In vitro culture of Boer goat mammary epithelial cells to form a monolayer constituting a tight barrier to drug movement

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Dissertation for the fulfilment of the Degree Magister Scientae (Master of Science)

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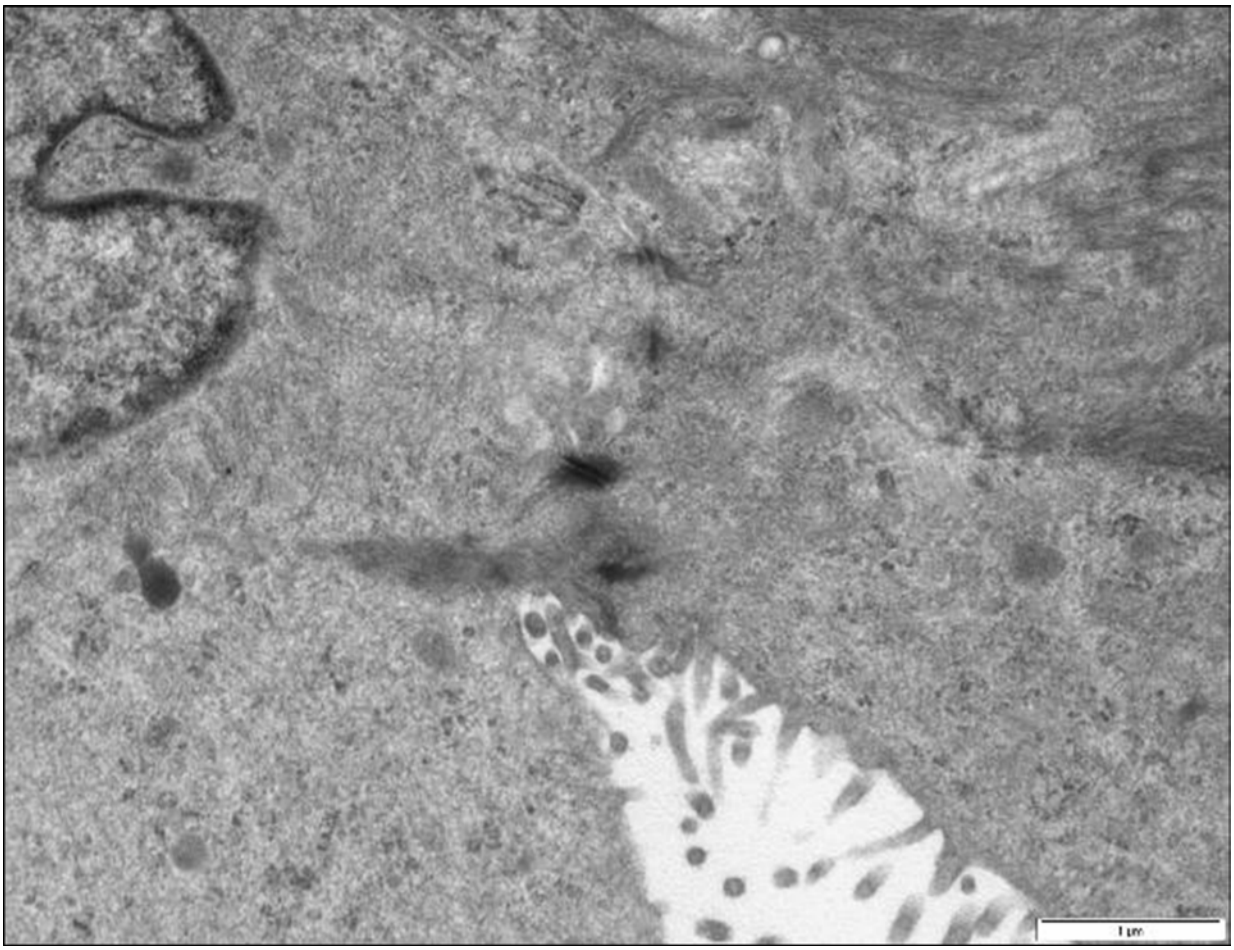

Boer goat mammary epithelial cells;
photo taken with electron microscope at 27500 times magnification.

Papers prepared from this dissertation

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List of frequently used abbreviations

CMEC/s - caprine mammary epithelial cell/s

Dextran-FITC - fluorescein isothiocyanate–dextran

HMEC/s – human mammary epithelial cell/s

TEER - transepithelial electrical resistance

T25 – growth area of 25 cm^2

MCF – Michigan Cancer Foundation-7, referring to the institute in Detroit where the human breast adenocarcinoma cell line was established

STDEV – standard deviation

FITC – fluorescein isothiocyanate

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Abstract

In rural areas of developing countries like South Africa, people typically depend on goat farming for both meat and milk production due to the shortage of grazing and the higher maintenance cost of cattle. An understanding of the functionality of the mammary gland and subsequent drug transport into milk are important factors in determining milk withdrawal periods and drug residues in milk intended for human consumption. Tight cellular monolayers, cultured to resemble the *in vivo* blood-milk-barrier, are used to evaluate the transepithelial transport of drugs into milk *in vitro*.

The aim of this study was to culture and maintain tight monolayers of primary Boer goat mammary epithelial cells that would be a barrier to paracellular drug movement. Cells were cultured and maintained similarly to the method described by Pantschenko and colleagues (2000), with some adaptations and with MCF10a as growth medium. The formation of tight barriers was evaluated by measuring transepithelial electrical resistance (TEER) and the paracellular movement of dextran-FITC.

An aggregated monolayer was established which had the characteristic cobblestone appearance, typical of epithelial cells, with no fibroblasts seen microscopically. On day 11 the monolayers appeared to be confluent under microscopic examination, they presented a significant barrier to the movement of FD70 dextran (Papp = 0.001), and the transepithelial electrical resistance (TEER) was greater than 200 $\Omega.cm^2$. At day 18 of culture, macroscopically the cells started to stack and cell debris formed, presumably due to overgrowing and cell differentiation, and the monolayers were no longer appropriate for use.

Furthermore, cryopreservation techniques were performed on the cells and these cells were frozen, stored, and regrown as viable epithelial cells.

Primary Boer goat mammary epithelial cells, cultured and maintained using the methods described in this dissertation, form tight monolayers that are a significant barrier to the paracellular movement of relatively large molecules like dextran70, with TEER values

appropriate for xenobiotic transcellular flux studies between day 11 and 18 of culture. This timeframe corresponds with the time in which drug transfer studies are typically done in cell cultures from other species.

Viable cryopreservation of Boer goat mammary epithelial cells is a useful tool that can be used to enhance these studies.

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The practical work was perfomed in the Tissue Culture Laboratory of the Department of Paraclinical Sciences, Pharmacology and Toxicology, Faculty Veterinary Science of University of Pretoria, under the guidance of Annette Venter.

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Chapter 1: Introduction

The global goat population is estimated at 861.9 million, with the largest number of goats found in Asia (59.7%) and Africa (33.8%); the goat population has shown a positive growth trend over the past three decades (Aziz, 2010). In developing countries, this growth has been attributed largely to a trend towards self-sufficiency by rural people; many rural areas in South Africa depend on goat farming for both meat and milk production, due to the shortage of grazing and the higher maintenance cost of cattle (Mmbengwa *et al.* 2000). Goat milk can also be processed into a variety of marketable products. In sub-Saharan Africa, less than 5% of the total milk produced by goats is currently marketed, and this is an area of great potential for future growth (Aziz, 2010; FAO, 2013). An estimated 3.4% of the global milk consumption of 696.5 million tons comprises milk derived from goats (FAO, 2012). Goats from developing countries produce approximately 83% of this total amount (Aziz, 2010). In South Africa in particular there is a demand for goat milk, not only due to the expanding tourism industry, but goat milk is also a popular source of nutrition for people with health problems (Olivier *et al*. 2005).

The majority of goats in the world (95%) are meat goats (Tolera *et al*. 2000). Small-scale farmers in South Africa's rural areas often do not have the financial means to acquire traditional dairy goats, like the Saanen breed, for milk production. These farmers rely on more hardy and affordable meat breeds, like Boer goats, Savannah goats and other indigenous breeds, as well as cross-breeds, for a dual purpose (Mmbengwa *et al*. 2000).

Despite the important role of goats in Africa and the importance of goat milk in the dairy industry, relatively few studies have examined drug metabolism and excretion in goats. Milk withdrawal periods for dairy goats are often erroneously extrapolated from data relating to cows, despite distinct physiological differences between the caprine and bovine mammary gland (Smith and Sherman, 2009). Furthermore, milk-drug related studies across the world

are primarily performed in traditional dairy breeds, like Saanen goats (Abo El-Sooud *et al*. 2004; Rule *et al*. 2001). Although Saanen goats are the primary commercial dairy goat breed found in South Africa, their numbers are few compared to Boer goats and mixed breeds. Recent figures for the total number of goats found in South Africa are not known but we know that there are a mere 432 registered Saanen females in South Africa versus 60 000 registered Boer goats (SA Milch Goat Association and SA Boer goat Breeders' Association, 2013).

An understanding of the functionality of the mammary gland and subsequent drug transport into milk are important factors in determining milk withdrawal periods and drug residues in milk intended for human consumption, and these aspects should therefore be explored in the Boer goat. Tight cellular monolayers, cultured to resemble the *in vivo* blood-milk-barrier, are used to evaluate the transepithelial transport of drugs into milk *in vitro* (Al-Bataineh *et al*. 2009). Developing cell culture models in humans, rats, bovine, caprine and other domestic species for *in vitro* pharmacokinetic and drug transfer studies has been a priority over the past decade (Al-Bataineh *et al*. 2009; Kimura *et al*. 2006).

Pantschenko and colleagues (2000) established and characterised a continuous caprine mammary epithelial cell (CMEC) line from Anglo-Nubian goat mammary gland biopsies. Ogorevc and colleagues (2009) did the same using Saanen goat tissue and studied various physiological pathways and gene expression in these cells. Ke and colleagues (2011) successfully immortalised a CMEC line with human telomerase reverse transcriptase (hTERT) gene, mainly for studying alveologenesis and lactogenesis. Tong and colleagues (2012) cultured lactating dairy goat mammary gland epithelial cells (DGMECs), studied milk synthesis in these cells and demonstrated the positive effect of prolactin on lactulose and β-casein secretion by these cell cultures.

To our best knowledge, no attempt has been made to evaluate the formation of tight barriers in any of these cellular monolayers. The formation of tight cellular monolayers, resembling

the *in vivo* blood-milk-barrier, is one of the essential pre-requisites for a model suitable to evaluate the transepithelial transport of drugs in an *in vitro* system (Al-Bataineh *et al*. 2009).

TEER, measured with a Volt-Ohm meter across cultured monolayers, gives an indication of the degree of tight junction formation of cells (Irvine *et al*. 1999). The confluency of a cellular monolayer is quickly determined by a sharp increase in TEER. Tracer permeability can then be used to confirm the formation of a tight barrier. Together, TEER and tracer permeability measurements reflect the paracellular pathway properties of a monolayer (Matter and Balda, 2003).

Chapter 2: Literature Review

2.1 The mammary gland

The udder of a goat is divided into two distinct halves separated by the median suspensory ligament. These two halves can easily be dissected by cutting along the medial suspensory ligament, but there are no evident gross anatomical barriers between either side of the udder. Each half is however an independend functioning gland and delivers synthesised milk through its own teat (Nickerson and Akers, 2002).

The parenchyma of the udder is composed of different types of tissue. The alveoli are tiny sac-like structures that are the milk-producing units. They have an internal lumen that is lined with milk-secreting epithelial cells and a single layer of cuboidal to columnar epithelium. Milk precursors are absorbed from blood capillaries adjacent to the alveoli by these mammary epithelial cells and converted into milk protein, lactose and fat. Each alveolus is surrounded by muscle cells which contract during 'milk let-down' (Nickerson and Akers, 2002). Clusters of alveoli are separated from other clusters by fibrous connective tissue and each cluster is referred to as a lobule. A cluster of lobules forms a lobe. The lobes makes up the glandular tissue of the udder. A series of tubular ducts drain the glandular tissue. The ducts draining the lobes are composed of a double-layered epithelium and are surrounded by myoepithelial cells. These ducts come together to form larger ducts that eventually drain into the teat cisterns. This structure of the mammary gland can be described as compound, branched and tubulo-alveolar. Mammary tissue also includes stroma or connective tissue, consisting of fibroblasts and fatcells (Nickerson and Akers, 2002).

Growth of the lactating tissue is dependent mainly on two hormones, estrogen and progesterone. Estrogen, which is cyclic, stimulates the duct development of the mammary gland. Progesterone is almost continuously secreted during pregnancy, causing secretory

tissue development. As the gestation period nears its end, the mammary glands become capable of producing milk. After parturition, the rate of milk secretion increases for some time, before it reaches a peak, and then it gradually declines. A net loss of secretory epithelial cells and a decline in their function occurs during involution (Nickerson and Akers, 2002).

2.2 Biological barriers

The mammalian body contains various rate-limiting barriers to xenobiotics. Such barriers include the blood-brain-barrier, the blood-GIT-barrier, the blood-testis-barrier and the blood-milk-barrier (although not technically correct, it is also sometimes referred to as the blood-mammary-barrier or blood-udder-barrier). These barriers are diffusion barriers, which prevent the influx of most compounds from blood into the protected organ. Suitable compounds can move across these barriers by a series of processes, including passive diffusion and more complex transport mechanisms, such as facilitated diffusion, active transport, ion-pair transport and pinocytosis (Schrickx and Fink-Gremmels, 2007). Active transport mechanisms in particular have received a lot of attention from researchers during preceding years.

The permeability of biological membranes, which differs substantially between different barriers, is one of the most important determinants of the rate at which drugs move through the body. Although it is often accepted that many drug substances cross biological barriers by passive diffusion, carrier-mediated mechanisms might account for the majority of transmembrane drug movement in biological systems (Sugano *et al*. 2010).

Mammary epithelial cells are found lining the alveoli of the udder. Tight junctions between these cells form the blood-milk-barrier, which is in part responsible for determining the composition of milk. This barrier is of particular importance as milk is consumed not only by offspring, but in the case of cow, sheep and goat milk, also by humans. Farm animals are

regularly treated for ailments with conventional medicines, for example antibiotics. The presence of residues from these drugs in milk could lead to adverse reactions in the consumer, with potential devastating consequences. It is therefore important to establish wich substances are able to cross the blood-milk-barrier and which are not (Wilson *et al*. 1980).

Several *in vivo* studies have indicated the presence of active transporters in the blood-milk-barrier. Milk:plasma ratios are higher than values calculated using the physiochemical properties of the drug such as the pH partition, partition coefficient into the milk lipid phase and the degree of protein binding (Al-Bataineh *et al.* 2010; Ito and Lee, 2003). Thus, incorporating carrier-mediated processes into the prediction models for drug secretion into milk has become essential. Cell culture models have proven very useful in studying carrier-mediated drug transport (Al-Bataineh *et al*. 2009; Barar *et al.* 2009; Kimura *et al.* 2006).

2.3 Drug transfer studies in goats

Historically, and in several aspects even today, pharmacological extrapolations are made between cattle and goats, this despite distinct physiological differences between the two species (Smith and Sherman, 2009). The mammary gland of the cow and doe differs in gross anatomy, histology, their response to hormones and in the composition of the milk they produce (Sejrsen *et al*. 2006).

The first extensive *in vivo* study investigating the pharmacokinetics of drugs and their excretion into milk in lactating does was done in the 1970's (Ziv and Rasmussen,1974). A few *in vivo* studies followed, in particular looking at the distribution and elimination of parenterally administered antibiotics (Jha *et al.* 1989; Patel *et al*. 2011; Rule *et al*. 2001; Verma and Roy, 2006). The majority of *in vivo* studies focused on studying intra-mammary compounds (Karzis, 2005). Despite the advantages of using an *in vitro* system to study drug transfer, CMECs for use in drug transfer has not been evaluated in goats to date. Recently

Halwachs and colleagues (2014) developed a novel MDCKII (Madin-Darby canine kidney II cells) *in vitro* model that expresses important drug transporters found in the caprine mammary gland, for assessing drug interactions. They consider this as an appropriate alternative to primary CMECs for studying the regulation of transporters and drug interactions (Halwachs *et al*. 2014)

2.4 Cell culture models for drug transfer

Epithelial cells grown as monolayers on permeable support structures can be used as *in vitro* models to investigate the movement of drugs across various epithelial barriers. These cell culture models need to express morphology and function similar to that of the *in vivo* cell layers (barriers) (Wilson *et al*. 1989). Models that have been developed successfully for this purpose include pulmonary epithelial cells as a model to investigate drug absorption across the human-air-blood barrier (Bur, 2007), a corneal epithelial cell culture model to investigate drug absorption across the human cornea (Toropainen, 2007) and an intestinal model for drug transport into the gut (Quaroni and Hockman, 1996).

The molecular understanding of the movement of nutrients and xenobiotics in the mammary gland is still incomplete. Kimura and colleagues (2006) developed an *in vitro* human mammary epithelial cell model in order to investigate this rate-limiting barrier for xenobiotic movement from plasma into milk (Kimura *et al*. 2006). Various researchers have cultured bovine mammary epithelial cells successfully to investigate their cellular behaviour (Matitashvili, 1999). Similar studies even include other bovid species like the Asian yak (Mingchao *et al*. 2014). Al-Bataineh and colleagues (2009) were the first to use bovine mammary epithelial monolayers for the purpose of drug transfer studies.

Although there are some limitations to the use of cell culture models, these models, when used for drug transfer studies, have considerable advantages compared to *in vivo* animal studies.

These advantages include:

- A controlled environment in which certain hypotheses regarding the function of tissues and biological barriers can be tested: environmental influences like temperature and physiological conditions of the animals are eliminated, resulting in improved experimental consistency and replication (Polikov *et al*. 2008).
- Characterization and homogeneity are achieved with subcultures. These almost identical experimental replicates simplify statistical analysis of variance (Freshney, 2010).
- A better insight into the mechanism of action of structures within cells and their interaction with selected compounds can be obtained (Polikov *et al*. 2008).
- Fewer financial implications; when the effects of more than one compound, or drug interactions are to be studied, or if a study needs to be repeated, a large test population is generally required. However, once cell culture facilities are established and continuous cell lines are maintained, the mentioned trials can be done repeatedly, efficiently and with little additional cost (*personal communication, R. Gehring*).
- Fewer ethical considerations; for the purpose of pharmacological studies cell culture models may be viewed as being more humane compared to animal studies; issues relating to receiving ethical approval and maintaining ethical standards are less complicated (*personal communication, R. Gehring*).

Disadvantages of cell culture include that after a period of continuous growth, cell characteristics can change, leading to cells that are different from the cells in the original starting population, this is called cell differentiation. Cells can also with time adapt to different culture environments. The monolayers normally used are composed of only one type of cell,

which makes it substantially different from the *in vivo* situation. Cell cultures are also systems that have been isolated from the overall structure and function of the animal's body and may therefore lack some features or have some different characteristics from the *in vivo* structure. It could further be considered a disadvantage that primary cell cultures do not have the same genetic heterogeneity that are found in a population of test animals (*personal communication, R. Gehring*).

In order to fully understand biochemical and physiological processes in the body and the body's interactions with compounds, extensive *in vitro* studies can be complemented with *in vivo* studies.

Primary cell cultures, that most closely resemble the *in vivo* cell functions, can be troublesome to establish and viably maintain in culture. In order to use such primary cells in trans-epithelial drug transfer studies, a range of characteristics need to be determined. One of the essential criteria of such culture is to establish a monolayer that forms a tight barrier to free drug movement (not allowing free paracellular drug movement). When such monolayers are successfully established, amongst others, transporter protein expression and functionality in the cells should then be demonstrated (*personal communication, R. Gehring*).

2.5 Cultured mammary epithelial cells used for drug transfer studies

In vitro drug transfer studies have been done using an immortalised bovine mammary epithelial cell line (BME-UV). An immortalised cell line is a population of cells which due to a mutation, does not undergo normal cellular senescence and instead can keep undergoing division. These cells can therefore be grown for prolonged periods *in vitro* (Freshney, 2010). These cell lines have been shown to form adequate tight junctions between adjacent cells and to significantly express transporter proteins, and therefore they are a good model representing the blood-milk barrier in bovids (Al-Bataineh *et al.* 2010).

Primary cell cultures are derived directly from animal tissue and after dissection, isolation, seeding and attachment, the cells are used without further passages (Freshney, 2010); passage changes the cells and causes eventual death. Despite being more labour intensive and having less repeatability during future experiments compared to immortalised cell lines, it is expected that these primary cells are more similar to *in vivo* tissue; they closely mimic the physiological state of *in vivo* cells. Primary cell cultures are used for investigation before being immortalised (Freshney, 2005; *personal communication, R. Gehring*).

To the author's knowledge Boer goat mammary epithelial cells have not yet been cultured and comprehensively investigated for their viability for drug transfer studies.

2.6 Culture of caprine mammary epithelial cells

During the past 15 years scientists cultured caprine mammary epithelial cells to investigate several different aspects.

Pantschenko and colleagues (2000) aimed to establish and characterize a continuous caprine mammary epithelial cell (CMEC) line. They were successful in culturing more than 560 population doublings (over 80 passages) without any sign of senescence while maintaining a normal or near-normal diploid chromosome modal number. They used Anglo-Nubian goat mammary gland tissue, harvested surgically. The methods used in the current study for dissociation of Boer goat mammary tissue and the culture and enrichment of the isolated cells were based largely on their methods (discussed in Chapter 8). Pantschenko and colleagues (2000) again derived and adapted their methods from those described by Cifrian and colleagues (1994) for culturing bovine mammary epithelial cells.

Ogorevc and colleagues (2009) and Tong and colleagues (2012) focused on lactation and milk synthesis in their studies. Their culture methods included the addition of lactating hormones and they encouraged cell differentiation. Both these studies were performed using

the tissue of dairy goats (Saanen and Guan Zhong dairy goats). The primary cells used were harvested during early lactation. Ke and colleagues (2011) also used the dissociation methods of Pantschenko and colleagues (2000) on Saanen goat mammary tissue, but for maintaining the cells in culture they used MCDB171 medium, supplemented with a commercial mammary epithelial growth hormone. Their aim was to immortalise a CMEC line, mainly for studying alveologenesis and lactogenesis.

The above-mentioned studies were all aimed at investigating aspects of mammary cell biology or physiology and to our knowledge no attempt has been made to evaluate the formation of tight barriers in these cellular monolayers. The formation of tight cellular monolayers, resembling the *in vivo* blood-milk-barrier, is essential if the transepithelial transport of drugs is to be evaluated in an *in vitro* system (Al-Bataineh *et al*. 2009).

2.7 Breed-specific cell cultures

It is well-known that there are species differences in the anatomy and physiology of the mammary gland. There are also differences in the pharmacokinetic and pharmacodynamic behaviour of administered compounds in different animal species. The differences related to drug transport have been shown to include differences at cellular level. The interactions between drug and transporters differ between species, between breeds, and even individual animals. These differences are most likely due to genetic variation, or transporter polymorphisms (Real *et al*. 2011; Sepponen *et al*. 2007; Toutain *et al*. 2010; Yıldırım and Şahin, 2010).

Keys and colleagues (1989) explained that differences in milk production between Hereford cattle (a meat breed) and Holstein cattle (a dairy breed), even when milked under similar conditions, are likely not only due to differences in cell numbers, but also function per cell. Akers and colleagues (2006) further hypothesised that the differences in milk production between beef and dairy animals not only depend on an increase in the relative parenchymal

mass, but also on enhanced activity of the majority of individual secretory cells. The presence and activity of transporters in these cells are likely to play a major role in differences seen with regards to milk yield and composition (Yıldırım and Şahin, 2010).

There are distinct differences between the volume of milk production, the sustainability of lactation and the milk composition of a traditional dairy goat like the Saanen goat and a meat goat like the Boer goat (Iaschi *et al.* 2004*)*. These differences, and potentially others, could influence drug secretion and milk residues *in vivo*. The question then arises if the mammary epithelium of the lactating Boer goat (a traditional meat breed, of which the milk is also used extensively for human consumption) is different in its physiological activity compared to that of a dairy breed like the Saanen goat.

Malreddy and colleagues (*personal communication*) have observed differences in the culture behaviour of the mammary epithelial cells harvested from cows, does and sows when cultured under the exact same conditions and applying the same methodology. It is not yet known if cells harvested from different breeds from the same species behave differently in cell culture.

Chapter 3: Aim, Objectives and Justification

3.1 Aim

The aim of this study was to successfully culture and maintain viable tight monolayers of Boer goat CMECs and to determine a range of TEER-values when monolayers had formed a tight barrier to paracellular movement of a tracer. Dextran-FITC, which is a standard tracer used to determine paracellular permeability (Matter and Balda, 2003), was used to indicate the formation and maintenance of a tight barrier in the monolayers.

In order to create a homogeneous population of cells and minimize culture aging and evolution, a prerequisite is that primary cells can be preserved (Freshney, 2005). Therefore, a secondary aim was to determine whether cryopreservation of primary Boer goat cells could yield viable cells for regrowth.

3.2 Objectives

a. Establish a suitable technique to culture and maintain viable primary Boer goat CMECs

b. Determine how the confluency/tightness of the monolayers developed over time in culture

c. Monitor TEER values for Boer goat CMEC monolayers over time in culture and determine appropriate TEER values for monolayers at confluency

d. Determine if the Boer goat CMEC monolayers form a tight barrier to dextran-FITC movement

e. Determine if there is a significant difference in TEER between single and multiple trypsinised (trypsin-resistant) Boer goat CMECs

f. Determine if Boer goat CMECs maintain viability after cryopreservation

3.3 Justification

The mammalian body contains various rate-limiting barriers to xenobiotics. One such barrier is the blood-milk-barrier. An understanding of the functionality of the mammary gland and subsequent drug transport into milk are important, as these factors determine the proportion of a systemically administered drug dose that is excreted in milk, consequently determining milk withdrawal periods and drug residues in milk intended for human consumption.

Cell culture models have proven very useful in studying drug transport at the cellular level. Only cellular monolayers that form a tight barrier to free paracellular drug movement can be considered for further evaluation.

Mammary epithelial cell cultures have been established successfully in various species, including dairy goats. However, none of these cell cultures have been evaluated for their suitability for drug transfer studies. The global goat milk consumption is estimated at 23.6 million tons per annum and yet we know little about drug excretion into milk in this species.

Goats from developing countries produce the bulk of the global goat milk yield. Boer goats and Boer goat crosses are the most abundant goat breeds in Africa and are popular globally. It is likely that there are differences in cell culture behaviour and drug-transporter interactions between different breeds. Despite this, primary mammary epithelial cell cultures in Boer goats have not yet been fully explored.

The importance of this study is therefore twofold:

- not only is this the first study reporting on the behaviour of Boer goat (a primarily meat breed) CMECs in culture,
- it is also the first study exploring the potential suitability of cultured CMECs for trans-epithelial drug transport studies; the first pre-requisites of monolayers to be used for such work is that they should form a monolayer that presents a significant barrier to the movement of ions and small molecules.

Chapter 4: Primary Boer goat Mammary Epithelial Cell Culture

4.1 Introduction

The first objective of this study was to successfully culture primary Boer goat mammary epithelial cells and to maintain these cells in culture to a point where tight monolayers, that would be appropriate for efflux studies, are formed.

Pre-trial work was done testing the response of isolated cells to two different culture mediums: MCF10a, typically used for HMECs (Soule *et al*. 1990) and a combination of Typical Bovine Medium and Bovine Mammary Apical medium, typically used for bovine mammary epithelial cells (Al-Bataineh *et al*. 2009). This pre-trial work was done on both Saanen CMECs and Boer goat CMECs.

CMECs proved to be robust cells, and after the second attempt viable cells were isolated, displaying typical epithelial cell behaviour – they attached to the bottom of the cell culture flasks, they also formed monolayers with a typical cobblestone appearance and in follow-up studies became selectively permeable. The cells responded well to MCF10a and bovine medium, with no obvious differences seen between the two. MCF10a medium is more cost effective and less time-consuming to prepare and therefore it was selected as the medium of choice.

4.2 Materials and Methods

4.2.1 Mammary tissue collection

Udder tissue samples were collected from lactating Boer goat does directly after humane slaughter at an approved abattoir. Approval from an ethics committee was not a requirement as samples were harvested from animals slaughtered for meat consumption. Does were all

past third parity and had recently weaned kids. Before slaughter the udders were palpated, the milk macroscopically evaluated for signs of clumping or discolouration and a California Milk Test performed to confirm the absence of mastitis. The udder was thoroughly rinsed with 70% ethanol before sample collection commenced. Areas in the udder that appeared consolidated or denser were avoided. Samples were taken from both the left and right half of each udder and only from parts of the udder that were still secreting milk (Figure 4.1).

Figure 4.1. Collection of udder tissue at abattoir directly after slaughter.

Each block of udder tissue collected (approximately 3 x 3 cm) was immediately immersed in ice-cold Hank's Buffered Saline Solution (HBSS, Sigma-Aldrich, Johannesburg) (50 mL), containing 25 mM hydroxyethyl-piperazine ethanesulfonic acid (HEPES, Sigma-Aldrich, Johannesburg) and put on ice for transportation to the laboratory (Figure 4.2).

Figure 4.2. Collected udder tissue placed in transport medium.

4.2.2 Cell isolation and enrichment

The dissociation of the mammary gland tissue from the fibrous udder material collected, as well as the retrieval and enrichment of the epithelial cells, were performed in a similar manner to the methods described by Pantschenko and colleagues (2000), with some adaptations. These adaptions include a shorter digestion time and a different growth medium.

The procedure was performed as follows:

→ Each block of collected udder tissue was cut into small pieces of approximately 5 mm diameter, using sterile surgical instruments (Figure 4.3).

Figure 4.3. Preparation of tissue for digestion by cutting it into smaller pieces.

Ten grams samples of this prepared tissue was then immersed in a HBSS solution, prepared as follows:

→ 30 mL HBSS (solution without calcium and magnesium) mixed with –
  - o 20 µg/ml gentamicin (Highveld Biological, Johannesburg)
  - o 100 IU/mL penicillin-streptomycin (Highveld Biological, Johannesburg)
  - o 2 µg/mL amphotericin B (stock solution prepared from Amphotericin B powder, Sigma-Aldrich, Johannesburg, using DMSO as a solvent)

→ The solution containing the tissue was then swivelled repeatedly in an orbital shaker incubator, at room temperature (approximately 25°C), for 10 minutes at 100 rpm.

→ The tissue was allowed to settle for a few minutes at room temperature before proceeding with digestion (Figure 4.4).

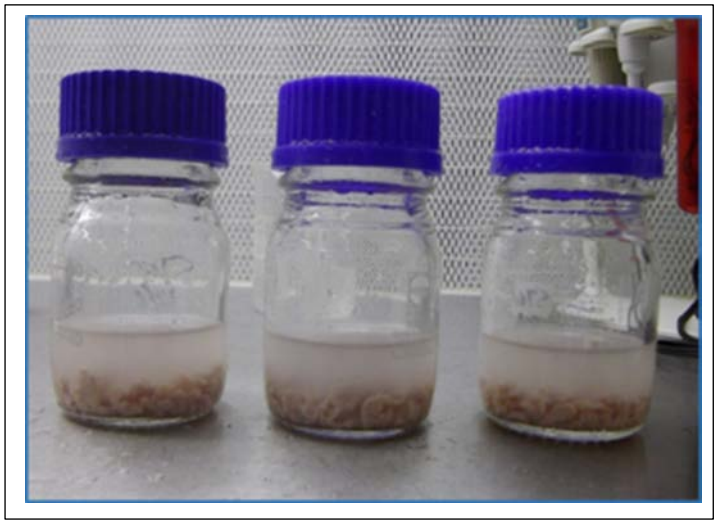


Figure 4.4. Tissue settling at room temperature after exposure to antimicrobial agents.

→ After visible settling of the material, digestion was done by replacing the supernatant with 20 mL fresh HBSS containing 200 U/mL collagenase Type IV (598 U/mg solid stock from Sigma-Aldrich, Johannesburg, filtered after preparation) and incubated at 37°C for 90 minutes while shaking continuously at 30 rpm.

→ After incubation, this homogenate was filtered through a sterilised 129 µm and then a 70 µm metal sieve to remove clumps (Figure 4.5).

Figure 4.5. Filtering of digested tissue through metal sieves to remove clumps.

→ The filtrate was centrifuged at 1000 x g (i.e. 1000 times Earth's gravitational force) for 5 minutes for the cells to form a visible pellet. The cells were then pipetted with approximately 1 mL HBSS until re-suspended and then centrifuged again at 1000 x g for 5 minutes to collect the cells (Figure 4.6).

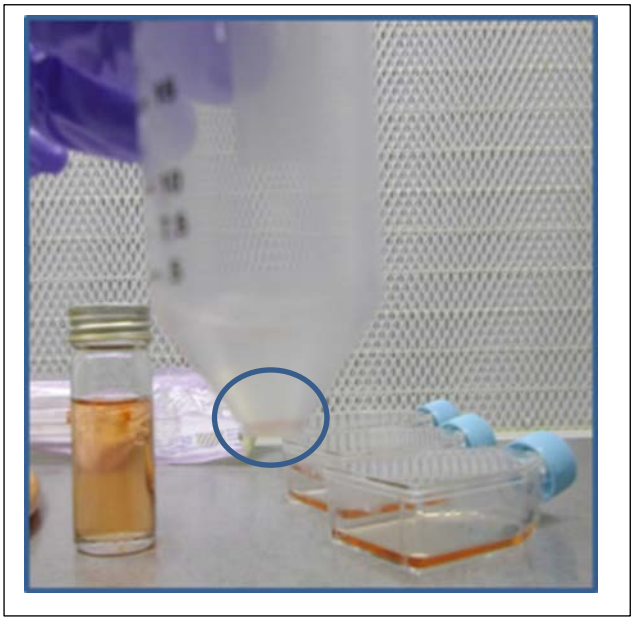


Figure 4.6. Layer of cells retrieved following centrifugation.

→ The collected cells were re-suspended in 1 mL growth medium typically used for human MCF10a mammary epithelial cells.

This medium was prepared as follow:

- DMEM/F12 (Sigma-Aldrich, Johannesburg) mixed with -
  - 5% inactivated horse serum (Whitehead Scientific, Boksburg)
  - 2 mM L-glutamine (Sigma-Aldrich, Johannesburg)
  - 100x insulin-transferrin-sodium selenite (Highveld Biological, Johannesburg)
  - 1% penicillin-streptomycin
  - 50 uM hydrocortisone (powdered cortisone from Sigma-Aldrich, Johannesburg, prepared into a stock solution using absolute ethanol as solvent)

- After mixing, the medium was filtered sterilely using a 32 µm syringe filter.
- After filtration, 20 ng/mL epidermal growth factor (EGF, Sigma-Aldrich, Johannesburg, reconstituted with 0.2 µm-filtered 10 mM acetic acid) was added.

→ The re-suspended cells were then transferred to the T25 (25 cm^2) culture flask which contained another 4 mL medium that had been incubating for a few hours in a 5% CO_2 incubator at 37°C.

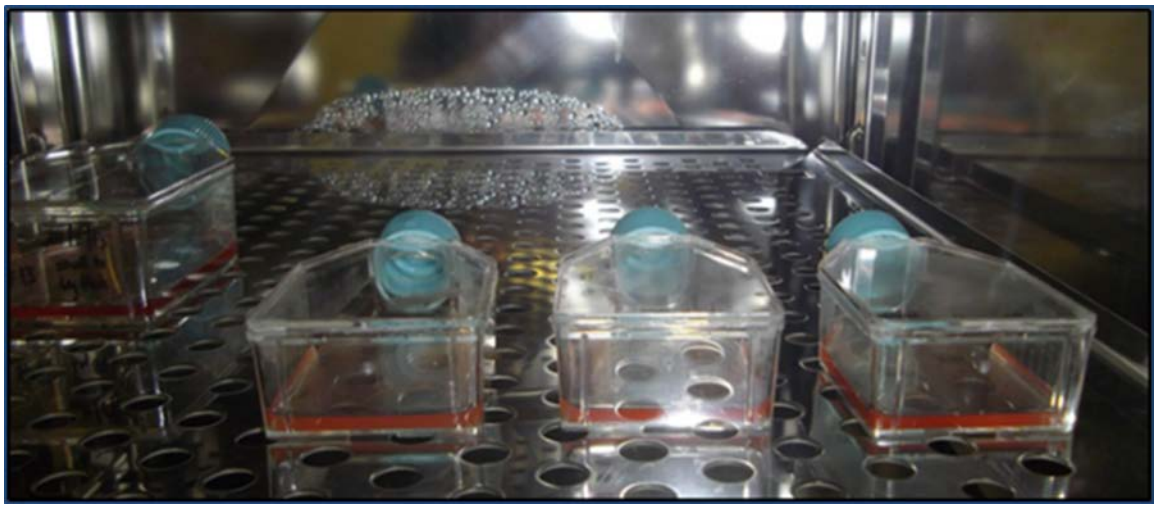


Figure 4.7. Cells in MCF10a-medium in T25 (25 cm^2) culture flask for incubation.

→ The flask was then left undisturbed for 72 hours (Figure 4.7).

After 3 days in culture, attachment of cells and growth of small colonies were evident. Hereafter medium was changed every 48 hours until 7 to 8 days after seeding when approximately 80% confluency was observed using a phase contrast inverted microscope (Ts100-F, Nicon Instruments Inc, Melville, USA).

4.2.3 Multiwell seeding

At 80% confluency cells were trypsinised, using the warm trypsin method (Freshney, 2005), for seeding into 6-well snapwells (Nunc plates and polycarbonate Membrane Cell Culture inserts, 0.4 µm pore size; 23/35 mm; Thermoscientific, Johannesburg).

The procedure was as follows:

→ The entire volume of medium from the T25 flasks was aspirated and the cells adhering to the base of the flask were rinsed with 5 mL warmed (at 37˚C) phosphate buffered saline (PBS, Sigma-Aldrich, Johannesburg).

→ Trypsin-EDTA (0.5%, 10x, Sigma-Aldrich, Johannesburg) was diluted with PBS in a 1:5 ratio and 500 uL was applied to the cells. The cells were then incubated at 37°C for 8 to 12 minutes, until only 20% of cells remained attached to the flask. The detached cells (i.e. other 80%) were re-suspended in about 1.5 mL of fresh medium for seeding into the snapwells

→ This retrieved cell suspension was counted using a haemocytometer and seeded at a density of 83 000 cells per well (20 000 cells/cm^2) (Kimura *et al*. 2006) onto the inserts of the multiwell plates.

A density of 83 000 cells per well was determined to be optimal in pre-trial experiments; higher densities of 166 000, 250 000 and 500 000 cells per well, as well as a lower cell density of 60 000 cells per well, gave less than optimal TEER readings.

→ Starting 48 hours after seeding the medium in the wells was changed every second day. Macroscopically the surface of the membrane in the multiwell had a clear grid-like appearance, compared to the smooth surface of an empty well.

After 5 days in culture the medium started to change colour rapidly within a 24 hour period, turning yellow from the normal light pink colour, with a pH of 6.7, versus the normal of 7.2 (measured using a Hach Sension156 pH/Conductivity Meter). No bacterial growth was

observed microscopically and so it was assumed this colour and pH change was due to high metabolic activity of the cells. Therefore, the medium was then changed daily for the remainder of the culture period. A working volume of 1.75 mL medium apically and 2.5 mL basolaterally was used in the flasks.

4.3 Results and Discussion

4.3.1 Cell culture

In total during this project tissue was collected from 9 goats. In the first experiment the cells collected appeared malformed and stayed in suspension (did not attach). The cause of this could not be confirmed. Refinements applied to the methodology hereafter included using the double-sieve during filtering and preparing new stock solution for the antimicrobial agents. This malformation and non-attachment did not happen again and cells collected from subsequent goats all formed attachments and colonies after seeding. Fungal contamination appeared in one batch of cells and the source of contamination was traced back to the HEPES we used. Contamination did not occur in any subsequent experiments.

4.3.2 Microscopic observation

Within 3 days after seeding into the T25-flasks, cells formed single attachments to the bottom of the flask. At a low density the Boer goat CMECs formed islands of small colonies spread over the flask surface (Figure 4.8.1). Visually there appeared to be no fibroblasts, with their characteristic spindle shape, following the trypsinisation and isolation method. The lack of spindle cells, which usually interfere with the formation of tight junctions, was later confirmed using the TEER and tracer-permeability experiments (see Chapter 5 and 6). When the mammary epithelial cells got closer to reaching confluency, the cells formed a monolayer and aggregated with the characteristic cobblestone morphological appearance of

epithelial cells (Figure 4.8.2). The cell nucleus and the nucleoli were prominent (Figure 4.8.3). As monolayers matured, dome-like (Figure 4.8.4) structures formed among the cells. These so-called domes, which are well documented, are a property of *in vitro* cultured transporting epithelia. These domes represent fluid filled blisters between the solid growth surface and the cell layer. The formation of these domes is regarded as a sign of an active transport processes; an intact epithelial barrier function due to functional tight junctional cell-cell contact (Lechner *et al.* 2011).

From day 18 onwards unusual macroscopic changes were observed in the cell cultures. Cultures formed thickened layers on top of the membrane and acquired a moth-eaten appearance (Figure 4.9). Droplets and cell debris were then visible on microscopic examination. Droplets are associated with cellular differentiation. Lipid droplets are an important component of apocrine secretion by the mammary gland and are observed in mature mammary epithelial cells in culture (Pauloin and Chanat, 2012).

Figure 4.8. Microscopic observations during culture of primary Boer goat CMECs. Magnification indicated in brackets.

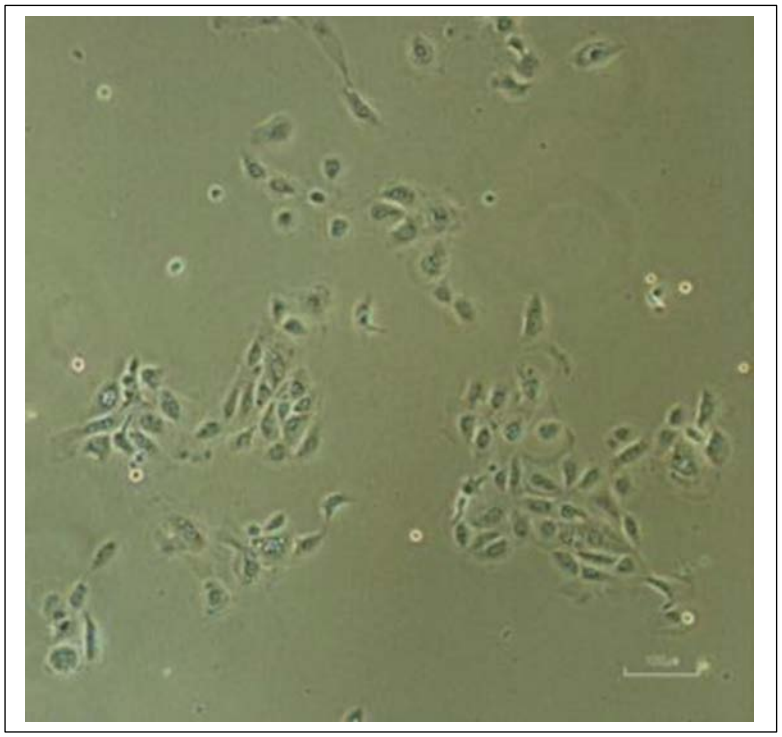


Figure 4.8.1. Islands of small colonies spread over the flask surface on day four of culture (100x).

Figure 4.8.2. Characteristic cobblestone morphological appearance of epithelial cells on day 9 of culture indicating confluency (40x).

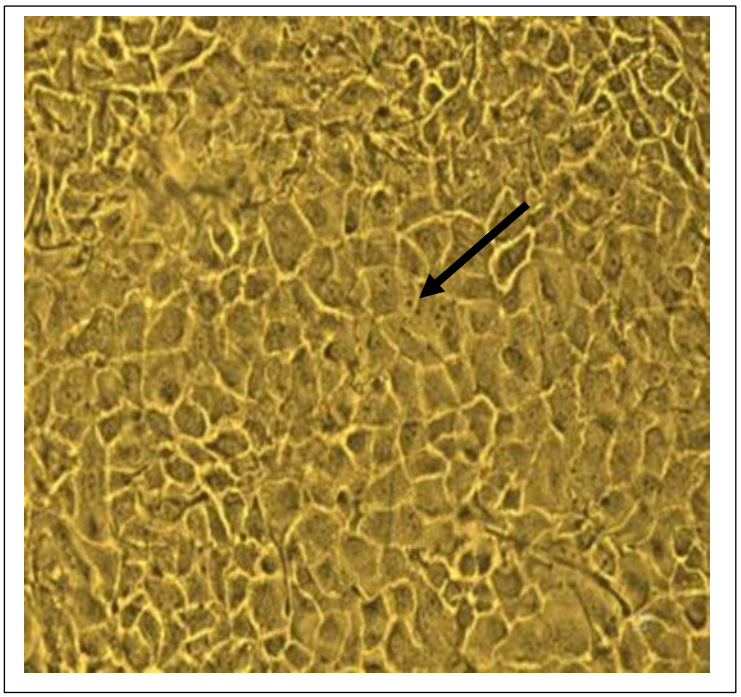


Figure 4.8.3. Prominent nucleoli (indicated by the arrow) of Boer goat CMECs (200x).

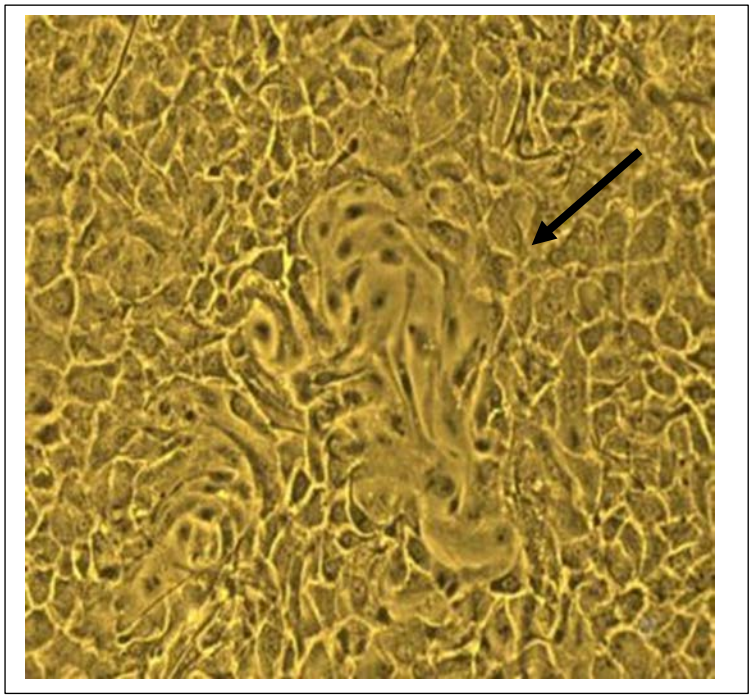


Figure 4.8.4. Dome-like structure (indicated by the arrow) seen in mature cultures of Boer goat CMECs (200x).

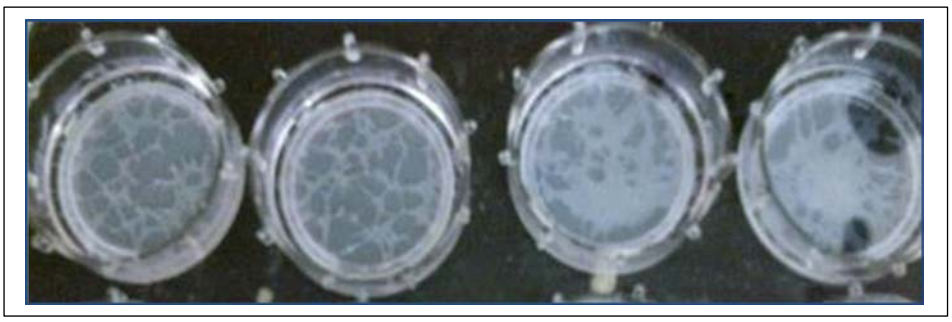


Figure 4.9. Macroscopically abnormal multiwell surfaces at 21 days in culture. Note the white moth-eaten film on the normally clear surface.

The microscopic morphological appearance of the Boer goat CMECs observed during the culture corresponded with those described during the culture of other dairy goat mammary epithelial cells (Tong *et al*. 2012). Abnormal macroscopic changes (Figure 4.9) that were visible after 18 days in culture were presumably due to cell differentiation, overgrowing, stacking of cells and the formation of cell debris (Tong *et al.* 2012).

Studies evaluating the epithelial transport of drugs in cell culture are normally only done on uniform cellular monolayers (Freshney, 2005). Therefore, after day 18 these cells were not considered to be appropriate for further experiments.

4.4 Conclusion

When Boer goat CMECs were isolated, enriched and cultured following the methods described in this chapter, they were viable and formed characteristic monolayers that could be maintained for up to 18 days in culture. Viable isolated cells attached to the bottom of the cell culture flasks within 3 days after being seeded and reached 80% confluency in T25 flasks after a week in culture, accompanied by frequent medium replenishment. Microscopic morphological observations during culture were similar to those described in the literature for DGMECs, including the formation of dome-like and lumen-like structures in mature cells.

After 18 days macroscopic changes occurred in these mature cells, with a white moth-eaten appearance seen on the membrane of the multiwell inserts. These changes indicated that the cells were not uniform monolayers at this point, rendering them inappropriate for the purpose of efflux or drug transport studies.

Chapter 5: Transepithelial Electrical Resistance and Multiple Trypsinisations

5.1 Introduction

TEER measurements can be used to monitor the growth and integrity of cellular monolayers in *vitro*. TEER indicates the ion-permeability of tight junctions between adjacent cells and therefore gives us an indication of the degree of tight junction formation in a cellular monolayer (Matter and Balda, 2003). TEER values are described for the most commonly used cell cultures (Maznah, 1999). Mean TEER values, following a certain amount of days in culture, vary between species and cell types.

The objective of this study was to monitor TEER values during days in culture and to measure how it changed over time until tight monolayers were established, so that these values can be used in the future to indicate when cultured Boer goat CMECs have formed monolayers that are a barrier to the movement of small molecules.

We further wanted to investigate the effect of trypsin treatments on TEER in Boer goat CMECs.

TEER (or TER) measurement technology was first introduced by World Precision Instruments in the mid-1980's and today it is widely used by scientists working with cultured epithelial cells. TEER measurement is a convenient, reliable and non-destructive method for evaluating and monitoring the growth of epithelial tissue cultures *in vitro* (World Precision Instruments, 2013). TEER is measured with a Volt-Ohm meter across cultured monolayers. TEER indicates the ion-permeability of tight junctions, and therefore it gives an indication of the degree of tight junction formation, and thus the integrity of cellular monolayers. The confluence of the cellular monolayer is quickly determined by a sharp increase in TEER (Irvine *et al*. 1999). Determining TEER is a widely used method to functionally analyse tight junction dynamics in cell culture models of physiological barriers (Blume *et al.* 2009).

Tight junctions restrict paracellular diffusion of ions and hydrophilic non-ionic tracers. Paracellular permeability to hydrophilic tracers can be monitored with compounds that are labelled fluorescently, such as dextrans (Matter and Balda, 2003). Once appropriate TEER values are reached for a specific cellular monolayer, tracer permeability can be used to confirm the formation of a tight barrier to paracellular movement of the molecule (Matter and Balda, 2003). Together TEER and tracer permeability measurements reflect the paracellular pathway properties of a monolayer with regards to the movement of ionised and hydrophilic non-ionic molecules (Matter and Balda, 2003).

Epithelial cells widely used for research purposes include Caco2-cells (human epithelial colorectal adenocarcinoma cells). When these cells are confluent (i.e. when all the cells in the culture are making contact and adhering to the bottom of the culture flask) and demonstrate a tight barrier to the paracellular movement of phenol red, TEER values are in the range of 250 to 260 $\Omega.cm^2$ (Maznah, 1999). These values are typically obtained between day 17 and 20 of culture. Human mammary epithelial cells that proved appropriate to evaluate transcellular drug movement (by using radioactive mannitol) measured TEER values of 227±11 $\Omega.cm^2$, with maximum values on day 19 of culture (Kimura *et al*. 2006).

Trypsin solutions are widely used for re-suspension in cell cultures by detaching cells adherent to the cell culture dish. Schmidhauser *et al*. (1990) investigated the differentiation of cultured mouse mammary epithelial cells after using trypsin. He found that trypsin-resistant cells had the ability to differentiate functionally with an increased ability, following an increased trypsin treatment period.

Kimura and colleagues (2006) investigated the effect of trypsin treatment on HMEC. They observed that the more a cell population is exposed to trypsin (becoming trypsin-resistant), the higher the maximal TEER reached; maximal TEER was achieved after three trypsinisations and only these cells were tight enough to be used for drug transport studies, with a TEER of 227±11 $\Omega.cm^2$.

5.2 Materials and Methods

5.2.1 Recording TEER

Monitoring the change in TEER over a typical growth period in Boer goat CMECs was done as follows:

→ The resistance across the epithelial layer on the membrane of the insert was measured using an Endohm chamber (Figure 5.1) (Endohm 24-snap, World Precision Instruments Inc., Florida, USA) connected to a Millicell® ERS-2 Volt-Ohm meter (Figure 5.2) (Merck, Massachusetts, USA).

→ The Endohm chamber was pre-soaked with cell culture medium 1 hour prior to taking the readings, to prevent erratic readings that typically follow after sterilization of the chamber with 70% ethanol. Such erratic readings were experienced during pre-trial experiments.

Resistance readings were also taken in wells without cells to serve as a control; empty pre-soaked wells typically showed readings between – 1 and 1 Ω.

Using 3 mL fresh warm medium (from the 37˚C incubator) in the Endohm chamber (Figure 5.1), each insert was transferred into the chamber for resistance measurement. Each measurement was taken twice to confirm accuracy and rule out human error. The insert was always placed centrally, with 2 to 3 mm between the electrode and the membrane.

Figure 5.1. Endohm chamber used to measure TEER.

Figure 5.2. Volt-Ohm meter used to measure TEER.

→ TEER was calculated by multiplying the resistance measured in ohm with the surface area of the insert (4.15 cm^2 for the Nunc inserts).

The Volt-Ohm Meter was balanced weekly with a Millicell@ERS test electrode probe (Merck, Massachusetts, USA) to 1000 Ω ±1.

5.2.2 Analysing TEER

TEER values were determined for cells isolated from three different goats, following a single trypsinisation. These cells were maintained in culture for 18 days, whilst no macroscopical changes were observed. Six technical replicates (six inserts cultured from the same cell stock) were cultured for each goat (n=3). To describe the change in TEER values over time, the data were fit to a sigmoid model (using the equation below) which assumes that after an initial lag, TEER values increase exponentially up to a plateau.

Equation: $\mathbf{TEER(t) = (TEER_{max} \times t^{\gamma})/(t^{\gamma}_{50\%} + t^{\gamma})}$

Where:

t = time

TEER(t) = TEER at time t

$TEER_{max}$ = predicted maximum TEER value

$t_{50\%}$ = time at which TEER achieves 50% of its maximum value

γ = Hill co-efficient, which represents the steepness of the time-TEER curve

The commercially available software program Monolix® (Lixoft, Paris, France) was used to fit the data from all the replicates for each individual to the model using nonlinear mixed effects modeling.

The coefficient of variation (CV %) was also determined for each set of replicas, by deviding the standard deviation with the mean TEER value.

5.2.3 Multiple trypsinisations

The difference in TEER between single and multiple trypsinisation of the cultured Boer goat CMECs (n=2) was also determined. When cells in the T25-flask were 70% to 80% confluent (around day 7 to 8 following seeding) the cells were trypsinised using the warm trypsin method as previously described (section 4.2.3). The 20% of cells that were still attached following the incubation period for the first trypsinisation received 5 mL of fresh media and were placed back into the incubator to regrow to 70% to 80% confluency (1t-Cells). To reach this confluency typically took only 3 to 4 days, compared to the initial 7 to 8 days following primary tissue isolation without trypsinisation. These cells were trypsinised again to form 2t- and 3t-Cells by repeating the trypsinisation process described above.

5.3 Results and Discussion

5.3.1 Single trypsinisation

Resistance was measured (in Ω) on different days in culture from 6 multiwells of 3 different goats (Tables 1, 2 and 3 respectively) and the corresponding TEER-value calculated (Ω x 4.15 cm^2).

Table 5.1. Goat 1, TEER-values (Ω.cm^2) measured over days in culture, with CV% calculated.

Replicate number	**1**	**2**	**3**	**4**	**5**	**6**	**Mean**	**Standard deviation**	**CV%**
Day	**TEER**	**TEER**	**TEER**	**TEER**	**TEER**	**TEER**			
4	29	37	29	46	50	33	37	9	24
8	145	133	154	183	149	141	151	17	11
11	415	382	390	440	291	407	388	51	13
14	560	498	506	440	403	510	486	56	12

18	564	552	535	544	544	573	552	14	3

Table 5.2. Goat 2, TEER-values (Ω.cm^2) measured over days in culture, with CV% calculated.

Replicate number	**1**	**2**	**3**	**4**	**5**	**6**	**Mean**	**Standard deviation**	**CV%**
Day	**TEER**	**TEER**	**TEER**	**TEER**	**TEER**	**TEER**			
5	62	83	100	71	87	58	77	16	21
11	245	257	174	257	249	232	236	32	14
14	606	606	407	589	647	481	556	92	17
17	789	776	548	623	776	689	700	99	14

Table 5.3. Goat 3, TEER-values (Ω.cm^2) measured over days in culture, with CV% calculated.

Replicate number	**1**	**2**	**3**	**4**	**5**	**6**	**Mean**	**Standard deviation**	**CV%**
Day	**TEER**	**TEER**	**TEER**	**TEER**	**TEER**	**TEER**			
4	25	25	25	29	25	25	26	2	8
8	87	71	104	129	75	83	92	22	24
11	228	228	199	212	241	203	219	16	7
14	390	415	336	398	378	365	380	28	7
18	743	751	789	743	793	730	758	26	3

TEER values measured in the primary cell cultures consistently and steadily increased over the entire growth period. TEER values on day 11 in all goats used in this study (n=3), as well as the two goats used in the Dextran-study (Chapter 6), were above 200 Ω.cm^2 (Table 1, 2, 3 and 7). TEER was determined over a period of 30 days, but only values measured from

the day of seeding to day 18 in culture are reported in this dissertation. TEER measurements after day 18 were accompanied by macroscopic changes in the cells, rendering them unsuitable for use in further studies.

The mean TEER measurement in all goats increased more than 10-fold between day one of culture and day eighteen of culture.

The estimated parameter values describing the change in TEER values over time are summarized in Table 4 and the model fit for each animal is shown in Figures 11.1 to 11.3.

Table 5.4. Parameter values for sigmoidal model of changes in TEER over time (mean ± (standard deviation))

Goat number	**$TEER_{max}$**	**$t_{50\%}$**	**γ**
1	1127.3 (40.3)	16.9 (0.3)	2.1 (0.1)
2	1396.6 (52.1)	16.9 (0.3)	3.2 (0.2)
3	1315.1 (53.9)	16.9 (0.3)	3.9 (0.2)
Average	1279.7	16.9	3.1

Figure 5.3. Fit of sigmoid model of TEER data measured in 6 replicates for goat 1.

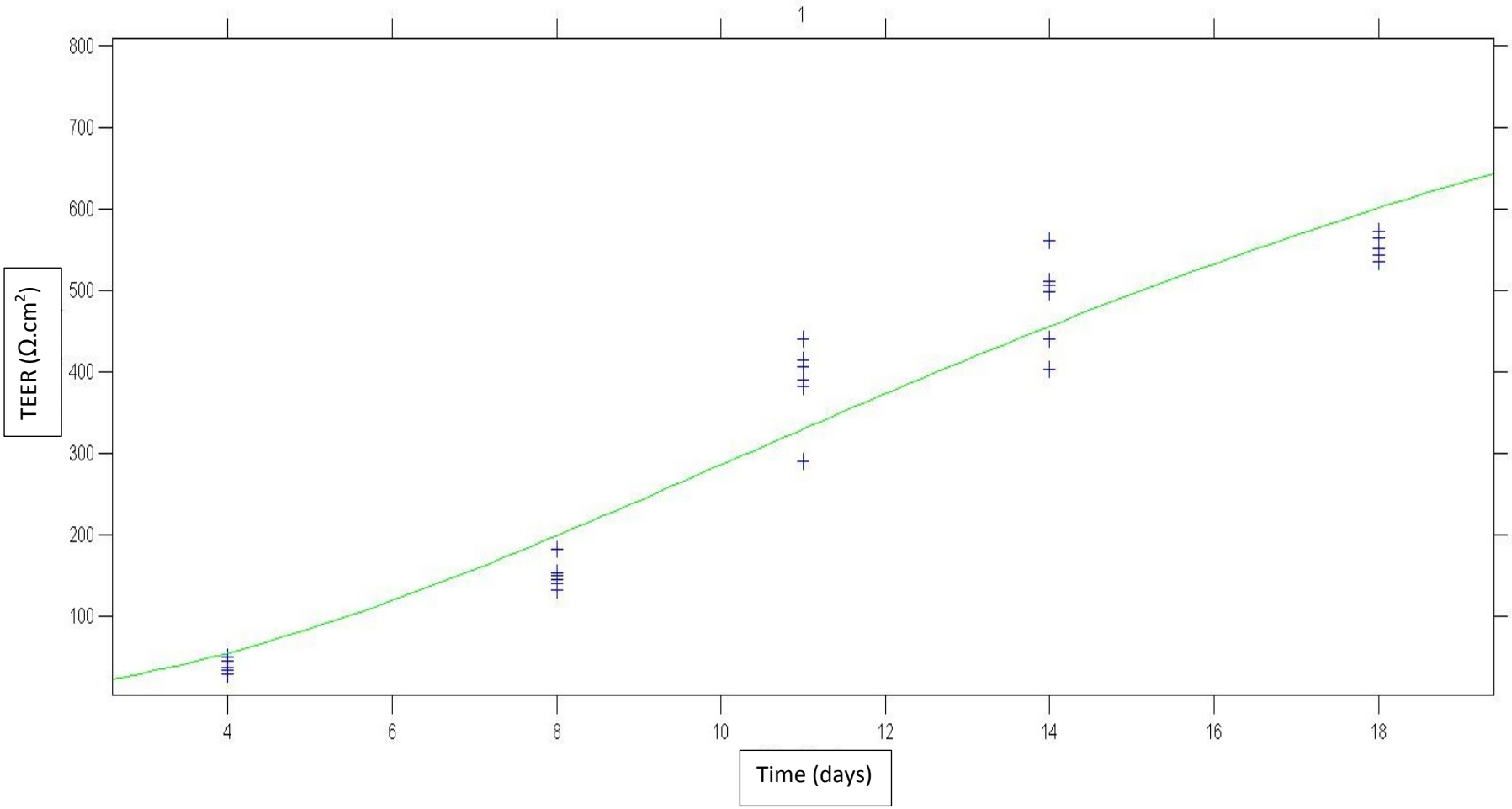


Figure 5.4. Fit of sigmoid model of TEER data measured in 6 replicates for goat 2.

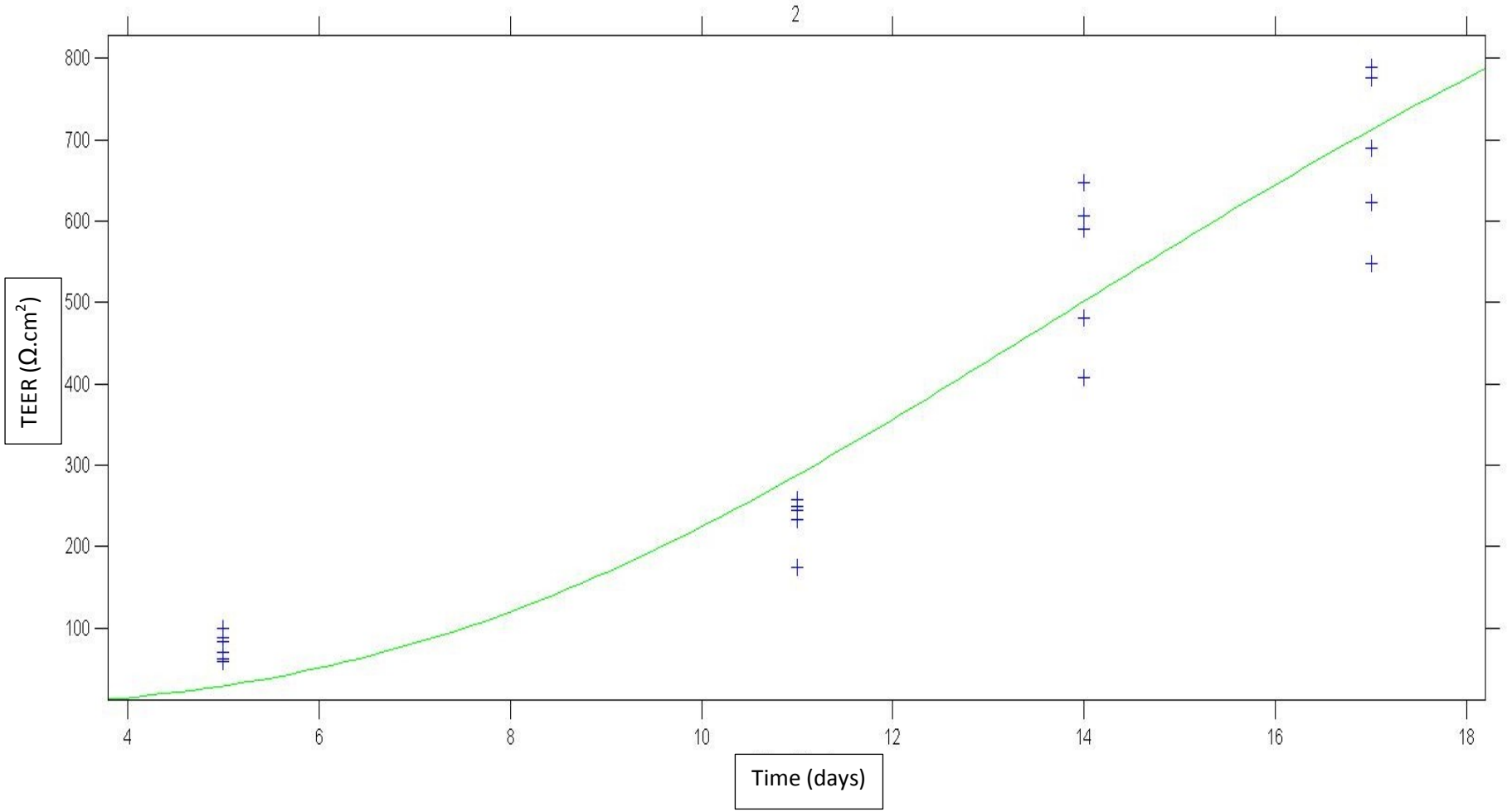
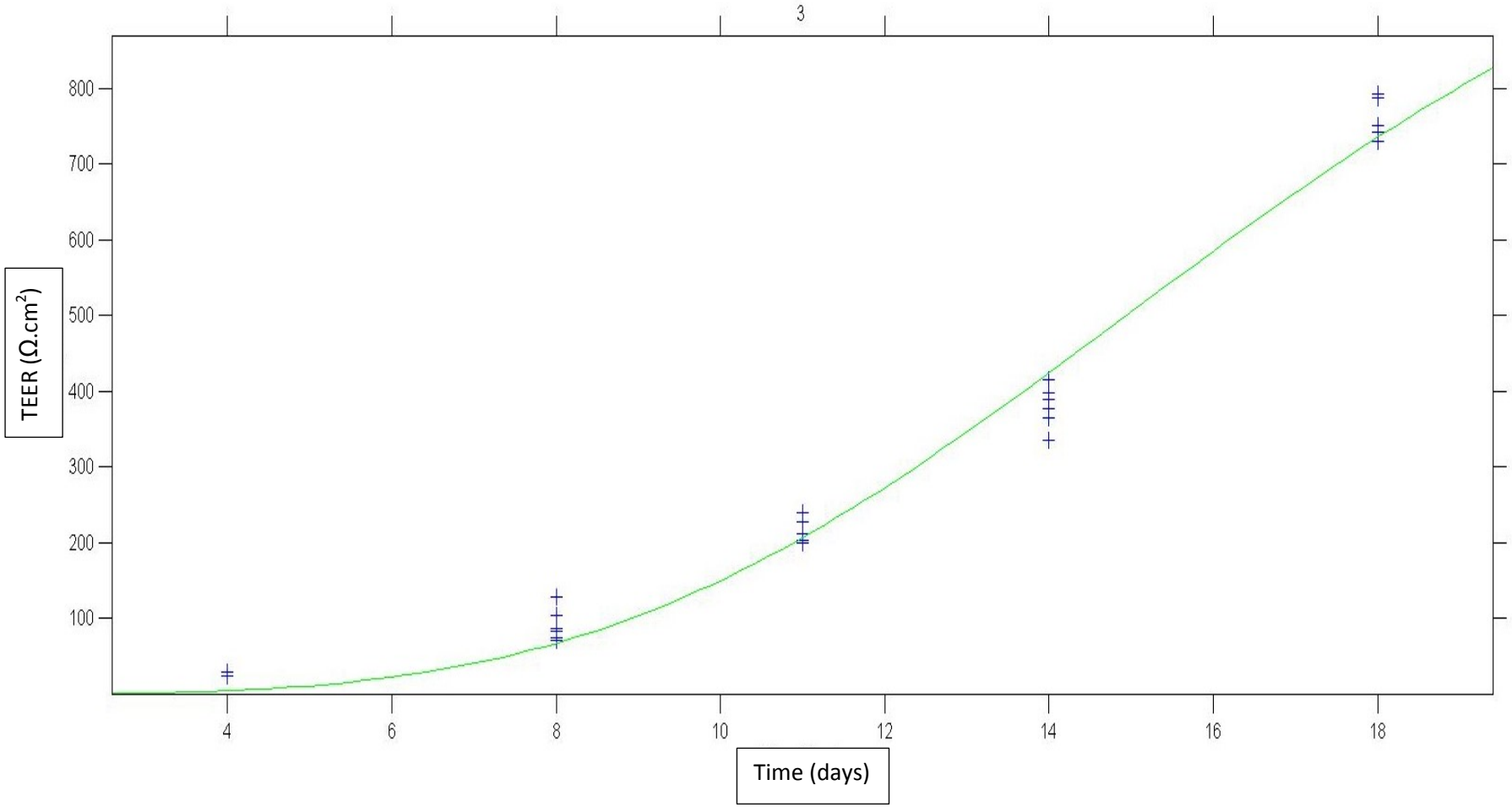


Figure 5.5. Fit of sigmoid model of TEER data measured in 6 replicates for goat 3.



The results of this analysis predict that the cultured monolayers will achieve an average maximal TEER of approximately 1300 Ω.cm^2, and that 50% of this resistance would be achieved by day 17 after initiation of the culture. The predicted average maximal TEER was higher than the TEERs measured on day 18 because the resistance was still increasing at this time. The higher the TEER, the greater the barrier to ion movement and therefore the tighter the barrier. But since ions are very small compared to most drugs, the TEER doesn't have to be extremely high for the monolayer to be a barrier to drug movement (*personal communication, R.Gehring*).

Variability of replicates within individuals as well as variability between individuals was very low (CV% < 20). Despite some variability seen between the different animals used for collecting primary cells, there was a similar growth trend between the animals. Values correspond with TEER readings of monolayers determined to be confluent and appropriate for drug transport studies in HMEC (Kimura *et al*. 2006), as well as values used as an indicator of sufficient tight barrier formation in commonly used cell lines like Caco-2 cells (Maznah, 1999). The TEER beyond 18 days, when abnormal macroscopic changes were seen, increased despite a layer of debris and dead cells accumulating on top of the confluent monolayer. This corresponded with the predictions of the sigmoidal model.

5.3.2 Multiple trypsinisations

The mean TEER (Ω.cm^2) readings after 1 (1t), 2 (2t) and 3 (3t) trypsinisations respectively were determined using 12 multiwells for 2 of the goats (Tables 5 and 6).

Table 5.5. TEER values after 1, 2 and 3 trypsinisations respectively, measured from cells cultured from goat 1 on different days in culture.

	TEER values		
Day in culture	**1t-Cells**	**2t-Cells**	**3t-Cells**
8	139	137	217
12	316	297	289
15	605	362	585
18	555	422	608
21	366	454	729

Table 5.6. TEER values after 1, 2 and 3 trypsinisations respectively, measured from cells cultured from goat 2 on different days in culture.

	TEER values		
Day in culture	**1t-Cells**	**2t-Cells**	**3t-Cells**
8	160	295	188
12	315	526	309
15	468	686	466
18	600	763	756
21	559	879	697

As seen in Table 5.5 and 5.6, Boer goat CMECs reached appropriate TEER values (>200 $\Omega.cm^2$) after one trypsinisation (1t-Cells). In contrast to the findings in HMEC where three trypsinisations were needed to reach appropriate TEER values, one trypsinisation (1t-Cells) was sufficient in the Boer goat CMECs. The formation of tight barriers in these 1-t monolayers was confirmed with a low dextran-FITC flux (Chapter 6).

Trypsinising cells more than once is time consuming and in the Boer goat CMECs offer no further advantage, therefore it is an unnecessary expense for the purpose of efflux or drug

transport studies. Furthermore, it is likely that primary cells undergo phenotypic and genotypic changes following multiple trypsinisations, as was demonstrated by the response in the expression of hOCT1 and hOCT3 mRNA in t-HMEC monolayers by Kimura and colleagues (2006). The 1t-Cells are more likely to closely resemble *in vivo* cells compared to cells that have undergone multiple trypsinisations and are therefore most appropriate for drug transfer studies.

5.4 Conclusion

When primary Boer goat CMECs were maintained in culture for 18 days (as per the methods described in Chapter 4), TEER values continued to increase steadily over the entire growth period. Initially this growth was exponential, but it slowed down after 11days as cells became more confluent. Statistical analysis showed that the experimental technique used was consistent and that the variability between replicates within an animal was low. Inter-individual variability was expected due to biological variability in primary cell cultures.

After 11 days in culture TEER readings were above 200 $\Omega.cm^2$. These values correspond with values in confluent and semi-permeable monolayers of other cell cultures which were appropriate for efflux and drug transfer studies. Although TEER values continued to increase after 18 days in culture, macroscopic changes to the monolayers rendered them unsuitable for further use.

Chapter 6: Dextran-FITC Permeability Testing

6.1 Introduction

TEER is an indicator of tight barrier formation (Irvine *et al.* 1999). Knowing expected TEER values for a particular cell culture at confluency is a useful indicator of the monolayer's appropriateness for efflux studies. However, before such values are known, a tracer substance can be used to confirm that an appropriate tight barrier has formed by testing the impearmeability of the monolayer to the tracer substance (Matter and Balda, 2003).

The objective in this chapter was to determine if the monolayers of Boer goat CMECs formed a tight barrier to dextran-FITC movement. Labeled dextrans, for example dextran-FITC, are hydrophilic polysaccharides that are used extensively in microcirculation and cell permeability research to confirm that barriers are tight and impearmeable to substances of which the movement across epithelial barriers is restricted to the paracellular route (Matter and Balda, 2003). Fluorescein isothiocyanate (FITC), a derivative of fluorescein, is easily measurable with a fluorescence plate reader. A widely used alternative tracer, radioactive mannitol, requires specialised equipment and radioactive safety facilities.

6.2 Materials and Methods

6.2.1 Paracellular movement of dextran-FITC

To determine the paracellular movement of dextran-FITC, a study similar to that described by Matter and Balda (2003) was performed.

This was done as follows:

→ Starting on day 0 (day of seeding) and repeating on subsequent wells every third day, 0.5 mg/mL of dextran-FITC (FD70, Sigma-Aldrich, Johannesburg, prepared to a

5 mg/ml stock solution in sterile reverse osmosis water) was applied to the apical medium of a well (Figure 6.1).

→ After 1 hour of incubation, three 500 µL samples were collected from the basolateral compartment of each well and transferred to sterile Eppendorf tubes. The tubes were put on ice until further analysis.

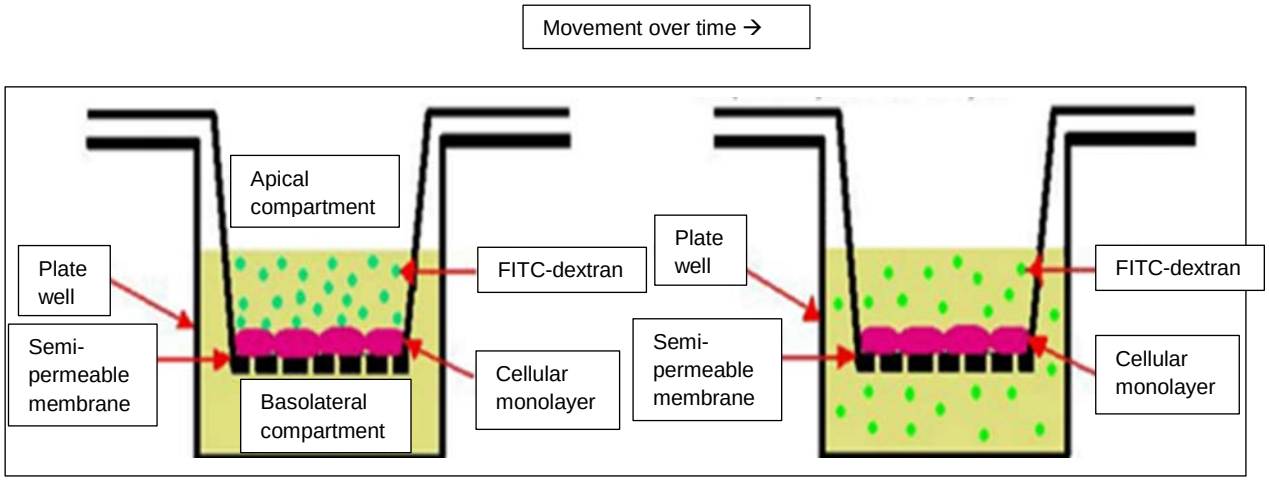


Figure 6.1. This schematic representation of the dextran-FITC movement shows the apical to basolateral flow of dextran through the semi-permeable membrane of the multiwell over time.

→ The samples were sent to an analytical laboratory (Pharmacology Department, Faculty Health Sciences, University of Pretoria), on ice and covered with foil to prevent exposure to light. The remaining samples were stored at -80 °C.

→ Three fluorescence readings (measured as relative fluorescence units (RFU)) were taken per sample, using a fluorescence plate reader (BMG Labtech FLUOstar Optima, Thermo Fisher Scientific, Massachusetts, USA) at an excitation of 485 nm and emmission of 544 nm.

→ The mean reading of each experiment was compared to the reading at day 0. Readings at day 0 were similar to readings from a control sample (taken from a membrane with no cells).

The degree of fluorescence represents the amount of dextran that moved across the monolayer to the basolateral compartment.

6.2.2 Apparent permeability

The apparent permeability coefficient is widely used as part of a general screening process to study drug absorption, and is routinely obtained from *in vitro* experiments. The index is defined as the initial flux of compound through the membrane, normalized by membrane surface area and donor concentration (Palumbo *et al*., 2008).

Apparent permeability was calculated for each dextran-FITC experiment using the following equation: **Papp = dQ / dt x A x C0**

Where:

Papp = apparent permeability coefficient

dQ/dt = slope* of RFU plotted against time (60 min)

A = area of the membrane (cm^2), 4.15 cm^2 for this study

C0 = initial concentration in the donor chamber (µg/ml), 500ug/ml for this study

*using the slope equation with the x-asis being time and the y-axis the measured RFU value

$$\text{slope} = \frac{(y_2 - y_1)}{(x_2 - x_1)}$$

A paired t-test was done using GraphPad software, to determine if there was a statistical difference between the apparent permeability coefficient in the sample collected from the two goats on the first day of seeding and at day 18 of culture.

TEER readings were recorded every time before the dextran-FITC was added, in order to establish TEER values in the well at that time.

6.3 Results and Discussion

The pooled average of the dextran flourescent counts measured (n=2), the corresponding Papp and average TEER values are reported in Table 6.1.

Table 6.1. Fluorescence readings following dextran movement, corresponding Papp values and average TEER values measured at each point in time.

	Pooled average dextran values (RFU)	**Papp**	**Average TEER ($\Omega.cm^2$)**
Day 0	10081	0,08097	1
Day 3	1086	0,00872	30
Day 6	371	0,00298	183
Day 9	146	0,00117	216
Day 12	155	0,00125	442
Day 15	156	0,00126	575
Day 18	147	0,00118	737

The apical to basolateral paracellular movement of FD70 dextran (expressed as the apparent permeability coefficient (Papp)), reduced by 40 fold between the day of seeding and day 6 in culture. From day 9 onwards the apparent permeability coefficient measured was more than 80 fold less than at the day of seeding. This low level of permeability (value of 0.001) was maintained for the remainder of the culture period as seen in Figure 6.2.

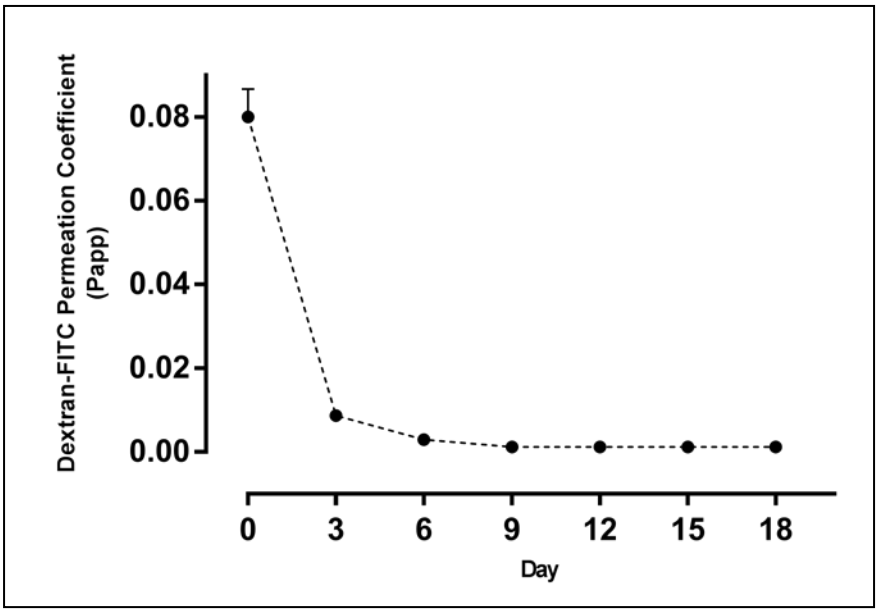


Figure 6.2. Apparent permeability of monolayer to dextran-FITC over 18 days in culture represented by mean ± STDEV (n=2).

There were no statistically significant difference between the two goats used in the experiment on either day 0 or day 18 (P = 0.502). The changes in the apparent permeability from day 0 to the rest of the days in which the tests were done were so profound and the results were so similar in the two goats that, due to the costs involved in these analysis, we did not do further tests.

6.4 Conclusion

In Boer goat CMECs, cultured and maintained as described in Chapter 4, the paracellular movement of the tracer Dextran FD70 rapidly decreased within 3 days (Figure 6.2). This time frame corresponds with the level of confluency seen microscopically at this time in 1t-Cells. The permeability was negligible after 9 days in culture (Papp = 0.001) and remained low over the remainder of the growth period (18 days).

These findings indicate that the monolayer presented a barrier to the paracellular movement of drugs and molecules bigger than Dextran FD70 (70 000 daltons). Further studies are needed to confirm whether the monolayer will be a significant barrier to drugs that have a smaller size than Dextran FD70.

On day 9 in culture TEER values in this experiment was in the range of 200 $\Omega.cm^2$, following a similar trend as seen in all the previous experiments (Chapter 5).

Chapter 7: Cryopreservation

7.1 Introduction

One of our objectives was to determine if Boer goat CMECs maintain viability after cryopreservation using a simple cryopreservation technique, with increased serum levels and DMSO as a cryoprotective agent in the medium (Freshney, 2010). Cryopreservation can be used as a tool to preserve isolated cells for future experiments. This tool is particularly useful when working with primary cells, as they generally need to be isolated each time a new experiment needs to be performed, which is time consuming and expensive. Thawing viable cryopreserved cells would negate these needs.

Cryopreservation is the use of very low temperatures to preserve structurally intact living cells and tissues. Spermatozoa were the first mammalian cells to be cryopreserved successfully (Polge *et al*. 1949). Since then, various cryopreservation methods have been developed for different types of cells, tissues and organs.

Using primary cells in research has advantages, but collecting the tissue and isolating the cells is a time-consuming and expensive procedure. At times primary tissue might not be readily available. It might also be necessary to repeat an experiment using tissue of the exact same origin. Considering that all cells are fated for senescence, it is vitally important that surplus cells can be frozen and preserved in storage for later use (*personal communication, R. Gehring*).

The best long-term cryopreservation results are achieved in liquid nitrogen at -196˚C (Freshney, 2010). Successful short-term storage can however be achieved in a -80˚C freezer (Freshney, 2010). It is important that the method for cryopreservation minimises the shock the cells are exposed to during freezing and thawing.

Cells are usually stored in complete medium and in the presence of a cryoprotective agent, such as DMSO. Cryoprotective agents reduce the freezing point of the medium and also allow for a slower cooling rate, greatly reducing the risk of ice crystal formation, which can damage cells and cause cell death. DMSO in particular penetrates inside the cell and prevents the formation of ice crystals that could result in membrane rupture (Freshney, 2010).

7.2 Materials and Methods

After trypsinisation with the warm trypsin method previously described (section 4.2.3), the primary cells were frozen in a -80°C freezer, using a medium containing 20% horse serum and DMSO in the following quantities:

- 37.5 mL MCF10a-medium
- 10 mL horse serum
- 2.5 mL DMSO

Serum levels were increased to improve the cells' post-freezing recovery and survival (Freshney, 2010).

A CoolCell (AEC Amersham, Kyalami) was used to ensure constant freezing at -1°C/min to avoid cold-shock to the cells.

Cells were defrosted four weeks later by immediate immersion into a 37°C warm bath; care was taken to avoid contamination. Each vial of cells was then added to 10 mL warm medium and centrifuged for 5 min at 2000 x rpm. The supernatant was removed and the pelleted cells re-suspended into medium containing 20% horse serum and seeded into T25 flasks.

After the formation of cellular attachments the horse serum levels in the medium were reduced to the usual 5% concentration. Cells were observed regularly for viability and growth behaviour.

7.3 Results and Discussion

Cryopreserved cells, which were stored at -80°C for four weeks and then thawed, exhibited similar growth characteristics as those primary cells that were collected from goats directly after slaughter and then seeded, but took slightly longer to reach confluency. Thawed cells formed single attachments after 3 days in culture, but at day 7 after seeding, although multiple colonies were evident, monolayers weren't yet near confluency (Figure 7.1).

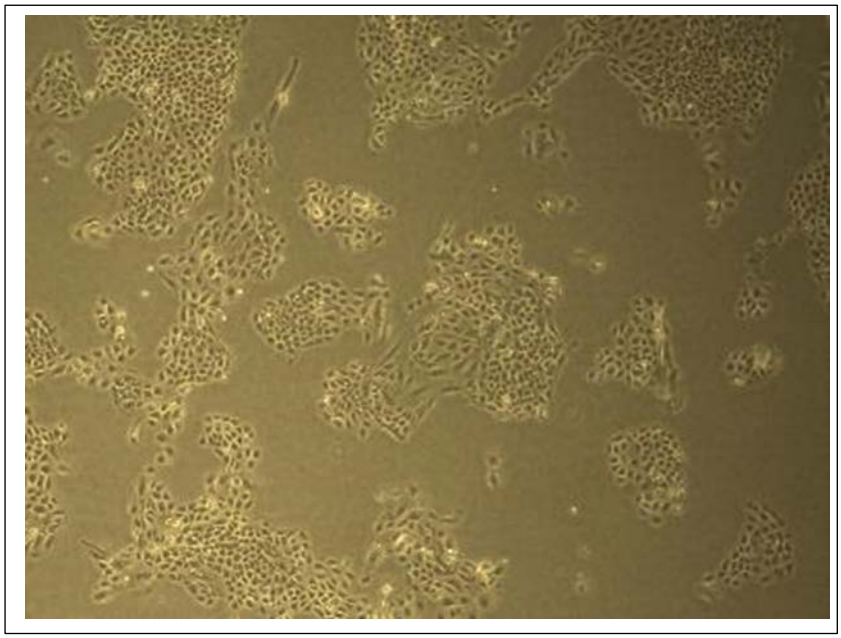


Figure 7.1. Boer goat CMECs at day 7 in culture
(original magnification electronically adapted to show the incomplete confluency).

Unfortunately cells were not counted during and after cryopreservation, nor was a trypan blue viability protocol done. This is a shortcoming of the experiment.

7.4 Conclusion

Boer goat CMECs maintain viability after being frozen in a medium containing increased levels of horse serum and supplemented DMSO for several weeks at -80°C. When thawed and re-grown, these cryopreserved cells attached to cell culture flasks and readily formed confluent colonies with the characteristic appearance of epithelial cells.

This is the first time cryopreservation has been reported on for epithelial cells collected from Boer goats.

Chapter 8: Conclusion

8.1 General Discussion

An estimated 3.4% of the global milk consumption, of 696.5 million tons, comprises milk derived from goats (FAO, 2012). Goats from developing countries produce approximately 83% of this total amount (Aziz, 2010). In South Africa, Boer goats and Boer goat crosses are numerous. The majority of these goats are found in rural areas and their exact number are not known. Breeding stock registered with the SA Boer goat Breeders' Association is in excess of 60 000 animals. Despite the important role of goats in Africa and the importance of goat milk in the dairy industry, relatively few studies have examined drug metabolism and excretion in goats. There are distinct physiological differences between the caprine and bovine mammary gland and extrapolations cannot be made without error (Smith and Sherman, 2009). Published findings of milk-drug related studies that have been done previously were primarily performed in traditional dairy breeds, like Saanen goats (Abo El-Sooud *et al*. 2004; Rule *et al*. 2001). Although Saanen goats are the primary commercial dairy goat breed found in South Africa, their numbers are few compared to Boer goats (SA Milch Goat Association and SA Boer goat Breeders' Association, 2013). It is very likely that there are breed differences, in particular between primary milk and primary meat breeds, with regards to the behaviour of the epithelial cells in culture and *in vivo*.

Developing cell culture models to investigate drug transport at cellular level has received a great deal of attention from scientists over the past few years. The understanding of the functionality of the mammary gland and subsequent drug transport into milk is essential. *In vitro* models, which represent the mammary epithelial barrier have, amongst others, been developed for humans and bovines (Al-Bataineh *et al*. 2009; Barar *et al.* 2009; Kimura *et al.* 2006). As far as the author is aware no such model has been developed for the purpose of

drug transport studies in caprine species. Furthermore, Boer goat mammary epithelial cells have not previously been cultured.

Models using mammary epithelial cell cultures to investigate drug transport at a cellular level must closely resemble the *in vivo* blood-milk-barrier (Al-Bataineh *et al*. 2009). To achieve this requirement, two main prerequisites for these models are:

a) The formation of tight cellular monolayers that provide a barrier to paracellular drug movement
b) The expression of functional drug transporters similar to those found in the udder

This study focused on the first prerequisite. The aim was to successfully culture and maintain viable tight monolayers of Boer goat CMECs and to determine a characteristic range of TEER-values at confluency. Dextran-FITC was used as a tracer of paracellular permeability to confirm the formation and maintenance of a tight barrier in the monolayers.

The methodology used to isolate, culture and maintain primary Boer goat CMECs allowed for the *in vitro* establishment of Boer goat mammary epithelial cell monolayers that were a tight barrier to the paracellular flux of FD70 dextran. Confluency of the monolayers on day 11 of culture was demonstrated by morphological observations, a low apparent permeability coefficient of FD70 dextran and appropriate TEER values of above 200 $\Omega.cm^2$. The maintenance of this integrity lasted until 18 days of culture, after which macroscopic changes, depicting cell differentiation, degradation and multiple layers of cells, indicated the monolayers to be unsuitable for further studies. Transport studies are generally only performed on uniform tightly-bound cellular monolayers (*personal communication, R. Gehring*). .

The methodology chosen for isolation of the primary cells from udder tissue was similar to that of previously published methods used by Pantschenko and colleagues (2000), with a simple isolation method, based on refinements developed in another cell culture laboratory (*personal communication, P.Malreddy*). The time period from the beginning of the isolation process after harvesting, to the initial seeding into a cell culture flask took approximately four hours. MCF10a medium was confirmed as an effective growth medium for Boer goat CMECs, as well as for Saanen CMECs used by the author in initial pre-trial work.

Key differences between the methods used in this study and that of Pantschenko and colleagues (2000) include:

- Pantschenko and colleagues (2000) collected three separate aggregated suspensions of cells during isolation. The first aggregate was collected before digestion, by putting the washed material through a cell strainer. The second was collected 1 hour after digestion and the third after another 2 hours of digestion.

  We collected a single suspension following a continuous digestion time of 90 min.

- Pantschenko and colleagues (2000) used a digestion medium consisting of phenol red-free DMEM/F-12, 300 U/mL type 1A collagenase, 100 units deoxyribonuclease, penicillin-G and streptomycin sulfate. We used HBSS containing 200 U/mL Collagenase Type IV as a digestion medium. Phenol red has estrongenic activity and can affect estrogen-responsive cells in culture (Berthois *et al*. 1986).

- The main differences between the growth medium used in the two methods was that in this study phenol-red containing DMEM/F-12 was used, while Pantschenko and colleagues (2000) used phenol-red free medium. In this study horse serum was used instead of foetal bovine serum; horse serum is a cost effective alternative to fetal calf serum and supports growth of most mammalian cells. Epithelial growth factor was added to the medium for the duration of the cells' lifespan.

Boer goat CMECs displayed the typical characteristics of epithelial cells and after attachment, within 3 days, started to aggregate in a cobblestone fashion. Microscopically there was no evidence of fibroblasts following trypsinisation and isolation. This observation was later confirmed in the results of the TEER and tracer- permeability experiments; fibroblasts would allow for the leakage of ions and tracers across the cell membrane (*personal communication, R. Gehring*).

The cells reached 70 to 80% confluency within the first week after seeding. The cells seemed to be metabolically active, requiring medium replenishment every day after 5 days in culture. Observations made during the culture of the Boer goat CMECs were similar to those made during the culture of other CMECs. The cells in the model developed by Pantschenko and colleageus (2000) formed island monolayer aggregates with the characteristic cobblestone morphology of epithelial cells when grown at low density on a plastic substratum. With increasing density, the cells organised into lumen-like structures. Post confluency, cells formed epithelial raised dome-like structures, which they determined was a process of contact-induced differentiation (Pantschenko *et al*. 2000). Cells in this study displayed the same morphological changes during the growth period. The cell cultures of Pantchenko and colleagues did grow slightly faster than the Boer goat CMECs in our study and we suspect that this is likely due to the fact that the cells used in their study were collected form high producing dairy goats in early lactation, or from the differences in the composition of growth mediums used in either study. Further studies are required to confirm this.

To monitor the growth and integrity of the Boer goat epithelial monolayers, TEER values were frequently measured across the monolayers over a growth period of 30 days with a Volt-Ohm meter. Following a single trypsinisation and seeding into 6-well snapwells, TEER values steadily increased over this period, first exponentially, then plateauing as the monolayer became more confluent, approximately 9 to 11 days in culture. This followed a

predicted trend which fitted a sigmoidal model. Only values measured from the day of seeding to day 18 in culture were reported in this dissertation, because the increased TEER measurement after day 18 were accompanied by microscopic and macroscopic changes. These changes indicated that they were not simple monolayers anymore at that point in time.

Statistical analysis showed that the experimental technique used was consistent and that the variability between replicates within an animal was low. Inter-individual variability was expected due to biological variability in primary cell cultures.

TEER values on day 11 in all goats (n=5) were above 200 $\Omega.cm^2$ (Table 5.1, 5.2, 5.3 and 6.1). These values corresponded with TEER readings of monolayers determined to be confluent and appropriate for drug transport studies in HMEC (Kimura *et al.* 2006), as well as values used as an indicator of sufficient tight barrier formation in commonly used cell lines like Caco-2 cells (Maznah, 1999).

Kimura and colleagues (2006) found in their culture of HMEC that trypsin-resistant epithelial cells formed more confluent monolayers and that only trypsin-resistant cells formed a barrier tight enough for drug transport studies. We wanted to determine if the same applied to Boer goat CMECs, but recorded high TEER values after one trypsinisation; multiple trypsinisation had no obvious benefit in improving the morphology or function of the cells. Primary cells are known to undergo changes following each trypsinisation and therefore t1-Cells mimic the *in vivo* blood-milk-barrier best (Kimura *et al*. 2006).

When using FD70 dextran as a tracer for paracellular movement in our study, the apical to basolateral paracellular movement of this molecule (expressed as Papp) rapidly decreased within 3 days. This time frame corresponded with the 70-80% confluency seen microscopically in 1t-cells (cells re-seeded after one trypsinisation). The apparent

permeability coefficient reduced by 40-fold from the day of seeding to day 6 in culture. The permeability was negligible after 9 days in culture (Papp = 0.001), being more than 80-fold less than at the day of seeding. This low level of permeability was maintained over the remainder of the growth period (18 days). The plateau seen after 9 days in culture at a negligible apparent permeability coefficient of 0.001 corresponded with the TEER values nearing 200 $\Omega.cm^2$.

TEER values recorded, in conjunction with the results of our tracer-experiments, suggest that Boer goat CMEC monolayers present a significant barrier to the paracellular movement of molecules. Further studies are needed to confirm whether the monolayer will be a significant barrier to drugs smaller than Dextran FD70.

The low number of replicates in both the TEER and tracer permeability experiments was a limitation of this study. This limitation was a consequence of limited time and resources.

The development of primary cell cultures is time consuming and expensive. Tissue from initial goats first had to be used to establish suitable culture techniques and reproducible data. Finding appropriate mammary tissue also posed a challenge. The majority of Boer goats in South Africa are slaughtered informally. Twelve abattoirs contacted in the greater Gauteng region could not supply a steady source of material. A further limiting factor was that does are mainly slaughtered because they are dry or suffering from chronic mastitis, udders not suitable for tissue collection. Surgically retrieving the required material from a live production animal was not an option as owners were not willing to accept the risks of infection to the udder that these procedures posed.

To conclude, primary Boer goat mammary epithelial cells cultured and maintained using the techniques described, form uniform and confluent monolayers with TEER readings above

200 Ω.cm^2, within a reasonable timeframe. Tracer-experiments confirmed that these monolayers could be considered for xenobiotic transcellular flux studies.

In order to create a homogeneous populations of cells and minimize culture aging and evolution, a prerequisite is that primary cells can be preserved. This study has shown that Boer goat CMEC can be successfully frozen and stored at -80°C for at least 4 weeks and when thawed became viable for culture. The cells exhibited similar growth characteristics as those primary cells that were collected from goats directly after slaughter. This finding is an important outcome if these cells are to be used in repeatable drug transfer studies.

Ideally scanning and transmission electron microscopy would have provided more detailed information on the structural features of these epithelial cells such as the presence of desmosomes, junctional complexes and the presence of keratin filaments. This was not performed due to cost constraints.

Successfully establishing the techniques used to culture and assess viable Boer goat mammary epithelial monolayers has paved the way for future drug transporter studies in this species and breed. Such studies will help gain an understanding of drug transport into milk at the cellular level, which is important when considering drug residues in milk intended for human consumption, in particular in countries where goats play a major role in milk production.

8.2 Recommendations for future work

The successful establishment of Boer goat CMEC monolayers has paved the way for further studies determining the appropriateness of using these monolayers in drug transporter studies. Importantly the expression of xenobiotic transporter proteins, similar to those expressed in tissue from *in vivo* samples, need to be identified in these monolayers. If viable, these studies will be of critical importance, especially in countries where goats play a major role in milk production.

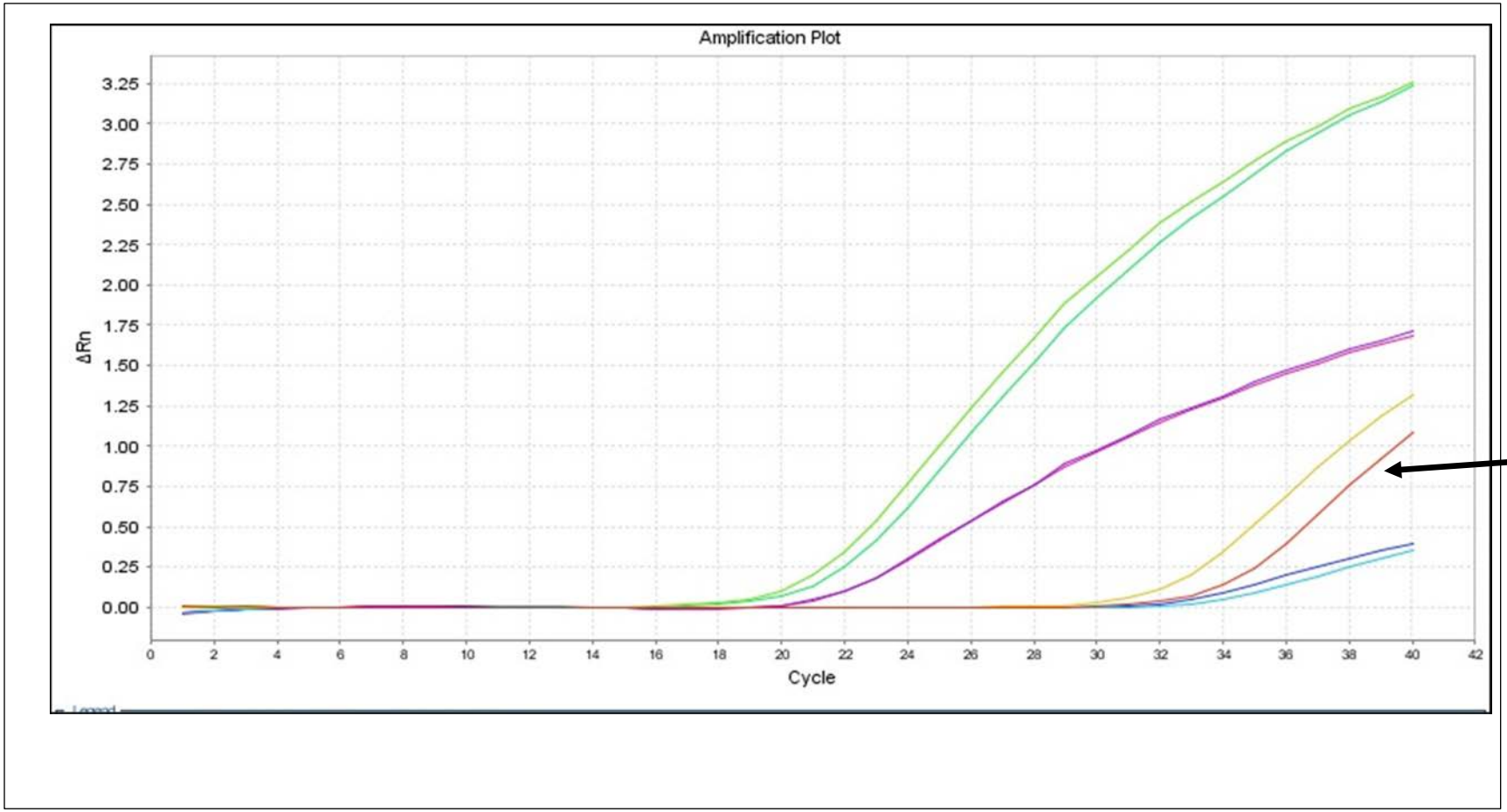
Pre-trial work to determine the likelihood of important transporters being expressed in the primary cell cultures generated in this study was done.

Breast cancer resistance protein (BCRP/ABCG2) is a very important determinant of active drug secretion into milk and seems to be a significant role-player of drug secretions in lactating mammary tissue of various species including mice, humans and cows (Herwaarden *et al*. 2006; Halwachs *et al*. 2014); it has been shown *in vivo* to be present in the lactating goat's mammary gland (Real *et al*. 2011).

Preserved Boer goat CMECs cells were sampled and total nucleic extraction was performed using MagMax reagents and instruments. Using the appropriate primers and probes and a fast rt-PCR protocol, BCRP m-RNA was detected (depicted as the brown graph in the amplification plot depicted in Figure 8.1). This indicates that future trials are worth doing to determine the -

a) degree of expression of BCRP in Boer goat CMECs
b) functionality of BCRP in Boer goat CMECs

Figure 8.1. Detecting BCRP m-RNA with a fast rt-PCR protocol; indicated with arrow.



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Addendumn A: Ethical clearance

Ref: V028/12

19 July 2012

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Dr L McGaw
Department Paraclinical Sciences
(lyndy.mcgaw@up.ac.za)

Dear Dr McGaw

PROTOCOL V028/12: COMPARING XENOBIOTIC TRANSPORTER PROTEIN EXPRESSION AND FUNCTIONALITY IN CAPRINE MAMMARY EPITHELIAL CELLS COLLECTED FROM LACTATING UDDERS OF THE BOER GOAT IN THE PRESENCE AND ABSENCE OF PROLACTIN – *L Le roux-Pullen*

I am pleased to inform you that the abovementioned protocol was approved by the Research Committee.

Kindly note that because there are no ethical issues involved in this project, approval from the Animal Use and Care Committee is not necessary and that you may commence with the project.

Please take note of the attached document.

Kind regards

NIESJE TROMP
SECRETARY: RESEARCH COMMITTEE

Copy: Prof JAW Coetzer, Deputy Dean: Research (koos.coetzer@up.ac.za)
Dr L le Roux, Researcher (lerica.leroux@up.ac.za)
Prof CJ Botha, HOD (christo.botha@up.ac.za)
Ms M Human, Student Administration (magda.human@up.ac.za)
Ms Elmarie Mostert, Animal Use and Care Committee (elmarie.mostert@up.ac.za)

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Full names of student: Lérica le Roux-Pullen

Student number: 23048299

Topic of work: In vitro culture of Boer goat mammary epithelial cells to form a monolayer constituting a tight barrier to drug movement

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SIGNATURE LLRPullen

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TO BE COMPLETED BY STUDENT:

Surname Le Roux-Pullen	**Initials** L	**Title** Dr.	
Student number 23048299	**Study completed in Month & Year**	10	15
E-mail address vivlericapullen@outlook.com	Should your e-mail address be made available on the UPSpace website? (YES) | NO		
Telephone number (124) 64 6046560	**Cell number** (726) 8 135117		
Department Paraclinical Sciences			
Faculty Veterinary Science	**Degree** MSc.		
Supervisor Dr. Leith Meyer	**Co-supervisor** Ass. Prof Renette Gehring		
Title of the study: In vitro culture of Boer goat mammary epithelial cells to form a monolayer constituting a tight barrier to drug movement.			
Please supply 5 keywords for the study:	1. Boer goat mammary epithelial cells		
2. barrier	3. primary cell culture		
4. TEER	5. Dextran-FITC		

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