Squire *et al*, 1976). It has been reported that epithelium accidentally implanted during surgery or trauma is capable of cyst formation (Abrams *et al*, 1977; Ettinger & Maderson, 1973; Papanayotou & Kayavis, 1977; Thaddeus & Shafter, 1958; Torres & Higa, 1970).

There is considerable evidence supporting the role of epithelial-mesenchymal interactions in the proliferation and differentiation of these tissues. Epithelial-mesenchymal interactions can be studied by tissue dissociation and

cross-recombination. In these studies, epithelium is separated from its original connective tissue and recombined with connective tissue from a different source (Mackenzie, 1984). Mackenzie *et al* (1979), Holmstrup *et al* (1981a-d), and Vedtofte *et al* (1982) built an experimental model by transplanting normal and leukoplakic human oral mucosa to subdermal sites in athymic mice. They demonstrated that the epithelial lining of the cysts retain the normal histological features of the human tissue during the growth and differentiation phases; this would allow researchers to investigate the role of epithelial-mesenchymal interactions in human oral diseases. This model can also be used to evaluate the influence of connective tissue on epithelium during chemically induced oral mucosal carcinogenesis (Rich & Reade, 2001).

Other models. Lindholm *et al* (1984) have transplanted buccal mucosa into various structures in the neck of Beagle dogs and produced experimental cysts. These cysts served as a tissue framework in which they studied the feasibility of combining biocompatible materials with mucosal membrane and which could be used as substitutes for parts of the larynx or trachea.

However, the availability of fresh human oral mucosa in sizes suitable for experimental purposes is limited. For this reason, fresh and/or frozen autopsy tissues, or tissue derived from animal models have been used as substitutes for fresh specimens of human oral mucosa (Holmstrup *et al*, 1981a-d; Mackenzie *et al*, 1979; Ramanathan & Philipsen, 1981; Soskolne *et al*, 1976; Vedtofte *et al*, 1982).

Because of the similarity between the histological, electron microscopic and permeability features of human vaginal mucosa and buccal mucosa, it has been proposed that human vaginal mucosa could be used as a substitute for buccal mucosa in *in vitro* and *in vivo* experimental studies (Thompson & Van Wyk, 1996; Thompson *et al*, 2000, Thompson *et al*, 2001b; Van Wyk & Thompson, 1992). These may include permeability and drug-absorption studies of a variety of chemical compounds, including carcinogens.

2.2 The case for human vaginal mucosa as a substitute of buccal mucosa in experimental models

Similarities: Buccal and vaginal epithelia have similar protective functions and histological characteristics. The protective functions include those that prevent mechanical damage to deeper lying tissues and act as barriers to the entry of microorganisms and noxious substances (Thompson *et al*, 2001a). The histological characteristics of the epithelial layers, epithelial thickness, and surface keratinisation are similar; the shared ultrastructural characteristics include the stacked intercellular lipid lamellae and membrane-bound intracellular granules containing internal lamellae, and the lipid compositions of two types of mucosa (Thompson *et al*, 2001a). Additionally no differences were observed in the morphology or growth patterns between the vaginal and buccal epithelium in cell cultures (Van Eyk & Van der Bijl, 1998). Similar permeability characteristics of fresh or frozen vaginal and buccal mucosa to water, 17ββ-estradiol, dextran, vasopressin, benzo[a]pyrene, and Sumatriptan have been demonstrated (Van der Bijl & Van Eyk, 1999; Van der Bijl *et al*, 2000; 1997; 1998a-c).

Differences: The glycogen content and the thickness of vaginal and buccal epithelium differ. The thickness of vaginal epithelium is influenced by hormonal stimulation and varies in particular during the menstrual cycle, pregnancy, post partum period, lactation period and in the post-menopausal female. Therefore, only vaginal epithelia from post-menopausal women on hormone replacement therapy are used in many studies to minimise hormonal influences (Van der Bijl & Van Eyk, 1999; Van der Bijl *et al*, 1997; 1998d; Van Eyk & Van der Bijl, 1998; Van Wyk & Thompson, 1992).

2.3 The methodology and benefits of establishing human vaginal mucosa cysts in nude mice as an animal model to study oral mucosal properties

Approximately 97% of all animals used in research, for teaching, and substance testing are mice or rats. This is because they are small and easily housed and bred, short lived, relatively inexpensive and they are genetically very similar to humans (FBR, 2003). The nude mouse, a hairless immunodeficient mutant, was discovered in 1962 and does not reject tissue or organs transplanted from other species. It lacks a thymus, and cannot produce T-lymphocytes which are an essential component of the immune system. Since they were discovered, nude mice have been widely used to study various aspects of tumour behaviour or in tissue transplantation studies. Lack of hair growth allows for visualization of subcutaneous transplants and the immunodeficiency state supports growth of many types of foreign cells (UCCAA, 2003). Immunodeficient mice have also not only been used in microbiological and parasitological experimental studies, but also for genetical, nutritional and environmental studies.

An implantation cyst model constructed from human vaginal mucosa has been established in athymic nude mice (Thompson & Van Wyk, 1996; Thompson *et al*, 2001b). It has been suggested that this cyst could be used as an *in vivo* biotest system to study oral diseases because the cyst lining had retained the structure and permeability features of the vaginal epithelium that is similar to oral epithelium. Moreover, the epithelial lining of the cyst remains associated with its own connective tissue and would thus not be subjected to messages from a foreign connective tissue (Thompson *et al*, 2001b, Van Wyk & Thompson, 1992). The model can thus be used to study the induction of pathological changes in the cyst linings, to test biomaterials and to investigate epithelial transformations such as hyperplasia and metaplasia.

The containment or prevention of disease is thus an important contribution to achieving reliable experimental results. One major problem encountered during

the establishment of this model has been the relative high failure rate of the implanted cysts due to inflammation and necrosis. Epithelial ingrowth from the surgical wound margins is also a problem (Mackenzie *et al*, 1979).

2.4 Putative causes of inflammation during the implantation cyst experiments

Because the susceptibility of immunodeficient mice to infection with pathogenic viruses and saprophytic bacteria is far greater than for normal animals, the implantation sites and cysts in athymic mice have often been described to be affected by acute pyogenic infection typically occurring 10-14 days following implantation (Holmstrup *et al*, 1981d; Mackenzie *et al*, 1979). This often leads to death of the animal or unsuccessful identification of the transplant tissue.

The literature not only lacks reports on proper peri- and post-operative anti-infective care, but also reveals that most of the experimental studies before 1980's on skin and oral mucosa implantation cysts have been performed without antibiotic cover and no specific measures were taken to prevent infection (Baker & Mitchell, 1965; Epstein & Kligman, 1957; Mackenzie *et al*, 1979). The fact that the animals did not receive antibiotic treatment and that the implants were rinsed in normal saline, often resulted in massive infection and necrosis of the implants. The "hat-shaped" polyethylene capsules, which were introduced by Mackenzie *et al* (1979) to protect oral mucosal implants by preventing contraction of the connective tissue bed and the ingrowth of the epidermis from the wound margins and surrounding surface only yielded a 44% success rate. Holmstrup *et al* (1981d) frequently noted that infection of the implant sites and loss of the capsules was associated with an open skin surface condition; however the presence of capsules did not appear to influence the recovery rate of human oral mucosa implants. A reduction of the infection rate was obtained by suturing and covering the incision wound with Histoacryl® as an antibiotic barrier to contamination. The use of Specified Pathogen Free (spf) inbred animals also reduced the infection risk.

When Terramycin or Gentamicin antibiotic cover was introduced, minimal infection was encountered and a higher yield of successful implantation cysts was obtained (Van Wyk & Thompson, 1992; Ramanathan & Philipsen, 1981).

However, it is still mandatory to establish and maintain a strictly clean general environment and in particular a sterile working environment for the animals. Because diseases may be transmitted from animal to animal by direct contact or via pathogens shed or excreted via their faeces, urine, saliva, exhaled air or dander, the experimental animals must be individually housed in isolators to avoid direct contact with each other or humans (Poole, 1987). All cages and materials used including bedding, food and water, must be autoclaved for sterilisation before use. The use of a laminar air-flow cabinet during the surgical procedure affords greater economy of time and effort in keeping the germ-free status of mice and no more contamination occurs compared to other methods (Taylor, 1975).

Previous studies have reported an improvement in the recovery rate of the transplant tissue from 47% to 87% when these measures were introduced (Holmstrup *et al*, 1981a; b; d; Mackenzie *et al*, 1979; Thompson & Van Wyk, 1996; Van Wyk & Thompson, 1992). However it is imperative to strive for an even better success rate since animal experiments are expensive and suffering of the animals should be kept to a minimum.

Other causes of infection and failure of the implants due to an inflammatory reaction can be attributed to contamination (ingrowth) of the implant site with murine cutaneous tissue and tissue reactions against absorbable suture material and other contaminants (Thompson, 2002). Because of these various features, the choice of suture material for any given wound closure should not be made arbitrarily, but rather with careful attention to the physical and handling properties of the suture, as well as its propensity for eliciting tissue reaction and promoting infection (J&J, 2004). For skin, non-absorbable sutures are usually used. Non-absorbable sutures are defined by the U.S. Pharmacopeia as "flexible strands

of material that are suitably resistant to the action of living mammalian tissue" (USPC, 2000). The most commonly used non-absorbable sutures are silk, nylon polypropylene, braided polyesters and polybutester (LaMorte, 2002).

Nylon (Ethilon, Dermalon) is the most widely used non-absorbable suture in skin surgery. It has high tensile strength, minimal tissue reactivity, excellent elastic properties and low cost. The major drawback to nylon is its high degree of memory. A greater number of knot throws (three or four) are required to hold a given stitch in place. Silk is a naturally occurring proteinaceous filament spun by silkworm larvae as they build cocoons. Silk is made into a braided suture, which has perhaps the best handling and tying characteristics of any suture material. In skin surgery, silk is used around the eyelids and lips, where it can lay flat, causes minimal irritation and has a low incidence of infection. (Moy *et al*, 1991; Lin *et al*, 2005)

Finally, it is highly probable that epithelial ingrowth from the surgical wound margins following wound dehiscence could be prevented by reducing the mechanical trauma of the stretched skin over an anatomically tight implantation site. Traditionally, implants have been subcutaneously inserted in the flank region of the experimental animals, however the skin in the neck region of nude mice is much looser and lends itself much better for implantation of rigidly supported mucosal transplants. (Thompson, 2002)

2.5 Putative microbiological contaminants of transplant models

Viruses.

The transplant tumours and tissues are the common source of murine viruses. The list of viruses includes Lactate DeHydrogenase virus (LDH), Minute Virus of Mice (MVM), Mouse Hepatitis Virus (MHV), Lymphocytic ChorioMeningitis virus (LCM), Polyoma virus, Reo3 and Sendai virus (Poole, 1987).

Bacteria.

Enterobacter species are rod-shaped bacteria that are found in the environment and also in the human intestinal tract. Some species are pathogenic, the most common being *E. cloacae* and *E. aerogenes,* which can cause opportunistic infections in immunocompromised patients. The urinary and respiratory tracts are the most common sites of infection with *Enterobacter* species. (HPA, 2006a)

Enterococcus species are commonly found in the bowel of normal healthy individuals. *Entercocci* can cause a range of illnesses including urinary tract infections, bacteraemia and wound infections. The two most common species are *E. faecalis* and *E. faecium* (HPA, 2006b).

Klebsiella species are rod shaped-bacteria that are found in the environment and also in the human intestinal tract. *K. pneumoniae* is the most common species isolated from hospital patients. A second species, *K. oxytoca* is an occasional cause of wound, bloodstream and urinary tract infections (HPA, 2006c).

Pseudomonas aeruginosa is a Gram-negative bacterium commonly found in soil and ground water. It rarely affects healthy people and most community-acquired infections are associated with prolonged contact with contaminated water. *P. aeruginosa* is increasingly important clinically as it is a major cause of both healthcare-associated infections and chronic lung infections in people with cystic fibrosis (HPA, 2006d).

Staphylococcus spp

Staphylococci are ubiquitous in the environment. Natural populations are associated with skin, skin glands and mucous membranes of warm-blooded animals. They have been isolated from animal products such as meat, milk and cheese, and other sources such as soil, sand, seawater, fresh water, dust and air. It is difficult to get rid of these commensals even by the vigorous routine scrubbing protocols that precede surgery. The main obvious effect of infection by *staphylococci* is the production of pus in boils, abscesses and suppurating wounds.

Staphylococci also produce toxins of great lethality; one of these toxins can cause food poisoning (Dawson, 1989; Pearse, 1997).

In 1884, Rosenbach described the two pigmented colony types of staphylococci and proposed the appropriate nomenclature: *Staphylococcus aureus* (yellow) and *Staphylococcus albus* (white). The latter species is now named *Staphylococcus epidermidis*. *Staphylococci* can be divided into two groups, depending on whether they produce the enzyme coagulase. Nearly all strains of *S. aureus* are coagulase-positive; nearly all strains of *S. epidermidis* lack this enzyme. *S. aureus* should always be considered a potential pathogen; most strains of *S. epidermidis* are nonpathogenic and may even play a protective role in their host as normal flora although they may be a pathogen in the hospital environment (Todar, 2005) as they have antibiotic resistance to penicillin.

Because of the involvement in some kinds of human and animal diseases, the coagulase-negative staphylococci (CNS) have been studied extensively. CNS lives naturally on the skin and mucous membranes of humans and is therefore often found in clinical specimens. Only half of the 32 coagulase-negative species have been found in specimens of human origin. They can be divided into two groups, depending on whether they are resistant or susceptible to novobiocin (Albamycin): a) **Novobiocin-susceptible:** *S. epidermidis, S. haemolyticus, S. auricularis, S. capitis subsp capitis, S. capitis subsp urealyticus, S. caprae, S hominis, S. lugdunensis, S. pasteuri, S. saccharolyticus, S. schleiferi subsp schleiferi, S. simulans, S. warneri*; b) **Novobiocin-resistant:** *S. saprophyticus subsp saprophyticus, S. cohnii subsp cohnii, S. sciuri,* and *S. xylosus.* (Von Eiff *et al*, 2001)

<u>*Streptococci*</u> spp.

Streptococci can be found in the majority of the human population, favouring colonisation of the throat, nose and mucous membranes of the perineum. They can cause a wide variety of diseases. (Dawson, 1989; Pearse, 1997)

Fungi

Superficial fungal infections of the skin are usually caused by dermatophytes, fungi which invade only 'dead' tissues of the skin or its appendages, such as the corneal layer of the skin, the nails, hair, etc.

Candida albicans may be present in the oral cavity under normal circumstances as part of the oral flora but will become pathogenic under certain circumstances to cause candidiasis, especially in suppressed immune conditions. It can also cause inflammation of the vagina (vaginal candidiasis), especially before puberty and after menopause, when the acidity of the vaginal fluids is such as to encourage its growth (Dawson, 1989; Pearse, 1997). It has long been recognised that fungi can be poisonous to man and animals.

Aspergillus flavus for instance produces aflatoxin that is highly carcinogenic in experimental animals as well as causing other diseases in animals and fish (Dawson, 1989).

2.6 General antibiotics, antimicrobials and disinfectants applied in animal research

Terramycin (oxytetracycline) is a broad-spectrum antibiotic compound with a wide range of activity against both Gram-positive and also to a lesser extend Gram-negative bacteria. It also has anti-inflammatory properties. Terramycin had a remarkably low toxicity and minimal side effects when taken by animals. (Todar, 2002)

Gentamicin is a broad spectrum aminoglycoside antibiotic widely used in veterinary medicine for the treatment of serious infections. Gentamicin is primarily active against gram-negative bacteria including: *Pseudomonas aeruginosa, Proteus* spp*., Escherichia coli, Klebsiella* spp*., Enterobacter* spp*.,*

Serratia spp*., Actinobacillus equuli, Haemophilus equigenitalis,* and many species of *Salmonella* and *shigella.* Gentamicin is also active against *Staphylococcus* spp. (including penicillin and methicillin-resistant strains) and *Rhodococcus equi*. (AL, 2004; Todar, 2002)

Peracetic acid ($C_2H_4O_3$) (PAA) is a mixture of acetic acid (CH_3COOH) and hydrogen peroxide (H_2O_2) in a watery solution. PAA is usually produced in concentrations of 5-15%. PAA is caustic topically, but is extremely germicidal due to its oxidation action. A special advantage of PAA is it has no harmful decomposition products (i.e., acetic acid, water, oxygen, hydrogen peroxide) and leaves no residue (OMRI, 2000a, b). PAA is a high-level hospital disinfectant with a proven efficacy of the rapid action against all microorganisms. PAA is an effective disinfectant on *Pseudomonas aeruginosa* (Chang, *et al*, 2005).

CHAPTER 3: RESEARCH DESIGN AND METHODOLOGY

3.1 Aims and objectives

3.1.1 To identify the commensals and contaminant microorganisms of this experimental mucosal implantation cyst model in athymic nude mice.

3.1.2 To distinguish the effect of two antibiotics coverage modalities to eliminate the risk of infection and failure of this model.

3.1.3 To evaluate two kinds of sutures: silk and nylon.

3.2 Nature of this study

This study should be considered as a pilot project due to the limited number of athymic nude mice used.

3.3 Animal husbandry and care

3.3.1 Animal husbandry and description of the isolators:

Athymic nude mice (MFI nu nu) of male sex were obtained in batches from the animal unit of Medical School of the University of the Witwatersrand (Wits). The animals were transported by airfreight in sterilised air-filtered boxes and upon arrival the mice were transferred and maintained in one of three isolators at the animal facilities of the Department of Medical Bioscience of the University of the Western Cape.

The polyvinyl chloride (PVC) isolator (Fig. 1) is suitable for small laboratory animals. It utilises room space more effectively and is particularly useful where accommodation is restricted. There are two ancillary equipments for the isolators: 1) an autoclavable stainless steel trolley for the sterility of the

food and bedding (Fig 3); 2) the laminar air-flow cabinet for surgical procedures (Fig 4).

Figure 1: Isolator

A→ Unidirectional airflow air filter: cotton filter media was used for sterilizing the air blowed into the isolators; the sterilized filters were changed every 3 weeks.

B→ Unidirectional airflow exhaust liquid filter: contains Glycerol [$C_3H_3(OH)_3$], to prevent the air flow back from outside to inside the isolator.

C→ A pair of sealed long sleeve gloves: Neoprene® gloves have sufficient chemical resistance to withstand decontaminating agents.

D→ A sealed Rapid Transfer Port unit allows the transfer of materials into/out of the isolator without compromising the sterility of the isolator. This procedure is one of the greatest potential sources of contamination.

Static Pass-through is the most basic using way: materials are placed into the port from outside and sterilised with peracetic acid (see Appendix I); the outside cover is closed and the materials are kept in the

port for two hours; then the inside cover can be opened and the materials passed through to the isolator (Fig 2). The port is sterilised with Peracetic Acid for 2 hours when the isolator is joined with the food autoclave unit and the laminar air-flow unit (Fig 5 and 6).

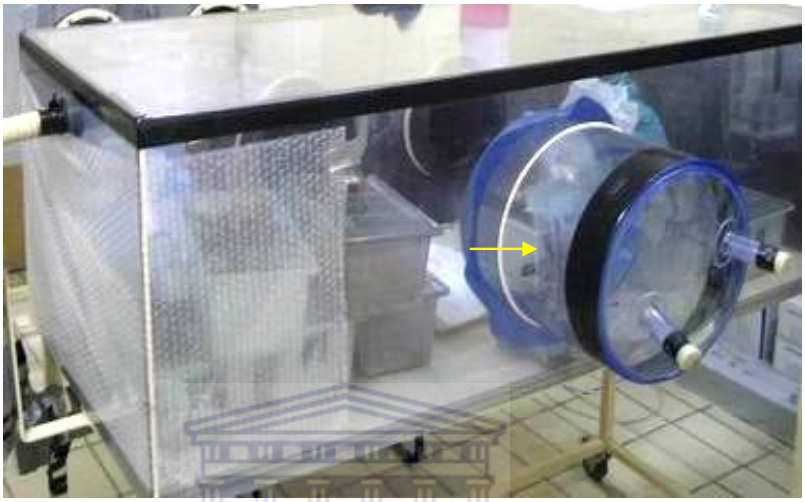


Figure 2: Materials kept in the port for sterilisation by peracetic acid.

Figure 3: The food autoclave unit, the sterilised food (thin arrow) and bedding (thick arrow) inside.

Figure 4: The laminar air-flow unit for implantation procedures.

Figure 5: The isolator joined with the food autoclave unit by a transfer tunnel.

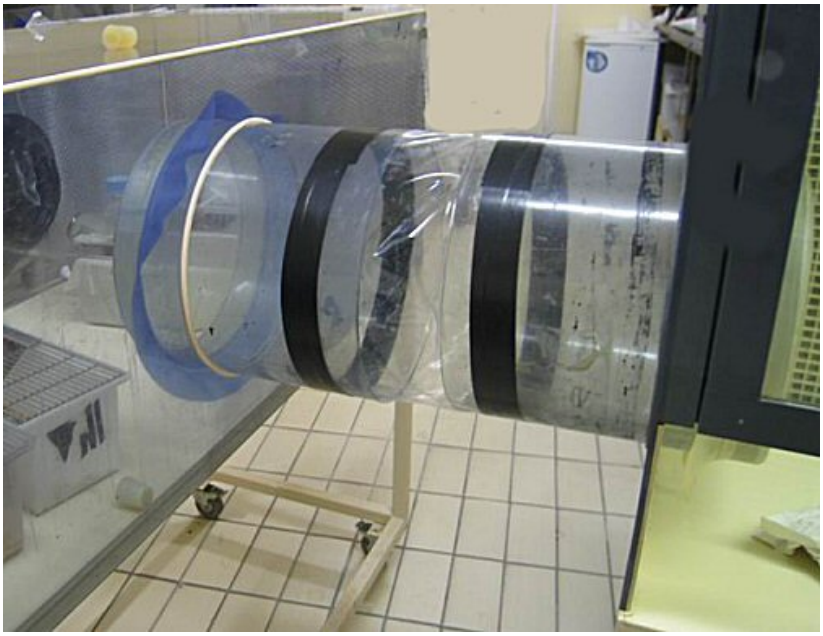


Figure 6: The isolator joined with the laminar air-flow unit.

3.3.2 Animal care

Before the implantation, the mice were accommodated in separate cages in batches of three on a bedding of autoclaved wood shavings and fed ad-lib with sterilised surgery water and autoclaved rodent breeder food. The autoclaving of the bedding and food was done in the Microbiology laboratory of the Department of Medical Bioscience.

After the implantation procedure, each animal was separately accommodated on a bedding of autoclaved wood shavings and fed ad-lib with normal sterilised surgery water or Terramycin/vitamin cocktail (see Appendix II) and autoclaved rodent breeder food for 9 weeks. The water and the cocktail supplies were refreshed every 2 days as to avoid contamination with fungi and protozoa.

The animals' health condition was closely observed and a short note was recorded every day. (See Appendix III)

The floor and the walls of the animal house were washed at regular weekly intervals with Biocide® (3g/l); the isolators were sterilised daily and the working surfaces and instruments were sterilised at all times with 1% potassium peroxsulphate solution (Virkon® 1g/l).

The temperature of the animal room was kept at 26 degrees Celsius and the light cycle was set for alternating 12 hours of light and 12 hours of darkness.

3.4 Antibiotic cover of the experimental mice

Immediately after implantation, all mice were administered with 2.000 units of Penicillin-G intramuscular. The mice were randomly allocated to three experimental groups and one control group as described below: ultimately there were 10 animals in each group.

Groups 1 and 2 were given an antibiotic (Terramycin)/vitamin cocktail in drinking water.
Groups 3 and the Control Group were given normal sterilised water.
Group 1 and 3 were administered subcutaneously with Gentamicin (1mg/100 bodyweight) twice daily for a postoperative period of 14 days.

Table 1: Experimental groups

Group 1: 10 mice Antibiotic (Terramycin)/vitamin cocktail. Gentamicin (I.M. b.d.) x14 days	Group 2: 10 mice Antibiotic (Terramycin)/vitamin cocktail
Group 3: 10 mice Gentamicin (I.M. b.d.) x14 days	Control Group: 10 mice No postoperative antibiotics except 1 penicillin injection

The body weight was as an important parameter of the health condition of all the experimental animals. Every animal was weighed before the operation, after 14 days of injections in case of Gentamicin injections (Groups 1 and 3), and at harvest.

3.5 Management of the vaginal mucosa specimens

Thirteen fresh human vaginal mucosal specimens were obtained from excess tissue removed during vaginal hysterectomies and corrective procedures in postmenopausal women at the theatres of the Department of Gynaecological Oncology, Tygerberg Hospital. The patients were briefly informed about the possibility of passively participating in this study by donating excess vaginal tissues resulting from the hysterectomy procedure for implantation into the nude mice. In particular it was made clear to the patient that no extra tissue would be harvested other than that is routinely included in hysterectomy specimens. All published data will remain anonymous (see Appendix IV: patient consent form).

The harvested vaginal tissues were clinically and macroscopically verified for any diseases before being prepared as xenografts. The specimens were immersed in Transport Medium (TM -see Appendix V), and transported to our laboratory (Fig 7). Each specimen was divided into 2 portions, one (A) to construct the experimental cyst and the other (B) for control by histological examination (Fig 8).

Specimen A was trimmed to pieces of approximately 18x8 mm and, by using a scalpel, the excess supporting connective tissue was separated as carefully as possible from the epithelium in order to preserve the integrity of the latter (Fig 9A, B). The specimen (consisting mainly of epithelium) was then placed in a sterile solution of 3mM disodium ethylenediaminetetraacetic acid (EDTA) in Dulbecco's calcium and magnesium-free phosphate saline (PBS) (see Appendix VI) and maintained for 2 hours in a shaker bath at 37°C in order to

achieve complete separation of the epithelial and connective tissue components (Fig 9C).

Specimen B (control portion) was fixed in 10% saline-buffered formalin and routinely processed for embedding, cutting, staining and histological examination by the NHLS histopathology laboratories at Tygerberg Hospital. The sections was evaluated for pathological abnormalities and kept for comparison purposes (see Appendix VII: Evaluation form of donated vaginal tissue).

The containers for TM (Fig7) and For EDTA (Fig9C) were sterilised by 1%Virkon before using.

Figure 7: Donated vaginal mucosa, immersed in transport medium.

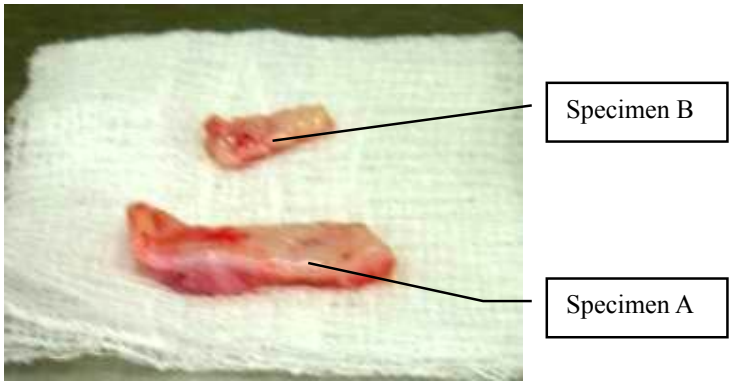
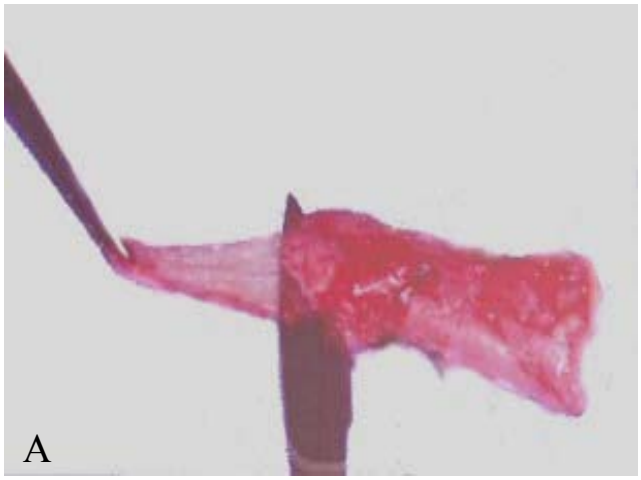
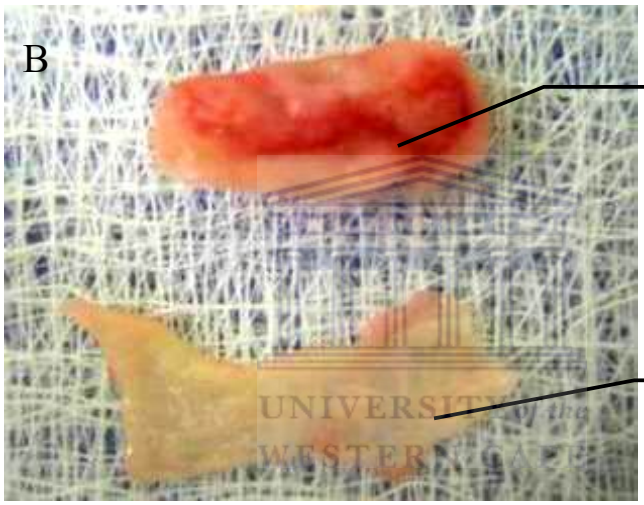


Figure 8: Two portions of donated vaginal mucosa





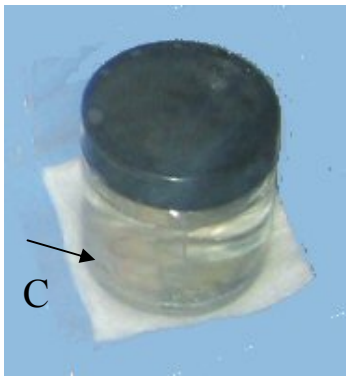


Figure 9A: Separating connective tissue from epithelium by using the scalpel

Figure 9B: Completed separation of connective tissue from epithelium.

Figure 9C: Epithelium was immersed in 3mM EDTA for 2 hours.

3.6 Artificial cyst creation and implantation

3.6.1 The construction of the artificial cysts (Fig 10)

The construction of the artificial cysts was accomplished at the sterilized laminar air-flow unit. Pre-cut 5mm segments of silicone tube of 5mm in diameter were gas-sterilised with ethylene oxide in glass containers enclosed in sealed envelopes at the Oral Health Center (Tygerberg) of the University of the Western Cape. These tube segments were placed on the surface of the epithelial specimens that were then wrapped around it and tied with a silk or nylon suture (5-0) tied so that the silicone tube (5mm) segment became enclosed in the artificial cyst. The artificial cysts were then placed in fresh transport medium and kept ready for implantation.

3.6.2 The procedures of the implantation (Fig 11)

Forty-three specimens were implanted during the study. The athymic mice (MFI nu nu ex University of the Witwatersrand) were transferred frome the isolator to the laminar air-flow unit where the operations were carried out. The mice were anaesthetised with a mixture of 10% dilution of Ketamine [25mg/kg] and Medetomidine [0.5mg/kg] in a single intraperitoneal injection (see Appendix VIII). The neck region of the mice, where the cysts were to be implanted, was cleaned/disinfected with Povidone-iodine solution before an incision of 10-15mm was made. A subcutaneous pocket was prepared under the skin by blunt dissection. The artificial cysts were carefully placed into the prepared pockets and the wound was sutured with 5-0 silk or nylon. The mice were administered intramuscularly once with Penicillin-G (100 units/g) immediately after the surgery.

Figure 10 A): The instruments of the implantation

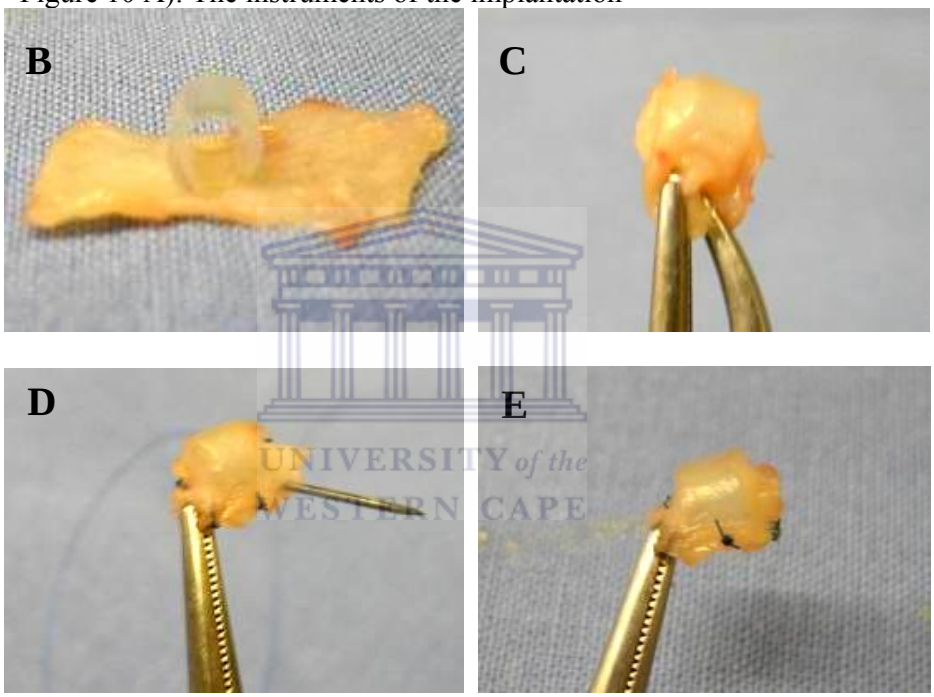


Figure 10 B): The silicone tube plated on the epithelium surface;
10 C-E): The construction of the artificial cyst

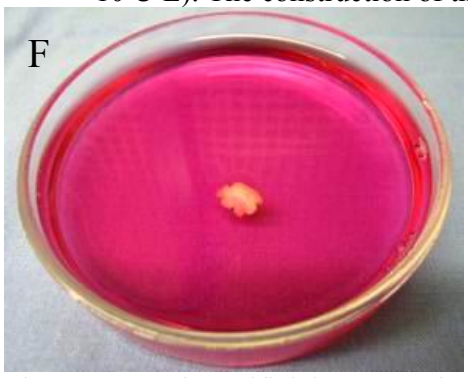


Figure 10 F) The artificial cyst was immersed into TM;

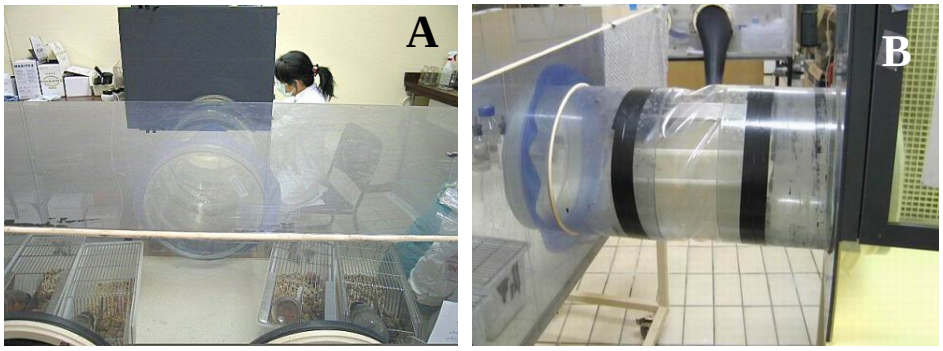


Figure 11 A, B: Transfer mice from the isolator to the laminar flow unit.

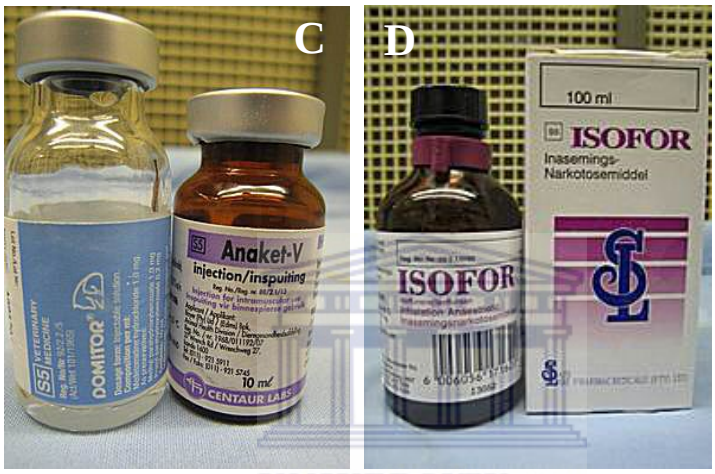


Figure 11 C, D: The applied anaesthetics in this study: Anaket-V® [ketamine 100mg/ml], Domitor® [medetomidine 1mg/ml], ISOFOR® [Isofluurane].

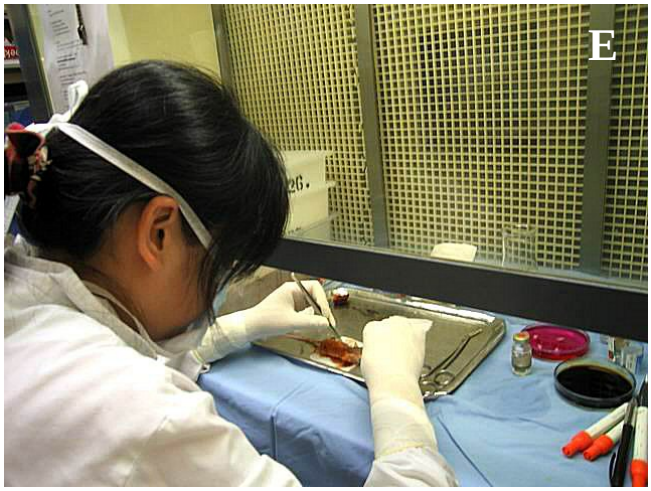


Figure 11 E: All operations were carried out in the laminar flow unit.

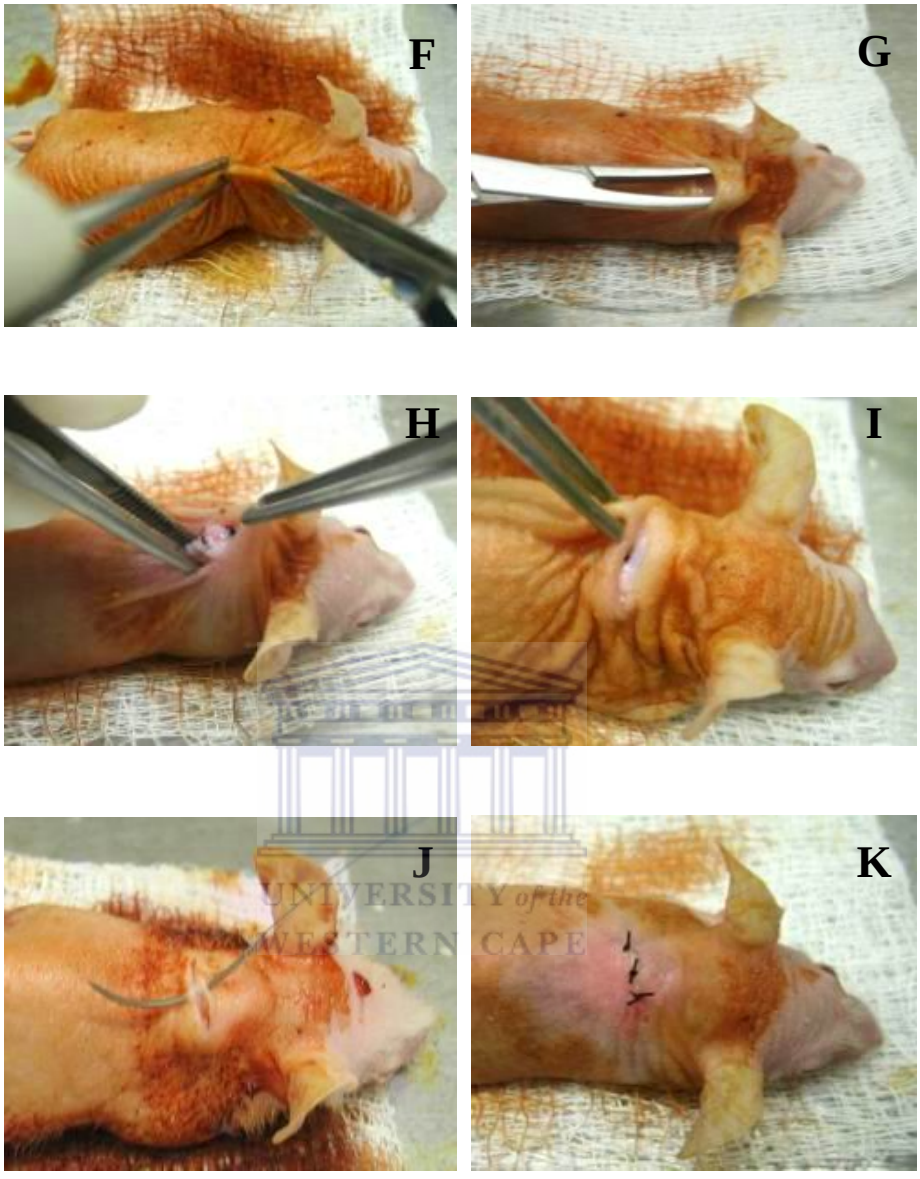


Figure 11 F-K: The implantation procedure.

3.7 Injection of Gentamicin

The 20 mice of Groups 1 and 3 were administered subcutaneously with Gentamicin (1mg/100 bodyweight) twice daily for a postoperative period of 14 days. The injections were performed inside the isolator. To prevent suffering, the animals were briefly anaesthetised with Isoflurane vapours (0.02% density -Fig 12A) before Gentamicin was administered.

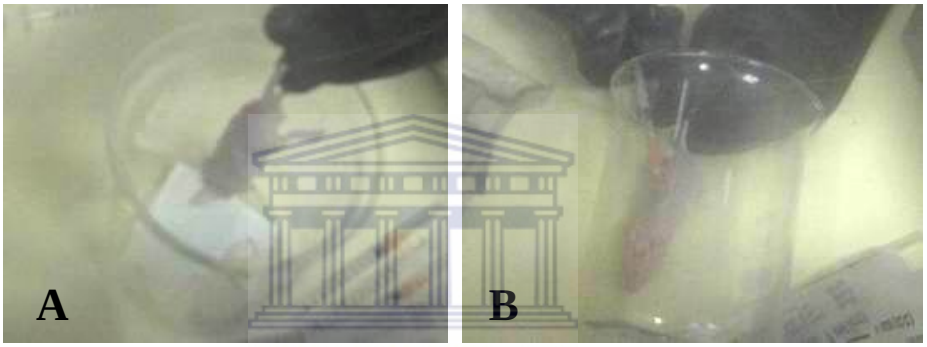


Figure 12: Photo showing gentamicin injection under anaesthesia.

A: Prior to the injection the animals were anaesthetized with 0.2 ml Isoflurane vapourised in a 1000ml beaker inside the isolator.

B: The Gentamicin was administered subcutaneously in the back (twice daily).

3.8 Microbiological testing

3.8.1 Microbiological testing of the animals

Microbiological samples of each experimental site were obtained by swabbing the following sites:

1) The skin of the neck prior to cleaning with Povidone-Iodine disinfectant and the implantation procedure.
2) The skin around the wound at sacrifice after the operation.
3) The inside of the cyst at donor site after harvesting.

3.8.2 Microbiological testing of the operation procedure

In order to assess the sterile conditions of the cyst preparation and implantation procedures, microbiology samples were obtained during the critical phases including the swabbing of the human vaginal tissue before separation, the epithelium after EDTA treatment, the artificial cysts before implantation, the wound and skin of the donor site, and the instruments used.

3.8.3 Microbiological testing of the investigators' nasal passages

The only one opportunity for animals to be in contact with humans was during the implantation operation. The investigators' nasal passages were swabbed to determine their staphylococcus carrier state.

3.8.4 Microbiological testing of the air of the animal room and isolators

Because of logistical problems with the animal housing facilities at Tygerberg campus, the study could only be carried out in a substandard animal room in the Department of Medical Bioscience on the campus of the University of the Western Cape. There were many examination and test papers stored at one side of the room (Fig 13). The ability of the isolators to keep strict germfree conditions was tested by taking microbiology samples of the air outside and

inside the isolator by placing culture medium dishes in the animal room and inside the isolators for 2 hours.

Fig 13: The substandard animal room. Stacks of examination papers and notes were stored at the corner of the room (arrow).

3.8.5 Microbiological testing of the sterilized rodent food and bedding

The sterilized food and bedding were transferred into the isolators usually 5 hours after autoclaving, allowing for 3 hours of cooling down and 2 hours of sterilization of the port between the autoclave and the isolator. The efficiency of the food autoclave unit was estimated at the beginning of the experiments by submitting small pieces of food and bedding taken 5 hours after autoclaving for microbiological examination.

All the samples were sent to the NHLS microbiology laboratories at Tygerberg for microscopical evaluation, culturing, subculturing (*Staphylococcus* and *Streptococcus* spp.) and further antibiotic sensitivity testing if bacterial growth had been identified.

3.9 Histological evalution

The tissue specimens from the all experimental groups and the control group were assessed for the success or failure of the xenograft procedure by recording/measuring the:

1. Vitality of the xenografts (absence or presence of necrosis),
2. Presence or absence of cyst formation by assessing the potency of the lumen and comparing the epithelial thickness and structure between the xenografted cysts and the original vaginal mucosa control tissues,
3. Presence or absence of fibrosis in the cyst wall and the adjacent donor site,
4. Establishment of new blood supply to the implanted connective tissue which has a bearing on the nutritional capacity of the cyst model and the correct functioning of the epithelium,
5. Presence or absence of infection and /or inflammation under the basal membrane of the cyst wall and in the cyst lumen. This was correlated to the routine microbiological information pertaining to each experimental animal. Gram stains were applied to the tissue sections when there has been clinical or histological evidence of infection.

The histological observation and comparison of the control vaginal mucosa with the cyst lining were determined by 2 observers using code-labelled slides. The procedure was performed in duplicate (see Appendix IX: Evaluation form of cyst wall).

3.10 The endpoint of the animal model

After 9 weeks of growth, when the healing around the cysts was complete, the mice were euthanatized by an overdose of Isoflurane (0.12% density). At harvest, the cysts were excised with overlying skin and surrounding soft tissues, the cysts were opened and the lumen swabbed for microbiological testing. Thereafter the tissues were fixed in 10% formalin for histological processing, sectioning and staining by the NHLS histopathology laboratories at Tygerberg hospital.

As already mentioned above, the endpoint of the experiments was reached after the 9 weeks post-implantation period. It was anticipated that a number of the artificial mucosal cysts, which were implanted in the neck region of the athymic nude mice, would become infected, especially in the control group and perhaps (but hopefully fewer) in the experimental groups. The animals were continuously and carefully assessed for their general health status; in particular they were monitored for 1) acute and chronic infection, 2) consistent or rapid body weight loss of 20% maintained for 72 hours, 3) signs of chronic pain and physiological stress: persistent anorexia, inability to move, unconsciousness, comatose or failure to respond to gentle stimuli. When any of the above signs had clinically crossed the humanely acceptable thresholds in a particular (single or group of) experimental animals before the 9 week post-operative healing period was over, the nude mice were fully anaesthetised (see above description) and killed by an overdose of Isoflurane. This was followed by post-mortem examinations and histological/ microbiological analyses of the (failed or diseased) implant. We anticipated a 25% loss of the experimental animals due to various causes; all lost animals were subjected to a post-mortem examination with histopathology.

3.11 Abbreviated research ethics statement

The laboratory animals were as humanely as possible taken care of during the experiments. The animals live, grow, reproduce and interact under conditions and circumstances in which their species' specific needs are met. Experimental procedures, which cause injury, disease, discomfort, fear, or pain, will keep exposure of animals to these conditions to a minimum. The care of experimental animals was under the direct control and supervision of a qualified laboratory animal technician. High standards were maintained in the daily care of experimental animals. The experimental animals were kept in optimal conditions at all times including good housing, correct environmental conditions with acceptable space for movement and provision for the gratification of their physical and etiological requirements. The professional animal technician was responsible for the pre- and post-operation care. All investigators in this study had experience with using animals in medical or dental research. At the end of the study, the animals were euthanised with Isoflurane (0.12% density in one 250ml flask).

Ethics clearances of the study were obtained from both the University of the Western Cape and University of Stellenbosch.

Project registration numbers: N05/04/061 (UWC) and P05/04/003 (US)

CHAPTER 4: RESULTS

4.1 Animal housekeeping and observations

Fifty-three athymic nude mice were transported to the laboratory at UWC and housed in the isolators. In general the animals survived the experiments quite well and no major problems were encountered in housekeeping. Aggression (climbing onto the cage cover and jumping), repetitive roaming behaviour, nervousness and handling difficulties were observed in some of the mice. Most of mice made their nest in the shadow of the food hopper and/or drinking bottle. The implantation sites at the neck region of some mice could be scratched with their rear paws or scraped by the steel food hopper.

Ten animals were lost before the implantation due to transport-associated problems and anaesthetic issues. Three mice with implants in the control group were euthanised after 3 weeks as severe inflammation around the opened wounds, persistent anorexia, and inability to move or impairment was observed. Another 3 implanted mice (two in Group 2 and one in the control group) were lost because of lung injury by peracetic acid respectively at 5 days, 22 days and 43 days (see table 2).

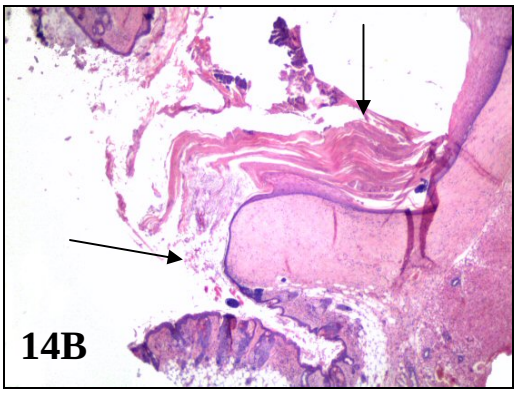
Of the other 37 mice with implants harvested at 9 weeks, 14 implants were re-established as intact cyst formation. There was no distinct difference between Terramycin/vitamin cocktail group and the control group. Meanwhile, 64.3% cases with intact cyst formation were administered with Gentamicin after the implantation. The histological structure of the harvested cysts will be described in detail in §4.3.

Eight implants resulted in partial cyst formation with partial necrosis or ingrowth of murine epidermis into the cyst cavity (Figs 14 A-C). Eight implants were associated with poor wound healing resulting in scabbed artificial cysts or full loss of the implanted cysts (Fig 15).

Table 2: Evaluation of the animals and implants

	Harvest at 9 weeks					Before harvest		Total
	Intact cyst formation	Partial Cyst formation	Atrophic cyst	Necrotic cyst	Poor wound healing	Eutha-nised	Lost	
Group 1 (Terramycin+Gentamicin)	5	1	1	1	2			10
Group 2 (Terramycin)	2	4			4		2	12
Group 3 (Gentamicin)	4	2	1	1	2			10
Control Group	3	1	1	2		3	1	11
Total	14	8	3	4	8	3	3	43
Lost before implantation								10

Fig.14A: The implanted site with ingrowth of the epidermis and nodular scar tissue around the wound.



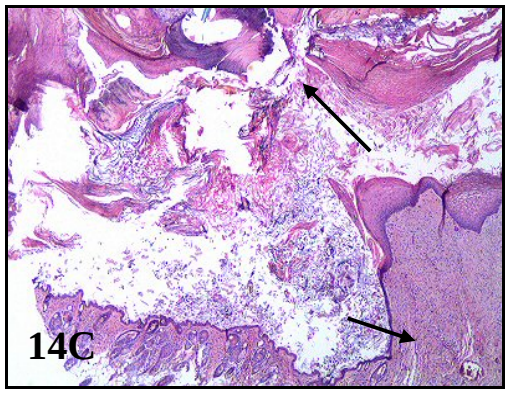
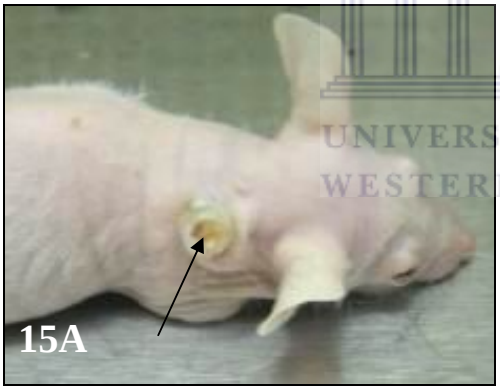


Fig.14B: Keratinized stratified squamous surface epithelium ingrowth into cyst cavity and lining scar tissue (arrows) (H&E, magnification x20).

Fig.14C: Keratinized stratified squamous surface epithelium of murine epidermal origin lining the cyst cavity and scar tissue (arrow) (H&E, magnification x20).



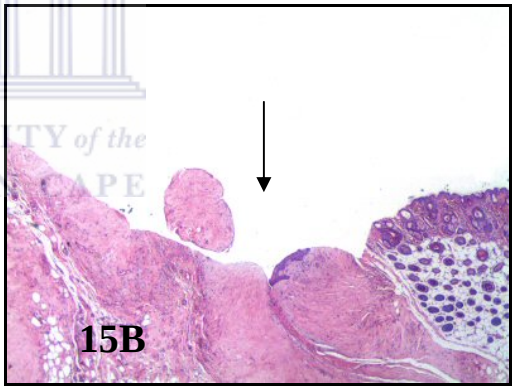


Figure 15A: Cyst necrosis and extrusion/sequestration of silastic tube (arrow) due to poor wound healing.

Figure 15B: No implanted cyst was present at the donor site (arrow) (H&E, magnification x 40).

Table 3: The outcome details of all of the implants.

Group	Vaginal Tissue No	Implants No	Batch	Suture*	Result**
1 (T&G)	T240806	A24080602	6	S	Necrotic
		A24080604	6	S	CF
	T250806	A25080601	7	S	Opened
		A25080603	7	S	Opened
		A25080605	7	S	CF
	T070906	A07090602	8	S	AC
		A07090604	8	S	CF
	T280906	A28090602	9	N	CF
		A28090604	9	N	CF
		A28090606	9	N	IG&PCF
2 (T)	T050506	A05050601	1	N	CF
	T180506	A18050603	3	N	CF
		A18050601	1	N	Opened
	T260506	A26050601	3	N	PCF
	T150606	A15060601	4	N	Lost
	T230606	A23060601	4	N	IG&PCF
	T060706	A06070601	4	S	Opened
		A06070603	5	S	IG&PCF
	T280706	A28070601	5	S	IG&PCF
	T030806	A03080601	5	N	Opened
	T110806	A11080601	6	S	Lost
	T240806	A24080606	7	S	Opened
3 (G)	T240806	A24080601	6	S	Opened
		A24080603	6	S	Necrotic
		A24080605	6	S	Opened
	T250806	A25080602	7	S	CF
		A25080604	7	S	IG&PCF
	T070906	A07090601	8	S	CF
		A07090603	8	S	CF
		A07090605	8	S	AC
	T280906	A28090601	9	N	IG&PCF
		A28090603	9	N	CF
CONTROL	T050506	A05050602	1	N	CF
	T180506	A18050605	3	N	PCF
		A18050604	1	N	Euthanised
	T250506	A25050601	3	N	Necrotic
	T260506	A26050602	3	N	CF
	T150606	A15060602	4	N	AC
	T230606	A23060602	4	N	Necrotic
	T060706	A06070602	5	S	Euthanised
		A06070604	5	S	Euthanised
	T030806	A03080602	5	N	Lost
	T280906	A28090605	9	N	CF

*Suture used: silk (S) and nylon (N)

**AC=atrophic cyst; ICF=intact cyst formation; PCF=partial cyst formation; IG=ingrowth of mouse epidermis; Necrotic= cyst epithelium necrosis; Opened = poor wound healing; Euthanised =mice were euthanised because of the severe inflammation with poor wound healing before 9 weeks; Lost=implanted mice died before the wounds healed completely.

4.2 Microbiological testing

4.2.1 The results of the skin swab before operation (Sample A)

The microbiological testing of animal skins before the implantation was undertaken in 43 mice. The presented rate of contaminants was 72.1% (31 mice). Organisms were isolated from 31 unoperated mice in 45 instances.

Table 4: Detail of the identification and antibiotic sensitivity testing of sample A

Group	Animal No	Batch	Identified contaminants	Penicillin	Gentamicin	Tetracycline	Vancomycin
1 (T&G)	A24080602	6	-				
	A24080604	6	-				
	A25080601	7	*Klebsiella pneumoniae*	S	S		
	A25080603	7	*Enterococcus* species	S			R
	A25080605	7	*CNS mixed*	S	S	R	S
	A07090602	8	*CNS mixed*	S	S	R	S
	A07090604	8	*CNS mixed*	S	S	R	S
	A28090602	9	*Klebsiella pneumoniae*	S	S		
	A28090604	9	*Enterococcus* species	S	S	S	R
	A28090606	9	*Enterococcus* species	S	S	S	R
			Klebsiella pneumoniae	R	S		
2 (T)	A05050601	1	-				
	A18050603	3	-				
	A18050601	1	-				
	A26050601	3	*Enterobacter cloacae*	R	S		
	A15060601	4	*Klebsiella pneumoniae*	R	S		
			Pseudomonas aeruginosa		S		
	A23060601	4	*Klebsiella oxytoca*	R	S		
			Pseudomonas aeruginosa		S		
	A06070601	4	*Klebsiella pneumoniae*	R	S		
			Pseudomonas aeruginosa		S		
	A06070603	5	*Staphylococcus sciuri*	S	S	R	S
	A28070601	5	*Staphylococcus sciuri*	R	S	R	S
	A03080601	5	*Staphylococcus aureus*	S	S	S	S
			Klebsiella pneumoniae	R	S		
			Pseudomonas aeruginosa		S		
	A11080601	6	*Enterobacter cloacae*	S			R
	A24080606	7	-				

Group	Animal No	Batch	Identified contaminants	Penicillin	Gentamicin	Tetracycline	Vancomycin
	A24080601	6	-				
	A24080603	6	*Proteus mirabilis*	S	S		
			Pseudomonas aeruginosa		S		
	A24080605	6	*Klebsiella pneumoniae*	R	S		
	A25080602	7	*Staphylococcus aureus*	S	S	S	S
	A25080604	7	-				
3	A07090601	8	-				
(G)	A07090603	8	*Pseudomonas aeruginosa*		S		
			β-haemolytic Strep grp A	S			S
	A07090605	8	*β-haemolytic Strep grp A*	S			S
			CNS mixed	S	S	R	S
	A28090601	9	*Staphylococcus aureus*	S	S	S	S
			β-haemolytic Strep grp A	S			S
	A28090603	9	*Klebsiella pneumoniae*	R	S		
	A05050602	1	*Enterococcus* species	S			
	A18050605	3	-				
	A18050604	1	-				
	A25050601	3	-				
	A26050602	3	*Staphylococcus aureus*	S	S	S	S
	A15060602	4	*Klebiella* species	R	S		
			Enterobacter cloacae	R	S		
C	A23060602	4	*Klebiella pneumoniae*	R	S		
			Pseudomonas aeruginosa		S		
	A06070602	5	*Klebiella pneumoniae*	R	S		
			Pseudomonas aeruginosa		S		
	A06070604	5	*Klebiella pneumoniae*	R	S		
			Pseudomonas aeruginosa		S		
	A03080602	5	*Staphylococcus gallinarum*				S
	A28090605	9	*Enterobacter cloacae*	R	S		

*s=susceptible; r=resistant.

Table 5: Summary table of the result of Sample A as identified per batch

Batch			Batch 1	Batch 2*	Batch 3	Batch 4	Batch 5	Batch 6	Batch 7	Batch 8	Batch 9	Total
Date of arriving			25/4	2/5	9/5	23/5	6/6	13/6	8/8	29/8	12/9	
ORGANISMS	No contaminants		3		3			3	2	1		12
	Identified contaminants	ENTCL			1	1		1			1	4
		ENTFE	1						1		2	4
		KLEOX				1						1
		KLEPN				4	3	1	1			9
		PSEAE				4	3	1		1	3	12
		STAAU			1		1		1		1	4
		STASC					2		1	3		6
		STAGA					1					1
		PROMI						1				1
		BHSA								2	1	3
		Total**	1		2	10	10	4	4	6	8	45
MICE	Implanted in Batch**		4		5	5	6	6	6	5	6	43
	Lost before microbiological testing		2	6		1				1		10

*All animals in Batch 2 died of low temperature during the airfreight on the second day after arrival.

**Unequal between total number of contaminants and the implants.

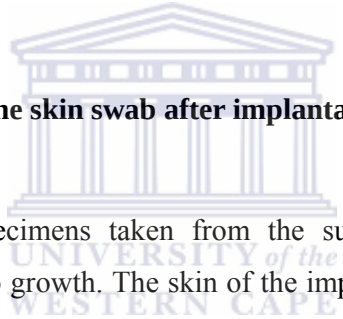
Abbreviations f Contaminants

ENTCL: *Enterobacter cloacae*
ENTFE: *Enterococcus faecalis*
KLEOX: *Klebsiella oxytoca*
KLEPN: *Klebsiella pneumoniae*
PSEAE: *Pseudomonas aeruginosa*
STAAU: *Staphylococcus aureus*
STASC: *Staphylococcus sciuri*
STAGA: *Staphylococcus gallinarum*
STAXY: *Staphylococcus xylosus*
PROMI: *Proteus mirabilis*
BHSA: *β-haemolytic streptococcus group A*

The contaminants were mainly normal commensals of healthy experimental animals: *Pseudomonas aeruginosa* (25.5%) was the most frequently isolated, followed by *Klebsiella pneumoniae* (20%), *Enterobacter cloacae* (8.9%), *Enterococcus faecalis* (8.9%), *Klebsiella oxytoca* (2.2%) and *Proteus mirabilis* (2.2%). *Staphylococus aureus and Streptococci* were only found in 3 mice (6.7%). Coagulase negative staphylococcus was the most commonly isolated pathogenic

organism (17.8%). High levels of *Klebsiella pneumoniae* were found in batches 4 and 5; *Pseudomonas aeruginosa* was mainly found in batches 4, 5 and 9, and *β-haemolytic streptococcus group A* was only collected from batches 8 and 9.

The antibiotic sensitivity test for these identified contaminants in Table 4 show that all kinds of contaminants, except for *Klebsiella* species, *Enterobacter cloacae,* and *Pseudomonas* species, were susceptible to Penicillin; only *Staphylococcus aureus* was susceptible to Terramycin (5% oxytetracycline); and all bacteria, except for *β-haemolytic streptococci group A,* were susceptible to Gentamicin. The latter were also found to be sensitive to Penicillin and Vancomycin. All *Staphylococci* species were sensitive to Vancomycin.



4.2.2 The results of the skin swab after implantation (Sample B)

The reports of all specimens taken from the sutured wound area after the implantation showed no growth. The skin of the implantation site was disinfected with Povidone-Iodine solution prior to incision.

4.2.3 The results of the cyst swab (Sample C)

The contents of the cysts of three mice were not swabbed because they had died from Peracetic acid poisoning before cyst growth and surgical wound healing took place.

Table 6: Detail of the identification and antibiotic sensitivity testing of sample C

Group	Cyst No	Result	Identified contaminants	Penicillin	Gentamicin	Tetracycline	Vancomycin
1	A24080602	Necrotic	*Staphylococcus sciuri*	S	S	R	S
	A24080604	CF	-				
	A25080601	Opened	*CNS mixed*	S	S		S
	A25080603	Opened	*Staphylococcus xylosus*	S	S	R	S
	A25080605	CF	*CNS mixed*	S	S		S
	A07090602	Necrotic	*Staphylococcus sciuri*	R	S	R	S
	A07090604	CF	-				
	A28090602	CF	-				
	A28090604	CF	-				
	A28090606	IG&CF	*Enterococcus faecium*	S			S
2	A05050601	CF	*Staphylococcus sciuri*	S	S	R	S
	A18050603	CF	*Staphylococcus xylosus*	R	S	R	S
	A18050601	Opened	*Staphylococcus xylosus*	R	S	R	S
	A26050601	CF	*Staphylococcus gallinarum*				
	A15060601	Lost	No collection				
	A23060601	IG&CF	*CNS mixed*	S	S		S
	A06070601	Opened	*CNS mixed*	S	S		S
	A06070603	IG&CF	*CNS mixed*	S	S		S
	A28070601	IG&CF	*Enterococcus faecalis*	S	S		
	A03080601	Opened	*Pseudomonas aeruginosa*		S		
	A11080601	Lost	No collection				
	A24080606	Opened	*Pseudomonas aeruginosa*		S		

Group	Cyst No	Result	Identified contaminants	Penicillin	Gentamicin	Tetracycline	Vancomycin
3	A24080601	Opened	*Staphylococcus aureus*	S	S		S
	A24080603	Necrotic	*CNS mixed*	S	S		S
	A24080605	Opened	*Staphylococcus gallinarum*	S	S		S
	A25080602	CF	-				
	A25080604	IG&CF	*CNS mixed*	S	S		S
	A07090601	CF	-				
	A07090603	CF	-				
	A07090605	Necrotic	*Staphylococcus gallinarum*	S	S		S
	A28090601	IG&CF	*Entercoccus faecalis*	S			S
	A28090603	CF	-				
C	A05050602	CF	*Staphylococcus aureus*	S	S	S	S
			Enterococcus faecalis	S			S
	A18050605	CF	-				
	A18050604	Euthasia	*Enterococcus faecalis*	S	R	S	
	A25050601	Necrotic	*CNS mixed*	S	S	S	S
	A26050602	CF	*Staphylococcus aureus*	S	S	S	S
	A15060602	CF	-				
	A23060602	Necrotic	*CNS mixed*	S	S	S	S
	A06070602	Euthasia	*Pseudomonas aeruginosa*		S		
	A06070604	Euthasia	*Enterobacter cloacae*	R	S		
	A03080602	Lost	No collection				
	A28090605	CF	*Enterococcus faecalis*	S			S

*R=resistant; s=susceptible.

Table 7: Summary table of the results of Sample C

Group		Group1 (T&G)	Group2 (T)	Group3 (G)	Control Group	Total
No contaminants		4		4	2	10
Identified contaminants	ENTFE	1	1	1	4	7
	PSEAE		2		1	3
	STAAU			1	2	3
	CNS mixed	2	3	2	2	9
	STASC	2	1			3
	STAXY	1	2			3
	STAGA		1	2		3
	Total*	6	10	6	9	31
Animal number *		10	10	10	10	40

*Unequal between total number of contaminants and the implants.

Abbreviations of Contaminants:

ENTFE: *Enterococcus faecalis*,
PSEAE: *Pseudomonas aeruginosa*,
STAAU: *Staphylococcus aureus*,
CNS: *Coagulase negative Staphylococcus*,
STASC: *Staphylococcus sciuri,*
STAGA: *Staphylococcus gallinarum,*
STAXY: *Staphylococcus xylosus*

Staphylococcus aureus, Coagulase negative staphylococcus spp *(S. sciuri. S. gallinarum, S. xylosus), Enterococcus faecalis,* and *Pseudomonas aeruginosa* were isolated at implanted sites where they induced wound infections. Only ten implant cases had no contamination inside the implanted cysts. There were 4 implants without contaminants in both Group1 and Group 3, which were both administered with Gentamicin injections. Twenty-one implants harboured *Staphylococcus* species and 18 of them contained coagulase-negative species. C*oagulase negative staphylococcus* species were isolated from groups 1 and 2, which were given Terramycin/vitamin cocktail, and were resistant to Terramycin. *Pseudomonas aeruginosa* was only isolated from opened wound sites. *Enterococcus faecalis* species were collected both from implants with

epidermis ingrowth and inside well healing wounds. *Klebsiella* species and β-haemolytic *strepoccci* group A were not detectable at the implanted sites after 9 weeks (See Table 6 for details).

4.2.4 Microbiological testing of the operation procedure and investigators' nasal passages

All samples taken from the constructed cysts, transport medium, instruments and worksurface during the operative procedures showed no growth. No *Staphylococci aureus* growth was isolated from the investigators' nasal passages.

4.2.5 Microbiological testing of the animal room and isolators

Two colonies of bacteria were grown on plate exposed to the ambient air of the animal room. The subculture and identification of the colonies were unfortunately not reported by the Microbiology laboratory. All reports on samples taken from the air inside the 3 isolators showed no growth.

4.2.6 Microbiological testing of the food and bedding

The reports on samples taken from the food and bedding also showed no bacterial growth.

4.3 The change of animals' body weight

The body weight was an important parameter for evaluating the health of all the experimental animals. Every animal was weighed before the operation, after 14 days of injections in case of Gentamicin injections (Groups 1 and 3), and at harvest.

Table 8: Weight change of Control Group

Implants Number	Weight at implant (g)	Weight at harvest (g)	Change (g)	Percent
A05050602	20	28	+8.0	+40.00%
A18050605	23	31.2	+8.2	+35.65%
A18050604*	18	23	+5.0	+27.78%
A25050601	28	36.4	+8.4	+30.00%
A26050602	24	33.7	+9.7	+40.42%
A15060602	27	30	+3.0	+11.11%
A23060602	27	27	0	0
A06070602**	29			
A06070604**	28			
A03080602***	22			
A28090605	28	33.1	+5.1	+18.21%
MEAN	24.9	30.3	+5.9	+23.79%
SD			3.3	

*Serious inflammation around the opened wound. Mouse was killed at 3 weeks.

**Serious inflammation around the opened wound. Mice killed at 3 weeks. Body weight lost respectively 1.3g and 0.5g.

***Dead of the lung injury by 4% Peracetic Acid at 22 days. Body weight increased 2g.

Table 9: Weight change of Group 2 (Terramycin/vitamin water)

Implants Number	Weight at implant (g)	Weight at harvest (g)	Change (g)	Percent
A05050601	21	29	+8.0	+38.10%
A18050601	23	32.4	+9.4	+40.87%
A18050603	25	34.6	+9.6	+38.40%
A26050601	17	27.1	+10.1	+59.41%
A15060601*	25			
A23060601	26	28	+2.0	+7.69%
A06070601	29	32	+3.0	+10.34%
A06070603	27	31.5	+4.5	+16.67%
A28070601	24	25	+1.0	+4.17%
A03080601	26	26.7	+0.7	+2.69%
A11080601*	28			
A24080606	24	28	+4.0	+16.67%
MEAN	24.2	29.4	+5.2	+21.61%
SD			3.7	

* Dead of the lung injury by 4% Peracetic Acid respectively at 43 days and 5 days after the operation. Body weight lost respectively 1.6g and 0.8g.

The mean increase of the body weight in the Terramycin/vitamin drink group (Group 2) was 5.2 grams, which is not significantly different to the 5.9 grams in the control group without any antibiotic coverage during the postoperative period.

Table 10: Weight change of Group 3 (Gentamicin)

Implants number	Weight at implant (g)	Weight after 14 days (g)	Change		Weight at harvest (g)	Change*	
			g	Percent		g	Percent
A24080601	30	28	-2.0	-6.67%	30.3	+0.3	+1.00%
A24080603	26	24	-2.0	-7.69%	25.3	-0.7	-2.69%
A24080605	22	21	-1.0	-4.55%	23	+1	+4.55%
A25080602	21	21	0	0	24	+3	+14.29%
A25080604	25	25	0	0	26	+1	+4.00%
A07090601	20	20	0	0	23	+3	+15.00%
A07090603	19	21	+2.0	+10.53%	28	+9	+47.37%
A07090605	16	19	+3.0	+18.75%	22	+6	+37.50%
A28090601	24	26	+2.0	+8.33%	30.8	+6.8	+28.33%
A28090603	26	28	+2.0	+7.69%	29.8	+3.8	+14.62%
MEAN	22.9		+0.4	+1.75%	26.2	+3.3	+14.50%
SD			1.8			3.1	

*The change is between the weight at harvest and the weight at implant time.

Table 11: Weight change of Group 1 (both antibiotics)

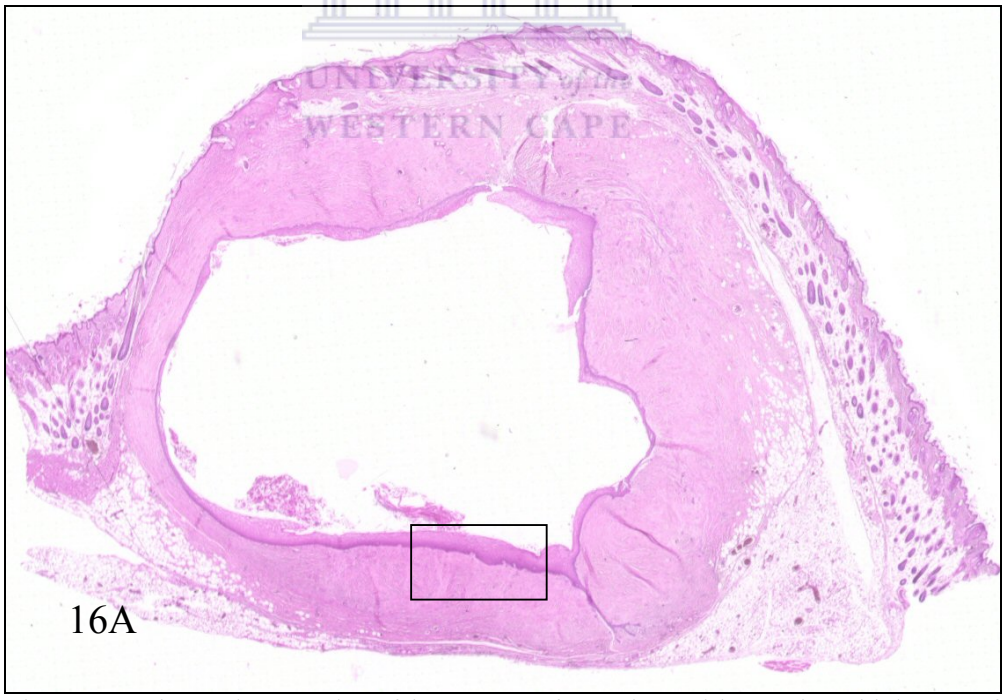
Implants number	Weight at implant(g)	Weight after 14 days (g)	Change		Weight at harvest (g)	Change*	
			g	Percent		g	Percent
A24080602	31	30	-1.0	-3.23%	32.5	+1.5	+4.84%
A24080604	21	21	0	0	20.4	-0.6	-2.86%
A25080601	24	23	-1.0	-4.17%	26.5	+2.5	+10.42%
A25080603	21	22	1.0	4.76%	24.4	+3.4	+16.19%
A25080605	25	25	0	0	28.4	+3.4	+13.60%
A07090602	18	20	2.0	11.11%	22	+4	+22.22%
A07090604	16	16	0	0	22	+6	+37.50%
A28090602	27	28	1.0	3.70%	32.5	+5.5	+20.37%
A28090604	27	27	0	0	31.4	+4.4	+16.30%
A28090606	24	24	0	0	27.1	+3.1	+12.92%
MEAN	23.4		0.2	0.85%	26.7	+3.3	+14.19%
SD			0.9		4.5	1.9	

*The change is between the weight at harvest and the weight at implant.

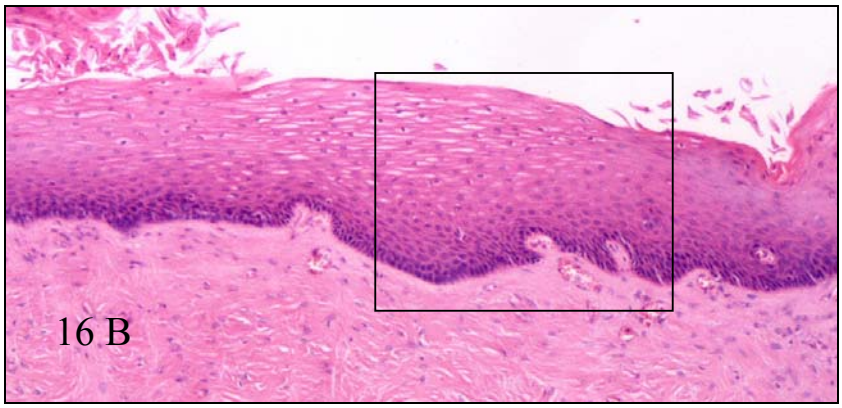
The mean increase in body weight in Groups 1 and 3 after both were administered with Gentamicin injections were very similar; the changes in body weight after 14 days injections were respective 0.4 grams and 0.2 grams. Among them, the weight of 7 mice increased, of 5 decreased and of 8 remained even. The increase in body weight was less than that for the groups without Gentamicin injections, but not statistically significant.

4.4 Histological evaluation of the cyst wall

Twenty-two of 37 implants which were harvested at 9 weeks yielded intact or partial cyst formation in this study. At 9 weeks the implanted artificial cysts were surrounded by mouse connective tissue. The non-keratinised stratified squamous epithelium (SSE) was microscopically similar to that of the vaginal mucosa from which the cysts were constructed (Fig 16).



Figs 16A: Photomicrographs of intact cyst formation with varying thickness of stratified squamous epithelium after 9 weeks growth (H&E, magnification x20)



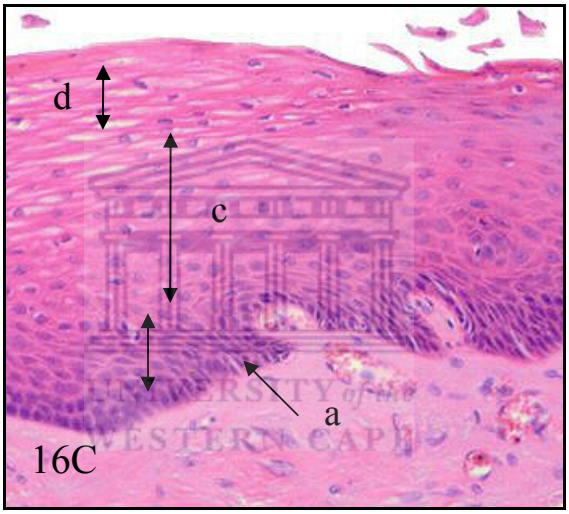


Figure 16B): Histology showing that the epithelium of the cyst maintained the structure of the human vaginal mucosa. (H&E, magnification x100)

Figure 16 C): Non-keratinised SSE of the cyst, a=basal cell layer, b=parabasal cell layer, c=intermediate cell layer, d=superficial, parakeratinising cell layer. (H&E, magnification x200)

The cyst epithelium was similar to the control vaginal tissue albeit flattened orthokeratotic or hyperparakeratotic, lacking rete pegs (Fig 17, 20) and exhibiting nodules of thickened epithelium in the cyst wall (Fig 19). However, a few rete pegs had developed in one cyst (Fig 21). The cell layers of the cyst epithelium are loosely arranged, and desquamation of the surface epithelial cells into the lumen is noticed in most cysts in this model (Fig 18). The majority of the thicker

epithelial regions of the cyst lining contained nodular or plaque-like epithelial thickenings (Fig 19), which were not deemed due to tangentially sectioning. The maximum and minimum cell layers were observed in 12 random foci of the epithelia of the control vaginal tissues and the derived cyst walls. There was no statistic difference in the number of the cell layers between the epithelial lining of cysts and the control vaginal epithelium. The results are recorded in Tables 12 and 13.

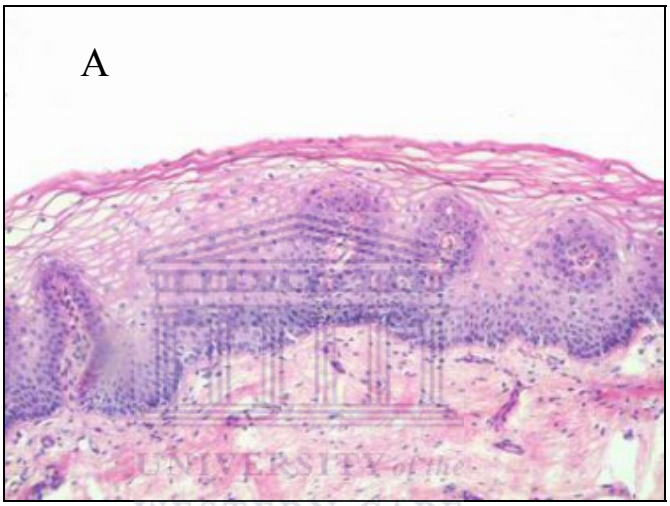


Figure 17 A: Photomicrograph of the vaginal mucosa processed as control tissue (H&E, magnification x100).

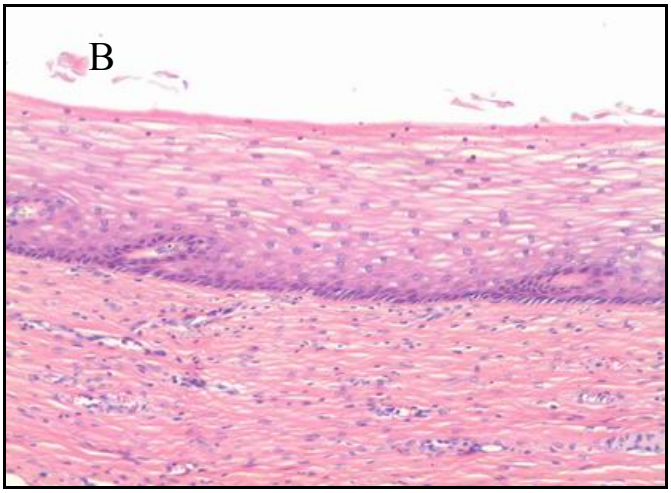
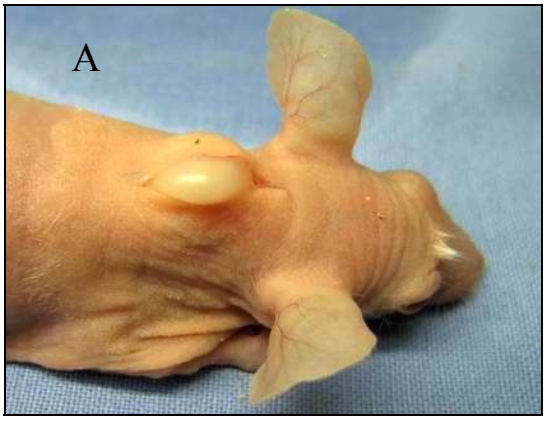


Figure 17 B: Cyst lining derived from the same vaginal mucosa specimen (A) after 9 weeks growth (H&E, magnification x100).



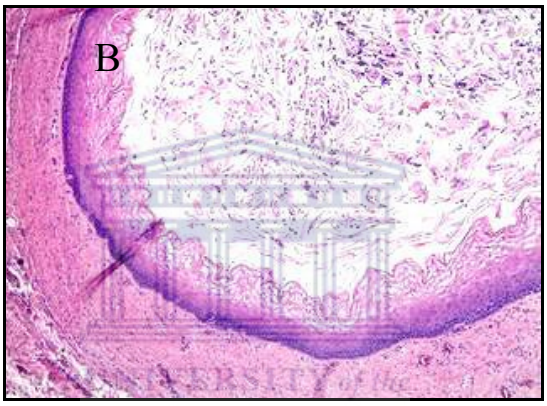


Figure 18A: Photo of a larger amount of keratin in the cyst lumen
Figure 18B: Photomicrograph of desquamation of the epithelial cells into the lumen. (H&E, magnification x40)

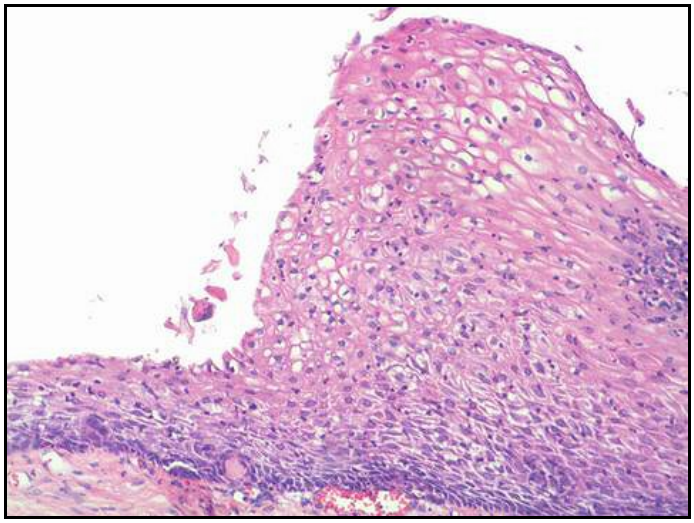


Figure 19: Photomicrograph of nodular epithelial thickening at the thick region of the cyst (H&E, magnification x100).

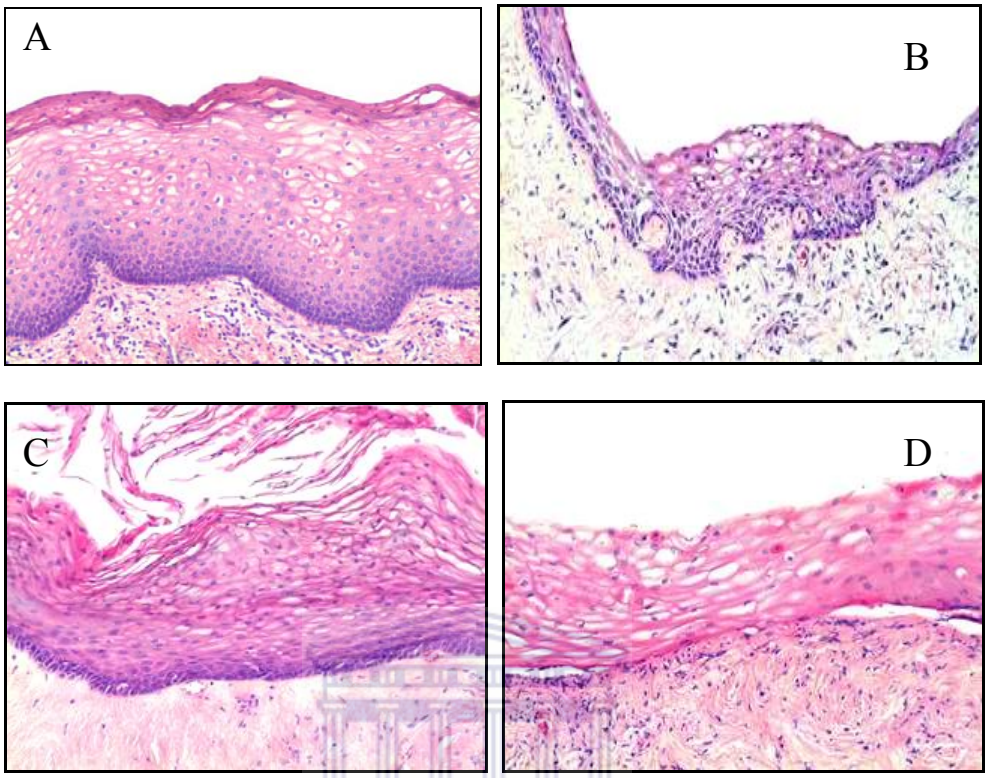


Figure 20: Photomicrograph of 3 cysts (B-D) constructed from one donated vaginal tissue (A). B&D) flattened and lifting off cyst epithelium; C) hyperkeratotic cyst epithelium (H&E, magnification x100)

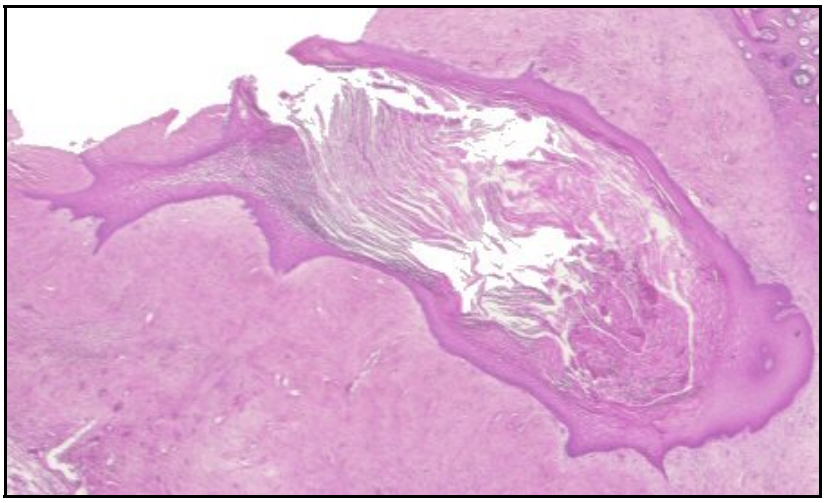
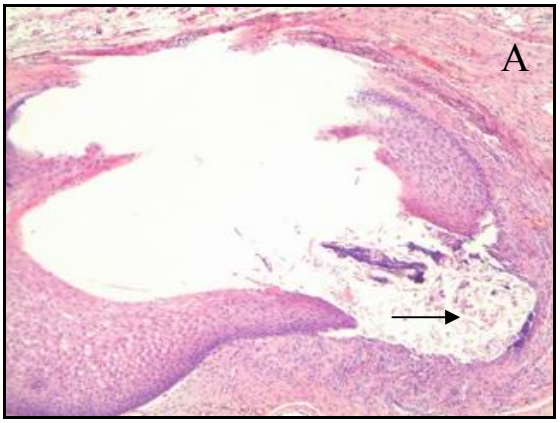
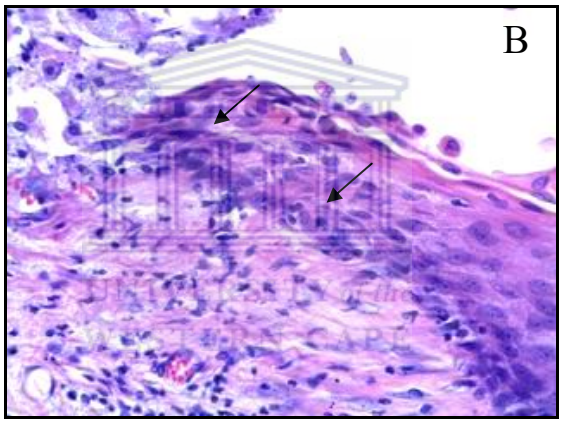


Figure 21: Photomicrograph of few rete pegs that had formed. (H&E, magnification x20)

Ulceration of the epithelium (Fig 22A), partial epithelium necrosis (Fig 22B) and ingrowth of the mouse epidermis (Fig 22C) were observed in the implants with partial cyst formation.





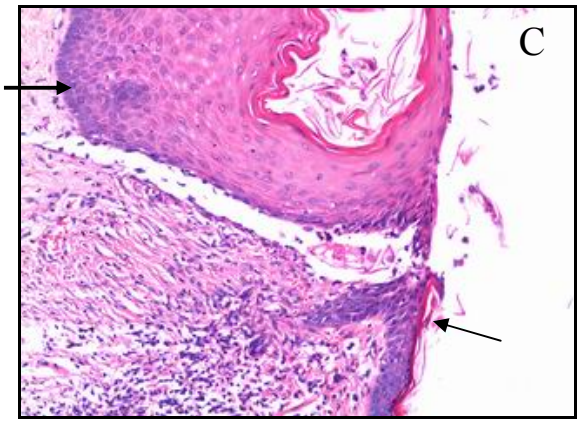


Figure 22A: Photomicrograph of ulcer of epithelium (arrow) (H&E, magnification x40)
Figure 22B: Photomicrograph of necrotic epithelium (arrow) (H&E, magnification x200)
Figure 22C: Photomicrograph of transformation between non-keratinized cyst epithelium (thick arrow) and keratinised epidermis (thin arrow). (H&E, magnification x100)

Table 12: The thickness of the control human vaginal epithelium

Tissue No	maximum number of cell layers														minimum number of cell layers													
	1	2	3	4	5	6	7	8	9	10	11	12	AV	SD	1	2	3	4	5	6	7	8	9	10	11	12	AV	SD
T05050601	19	21	20	22	24	21	21	23	19	21	22	23	21.3	1.56	10	8	8	9	11	14	10	10	7	6	8	7	9.0	2.17
T18050601	21	20	24	23	22	24	24	21	23	20	22	24	22.3	1.56	6	12	6	7	8	8	7	9	7	11	7	9	8.1	1.88
T25050601	21	24	25	24	21	20	23	20	26	23	20	22	22.4	2.07	12	12	8	11	5	13	11	8	8	6	9	10	9.4	2.50
T26050601	21	25	28	27	23	29	24	26	29	22	21	23	24.8	2.95	14	7	9	10	11	11	12	9	10	11	13	12	10.8	1.91
T23060601	20	21	19	20	24	22	21	20	21	24	20	25	21.4	1.93	12	11	12	8	7	10	8	13	9	9	12	9	10.0	1.95
T06070601	21	23	20	27	23	22	24	23	22	20	22	26	22.8	2.14	12	10	13	14	12	10	15	14	12	14	10	13	12.4	1.73
T28070601	24	25	23	26	22	23	21	23	21	22	20	20	22.5	1.88	14	13	11	12	10	9	11	14	9	12	9	11	11.3	1.82
T03080601	27	19	25	27	21	22	24	22	24	25	20	22	23.2	2.59	10	12	15	13	14	10	13	10	12	13	11	9	11.8	1.85
T24080601	24	26	28	25	30	29	31	27	26	28	24	25	26.9	2.31	13	9	6	10	10	7	11	14	12	9	11	13	10.4	2.43
T25080601	22	27	25	22	23	20	23	26	28	25	27	24	24.3	2.42	13	14	13	15	12	12	11	15	11	13	11	12	12.7	1.44
T07090601	23	25	19	20	21	19	18	22	21	20	23	24	21.3	2.18	12	10	11	8	10	11	13	9	12	12	8	8	10.3	1.78
T28090601	31	29	28	32	25	35	33	30	27	29	31	33	30.3	2.83	16	18	13	15	14	17	16	15	13	8	13	11	14.1	2.75
MEAN													**23.6**														**10.9**	
SD													**2.66**														**1.7**	

Table 13: The thickness of the cyst epithelial lining

	maximum number of cell layers														minimum number of cell layers													
Cyst No	1	2	3	4	5	6	7	8	9	10	11	12	AV	SD	1	2	3	4	5	6	7	8	9	10	11	12	AV	SD
C05050601	25	24	26	17	19	17	25	23	25	16	17	19	21.1	3.9	12	9	7	5	6	10	10	11	6	7	9	6	8.2	2.29
C05050602	26	26	28	28	25	30	31	29	25	28	26	27	27.4	1.93	12	8	7	14	12	9	10	12	9	12	11	13	10.8	2.14
C18050603	27	26	28	29	28	24	25	25	28	24	30	28	26.8	1.99	10	8	6	9	13	11	9	7	12	11	9	8	9.4	2.07
C18050605	25	27	25	26	28	22	30	22	24	28	27	25	25.8	2.42	9	10	7	8	8	11	7	6	14	11	12	9	9.3	2.35
C26050601	28	24	25	29	21	26	29	26	28	30	25	24	26.3	2.63	9	10	13	8	7	11	10	11	14	11	12	13	10.8	2.09
C26050602	23	21	20	22	21	22	19	23	21	19	22	20	21.1	1.38	9	8	8	13	12	8	11	9	10	8	11	10	9.8	1.71
C23060601	25	26	25	27	24	26	28	24	28	25	27	26	25.9	1.38	5	6	7	4	3	5	5	6	4	5	6	3	4.9	1.24
C06070603	25	24	27	26	25	22	21	20	26	23	19	18	23.0	2.98	9	10	7	9	8	11	9	7	11	6	9	7	8.6	1.62
C28070601	8	10	12	14	11	9	11	13	14	10	12	9	11.1	1.98	4	3	5	7	3	4	6	5	4	3	6	5	4.6	1.31
C24080604	21	26	19	29	23	30	21	18	29	25	27	30	24.8	4.34	6	4	5	5	6	6	8	11	8	5	6	10	6.7	2.15
C25080602	17	15	14	14	16	18	17	18	14	15	19	16	16.1	1.73	7	7	7	8	9	7	9	10	6	8	9	7	7.8	1.19
C25080604	27	30	24	29	31	25	31	26	27	28	30	24	27.7	2.57	11	13	10	12	9	13	14	11	10	9	11	13	11.3	1.67
C25080605	27	25	20	19	18	22	26	20	18	23	24	21	21.9	3.09	12	5	7	6	11	7	8	10	9	5	8	6	7.8	2.29
C07090601	22	19	23	26	20	23	22	16	24	23	21	18	21.4	2.78	5	6	6	9	7	5	8	7	10	11	9	7	7.5	1.93
C07090603	20	24	19	18	21	18	19	20	22	25	23	21	20.8	2.29	6	6	8	7	6	4	5	4	4	6	5	8	5.8	1.42
C07090604	23	22	32	31	28	22	24	26	28	24	25	26	25.9	3.29	10	12	11	14	13	12	14	9	11	13	11	14	12.0	1.65
C28090601	25	30	29	27	26	23	27	30	26	23	25	29	26.7	2.46	12	11	6	13	12	12	6	7	14	8	7	13	10.1	3.03
C28090602	18	19	17	21	14	16	18	20	21	17	18	19	18.2	2.04	5	7	10	6	8	7	10	9	8	6	5	8	7.4	1.73
C28090603	22	21	28	29	24	25	23	27	26	24	21	28	24.8	2.79	6	7	9	10	12	8	8	7	9	11	7	6	8.3	1.92
C28090604	19	26	18	24	20	25	21	18	23	17	22	25	21.5	3.12	3	12	10	9	6	4	9	7	9	11	9	6	7.9	2.75
C28090605	20	15	21	16	16	25	18	22	25	23	19	22	20.2	3.43	4	7	4	9	6	7	6	7	5	6	9	9	6.6	1.78
C28090606	20	21	19	19	18	22	20	18	18	21	20	19	19.6	1.31	11	7	10	7	6	10	5	6	9	10	11	6	8.2	2.21
MEAN													**22.6**														**8.3**	
SD													**4.14**														**1.9**	

Blood supply has a bearing on the nutritional capacity of the cyst model. The thickened regions of the epithelial layer of the cyst usually were associated with new blood supply (Fig 23). The re-establishment of the new blood supply to implanted artificial cysts might influence the thickness of the cyst epithelium.

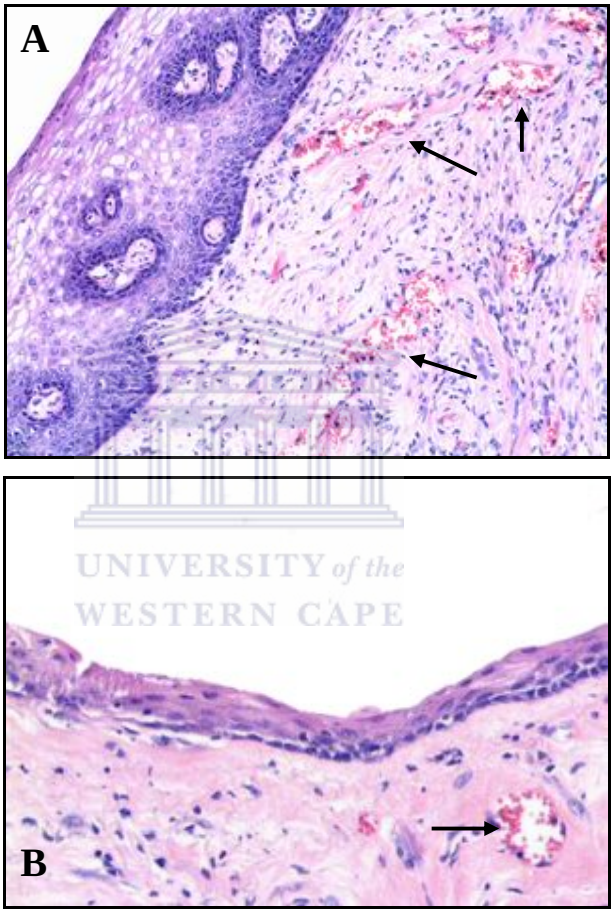


Figure 23: Photomicrograph of the new blood supply to implanted epithelium. A) Thickened regions of epithelium associated with vessel-enriched connective tissue. (H&E, magnification x 40) B) Thinner epithelium region with fewer blood vessels (arrows). (H&E, magnification x 100)

The inflammation was still serious even though there were two antibiotic covers used in this study. Only 10 of the implanted cysts harvested after 9 weeks growth had no contaminants. The other 30 cases contained contaminants at the sites of implantation, either in the opened wounds or inside the well healing cysts.

In the cases with opened wounds and/or epidermis ingrowth, the infections were due to the uncompleted epidermal wound healing (Fig 24). It was common in the well-healed cases that the inflammation was located under the epidermis; especially around the skin wound sutures (Fig 25). The ulcerated regions of the cysts also contained dense inflammation in the underlying cyst wall (Fig 26).

In those cases with serious inflammation, the inflammatory cells were distributed throughout the entire cyst wall and the lumen (Fig 27A, B). In individual cases with less inflammation, the inflammatory cells were only located at the base of the epithelium (Fig 28).

Sections of the implants from which contaminants were isolated were stained with the Gram stain and examined by light microscopy. Colonies of Gram-negative Gram-positive bacteria were present in the inflamed cysts (Fig 29).

Serious inflammation was observed in those cases in which silk sutures were used. This observation will be described in §4.4 and compared to the cases in which nylon sutures were used.

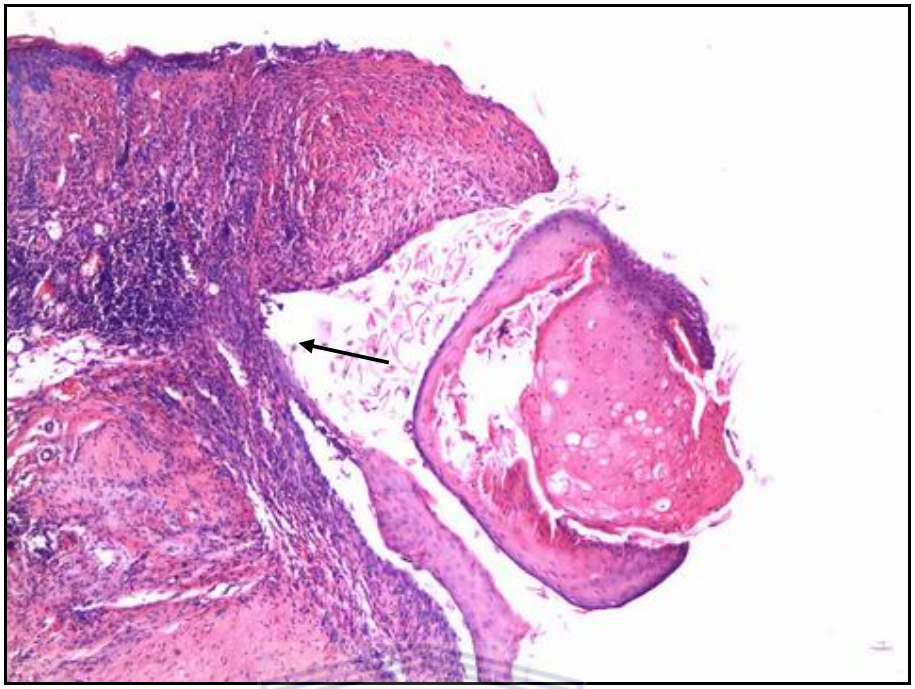


Figure 24: Photomicrograph of the epidermis ingrowth and inflammation around the wound (arrow). (H&E, magnification x 20)

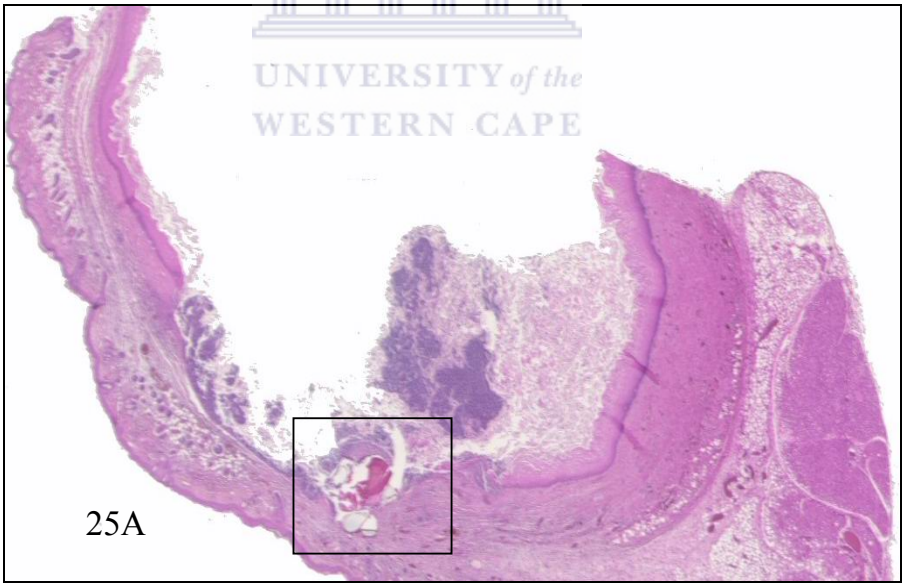


Figure 25A: Low-power photomicrograph of the inflammation in the cyst wall close to the murine dermis. (H&E, magnification x 20)

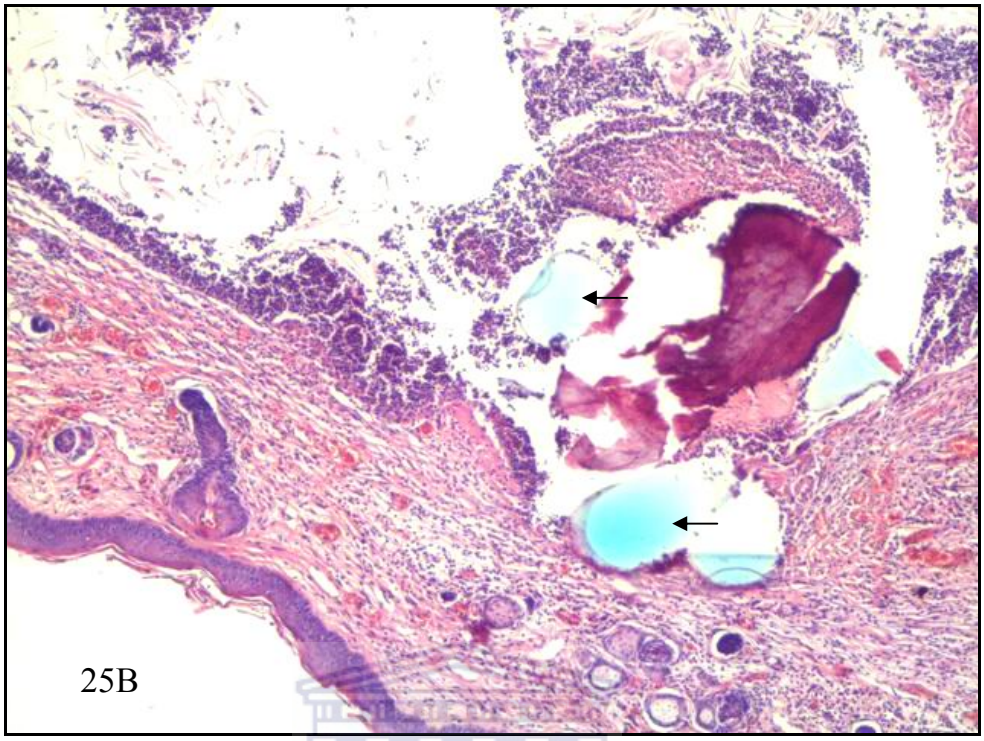


Figure 25B: high-power photomicrograph with details of the inflammation around the wound suture (arrow). (H&E, magnification x 40)

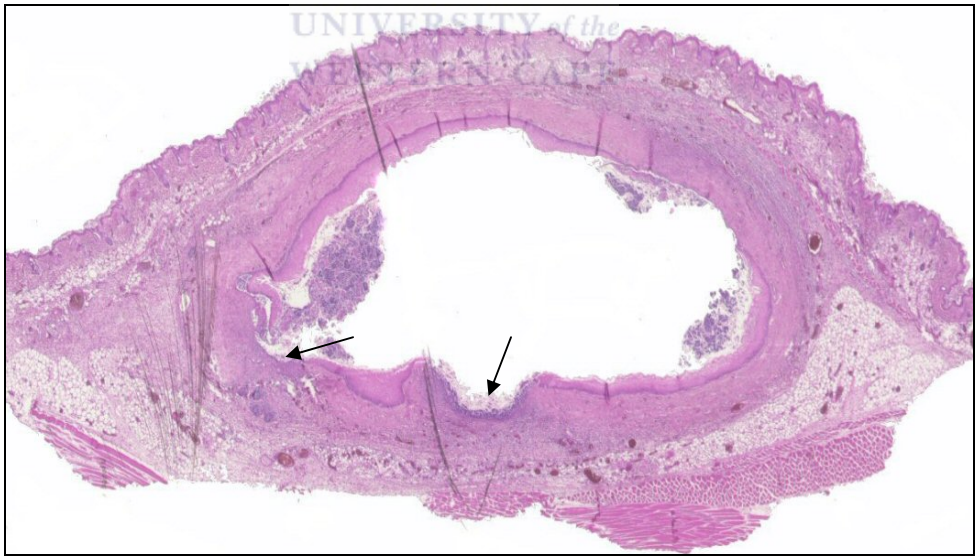


Figure 26: Photomicrograph of the inflammation at ulcerated regions in the cyst (arrows) (H&E, magnification x 20)

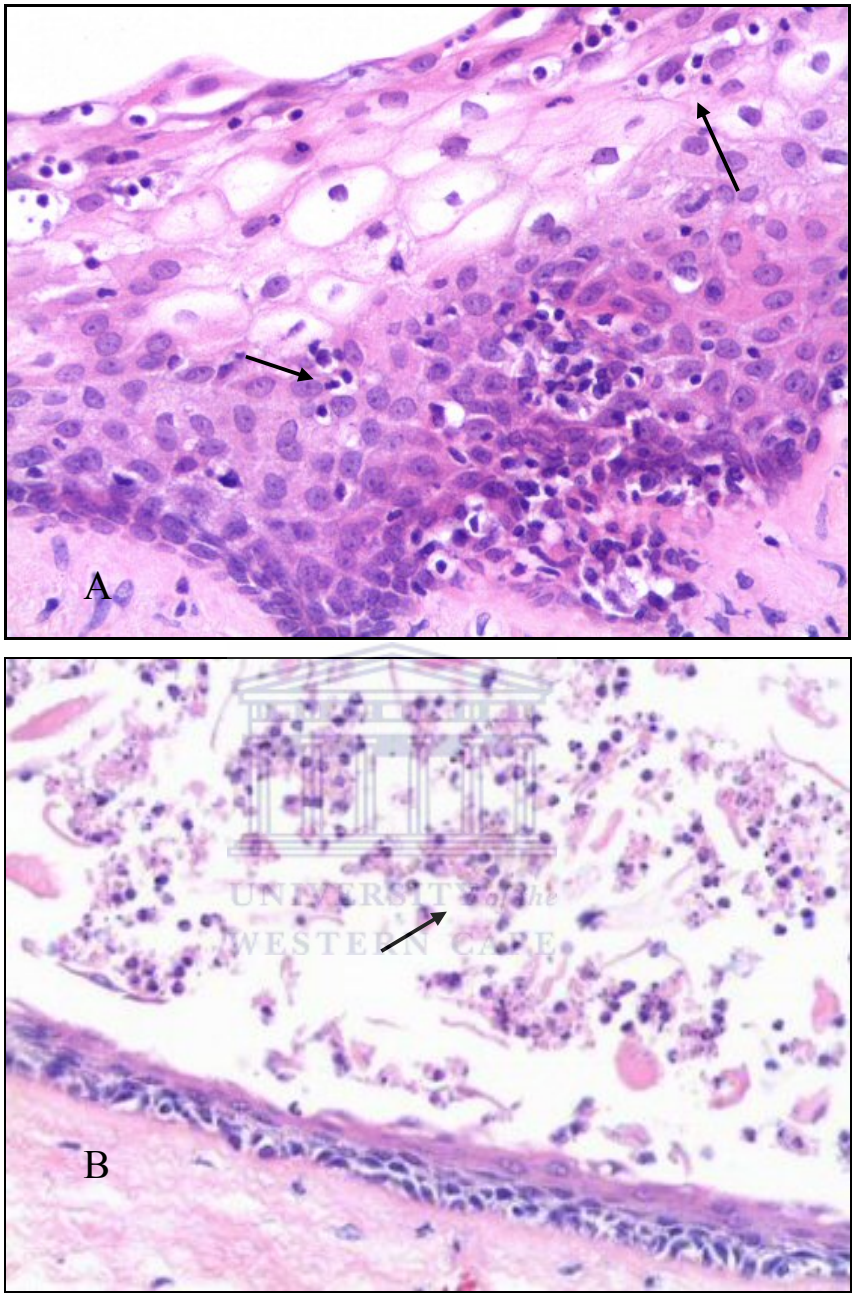


Figure 27: A) Photomicrograph of moderately dense inflammatory cell exocytosis in the cyst epithelium (H&E, magnification x 200); B) Photomicrograph of the inflammatory cells (arrow) accumulated in the cyst lumen (H&E, magnification x 100)

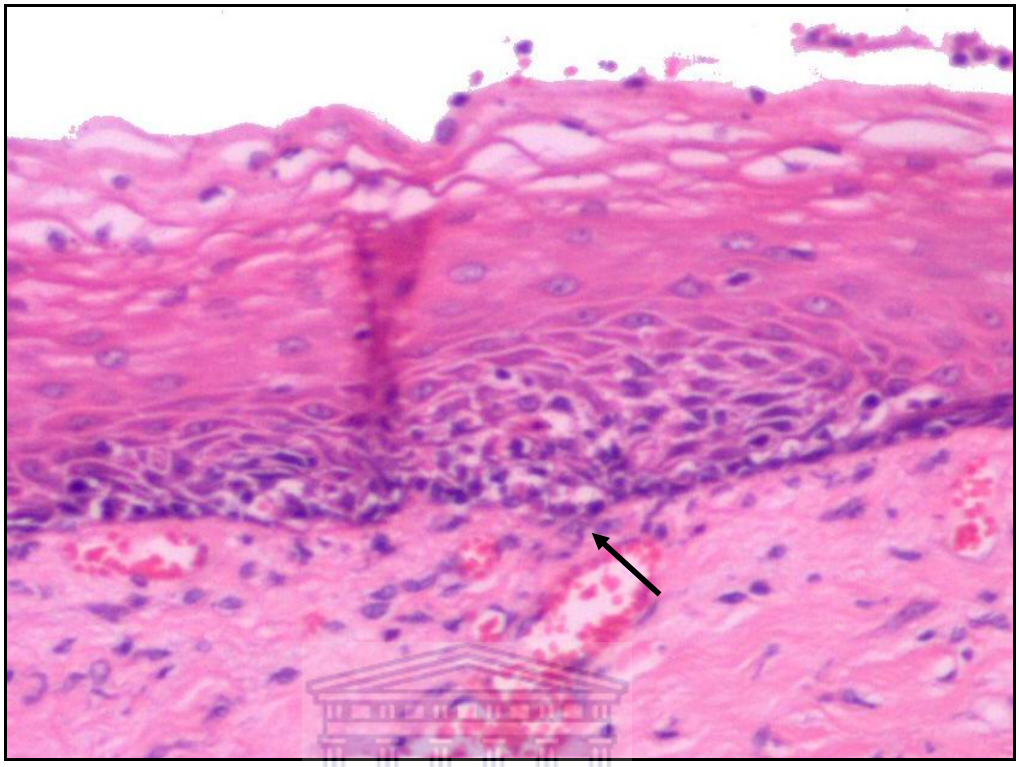


Figure 28: Photomicrograph of mild presence of inflammatory cells near base of the epithelium (H&E, magnification x 200).

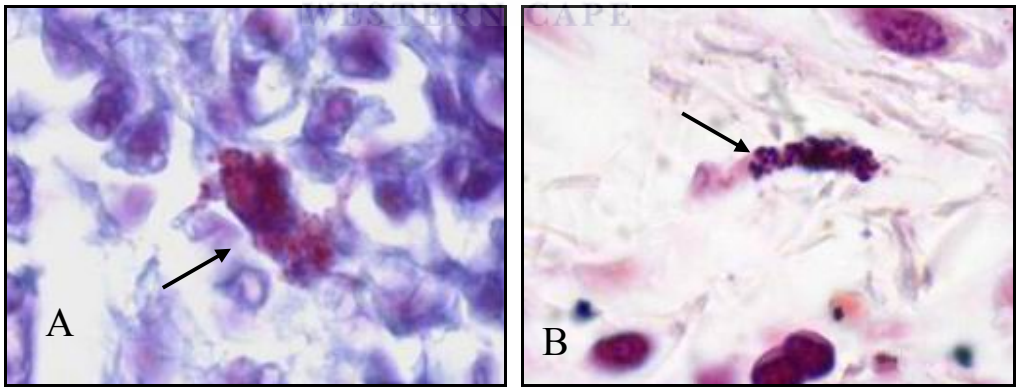


Figure 29: Photomicrograph of Gram stains. A) Gram-negative bacteria (pink colour); B) Gram-positive bacteria (purple colour) (arrows) (Gram, magnification x 1000)

A continuous layer of fibrosis was present between the host connective tissue and the implanted cyst in those implants that were constructed from the vaginal tissue without 3mM EDTA treatment due to time constraints (Fig 30).

Fibrosis was also present in the sutured areas of the implants (Fig 31). Moreover fibrotic human connective tissue was observed filling the lumen of one cyst; the epithelial lining was substituted by granulation tissue (Fig32).

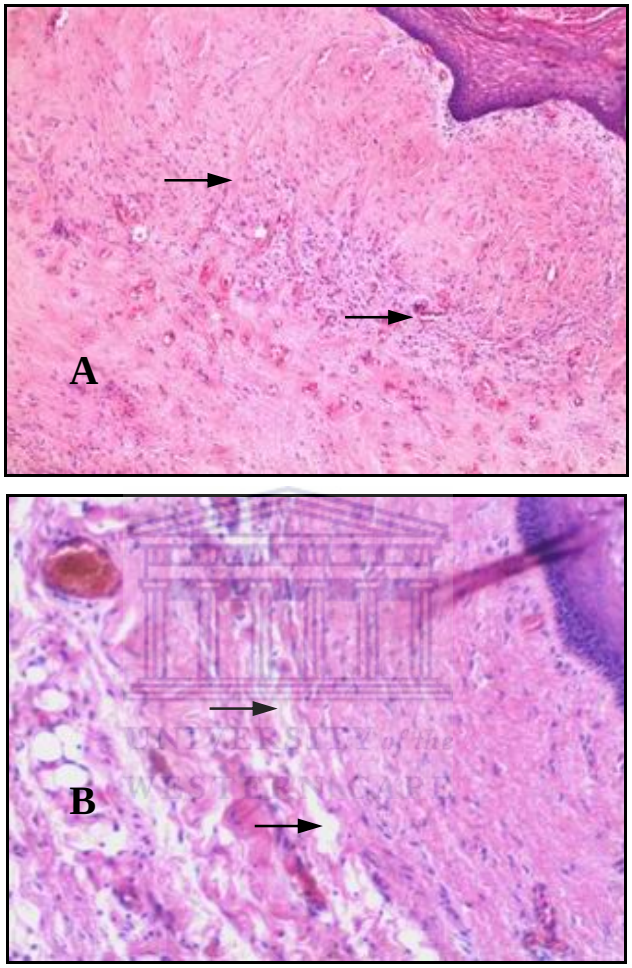


Figure 30: Photomicrographs of the junction between the murine host connective tissue and the human connective tissue of the artificial cyst. A) Fibrosis in the implants without EDTA treatment (arrows); B) No fibrosis at same area in the implants with EDTA treatment (H&E, magnification x 40)

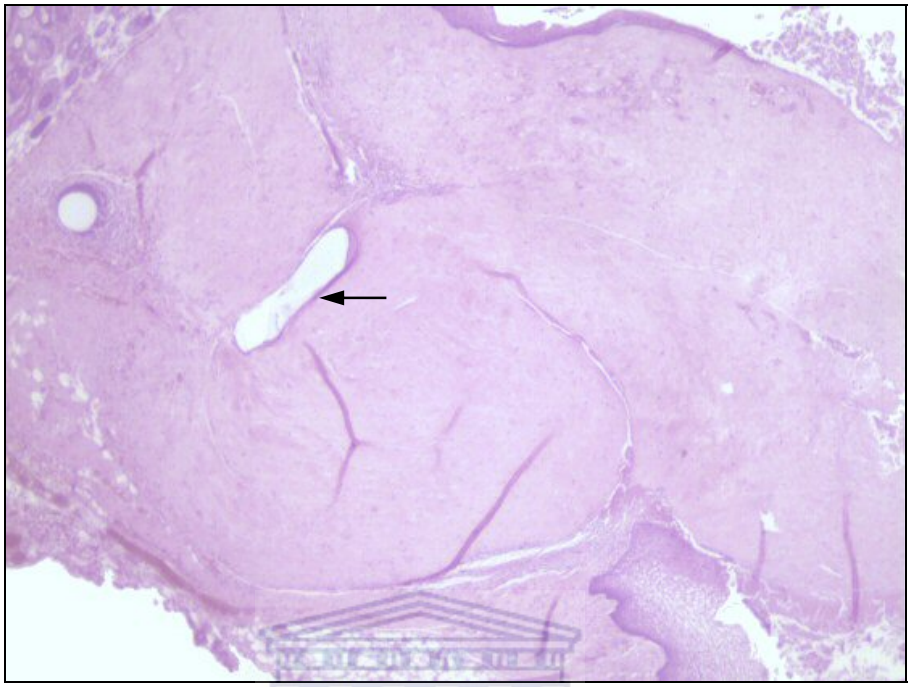


Figure 31: Photomicrograph of the fibrosis around the suture (arrow). (H&E, magnification x 20)

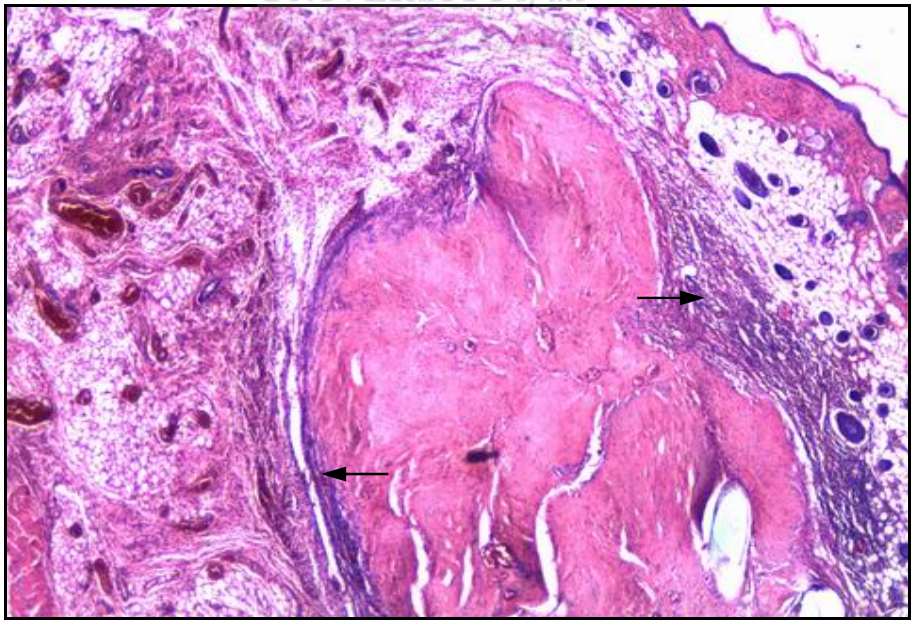


Figure 32: photomicrograph of the granulation tissue wall (arrow) surrounding the fibrotic lumen contents (H&E, magnification x 20)

Three implants with well healing wounds (in Groups 1, 2, 3 each) resulted in atrophic cyst formation with an average 3-5 cell layers of cyst epithelium, no epithelium necrosis, and without Coagulase–negative staphylococci infection (Fig33). In individual cases, epithelial islands were observed in the connective tissue of the cyst wall (Fig 34).

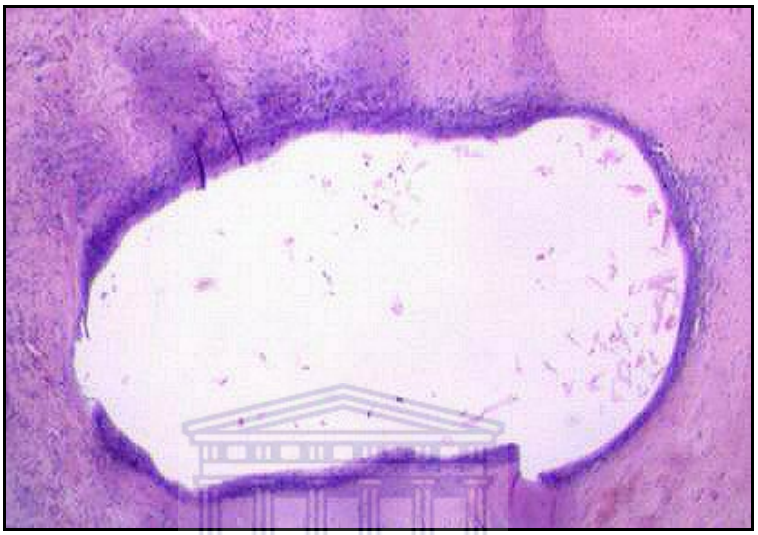


Figure 33: Photomicrograph of the atrophic cyst. (H&E, magnification x 20)

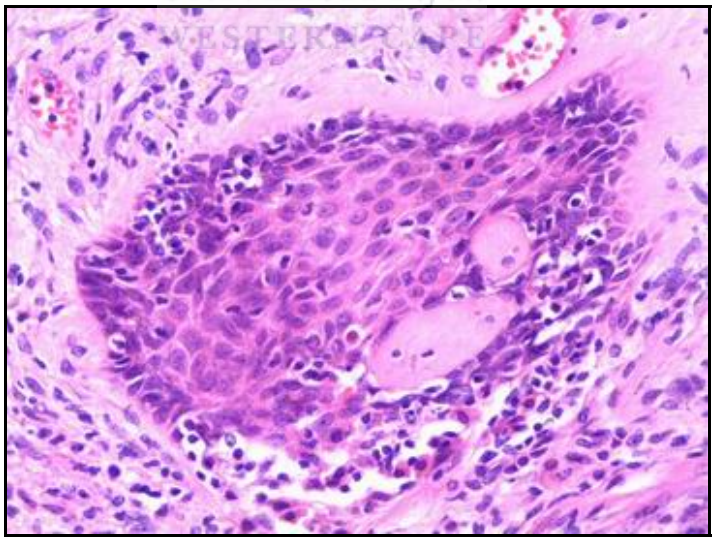


Figure 34: Photomicrograph of the epithelial island in cyst wall. (H&E, magnification x100)

4.5 Comparison of silk suture and nylon suture

Table 14: Evaluation of two kinds of sutures.

		Nylon Suture	Silk Suture	Total
Wound healing with intact or partial cyst formation	Without contaminants	5	5	10
	With contaminants	6	1	7
Wound healing without cyst formation		2	4	6
Ingrowth of mouse epidermis with partial cyst formation		3	3	6
Poor wound healing		3*	8	11
Animal died before wound healing		2	1	3
Total		21	22	43

* Poor wound healing of implants in two mice was due to the wound being compromised by forcible restraint when the animals were administered with Gentamicin injection without anaesthesia in the beginning of the experiment.

The number of poor wound healing cases which were stitched with silk sutures was nearly three times higher than that observed in those cases when nylon sutures were used. The ingrowth of murine epidermis was also more pronounced in the silk suture groups than in the nylon suture groups (Fig 35 C, D). Even though there were same numbers of cases without bacterial contamination in both suture groups, the inflammation around silk sutures was generally more severe than that around nylon sutures (Fig 36 C, D). A foreign body reaction and acute inflammatory infiltrate usually surrounded the silk sutures (Fig 37).

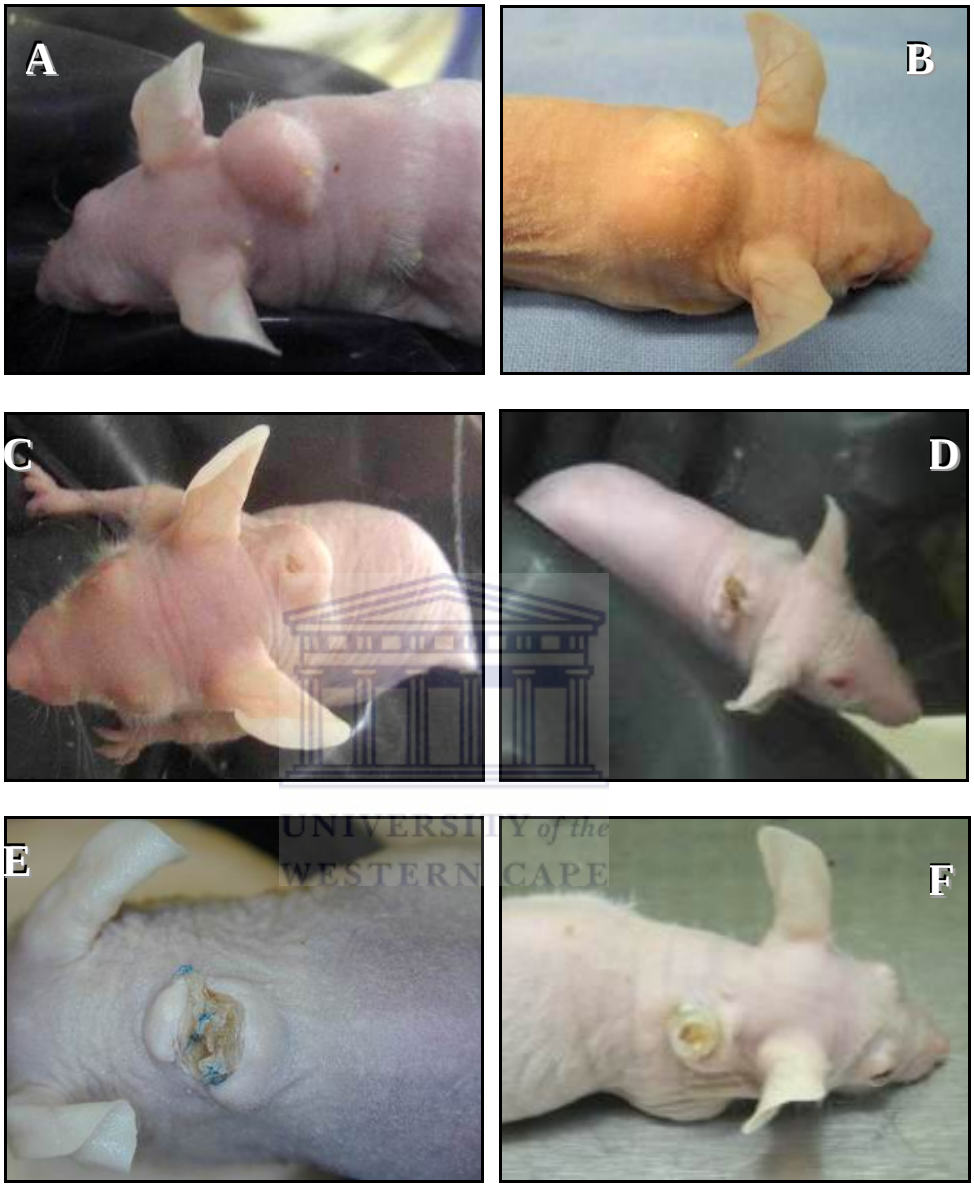


Figure 35: Photos of skin wounds. A) wound healing (nylon suture), B) wound healing (silk suture), C) epidermal ingrowth (nylon suture), D) serious epidermal ingrowth (silk suture), E) poor wound healing (nylon suture), F) poor wound healing (silk suture)

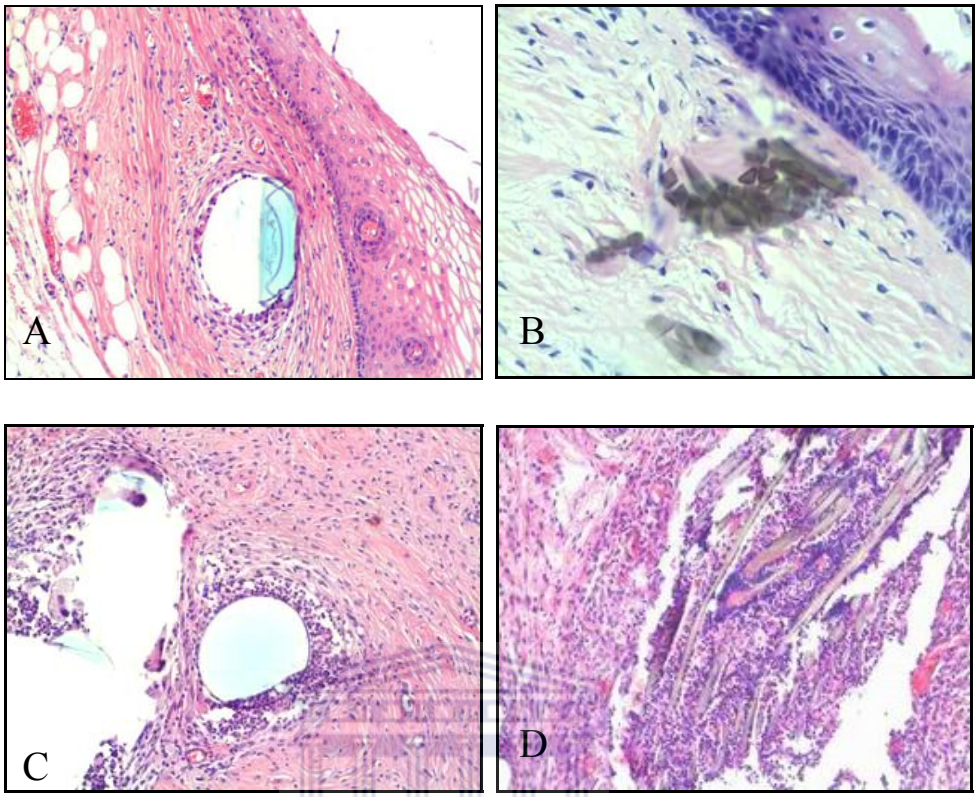


Figure 36: Photomicrograph of the sutures. (H&E, magnification x 100)

A) Mild inflammation around nylon suture in the implant without contaminants.

B) Mild inflammation around silk suture in the implant without contaminants.

C) Inflammation around nylon suture in the implant with contaminants.

D) Severe inflammation around silk suture in the implant with contaminants.

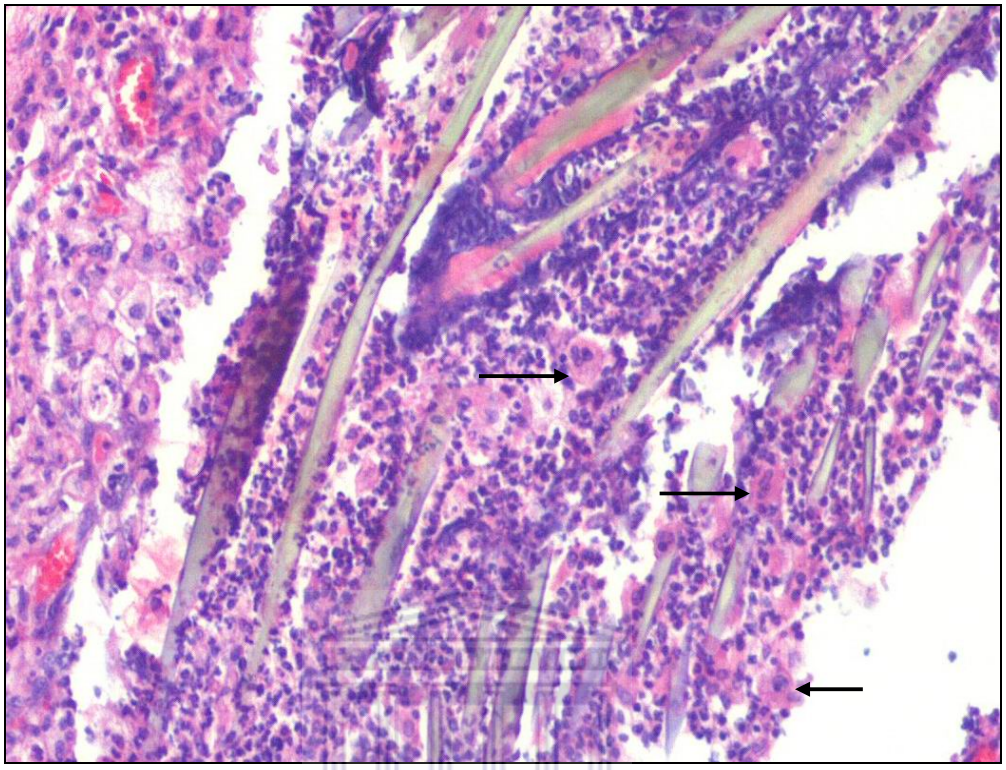


Figure 37: High-power photomicrograph of a foreign body reaction around a silk suture. Foreign body giant cells (arrows) were present. (H&E, magnification x 200)

CHAPTER 5: DISCUSSION AND CONCLUSION

5.1 Identification of the contaminants and evaluation of antibiotic cover

5.1.1 Common pathogenic organisms

In the present study, *Enterobacter cloacae, Enterococcus faecalis, Klebsiella oxytoca, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Proteus mirabilis* were isolated from the neck regions of the nude mice before operation. These organisms are the normal commensals of healthy animals (ILAR, 1996). *Staphylococus aureus and Streptococci* were only carried by 3 mice. Coagulase negative staphylococci (CNS) were the most commonly isolated organisms (17.8%) before the implantation; they are considered potentially pathogenic in this model.

After the implantation of the artificial cyst, CNS were still the most frequently isolated pathogenic organisms (58.1%), followed by *Enterococcus faecalis* (22.6%). This is in agreement with the recent data of the National Nosocomial Infection Survey (NNIS), in the USA, which CNS and Enterococci are ranked second and third most common cause of postoperative surgical site infection (CDC NNIS, 1997; Mangram *et al*, 1999).

Over the last two decades, it has become apparent that CNS is the most significant pathogen in medical-device-related infections and in immunocompromised patients (Wieser & Busse, 2000). NNIS found that from 1980 to 1989, the incidence of CNS as a common cause of nosocomial bacteremias increased from 9 to 27%. Hence, it has become the single most common cause of these infections (Archer & Climo, 1994). In another recent study (Edmiston, *et al*, 2005), CNS was recovered from 86% of air-samples isolated from the operating theater. CNS produced multiple infections, such as bacteremia, catheter-related infections, central nervous system shunt infections, endocarditis, urinary tract infection, surgical site infection and endophthalmitis (Huebner & Goldmann, 1999). Almost

all cases of bacteremia due to CNS were nosocomial in origin and are related to an indwelling foreign body.

The past few years have witnessed increasing interest in enterococci which tolerate a wide variety of growth conditions, including temperatures of 10°C to 45°C, and hypotonic, hypertonic, acidic, or alkaline environments (Huycke, 1998). Enterococci have become recognized as leading causes of nosocomial bacteremia, surgical wound infection, and urinary tract infection (CDC NNIS, 1997).

5.1.2 Putative source of contaminants

In this study, the athymic nude mice (MFI nu nu) that were used were immunodeficient animals, but not germ-free. It is believed that the contaminant organisms of these animals were acquired at the University of the Witwatersrand (Wits) Animal Unit (Johannesburg) and transmitted from animal to animal in same transport batch. The mice were transported from the Wits Animal Unit in Johannesburg to the laboratory on main campus of UWC in Cape Town, by road and air in a single sterilized air-filtered cage in batches of six. On arrival at the laboratory, the whole cage was transferred into an isolator. The animals were then accommodated into two separate cages in batches of 3 each. The floor of the cages was covered with a bedding of autoclaved wood shavings. The animals were fed ad-lib with normal sterilised water and autoclaved rodent breeder food. Up to this stage, there was no opportunity for the animals to have been in human contact in the laboratory before the implantation procedure. In support of our assumption that the animals were infected at Wits animal unit, bacteria species were isolated from the individual batches. *Klebsiella pneumoniae* was isolated in larger numbers from batches 4 and 5; *Pseudomonas aeruginosa* was isolated in larger numbers from batches 4, 5 and 9; and *β-haemolytic streptococcus group A* was isolated only from batch 8 and 9.

It is believed that CNS which was isolated from the cyst after 9 weeks of growth, might have been introduced by the ventilated air of the substandard animal room. Microbiology of room air samples only isolated two bacterial colonies.

5.1.3 Inflammation and infection of the cysts

The contamination, inflammation and infection control in this experiment was not deemed entirely satisfactory even though isolators were used and antibiotic cover was instituted. It was observed, in cases with good healing, that the inflammatory focus was located at the cyst wall closest to the mouse epidermis (Fig 25). In cases of poor wound healing, there was marked contamination and inflammation. This was noted in 6 mice which showed epidermal ingrowth, 11 mice presented with opened wound and 3 mice died as a result of euthanasia. The infections that resulted in poor wound healing could be surgical site infections (SSI). These are defined as infections involving only the skin or subcutaneous tissue at the incision sites. In a few mice, "Stitch abscess" also presented which is defined as minimal inflammation and discharge confined to the points of suture penetration (Mangram *et al*, 1999). In addition, mice in which the silk suture was used showed marked inflammatory reaction at the suture site. This inflammatory tissue response was thought to be elicited by the use of silk suture material (Moy *et al*, 1991).

In the last two decades, SSIs has remained a major cause of nosocomial (hospital-acquired) infections with increasing global rates. In epidemiologic studies, risk factors of SSIs were identified by multivariate analyses. These included patient or operation features, such as the patient's age, nutritional status, altered immune response, *etc*. Operation factors included duration of operation, preoperative preparation, skin antisepsis, antimicrobial prophylaxis, foreign material in the surgical site, *etc* (Cruse, 1992; SHEA, 1992). The implantations of the cysts were preformed in a substandard animal room with no scrub suits or gowns worn by the operator. This is in direct contradiction to the SSI prevention measure suggested by the Hospital Infection Control Practices Advisory Committee, USA (Mangram *et al*, 1999). However, there is limited data describing the use of scrub suits or gowns in terms of SSI's risk in general.

Although CNS can produce serious postoperative infection, especially in patients with implanted foreign material, there may be a tendency to attribute SSI's to organisms that are merely skin-flora contaminants in culture specimens (Boyce *et al*, 1990). In the present study, the contraction of contaminants is possibly due to a lack of an efficient cover dressing after the implantation of the cyst. An ideal wound dressing should maintain a moist environment, enable gaseous exchange, and protect from secondary infection. The dressing should also be comfortable, and able to be removed without causing trauma (Dale, 1997). The ideal surgical dressing also aims to stimulate superficial epithelialisation when placed over a surgical incision wound. In so doing, it intends to protect underlying structures from contamination and infection (Wynne *et al*, 2004). Vapour-permeable, adhesive-coated dressings such as Opsite™ and Tegaderm™ are sterile, thin, vapour-permeable, hypoallergenic dressings that are sometimes used on surgical incisional wounds (Pudner, 2001). These dressings require care when being removed as their adhesive borders can cause possible tissue damage if their removal is not according to manufacturer's recommendation. Opsite Post-Op™ dressing has a vapour-permeable film border which can be removed by gently stretching the material horizontally away from the wound (Thomas, 1996).

5.1.4 Efficiency of antibiotic coverage

In this study, Terramycin and Gentamicin were used as antibiotic cover for CNS. Terramycin was efficient in preventing Staphylococcus aureus infection, but did not produce the desired efficiency to CNS. There was no difference in the number of the cases with intact cysts, between the Terramycin/vitamin cocktail group and the control group. Gentamicin proved to be more efficient against Gram-negative contaminants than Terramycin. This was confirmed by the microbiological results in this study. The body weights of the mice in groups 1 and 3, in which Gentamicin injections were administered, increased to a lesser degree than that of the mice in groups 2 and 4, where Gentamicin injections were not administered. Despite their discomfort, subjecting the mice to twice daily injections is definitely advisable

than just adding an antibiotic cocktail in drink water. This is acceptable because the body weight of the mice continued to increase after the injections.

The proportion of nosocomial CNS resistant to methicillin, oxacillin and nafcillin increased from 20 to 60% in the 1980's in the United States. Most of these methicillin-resistant CNS were also resistant to multiple additional antimicrobial agents (Archer & Climo, 1994). When CNS isolates are resistant to commonly used antibiotics, the glycopeptide vancomycin has been considered to be the antibiotic of choice (Kloos & Bannerman, 1994). Vancomycin is recognized as one of the most potent anti-staphylococcal drugs available, especially to hospital acquired infections caused by antibiotic resistant strains. It is the drug-of-choice in the treatment of serious methicillin-resistant *S. aureus* infections (Chang *et al*, 2003). It has been suggested by Shelburne, *et al* (2004) that by adding low concentrations of gentamicin to vancomycin, led to substantial synergistic bactericidal activity against methicillin-resistant *Staphylococcus aureus*. Vancomycin-Rifampin-Gentamicin is the standard treatment for deep *S. epidermidis* infection (Secasan, 2005). Combined treatment with vancomycin-gentamicin is also highly efficient in patients with endocarditis caused by penicillin-resistant *Streptococcus sanguis* (Martinez. *et al*, 1995). The combination of gentamicin plus vancomycin was the most effective regimen in the experimental endocarditis animal model (Righter, 1987). In the present study, the reports of antibiotics sensitivity testing on the isolated organisms showed that all strains of CNS were sensitive to vancomycin. The recent emergence of vancomycin intermediate *S. aureus* (VISA) and vancomycin-resistant *S. haemolyticus* emphasizes the importance of using this agent selectively and appropriately (Huebner & Goldmann, 1999). Another glycopeptide, Teicoplanin has been used with some success, as an alternative to vancomycin against some vancomycin-resistant strains of *Enterococci*, and in the treatment of moderate and severe infections by CNS (Kloos & Bannerman, 1994).

The past two decades have witnessed the rapid emergence of multiple-drug resistant (MDR) *Enterococci* (CDC NNIS, 1997). The therapeutic challenge of

MDR *Enterococci* - those strains with significant resistance to two or more antibiotics, often including, vancomycin - has brought their role as important nosocomial pathogens into sharper focus. *Enterococci* are intrinsically resistant to many antibiotics. Unlike acquired resistance and virulence traits, intrinsic resistance is based in chromosomal genes, which typically are nontransferrable. Penicillin, ampicillin, piperacillin, imipenem, and vancomycin are among the few antibiotics that show consistent inhibitory, but not bactericidal, activity against *E. faecalis* (Huycke, 1998). MDR *Enterococci* that had lost susceptibility to vancomycin were reported in Europe (Leclercq *et al*, 1988) and the United States (Sahm *et al*, 1988).

5.2 Wound dehiscence and comparison of two non-absorbable sutures

In this study, the non-absorbable suture materials, silk 5-0 and nylon 5-0, were used as a substitute for absorbable sutures which produced a foreign body reaction and acute inflammation noted by Thompson (2002). Silk suture was booked in these experiments as it was thought be less irritative near the wound edges and easier to apply than nylon sutures. However, the wounds of eight mice (20%), whose skin was stitched with silk sutures, showed wound dehiscence or poor wound healing. Twice as many mice presented with the features of dehiscence and poor healing than those mice stitched with nylon sutures. Marked inflammation was also noted around silk sutures of the artificial cyst. The infection risk associated with silk sutures was significantly higher than that with nylon sutures.

Silk used for suturing has the lowest tensile strength of any material tested, and elicits a considerable tissue reaction. Adamsons and Kahan (1970) demonstrated that rabbit skin wounds closed with a continuous 4-0 silk suture, regained only 40% of the strength of normal tissue 120 days after wound initiation. Van Winkle and associates (1975) noted that skin wounds approximated by different percutaneous sutures developed 70% of their normal strength by 120 days in the dogs. Superior tensile strength and knot security not only minimizes the risk of

suture line disruption, but also potentially reduces the amount of foreign material left in the wound (Moy *et al*, 1991).

Postlethwait and colleagues (1975) found that multifilamentous natural fibers, such as catgut and silk, cause the most intense inflammatory reactions, whereas monofilamentous synthetic materials, such as nylon and polypropylene, generate less reaction. Silk also has a high degree of capillarity because of its braiding and should not be used in wounds where there is increased potential for infection. In skin surgery, silk is used around the eyelids and lips, where it lies flat, causes minimal irritation and has a low incidence of infection (Moy *et al*, 1991).

In a study completed by Chu *et al* (1987), it was found that a new type of braided nylon thread with a silver compound coating exhibited moderate to good bactericidal property against the following seven bacterial species: *S. aureus, E. coli, P. aeruginosa, K. pneumoniae, S. dysenteriae, S. maruslene*, and *P. mirabilis*.

It was well documented that the diminished tensile strength of wounded skin compared to the normal skin, correlated with the appearance of narrower collagen fibres present in the wound as opposed to normal collagen (Lin & Long, 2005). The continuous production of keratin by the cyst epithelium may not only lead to the increased intraluminal mechanical pressure, but also produce pressure to the wounded skin due to the increase in size of the cyst. Wound dehiscence and resulting necrosis was observed in 4 mice after 6 weeks of cyst growth.

5.3 Evaluation of the animal model

5.3.1 Observation of animal housekeeping

In general the nude mice survived the experiments quite well and no major problems were encountered in housekeeping them during this study. Nevertheless, ten mice were lost before the implantation and 6 mice were lost after the implantation procedure. Low ambient temperature during airfreight or in the animal room due to a power shutdown accident could have possibly resulted in the death of 6 mice in batch 2. An overdose of anaesthetic was another reason of the death of 2 mice in batch 1. The loss of three post-implantation mice could be attributed to the toxicity of peracetic acid, a strong disinfectant for the sterilisation of the port of the isolators. Another 3 mice with implants in the control group were euthanised after 3 weeks as severe inflammation around the opened wounds, persistent anorexia, and inability or impairment to move was observed.

Aggression (climbing the cage cover and jumping), repetitive roaming behaviour, nervousness and handling difficulties were observed with some of the mice during this study. These symptoms are defined as the behavioural patterns of 'isolation stresses' which were present in the mice as they were accommodated individually in single cage (Balcombe, 2006). Other physiological symptoms including lower immunocompetence, higher tumour incidence, gastric ulcerations, hyper-sensitivity to toxin and increased pathology are also present in single-housed mice (Van Loo *et al*, 2003). However, in this study, the death of three mice was associated with the toxicity of peracetic acid.

Preferences for hiding shelters and nesting materials have been repeatedly demonstrated in mice. Mice are strongly motivated to build nests not only for breeding, but also for temperature and light regulation. In this study, the mice often made their nest in the shadow of the food hopper and/or drinking bottle as noted in the previous study (Van Loo, *et al*, 2004; Balcombe, 2006). The implantation sites at the neck region of some of the mice were scratched by the

steel food hopper; this could be due to the small size of standard cages (14 cm in width, 30 cm in length, and 15 cm in depth). This could have possibly aggravated the dehiscence of the wound stitched with silk sutures.

5.3.2 Shortcomings of the isolator

In all likelihood, *Staphylococci* were transmitted between the mice within the isolators after implantations. An accident of Glycerol flowing back from the liquid filter into the isolator could have been the reason that nine of 14 mice showed *Staphylococcus species*. Two mice showed bacteria isolates of *S. aureus* and 7 mice showed isolates of CNS from inside the cysts at harvest. Eight of 10 mice that were maintained in another isolator, also showed isolates of *Staphylococcus species* at harvest. These included 1 mouse from which *S. aureus* was isolated and 7 mice from which CNS was isolated. In these 17 mice, *S. aureus* was isolated only from one mouse and CNS was isolated form another mouse before implantation.

Microbiological reports on the random samples of air from inside the isolator showed no bacterial growth. It should be noted that the microbiological sampling of air was not optimal due to a lack of an effective sampling protocol and a quantifiable measure of success of the decontamination for monitoring of the isolators in this study.

The isolator filters were dust filters constructed from three layers of fiberglass filter material. The filters were then wrapped in paper bags and sterilized by an autoclave. A plastic shield carrying a hose adaptor was placed around the filter and sealed by 3M® stretchable tape. It is possible that the plastic shield might not have provided an adequate seal and hence prevention of microbiological contamination could have been compromised.

The type of filter used also makes a difference in the degree of decontamination of isolators. The high efficiency particulate air (HEPA) filters which have an efficiency rate of 99.97% are being used in various high technology industries, such as aerospace, pharmaceutical processing, hospitals, healthcare, nuclear fuels, nuclear power, and electronic microcircuitry (computer chips) (DOE, 1997). These filters have the ability of removing particles the size of 0.3 microns from the air. These filters filters are critical in the prevention of the spread of airborne bacterial and viral organisms. When the HEPA filters are used in a medical environment, the filtration systems also incorporate high-energy ultra-violet light units which kill off the live bacteria and viruses that might be trapped by the filter media. Some of the best-rated HEPA units have an efficiency rating of 99.995%, which assures a very high level of protection against transmission of the airborne disease (Zajac, 2006).

5.4 Comparison of the cyst model with the Thompson studies

In this study, the non-keratinised stratified squamous epithelial lining of the implanted cysts was microscopically similar to that of the vaginal mucosa from which the cysts were constructed. It is well documented that the cyst lining forms at 6 weeks after the implantation, but the sutured edges of the cysts healed completely at 9 weeks (Thompson *et al*, 1996). There was not a remarkable difference in the number of the cell layers between the 9 weeks old cysts and the control vaginal epithelium. However, there was a statistical difference in the number of cell layers between the 10 weeks old cysts and the donor vaginal epithelium, as noted in the studies by Thompson and his colleagues (1996, 2001b, 2002). In some individual cysts which contained larger amounts keratin, the cyst lining was thinner than that of the donor vaginal epithelium. This could possibly be attributed to an increased intraluminal mechanical pressure on the cyst epithelium due to the continuous production of keratin (Fig 18A). This increased pressure may also result in the lack of rete pegs at the epithelium/ connective tissue junction. The feature of the thinner cyst lining could also

possible be due to the lack of re-establishment of the new blood supply which was as representative of the nutritional capacity of the cyst model. Marked new blood supply resulted in the thicken regions of the epithelial layer of the implanted artificial cyst.

The goals of the present study were to identify the common pathogens and factors that could adversely affect the establishment and maintenance of the mucosal cysts and to offer significant improvements in infection control of the cyst model constructed from human vaginal mucosa in athymic nude mice, which was established by Thompson and co-workers (1996, 2001b, 2002). However, the successful rate of this study was not better than that of Thompson studies and the contaminants were isolated from 75% mice after 9 weeks. It could be attributed to the shortcomings of the isolators in the substandard animal housing room.

5.5 Toxicity of peracetic acid (PAA)

Toxicity of PAA is high via oral administration to guinea pigs, and moderate via oral and dermal routes for rats and rabbits. Skin, Oral and Eye Irritation Data: skin-rabbit LD50 1410 mg/kg; oral-rat LD50 1540mg/kg, eye-rabbit 1 mg SEV (OMRI, 2000a, UPSC, 2000). It is caustic and corrosive at concentrations >10% and irritative concentrations below 2%. The irritant of the skin, eyes, mucous membranes, and respiratory tract produces the burns, inhalation and ingestion injuries.

In this study, PAA proved to be toxic by inhalation and eye irritation, but was most probably also absorbed through the skin. One mouse that was transferred from the laminar air-flow unit to the isolator via the port sterilized with PAA, became blind after being present inside the port for 2 minutes. The animal subsequently died 5 days later. The death of two other mice could also be attributed to the possible leakage of PAA from the port cover due to their frequent proximity to the port. These mice presented with persistent anorexia and lethargy.

For the present study, PAA was acquired as acetic acid (100%) and hydrogen peroxide (30%). These two chemicals were mixed 10 days prior to use. It is also believed that there could have been an error in the strength of PAA mixed in our laboratory. Enviro Tech Chemical Services, Inc., USA (2006) described the process of determining hydrogen peroxide and peracetic acid content of a PAA sample at equilibrium as an extremely difficult process.

A new formulation has recently been developed: Perasafe®. This consists of a fine powder which contains a combination of peroxygen compounds, organic acids and stabilizers. This formulation liberates peracetate ions equivalent to 0.26% PAA into the medium. It can be manipulated easily using with gloves and there is no release of toxic residues (Vizcaino-Alcaide, *et al*, 2003). Health and Hygiene Inc. (2004) have also released another low irritative disinfectant, F10 SC Veterinary Disinfectant. This has been introduced to use at animal facilities in recent years.

5.6 Other problems encountered in this study

Ulceration of cyst linings without infection: This feature was observed in 8 mice in which there was partial cyst formation (Fig 22A). This could be attributed to the destruction of the vaginal epithelium by forceps during the vaginal hysterectomies or during manipulation and manufacture of the artificial cyst.

Atrophic cysts: This feature could possibly have been due to pressure exerted by the silastic tube on the vaginal mucosa, which were relatively small in size, with a resultant decreased blood flow and possibly ischemia.

Intracystic fibrosis: Unusual fibrosed human connective tissue was present in a single cyst cavity; the cyst surrounded by granulation tissue. This phenomenon could possibly have resulted from reversal of the vaginal epithelium during the construction of the artificial cyst; hence the vaginal epithelium faced the murine connective tissue directly.

Epithelial islands in connective tissue This phenomenon is believed to be due to the vaginal epithelium of the artificial cyst at the edges of the sutures being implanted into the connective tissue and is unlikely to affect the cyst growth.

Fibrosis at junction of the host connective tissue and implanted connective tissue: It was observed that marked fibrosis developed at the junction of mouse connective tissue and implanted human vaginal connective tissues that of three specimens were not treated with EDTA prior to cyst construction and implantation due to time constraint. The resultant fibrosis is a normal defence mechanism of the mouse and is not likely to affect the cyst lining.

5.7 Conclusion

We have provided histological evidence that the structure of the epithelium of the implanted cyst retained the main characteristics of the vaginal epithelium from which it was constructed. We have also reaffirmed that this experimental cyst model is potentially suitable as an *in vivo* biotest model for studies involving the oral mucosa. In this study, we showed that the coagulase negative *staphylococci* were the most common pathogenic microorganisms and caused necrosis of the cyst epithelium. Severe inflammation was seen around the suture area of the skin wounds due to the contamination. Gentamicin proved to be more efficient against Gram-negative contaminants than Terramycin. It was also found that the nylon suture was the appropriate non-absorbable suture in minimizing the infection around the experimental cysts. An alternative low irritative disinfectant should be chosen and the prevention of microbiological contamination was compromised when the isolators were used in the substandard animal room.

CHAPTER 6: RECOMMENDATIONS

Further recommendation for future research:

1) Substandard housing condition of the isolators must be avoided at all times;
2) An effective protocol should be designed to monitor the isolators;
3) The high efficiency particulate air (HEPA) filter could be a suitable filter for sterilizing the air of the isolator;
4) Peracetic acid should be used at concentrations blow 2%; and alternative low irritative disinfectant should rather be used;
5) Vancomycin plus Gentamicin could be applied to minimize the infection of *coagulase negative staphylococci*;
6) Opsite dressing could be used post-operative period to limit the leakage of the wound.

This model could be used as a *in vivo* biotest model to perform studies on:

1) The combined chemical and viral carcinogenicity of the oral and upper airway mucosa; of particular interest are:
   a) the absorption and distribution characteristics of these carcinogens in the sub-epithelial tissues of the cyst,
   b) the proliferative and (pre-) malignant epithelial changes in response to these viruses and carcinogens,
   c) the molecular biological mechanisms underling these changes.
2) The pathogenesis of various other mucosal conditions, in particular of inflammatory, viral and immunopathological nature.
3) The molecular genetics, proteonomics and growth factors in a cyst model.
4) The receptor of virus on the oral and vaginal mucosa
5) The local and systemic effects of drugs and immunisations on diseases that involve oral mucosa.

NOTE ABOUT COPYRIGHT

I, Meng Wang, hereby cede to the University of the Western Cape the entire copyright related to any aspect or results of this study that may in future be part of/included in any research report or thesis submitted by me to the department of Immunology of the University of Sherbrooke (Quebec) in [partial] fulfilment of the requirements for the degree of PhD.

Meng Wang

Date: 2007-6-25

REFERENCE

Abrams, M.B., Andrews, J. E., and Laskin, D. M. (1977): Epidermoid (implantation) cyst after temporomandibular joint surgery. *Journal of Oral Surgery*, 35:587-589.

Adamsons, R. J. and Kahan, S.A. (1970): The rate of healing of incised wounds of different tissues in rabbits. *Surgery Gynecology & Obstetric* 130: 837-846.

AL (Apex Laboratories) (2004): GENTAPEX-50 ANTIBIOTIC INJECTION. Apex Laboratories Pty. Ltd, Somersby, Australia. [Online] Available http://www.apexlabs.com.au/veterinary/

Archer, G. L. and Climo, M. (1994): Antimicrobial susceptibility of coagulase-negative staphylococci. *Antimicrobial Agents and Chemotherapy*, 38 (10): 2231-2237.

Baker, B. R. and Mitchell, D. F. (1965): The pathogenesis of epidermoid implantation cysts. *Oral Surgery, Oral Medicine, and Oral Pathology*, 19:494-501.

Balcombe, J. P. (2006): Laboratory environments and rodents' behavioural needs: a review. *Laboratory Animals*, 40 (3): 217-235.

Bartlett, P.F., Radden, B.G. and Reade, P.C. (1973): The experimental production of odontogenic keratocysts. *Journal of Oral Pathology*, 2:58-67.

Boyce, J.M., Potter-Bynoe, G., Opal, S.M., *et al*. (1990): A common source outbreak of *Staphylococcus epidermidis* infections among patients undergoing cardiac surgery. *Journal of Infectious Diseases,* 161:493-99.

CDC NNIS System (Centers for Diseases Control and Prevention, National Nosocomial Infections Surveillance system) (1997): National Nosocomial Infections Surveillance (NNIS) Report, Data Summary from October 1986 to April 1997. *American Journal Infection Control* 25: 477-87.

Chang, F-Y., McDonald, B.B., Peacock, Jr. J.E., *et al* (2003): Staphylococcus aureus bacteremia: recurrence and the impact of antibiotic treatment in a prospective multicenter study. *Medicine* 82:333-339.

Chang, W., Small, D. A., Toghrol, F., *et al* (2005): Microarray Analysis of Toxicogenomic Effects of Peracetic Acid on *Pseudomonas aeruginosa*. *Environmental Science and Technology*, 39(15): 5893-5899.

Chu, C.C., Tsai, W.C., Yao, J.Y., *et al* (1987): Newly made antibacterial braided nylon sutures. I. *In vitro* qualitative and *in vivo* preliminary bio-compatibility study. *Journal Biomedical Material Research,* 21 (11): 1281- 1300.

Cruse, P. J. (1992): Surgical wound infection. In: Wonsiewicz, M.J., ed. *Infectious Diseases,* Philadelphia: W.B. Saunders Co; pp. 758-64.

Dale, J. (1997): Wound dressings. *Professional Nurse* 12: S12–S14, S18.

Dawson, J. (1989): *Infection control, the British Medical Association Guide*. Edward Arnold, Great British, 1989: pp41-65.

DOE (Department of Energy, USA) (1997): DOE STANDARD specification for HEPA filters used by DOE contractors. Washington D.C., United States [Online] Available http://www.eh.doe.gov/techstds/standard/std3020/std3020.pdf

Edmiston, C.E., Seabrook, G.R., Cambria, R.A., *et al*. (2005): Molecular epidemiology of microbial contamination in the operating room environment: Is there a risk for infection? *Surgery*, 138 (4): 573-582.

Enviro Tech Chemicals Services, Inc (2006). Determination of hydrogen peroxide and peracetic acid in solutions. [Online] available http://www.envirotech.com/peraceticacid

Epstein, W. L. and Kligman, A. M. (1957): Epithelial cysts in buried human skin. *Archives of Dermatology*, 76: 437-445.

Ettinger, R. L. and Manderson, R. D. (1973): Implantation keratinizing epidermoid cysts. *Oral Surgery, Oral Medicine, and Oral Pathology*, 36:225-230.

FBR (Foundation for Biomedical Research) (2003): Rats and Mice. The Essential Need for Animals in Medical Research. [Online] Available http://www.fbresearch.org/Education/pdf/RatsAndMice.pdf

Health and Hygiene Pty Ltd (2004): F10 SC veterinary disinfectant. [Online] Available http://www.healthandhygiene.net/products

HPA (Health Protection Agency, UK) (2006a): Enterbacter spp. [Online] Available http://www.hpa.org.uk/infections/topics_az/enterobacter

HPA (Health Protection Agency, UK) (2006b): Enterococcus spp and GRE. [Online] Available http://www.hpa.org.uk/infections/topics_az/enterococci

HPA (Health Protection Agency, UK) (2006c): Klebsiella. [Online] Available http://www.hpa.org.uk/infections/topics_az/klebsiella

HPA (Health Protection Agency, UK) (2006d): Pseudomonas. [Online] Available http://www.hpa.org.uk/infections/topics_az/pseudomonas

Heaney, T.G. (1977): A histological investigation of the influence of adult porcine gingival connective tissues in determining epithelial specificity. *Archives of Oral Biology*, 22:167-174.

Holmstrup, P., Dabelsteen, E. and Roed-Petersen, B. (1981a): Oral leukoplakia transplanted to nude mice. *Scandinavian Journal of Dental Research,* 89:275-282.

Holmstrup, P., Dabelsteen, E., Reibel, J. and Harder, F. (1981b): Normal keratinized mucosa transplants in nude mice. *Acta Odontology Scandinavia*, 39:187-193.

Holmstrup, P., Dabelsteen, E., Stoltze, E. and Worsaae, N. (1981c): Epithelial outgrowths from human buccal mucosa transplants in nude mice. *Scandinavian Journal of Dental Research*, 89:417-423.

Holmstrup, P., Stoltze, E., Bretlau, P. and Dabelsteen, E. (1981d): Human buccal mucosa transplants in nude mice. *Scandinavian Journal of Dental Research,* 89:89-96.

Huebner, J. and Goldmann, D. A. (1999): Coagulase-Negative Staphylococci: Role as Pathogens. *Annual Review of Medicine,* 50: 223-236.

Huycke, M.M., Sahm, D.F., and Gilmore, M.S. (1998): Multiple-Drug Resistant *Enterococci*: The Nature of the Problem and an Agenda for the Future. *Emerging Infectious Diseases,* 4 (2): 239-249.

ILAR (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, USA) (1991): Companion Guide to Infectious Diseases of Mice and Rats. National Academy Press, Washington, D.C.

ILAR (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, USA) (1996): Guide for the care and use of laboratory animals. National Academy Press. Washington, D.C. pp 21-70.

J&J (Johnson and Johnson Inc) (2004): Ethicon suture. Johnson and Johnson Gateway, [Online] Available http://www.jnjgateway.com/public/USENG/Ethicon_WCM_Feb2004.pdf

Klein, J., Secosky, W.R. and Klein, D. (1971): Tooth transplantation in the mouse-1: the use of porcion dyes and tritiated proline in a study of syngenic tooth germ transplantation. *American Journal of Anatomy*, 131:371-386.

Kloos, W. E. and Bannerman, T. L. (1994): Update on Clinical Significance of Coagulase-Negative Staphylococci. *Clinical Microbiology Reviews*, 7 (1): 117-140.

LaMorte, W.W. (2002): Basics of Wound Closure and Healing. Department of Surgery, Boston University Medical Campus, [Online] Available http://www.bumc.bu.edu/Dept/Content.aspx?DepartmentID=69&PageID=6067

Leclercq, R., Derlot, E., Duval, J., *et al* (1988): Plasmidmediated resistance to vancomycin and teicoplanin in *Enterococcus faecium*. *National English Journal of Medicine*, 319:157-61.

Lofgren, L.A., Lindholm, C-E. and Jansson, B. (1984): The autogenous mucosal cyst. An experimental study with special reference to reconstructive surgery of the airway. *Acta Otolaryngology*, 98:171-184.

Lin, K. Y. and Long, III, W. B. (2005): Scientific Basis for the Selection of Sutural Skin Closure. Dannemiller Memorial Educational Foundation. [Online] Available http://www.woundclosures.com/home.cfm

Mackenzie, I.C. (1984): Epithelial-connective tissue relationships and the development and maintenance of structure. In: Meyer, J., Squier, C.A., Gerson, S.J. (ed.): *The Structure and Function of Oral Mucosa.* Pergamon Press 1984, pp.119-140.

Mackenzie, I.C., Dabelsteen, E., and Roed-Petersen, B. (1979): A method for studying epithelial-mesenchymal interactions in human oral mucosal lesions. *Scandinavian Journal of Dental Research*, 87:234-243.

Mangram, A.J., Horan, T.C., Pearson, M.I., *et al* (1999): Guideline for prevention of surgical site infection, 1999. *Infection Control and Hospital Epidemiology*, 20 (4): 247-278.

Martinez, F., Martin-Luengo, F., Garcia, A., *et al* (1995): Treatment with various antibiotics of experimental endocarditis caused by penicillin-resistant *Streptococcus sanguis. European Heart Journal,* 16 (5): 687-691.

Moy, R.L., Lee, A., Zalka, A. (1991): Commonly used suture materials in skin surgery. *American Family Physician,* 44(6): 2123-8. Review.

OMRI (Organic Material Review Institute) (2000a): Peracetic Acid - livestock. *NOSB TAP Review.* [Online] Available http://www.ams.usda.gov/nop/NationalList/TAPReviews/PeraceticAcid2.pdf

OMRI (Organic Material Review Institute) (2000b): Peracetic Acid - processing. *NOSB TAP Review.* [Online] Available http://www.omri.org/peracetic_acid3.pdf

Papanayotou, P. H. and Kayavis, J.G. (1977): Epidermoid implantation cyst of the lower lip: report of case. *Journal of Oral Surgery*, 35:585-586.

Pearse, J. (1997): *Infection control manual.* Jacana Education, Republic of South Africa, 1997: pp51-107.

Poole, T. B. (1987): *The UFAW handbook on the Care and Management of Laboratory Animals*. –Sixth Ed. Longman Scientific & Technical, Bath Press, Avon, UK. 1987.

Postlethwait, R.W., Willigan, D.A., Ulin, A.W. (1975): Human tissue reaction to sutures. *Annal of Surgery*, 181: 144-150.

Pudner, R. (2001): Hydrocolloid dressings in wound management. *Journal of Community Nursing,* 15 (4): 44–48.

Ramanathan, J. and Philipsen, H. P. (1981): In vivo behaviour of intraosseously implanted oral epithelium in rats. *International Journal of Oral Surgery,* 10:180-188.

Rich, A. M. and Reade, P. C. (2001): Epithelial-mesenchymal interactions in experimental oral mucosal carcinogenesis. *Journal of Oral Pathology and Medicine,* 30:389-397.

Righter, J. (1987): Treatment of coagulase-negative staphylococcal infections: dilemmas for laboratory and clinician. *Journal of Antimicrobial chemotherapy*, 20:459-465.

Riviere, G.R. and Sabet, T.Y. (1973): Experimental follicular cysts in mice: a histological study. *Oral Surgery, Oral Medicine, and Oral Pathology,* 36: 205-213.

Sahm, D.F., Kissinger, J., Gilmore, M.S., *et al* (1989): In vitro susceptibility studies of vancomycin-resistant *Enterococcus faecalis*. *Antimicrobial Agents and Chemotherapy*, 33:1588-91.

Secasan, I., Pop, D.I., Secasan, C.C. (2005): Treatment of Acute and Chronical Bacterial Prostatitis Caused by Staphylococcus Epidermidis. *Science Blog* [Online] available http://www.scienceblog.com/cms/node/8838

SHEA, APIC, CDC, SIS. (1992): Consensus paper on the surveillance of surgical wound infections. *Infection Control and Hospital Epidemiology*, 13 (10): 599-605.

Shelburne, S.A., Musher, D.M., Hulten, K., *et al* (2004): In-vitro killing of community-associated methicillin-resistant *Staphylococcus aureus* with drug combinations. *Antimicrobial Agents and Chemotherapy*, 48:4016-4019.

Soskolne, W.A., Bab, J. and Sochat, S. (1976): Production of keratinizing cysts within mandibles of rats with autogenous gingival epithelial grafts. *Journal of Oral Pathology*, 5:122-128.

Squire, C.A., Johnson, N.W. and Hopps, R.M. (1976): *Human oral mucosa. Development, structure and function.* Blackwell Scientific Publications, Oxford 1976.

Taylor, D.M. (1975): The use of laminar flow for obtaining germ-free mice. *Laboratory Animals*. 9 (4): 337-43.

Taylor-Robinson, D. and Furr, P.M. (2000): Observations on the antibiotic treatment of experimentally induced mycoplasmal infections in mice. *Journal of Antimicrobial Chemotherapy*. 45 (6): 903-7.

Thaddeus, G. G. and Shafter, W. G. (1958): Surgical ciliated cyst of the maxilla: report of case. *Journal of Oral Surgery*, 16:251-253.

Thomas, S. (1996): Vapour-permeable film dressings. *Journal of Wound Care*, 5 (6): 271–274.

Thompson, I.O.C. (2002): The establishment, development and evaluation of a non-keratinised experimental cyst as a biotest model. *Unpublished PhD's Thesis. Stellenbosch: University of Stellenbosch.*

Thompson, I.O.C. and Van Wyk, C.W. (1996): An experimental cyst model established from human unkeratinized vaginal mucosa. *British Journal of Oral and Maxillofacial Surgery*, 34:530-532.

Thompson, I.O.C. and Van Wyk, C.W. (1989): Separation of viable epithelium and lamina propria with ethylenediamine tetraacetate (EDTA): an evaluation. *South African Journal of Science*, 85:537-539.

Thompson, I.O.C., Van der Bijl, P., Van Wyk, C.W., *et al* (2000): Comparison of the permeability of vaginal mucosa and the wall of an experimental cyst developed from it. *South African Journal of Science*, 96:385-386.

Thompson, I.O.C., Van der Bijl, P., Van Wyk, C.W., *et al* (2001a): A comparative light-microscopic, electron-microscopic and chemical study of human vaginal and buccal epithelium. *Archives of Oral Biology*, 46:1091-1098.

Thompson, I.O.C., Van Wyk, C.W. and Daling, M. R. (2001b): Human vaginal epithelium and the epithelial lining of a cyst model constructed from it: a comparative light microscopic and electron microscopic study. *South African Dental Journal*, 56:517-520.

Todar, K (2002): Antimicrobial Agents Used in Treatment of Infectious Disease. *Todar's Online Textbook of Bacteriology*. Department of Bacteriology, University of Wisconsin-Madison. [Online] Available http://textbookofbacteriology.net/antimicrobial.html

Todar, K. (2005): Staphylococcus. *Todar's Online Textbook of Bacteriology*. Department of Bacteriology, University of Wisconsin-Madison. [Online]Available http://textbookofbacteriology.net/staph.html

Torres, J. S. and Higa, T. T. (1970): Epidermoidal cyst in the oral cavity. *Oral Surgery, Oral Medicine and Oral Pathology,* 30:592-600.

UCCAA (University of California, Center for Animal Alternatives) (2003): *The Mouse in Science.* University of California, Davis, California, USA [Online] Available http://www.vetmed.ucdavis.edu/Animal_Alternatives/mouse.htm

UPSC (The United States Pharmacopeial Convention, Inc) (2000): UPS24/NF19. *U.S. Pharmacopoeia and National Formulary*. Published by the Board of Trustees. National Publishing, Philadelphia, PA, 1999. pp1585-1586.

Van der Bijl, P. and Van Eyk, A. D. (1999): Penetration of benzo[a]pyrene through human buccal and vaginal mucosa. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontics,* 87:452-455.

Van der Bijl, P., Penkler, L. and Van Eyk, A. D. (2000): Permeation of Sumatriptan through human vaginal and buccal mucosa. *Headache,* 40:137-141.

Van der Bijl, P. and Thompson, I.O.C. (1998): Effect of aqueous areca nut extract on the permeability of mucosa. *South African Journal of Science,* 94:241-243.

Van der Bijl, P., Thompson, I.O.C. and Squier, C. A. (1997): Comparative permeability of human vaginal and buccal mucosa to water. *European Journal of Oral Science,* 105:571-575.

Van der Bijl, P., Van Eyk, A. D. and Thompson, I.O.C. (1998a): Permeation of 17β-estradiol through human vaginal and buccal mucosa. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontics,* 85:393-398.

Van der Bijl, P., Van Eyk, A. D. and Thompson, I.O.C. (1998b): Diffusion rates of vasopressin through human vaginal and buccal mucosa. *European Journal of Oral Science,* 106:958-962.

Van der Bijl, P., Van Eyk, A. D. and Thompson, I.O.C. (1998c): Penetration of human vaginal and buccal mucosa by 4.4-kd and 12-kd fluorescein-isothiocyanate-labeled dextrans. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontics,* 85:686-691.

Van der Bijl, P., Van Eyk, A. D. and Thompson, I.O.C. (1998d): Effect of freezing on the permeability of human buccal and vaginal mucosa. *South African Journal of Science*, 94:499-502.

Van Eyk, A. D. and Van der Bijl, P. (1998): The culture of human buccal and vaginal epithelial cells for permeability studies. *South African Dental Journal*, 53:497-503.

Van Loo, P. L. P., Van de Weerd, H. A., Van Zutphen, L.F.M., *et al*, (2004): Preference for social contact versus environmental enrichment in male laboratory mice. *Laboratory Animals*; 38 (2): 178-188.

Van Loo, P. L. P., Van Zutphen, L.F.M., and Baumans, V. (2003): Male management: coping with aggression problems in male laboratory mice. *Laboratory Animals*; 37 (4): 300-313.

Van Winkle, Jr. W., Hastings, J.C., Barker, E., *et al* (1975): Effect of suture materials on healing skin wounds. *Surgery Gynecology & Obstetric* 140:7-12.

Van Wyk, C.W. and Thompson, I.O.C. (1992): A model for epithelial growth and epithelium-connective tissue interaction studies. *Journal of the Dental Association of South Africa,* 47:234-236.

Vedtofte, P., Holmatrup, P., and Dabelsteen, E. (1982): Human odontogenic keratocyst transplants in nude mice. *Scandinavian Journal of Dental Research*, 90:306-314.

Vizcaino-Alcaide, M. J., Herruzo-Cabrera, R., and Fernandez-Acenero, M. J. (2003): Comparison of the disinfectant efficacy of Perasafe and 2% glutaraldhyde in *in vitro* test. *Journal of Hospital Infection*, 53:124-128.

Von Eiff, C., Proctor, R.A. and Peters, G. (2001): Coagulase-negative staphylococci. Postgraduate Medicine, vol 110, no 4. [Online] Available http://www.postgradmed.com/issues/2001/10_01/eiff.htm

Wieser, M. and Busse, H-J. (2000): Rapid identification of *Staphylococcus epidermidis. International Journal of Systematic and Evolutionary Microbiology*, 50 (3): 1087–1093.

Wynne, R., Botti, M., Stedman, H., *et al* (2004): Effect of three wound dressings on infection, healing comfort, and cost in patients with sternotomy wounds: A Randomized Trial. *Chest,* 125: 43-49.

Zajac, T. (2006): HEPA air purifiers. Clearfilte Air Purifiers, Inc. [Online] Available http://www.airpurifiers.com/technology/hepa.htm

APPENDIX I: Preparation of peracetic acid (PAA)

- Acetic Acid (100%) (CH_3COOH, 1L=1.05Kg) (Merck® [Pty] Ltd, Wadeville, SA. Licence from Merck, German)

- Perhydrol (30%) (H_2O_2, 1L=1Kg) (Merck® [Pty] Ltd, Wadeville, SA. Licence from Merck, German)

Recipe

- 450ml Acet acid
- 100ml Perhydrol
- Prepared 10 days prior to use

APPENDIX II: Terramycin/ Vitamin cocktail

- Terramycin (5% oxytetracyclin) powder (Highveld Biological [Pty] Ltd, Sandringham, SA)

- Vitamins MEM (100x) (Cat 216, Highveld Biological [Pty] Ltd, Sandringham, SA)

Recipe

Given after implantation

- 1g Terramycin powder in 100ml sterilized water
- 1ml Vitamin MEM / 100ml drink water
- 50ml per mouse every third day- freshle prepared,

APPENDIX III: Animal husbandry information form

Animal no:
Ref no:
Date of arrival
Date of implantation:

Date	Weight	Food and water	Injection	Health	Room temperature

APPENDIX IV

Participant information leaflet and consent form

TITLE OF THE RESEARCH PROJECT:
"*Contamination, infection and inflammation control in an experimental mucosal cyst model using athymic nude mice*"

REFERENCE NUMBER: N05/04/061
PRINICIPAL INVESTIGATORS: Prof J Hille, Dr M Wang
ADDRESS:
UWC Academic Oral Health Centre, Tygerberg
Anatomical Pathology Laboratories, Tygerberg Hospital 10th Floor, Tygerberg Hospital
Private Bag X1, 7505 Tygerberg,
Cape Town, South Africa
CONTACT NUMBER: 021-9386159 / 082-5560703

You are being invited to take part in a research project. Please take some time to read the information presented here, which will explain the details of this project. Please ask the study staff or doctor any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research entails and how you could be involved. Also, your participation is **entirely voluntary** and you are free to decline to participate. If you say no, this will not affect you negatively in any way whatsoever. You are also free to withdraw from the study at any point, even if you do agree to take part.
This study has been approved by **the Committee for Human Research at Stellenbosch University** and will be conducted according to the ethical guidelines and principles of the international Declaration of Helsinki, South African Guidelines for Good Clinical Practice and the Medical Research Council (MRC) Ethical Guidelines for Research.

What is this research study all about?

- *There is a need for a stable experimental model, which offers a closed environment similar to the oral cavity and which allows for controlled studies on the mucosal absorption mechanisms and local reaction patterns to introduced agents such as carcinogens, viruses, drugs and vaccines.*
- *Human vaginal tissue has been used as a substitute for oral mucosa in in-vitro and in-vivo experimental studies. Our implantation cyst model which is constructed from human vaginal mucosa and grown in athymic nude mice could be used as an in-vivo biotest system to study oral diseases. This project seeks to identify the contaminant microorganisms and to successfully prevent the risk of infection and failure of this cyst model by applying various antibiotic coverage modalities and eliminating other possible contaminants or irritating factors.*

Why have you been invited to participate?

- *As you are about to undergo a vaginal hysterectomy or other corrective surgical gynaecological procedure, there very likely will be some excess vaginal tissue available which otherwise will be sent to the pathology laboratory (by law) with the uterus for routine investigations.*
- *We are asking you for permission to examine this immediately after the operation and, if it looks normal, separate your healthy vaginal tissue from the main specimen before it is being stored in a fixative. The vaginal tissue will be specially treated and prepared in the laboratory and inserted into nude mice to grow an experimental cyst from it. Any tissues that do not appear normal will be examined with the uterus in the histopathology laboratory and not used for the experiment.*

What will your responsibilities be?

Your responsibilities as a participant are solely passive and consist only of agreeing to donate the excess tissues from your hysterectomy specimens for implantation into experimental nude mice.

Will you benefit from taking part in this research?

You as a participant will not directly benefit from this project.

If successful, research using this in-vivo biotest model may lead to establishment of improved medical services.

Can you sustain injury as result of your taking part in this research study?

Are there any other risks involved in participating in this study?

No, absolutely not. You will bear no more risk other than that associated with the routine hysterectomy procedure. We would like to reassure you that under no circumstances any extra tissue will be harvested other than that is included in routine hysterectomy procedures.

Who will have access to your medical records?

Only the investigators will keep a confidential laboratory logbook in which your absolute minimum data will be recorded e.g. the hospital number, age and surgical indication for the hysterectomy. All information collected will be treated as confidential, and it will for instance be included in a thesis, a publication in a professional journal, etc, without disclosing your identity.

Will you be paid to take part in this study and are there any costs involved?

No, you will not be paid to take part in the study.

There will be no costs involved for you, if your tissue is used in the experiment.

The hysterectomy procedure and the routine pathology examination are not part of the research project.

Is there any thing else that you should know or do?

You can contact Prof Jos Hille at tel 021-938 6159 if you have any further queries or encounter any problems.

You can contact the Committee for Human Research at 021-938 9207 if you have any concerns or complaints that have not been adequately addressed by your study doctor.

You will receive a copy of this information and consent form for your own records.

By signing below, I…….. agree to take part in a research study entitled:- "Contamination, infection and inflammation control in an experimental mucosal cyst model using athymic nude mice"

I declare that:

- I have read or had read to me this information and consent form and it is written in a language with which I am fluent and comfortable.
- I have had a chance to ask questions and all my questions have been adequately answered.
- I understand that taking part in this study is **voluntary** and I have not been pressurised to take part.
- I may choose to leave the study at any time and will not be penalisd or prejudiced in any way.
- I may be asked to leave the study before it has finished, if the study doctor or researcher feels it is in my best interests, or if I do not follow the study plan, as agreed to.

Signed at (*place*)……..on (*date*) …….. 2006

…….. ……
Signature of Participant Signature of Witness.

Declaration by Investigator

I (name) ……declare that:-

- I explained the information in this document to ……..
- I encouraged her to ask questions and took adequate time to answer them.
- I am satisfied that she adequately understands all aspects of the research, as discussed above
- I did/did not use a translator. (*If a translator is used then the translator must sign the declaration below.*

Signed at (*place*)……..on (*date*) …….. 2006

…….. ……
Signature of Investigator Signature of Witness.

Declaration by Translator

I (*name*) ……declare that:-

- I assisted the investigator (*name*)……. to explain the information in this document to (*name of participant*)…….. using the language medium of Afrikaans/Xhosa.
- We encouraged her to ask questions and took adequate time to answer them.
- I conveyed a factually correct version of what was related to me.
- I am satisfied that the participant fully understands the content of this informed consent document and has had all his/her question satisfactorily answered.

.
Signed at (*place*)……..on (*date*) …….. 2006

…….. ……
Signature of Translator. Signature of Witness.

APPENDIX V: Transport Medium

- Dulbecco's Minimum Essential Medium (DMEM) with 4.5g/l glucose without L-glutamine (Cat no L02-cm, Highveld Biological [Pty] Ltd, Sandringham, SA)

- 10% Fetal calf serum (Cat no 306, Highveld Biological [Pty] Ltd, Sandringham, SA)

- Antibiotic-Antimycotic mixture consisting of streptomycin (10 000 µg/ml), penicillin (10 000 units/ml), and Fongizone (25 µg/ml) (Cat no 228-cm, Highveld Biological [Pty] Ltd, Sandringham, SA),

Recipe

- 900ml DMEM
- 100ml Fetal calf serum
- 10ml Antibiotic-Antimycotic

APPENDIX VI: EDTA

- Dulbecco's phosphate Buffered Saline (DPBS) with, with (Highveld Biological [Pty] Ltd, Sandringham, SA)

- EDTA (Versene®) 0.1% (in Ca^{++} and Mg^{++} free PBS) mix 1:1 with trypsin (0.25%) (Highveld Biological [Pty] Ltd, Sandringham, SA)

Recipe: 3mM EDTA (100ml)

- 37.2ml 0.1%EDTA
- 62.8ml DPBS

APPENDIX VII

1. Evaluation form of donor vaginal tissue

Vaginal tissue number:

	Maximum num of cell layers	Minimum num of cell layers	Inflammation +/-
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			
AV			
SD			

2. Evaluation form of cyst wall

Tygerberg Reg No:
Animal number:
Ref number:
Harvest Date:

	Cyst formation or Cyst necrosis	Maximum num of cell layers	Minimum num of cell layers	Inflammation under basement +/-	Contaminant G stain +/-	Fibrosis in connective tissue +/-	Blood supply +/-
1							
2							
3							
4							
5							
6							
7							
8							
9							
10							
11							
12							
AV							
SD							

APPENDIX VIII: Anaesthetics

(1) ISOFOR® (100ml) (Safe Line Pharmaceuticals [Pty] Ltd, SA)

(2) Anaket-V® (10ml) (Ketamine 100mg/ml) (Bayer [Pty] Ltd, SA)

(3) Domitor® (10ml) (Medetomidine hydrochloride 1.0mg/ml) (Novartis South Africa [Pty] Ltd)

Dilution protocol

Take 0.1ml (2) and add 0.9ml saline (Ketamine = 10mg/ml)

Take 0.1ml (3) and add 0.9ml saline (Medetomidine = 0.1 mg/ml)

Administration

Inject subcutaneously into mouse

0.05-0.07ml (4) + 0.09-0.13ml (5) per mouse at the dose of Ketamine [25mg/kg] + Medetomidine [0.5mg/kg]

APPENDIX IX: Other antibiotics and disinfectants

- Penicillin-G solution (10,000μ/ml) (Cat no 214-cm, Highveld Biological [Pty] Ltd, Sandringham, SA)

- Gentamicin solution (1.32mg/ml) (Cat no 208-cm, Highveld Biological [Pty] Ltd, Sandringham, SA)

- Povidone Iodene (Betadine®) (Kenndon Medical Supplies [TVL] [Pty] Ltd, SA)

- Biocide® D Extra-30gm (General Medical Supplies, SA)

- Virkon® (Adcock Ingram Critical Care [Pty] Ltd, SA)