SPERMATOGONIAL STEM CELL CULTURE, TRANSFECTION, AND INTRA-TESTICULAR TRANSPLANTATION AS A PRELIMINARY TOOL TOWARDS OBTAINING GENETIC MODIFICATION OF KENYAN GALLA GOAT

A thesis submitted in fulfillment of the requirements for the Doctor of Philosophy Degree of the University of Nairobi (Clinical Studies)

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university

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DEDICATION

To

My fabulous husband Maurice, lovely daughter Nerissa Kendi

And

Our families.

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LIST OF ABBREVIATIONS

SSC	Spermatogonial stem cells
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
LIF	Leukaemia inhibitory factor
SDF	Stromal cell-derived factor
SCF	Stem cell factor
EGF	Epidermal growth factor
IGF	Insulin-like growth factor
CD9	Cluster of differentiation 9
CSF-1	Colony Stimulating Factor 1
IGF-I	Insulin-like growth factor I
FGF2	Fibroblast growth factor 2
GDNF	Glial cell line-derived neurotrophic factor
EGF	Epidermal growth factor
ID4	Inhibitor of differentiation 4
PLZF	Promyelocytic leukaemia zinc finger
GFRα1	The glial cell line-derived neurotrophic factor (GDNF) family receptor alpha-1
THY1	Thymocyte differentiation antigen 1

UCH-L1	Ubiquitin carboxy-terminal hydrolase 1
PGP 9.5	Protein gene product 9.5
SSEA	Stage-specific embryonic Antigen
CXCR4- C-X	Chemokine receptor type 4
OCT-4	Octamer-binding transcription factor 4
DBA	Dolichos biflorus agglutinin**;** BIO,6-bromoindirubin-oxime
c-Kit- KIT	Proto-oncogene receptor tyrosine kinase
SOX2-SRY	Sex determining region Y)-box 2
BCL6B6-B	Cell CLL/lymphoma 6
SFM	Serum-free medium
FBS	Fetal bovine serum
FCS	Fetal calf serum
MEM	Minimum essential media
KSR	Knock out serum replacement media
BEF	Bovine embryonic fibroblasts
BFF	Bovine fetal fibroblasts
BSC	Bovine somatic cells
STO	Sando’s inbred mouse (SIM)-derived 6-thioguanine- and ouabain-resistant cells
RT-PCR	Real-time Polymerase Chain Reaction

Egfp	Enhanced green fluorescent protein
CMV	Cytomegalovirus promoter
GFF	Goat fetal fibroblasts
AI	Artificial insemination
PGC	Primordial germ cells
TGF	transforming growth factor
ZFN	Zinc-finger nucleases
TALEN	Transcription activator-like effector nucleases
CRISPR	Clustered regularly interspaced short palindromic repeats
Cas 9	CRISPR-associated protein 9
DSB	Double-stranded breaks
LV	Lentivirus
AAV	Adeno associated virus
ILRI	International Livestock Research Institute
PBS	Phosphate-buffered saline
HBSS	Hank’s balanced salt solution
MACS	Magnetic activated cell sorting
BSA	Bovine serum albumin
DMEM/F-12	Dulbecco’s modified Eagle’s medium F12
EAA	Essential amino acids

NEAA	Non-essential amino acids
GSC	Goat testicular somatic cells
DMSO	Dimethyl sulphoxide
PBST	Phosphate buffered saline with Triton
DDX4	DEAD-box helicase 4
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
ANOVA	One-way Analysis Of Variance
DNA	Deoxyribonucleic acid
cDNA	Complementary or copy-DNA
RNA	Ribonucleic acid
mRNA	Messenger RNA
PM	Pronuclear microinjection
SNCT	Somatic Cell Nuclear transfer
TAE	Tris-acetate-EDTA

ABSTRACT

Spermatogonial stem cells (SSCs) are the germline stem cells responsible for continuous spermatogenesis. The continuous production of spermatozoa relies on the capacity of SSCs to undergo self-renewal to maintain a reservoir for future production. SSC has been previously isolated from testes and transplanted to homologous recipients, successfully re-establishing donor-derived spermatogenesis. This unique characteristic of SSC can be exploited as a reproductive tool in livestock production to propagate desirable genetics through SSC transplantation to surrogate sires. However, the initial population of SSC isolated from the testis is usually low; therefore, there is a need to optimize methodologies for their *in vitro* propagation to generate enough numbers for their use in these reproductive technologies. Surrogate sires are ideal recipients for SSC transplantation since they do not possess an endogenous germline layer, but they have functional somatic cell structural support. The aim of the current study was (a) to do Systematic review of literature for in vitro culture of spermatogonial stem cells (SSC) and their applications in livestock species, (b) to establish long-term SSC culture system for indigenous Galla goats in Kenya and characterize the SSC through morphology, immunochemistry, and molecular markers, (c) to optimize gene transfection protocols for the *in vitro* cultured SSC and (d) to transfer (transplant) SSC to germline intact prepubertal bucks and evaluate their ability to colonize the recipient seminiferous tubules.

The literature search on spermatogonial stem cell culture was performed. Relevant data were screened and extracted. There was limited data on *in vitro* culture of SSC from goats and also none of the studies had been done on livestock in Africa.

The SSC was isolated from prepubertal goat testes via a two-step enzymatic digestion method followed by testicular cell enrichment for SSC through a multiparameter selection approach. The isolated SSC had a viability mean of 77.4 ± 1.2 %. The multiparameter selection yielded a population of cells enriched for SSC with higher *in vitro* colony formation, cells of uniform size, cultures with very few somatic cells, and a majority (69.20 ± 1.0 %) of the cells stained positive for promyelocytic leukaemia zinc finger factor (PLZF), which is a specific SSC marker to ascertain their stem status through immunocytochemistry and real-time polymerase chain reaction (qPCR). The single enriched procedure of differential plating on gelatin-coated plates results in about 25.62±1.76% cell population of PLZF-staining cells. The study demonstrated a thriving goat SSC culture and proliferation on a feeder-free system with goat fetal fibroblasts (GFF) pre-conditioned Stempro medium for 45 days. Notably, the immunostaining of the feeder-free cultured SSC germ cell clumps invariably expressed PLZF staining, which indicated maintenance of undifferentiated spermatogonial phenotype through immunochemistry and RT-PCR. The goat SSC culture also exhibited typical germ cell clump morphology similar to what has previously been reported in rodent SSC.

The cultured SSC were transfected with enhanced green fluorescent protein (eGFP) reporter gene plasmid bound to cytomegalovirus (CMV) promoter and delivered to the cell cytosol through lipofectamine reagents and electroporation. The use of Lipofectamine™ stem reagent carrier had a higher number of SSC colonies expressing the eGFP gene (25.25%) compared to Lipofectamine™ 2000 carrier molecule (22.25%). Electroporation of the SSC resulted in the highest transfection efficiency of 15% with a viability rate of 50% cells. The high voltage of electroporation resulted in SSC death. The two transfection methods yielded promising results for utilizing the techniques for gene transfer of genetic material into goat SSC. However, further studies are required to modify the parameters and transfection conditions

for improvement of the overall transfection efficiency and the viability of cells after transfection.

The SSC are the only cells with the ability to migrate to the basement membrane of the seminiferous tubules and colonize the membrane through the occupation of stem cell niches. An enriched population of cultured and eGFP transfected SSC has successfully been transplanted to prepubertal buck testes through mediastinum testis in an ultrasound-guided injection. The presence of eGFP-expressing cells in seminiferous tubules of recipient testis following transplantation in prepubertal bucks indicates that the ultrasound-guided transplantation of donor cells was successful, but whether these cells would persist long enough to colonize seminiferous tubules and donor-derived genotype was not evaluated. There was no inflammatory reaction or infection to transplantation of SSC in recipient bucks.

The establishment of a robust long-term culture system for SSC can unlock possibilities of their use for transplantation technology in livestock production and a generation of transgenic animals. In conclusion, the study was the first report of a combined serum-free feeder-free *in vitro* culture system of goat SSC. The devised goat SSC culture system also marks the first report of culturing SSC in livestock in Africa. The established conditions can be used as a benchmark for further studies in the long-term expansion of goat SSC that will provide enough numbers for SSC application in transgenesis and surrogate sire breeding technology. The established protocols for transfection parameters, contribute significantly to knowledge of successful gene manipulation of SSC in generating transgenic animals with better traits for disease resistance, fertility, or production.

CHAPTER ONE

1.0 GENERAL INTRODUCTION

Livestock production is a significant economic and food security contributor in Sub-Saharan Africa. It has been projected that in Africa, the human population will double by 2050, which will subsequently increase the demand for animal-based products by 70%. To meet this demand with minimal impact on the environment, advanced and efficient reproductive technologies will be necessary for use in livestock farming systems to increase production per animal (Mcfarlane *et al*., 2019). The Indigenous goat production system accounts for about 30% of Africa's ruminant livestock population and produces about 17% and 12% of the continent's meat and milk respectively. Unlike cattle, goats are ubiquitous, they are kept in a very broad range of agro-ecological zones, where they contribute considerably to the low input production systems and rural economies as a source of income, meat, and other animal products (Bjornlund *et al.,* 2020).

In Kenya, large populations of indigenous goats are kept by pastoralist communities in the arid and semi-arid areas. Despite the socio-economic significance of goats in Kenya, sustainable genetic improvement through the use of reproductive technologies has been minimal, thus the potential for the goat production system remains largely unexploited (Amayi *et al.,* 2016). Artificial insemination (AI) and other technologies such as embryo transfer have not been fully adopted in small ruminants in Kenya. The greatest hurdles are the lack of technical and infrastructural framework for sustainable adoption of these technologies. Breeding of the goat can be revolutionized by A.I, similar to the cattle industry

where it doubled milk production and easily availed genetically proven pure breeds of dairy cattle in Kenya (Odero-Waitituh *et al.,* 2017). Low utilization of artificial insemination limits faster and more efficient genetic gains in indigenous goat production systems (Otieno *et al.,* 2015).

Despite technological advancements, the livestock industry still faces myriads of challenges, for instance: animal diseases, inadequate and low-quality pastures, low uptake of breeding technologies and, low genetic potential breeding stock (Kyalo *et al.,* 2016). Since the roll-out of A.I, other advances in reproductive technologies developed such as embryo transfer, in vitro embryo production (IVEP), animal cloning, and genetic engineering have been developed. In Kenya, with the exception of AI, the use, uptake, and adoption of other reproductive technologies has been minimal and some of them such as animal cloning has only been experimental. An increase in the human population subsequently leads to an increase in the urban population, income generation, and consumption of animal products and by-products. This demand for increased consumption of animal products cannot be met by traditional livestock breeding systems. It necessitates the development and implementation of cutting-edge reproductive technologies that will hasten genetic improvement over generations to ensure the few numbers of animals that kept maximize their reproductive performance. Additionally, the genome-edited animals may carry transgenes that impart superior production, fertility, or disease resistance traits, hence accelerating genetic gains for livestock.

The use of gene-editing tools in the genetic improvement of livestock will allow breeders to improve animal reproductive performance, production efficiency and paves the way for a

more sustainable livestock sector (Giassetti, *et al.*, 2019). Currently, the gene manipulation technology in livestock is mainly confined to laboratory experiments due to the complexity of techniques available for the delivery of genome editing reagents into reproductive cells. Another emerging technology that has potential as an alternative breeding technology, is the use of surrogate sires that carry superior germplasm from other males (Gottardo *et al*., 2019). The surrogate sire breeding technology will provide livestock breeders with a new toolkit of delivery strategies for genome editing. The simplicity of this technology will enable widespread on-farm application in major livestock species by seamlessly integrating it into current breeding systems (Mcfarlane *et al.*, 2019).

The current Ph.D. research project is part of a larger project whose aim is to develop a breeding technology involving the use of genetically edited Galla goat surrogate sires in Africa. These sires are incapable of producing their semen due to a lack of endogenous germline layer, but have testicular ultrastructure necessary for the support of spermatogenesis when spermatogonial stem cells (precursor cells for spermatozoa production) are transplanted into them (Park *et al.*, 2017). Utilization of the surrogate sire technology necessitates the establishment of in vitro culture systems for goat spermatogonial stem cells, which enables the multiplication of these cells into millions required for transplantation. Spermatogonial stem cells (SSC) are a rare type of cells within the total population of testicular cells. Their isolation and culture have been the main hurdle in the utilization of the surrogate sire technology. This Ph.D project focused on carrying out the research for several objectives, which included: a systematic review of the in vitro culture systems for spermatogonial stem cells and their practical application in livestock populations, short term and long term culture of Galla goat spermatogonial stem cells, molecular characterization of goat spermatogonial

stem cells by use of specific stem cell markers, transfection of the cells with Green Fluorescent Protein reporter gene and transplantation of SSC to recipient germline intact Galla goats.

1.1 General objective

To experimentally carry out spermatogonial stem cell culture, transfection and intra-testicular transplantation as a preliminary study of gene transfer method for attempted genetic modification of the Kenyan Galla goat.

1.2 Specific objectives

1. Systematic review of literature for in vitro culture of spermatogonial stem cells (SSC) and their applications in livestock species.
2. To optimize techniques for short-term and long-term culture of spermatogonial stem cells (SSC) of the Galla goats and their molecular characterization.
3. To evaluate efficiency of spermatogonial stem cell (SSC) transfection with enhanced Green Fluorescent Protein (eGFP) gene through lipofectamine and electroporation methods.
4. To evaluate the efficiency of spermatogonial stem cell (SSC) transplantation into the testes of the germline intact prepubertal bucks.

1.3 Hypothesis

Spermatogonial stem cells can be successfully cultured *in vitro*, transfected with eGFP plasmid, transplanted through ultrasound-guided technique and transferred into the testicles of germline intact prepubertal bucks.

CHAPTER TWO

2.0 LITERATURE REVIEW

Reproductive technologies have focused on using the male germline to disseminate elite genetics within the animal population. Improvement in the male reproductive efficiency or having the male carrying genetically proven semen translates to greater improvements in production efficiency and profitability in the livestock sector through breeding. Greater genetic improvement through breeding using high genetic value semen is the major factor in the improvement of livestock production efficiency, but the management of the animals has a contributory factor (Gottardo *et al*., 2019). Male gametes (spermatozoa) are formed through a process referred to as spermatogenesis that takes place in the testis. Spermatozoa are produced from the differentiation of spermatogonial stem cells within the testis and progress to become mature gametes that are capable of fertilization. Continuous availability of spermatogonial stem cells through a balance between the processes of self-renewal and differentiation, ensures that spermatogenesis continues throughout a male's life (de Rooij *et al*., 1997; de Rooij, 2001).

2.1 Testicular structure and function

The testis is the male reproductive organ whose main functions are gametogenesis (production of spermatozoa) and androgenesis (production of androgens). The androgenic function of the testis begins from fetal life to support male reproductive organ development in utero and continues throughout a male's life for the development of secondary male structures as well as maintenance of the spermatogenic process (Murta *et al*., 2010). In livestock, a pair of testes are found in a sac located outside the body and is called the

scrotum. Each testis consists of a fibrous highly collagenous tunica albuginea covering. The testis is composed of a seminiferous tubule compartment where germ cells and Sertoli cells are located, and the interstitial tissue compartment (Fig. 2.1:). The Leydig cells, fibroblasts, immune cells, blood vessels and nerves are found in the interstitial tissue compartment (Griswold and McLean, 2005).

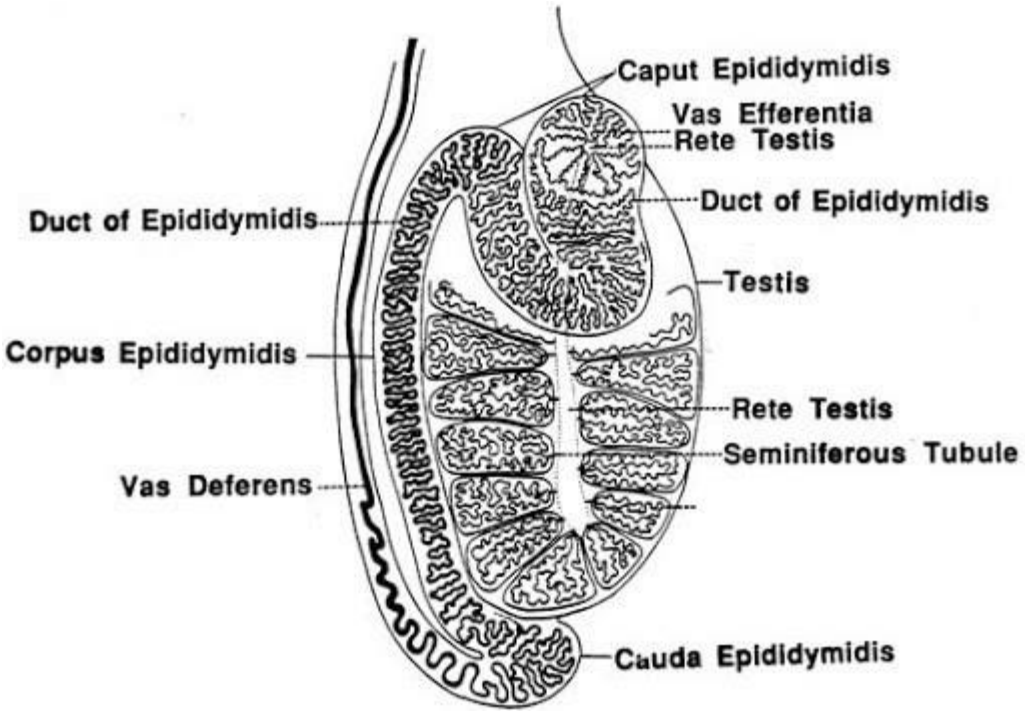


Figure 2.1: Buck testicular structure (http:/www.ansci.wsci.edu/ansci_repro/male_anatomy)

2.1.1 The seminiferous tubules

The seminiferous tubules are highly coiled tubular structures within the testes where spermatogenesis occurs. Each tubule forms a convoluted loop with both ends connecting to a continuous channel called the rete testis, a centrally located area within the testis through which the spermatozoa pass as they progress into the epididymis. Efferent ductules arise from the rete testis, which is the passage of semen to the epididymis (Staub and Johnson, 2018).

The seminiferous epithelium can be divided into two compartments: the basal and the, adluminal compartment (Oatley and Brinster, 2012). Other than the provision of structural support to the tubules the basal lamina also regulates spermatogonia functions. Spermatozoa are continuously produced and the mature ones spermiated into the lumen for transportation to the rete testis, the efferent ducts, the epididymis, and finally the vas deferens for ejaculation (de Rooij, 2015).

2.1.2 Sertoli cells

The Sertoli cells play a significant role in the spermatogonial stem cell niche and regulation of their activities. Morphologically, the Sertoli cell is polygonal with numerous filamentous processes that extend to make contact with the adjacent Sertoli cell (Hai *et al*., 2014). Sertoli cells are in contact with the basal lamina and their filamentous processes extend to the lumen of the tubules. The filamentous processes of adjacent Sertoli cells are usually in contact to form junctions separating the tubules into the basal compartment and the ad luminal compartment (Hai *et al*., 2014; Oatley and Griswold, 2017). Separation of the basement and ad luminal compartments creates an immune-privileged area (blood-testis barrier), avoid haploid spermatocytes in the ad luminal compartment from being treated as foreign, thus preventing erroneous destruction by the blood immune cells (Russell *et al.*,1993; Murta *et al*., 2010; Staub and Johnson, 2018) (Fig. 2.2). The differentiating spermatogonia up to the spermatocyte stage just before meiosis is found within the basal compartment. Sertoli cells secrete cytokines, which determine the decision of SSCs to produce daughter stem cells or differentiating daughter cells. Glial cell line-derived neurotrophic factor (GDNF) is produced by Sertoli cells and is essential for SSC self-renewal. Sertoli cells maintain the homeostasis in

the SSC niche, regulate mitosis/meiosis switch processes of the spermatogonia and mediate the biological signals of the hypothalamus-pituitary-gonadal axis. *In vivo*, germ cells cannot survive without the support of Sertoli cells (Oatley and Brinster, 2008).

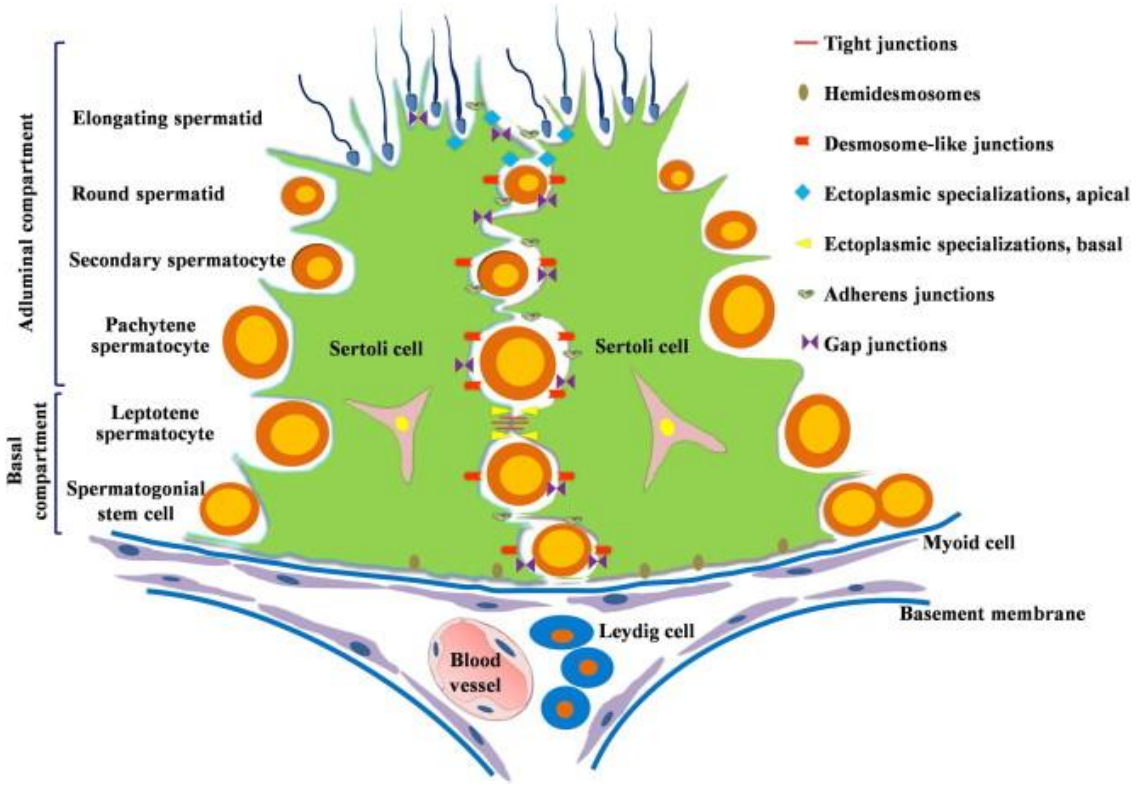


Figure 2. 2: Sertoli cell functional structure (adapted from Hai *et al.*, 2014)

2.2 Spermatogonial stem cells

Stem cells are cells with the capacity to either divide into daughter cells committed to differentiate (differentiating daughter cells) or into new stem cells to maintain the original pool size (daughter stem cells). If the cell division produces a differentiating daughter cell, then it is referred to as symmetric division and if it results in a daughter stem cell it is referred to as asymmetric division. Spermatogonial stem cells are the progenitor cells for spermatozoa production in males. They arise in the testis from the differentiation of gonocytes derived

from primordial germ cells (PGC). The PGCs originate from the epiblast and migrates to the genital ridge during the embryogenesis period (McLaren, 2003). Sexual differentiation of the PGC occurs at the genital ridge following signal-associated somatic cells. The female embryo PGC undergoes meiosis, which gets arrested at the prophase I phase, when they are termed oogonia. In male embryos, meiosis does not occur and the PGCs undergo a few mitotic divisions, to transform to spermatogonia (gonocytes) and enter a quiescence state until after birth (Hermann *et al*., 2018). The duration when the gonocytes resume proliferating and transformation differ in mammals. Prespermatogonia cells differentiate into SSCs within specific periods in mammalian post-birth development. For example in bovine, gonocytes transform to SSC between 12-14 weeks (Curtis and Amann, 1981) and in porcine most gonocytes are already differentiated to SSC by 12 weeks (Murta *et al*., 2010). Before the differentiation of gonocytes to SSC, the cells migrate from the center the seminiferous cords to the basement membrane. This is followed by differentiation with a change in morphology to be called undifferentiated spermatogonia. The SSC sustain the male germ-line lineage by transmitting genetic information from one generation to the next (Beedle and Griswold, 2019). The single type A spermatogonia (As), make up the SSC population.

At puberty, the spermatogenesis process is initiated following the division of SSCs to produce daughter cells destined for differentiation (Oatley and Griswold, 2017). The differentiating daughter cells remain interconnected through intercellular bridges and are referred to as A paired (Apr) spermatogonia. Mitotic division of the Apr spermatogonia produce A-aligned (Aal) spermatogonia. At the same time, self-renewing divisions of SSCs generate more type A (As) for maintaining an SSC pool for future spermatozoa production.

The Aal spermatogonia proliferate to produce Type A1-A4 spermatogonia in mammals. The differentiating A4 spermatogonia further proliferate to intermediate and type B spermatogonia. These cells undergo meiotic division to produce primary and secondary spermatocytes followed by differentiation to haploid spermatids. The round spermatids undergo spermiogenesis to produce spermatozoa (Aponte, 2015; Aponte and de Rooij, 2018).

The undifferentiated spermatogonia population which are the spermatogonial stem cells are made up of the As, Apr, and Aal germ cells. The biochemical and molecular characteristics are likely to be similar. Currently, they are collectively identified using similar markers with unknown molecular or biochemical characteristics that differentiate among them. Conversely, the differentiating spermatogonia (A1–A4) committed to spermatogenesis can be easily distinguished from the undifferentiated spermatogonia population through the use of specific SSC markers (Oatley *et al.*, 2016). The proliferation activity of SSC is dependent on cues from the SSC niche to ensure they undergo the required number of divisions (Griswold and Hogarth, 2018).

2.3 Spermatogonial stem cell niche

A stem cell niche has been described as a microenvironment with anatomical and chemical specifics having a group of support cells, which produce growth factors that regulate fate decisions of stem cell self-renewal (de Rooij, 2009). Stem cells reside in specific areas called niches, where the surrounding support cells maintain their self-renewal (de Rooij, 2015). To maintain homeostasis within the niche, half of the daughter cells of the stem cells migrate

from the niche and differentiate into specialized cells for tissue function (Yoshida *et al.*, 2007; Gómez-Gaviro *et al*., 2012).

Secretions and signaling mechanisms from the stem cell niche support cells provide cues that influence the self-renewal of stem cells or differentiation depending on the state of tissue function. It has been postulated that SSC self-renewal takes place within the niche and differentiating cells are adjacent and separated from the SSC niche (de Rooij, 2017). Continuous and robust spermatogenesis relies on SSC self-renewal to maintain a pool of SSC and differentiation for spermatogenesis to take place (Oatley and Brinster, 2012; de Rooij, 2015; de Rooij, 2017). During post-neonatal and prepubertal period in males, SSC undergo a series of self-renewal divisions to establish an SSC pool. In post pubertal period, the rate of SSC renewal is reduced due to active spermatogenesis taking place (Kanatsu-Shinohara *et al.*, 2005; Law and Oatley, 2018). Sertoli cells, Leydig cells and peritubular myoid cells (Fig 2.2) are somatic cells that play a significant role in SSC niche support. Sertoli cells being in contact with germ cells imply they have a significant role in the SSC niche (Law and Oatley, 2018).

2.4 Factors governing SSC self-renewal and differentiation

Spermatogonial stem cells continuously replenish themselves through undifferentiated cell division through a self-renewal process. In the absence of self-renewal process, the SSCs would be depleted through the course of differentiation and would lead to premature sterility of the male (Oatley and Brinster, 2006). Several research projects have been done for more than a decade on factors that control stem cell renewal and, in each research, more details about them are emerging. Glial cell-line derived neurotrophic factor (GDNF) produced by Sertoli cells is essential in SSC self-renewal. Experimental studies *in vivo* have shown

evidence of the role of GDNF in promoting SSC self-renewal among mammalian species (Meng *et al*., 2000; Meng *et al.*, 2001; Oatley and Brinster, 2012; de Rooij, 2015). *In vitro* culture studies of SSC have revealed that supplementing culture medium with GDNF enhanced long-term proliferation of SSC and prevented differentiation. It also promoted the maintenance of rodent SSC in culture (Kubota *et al.*, 2004b). In culture of SSC of livestock species, GDNF has been the main growth factor among a cocktail of others added to the cell culture medium (Aponte *et al.*, 2006; Crouse, 2012; Oatley *et al.*, 2016; Suyatno *et al.*, 2018). Sertoli cells are responsible for the production of GDNF and it acts through binding to its receptors GFRA1 and c-RET on the plasma membrane of spermatogonia (Tadokoro *et al.*, 2002). Evidence of GDNF acting as a chemoattractant to induce migration of SSC out of the stem cell niche to has been documented (Dovere *et al*., 2013). Cell migration of SSC is essential to maintain homeostasis within the niche.

The Fibroblast Growth Factor 2 (FGF2), which is also produced by Sertoli, peritubular, Leydig, and germ cells is essential for SSC renewal. The FGF2 has been shown to increase SSC proliferation and colony forming of SSC in culture for livestock similar to the one in rodents (Aponte *et al.*, 2008; Oatley, 2010). FGF2 activates a signaling pathway, which, in turn, upregulates the expression of proteins associated with SSC self-renewal (Oatley *et al*, 2006). Sertoli cells also produce chemokine (C-X-C motif) ligand 12 (CXCL12), which has been revealed to take part in the maintenance of SSC *in vitro* in mice (Yang *et al*., 2013) as well as maintenance of SSC in bovine (Oatley *et al.*, 2016). Kanatsu-Shinohara et al. (2012) showed that both GDNF and CXCL12 act as essential SSC chemotactic factors *in vitro* and *in vivo* (Kanatsu-Shinohara *et al*., 2012). Colony-forming Factor 1(CSF1) has been associated

with signalling mechanisms that promote SSC self-renewal. This cytokine is produced by Leydig cells and some peritubular myoid cells. Studies in mice documented the importance of CSF 1 in the self-renewal of SSC (Oatley *et al*.,2009) and bovine SSC (Oatley *et al.*, 2016).

In addition to GDNF, CSF-1, FGF2, and CXCL12, research in mice SSC indicated that leukemia inhibitory factor (LIF) and insulin-like growth factor I (IGF-I) may be important in SSC self-renewal. Some studies have documented a significant role of LIF in SSC renewal when used with other growth factors although its omission in cultures did not stop SSC proliferation (Kanatsu-Shinohara *et al.*, 2007). Collectively, evidence from in vitro studies has identified GDNF, CXCL12, LIF, CSF-1, IGF-I, and FGF2 as regulators of SSC functions, specifically self-renewal. GDNF and FGF2 have been listed as the main signaling factors as a cocktail with other growth factors to promote the proliferation of SSC in vitro.

2.5 The testicular interstitial space

The interstitial space, which lies in between the seminiferous tubules is occupied by the Leydig cells, myoid cells, macrophages, and the vascular network. These have specialized functions that are crucial for germ cell development. Leydig cells produce mainly testosterone, which is the main androgen in the male that is responsible for the development of the male sexual characteristics (de Rooij, 2017). Leydig cells together with myoid cells secrete CSF-1, which stimulates the proliferation of As and Apr spermatogonia, thereby promoting self-renewal. On the other hand, myoid cells are flat mesenchymal cells lining the outer seminiferous tubules, important in structural support (Oatley *et al*., 2009).

2.6 Spermatogenesis process

Spermatogenesis is the process in which SSC undergo proliferation and differentiation to produce haploid spermatozoa from puberty and throughout a male's life. Spermatogenesis includes a series of cell divisions, which begins at the level of the spermatogonial stem cells (SSC) and many subsequent steps, leading to the production of spermatozoa, the highly specialized differentiated gametic cells (Oatley and Brinster, 2006). Through fertilization of the ova in the female, the spermatozoa are capable of transmitting genetic material to the offsprings. Spermatogenesis yields high numbers of spermatozoa and since the quality of their genetic material is crucial for future generations, the maintenance of DNA integrity is of fundamental importance in this process. Hence spermatogenesis is a highly regulated three-stage process including the proliferative phase (spermatogoniogenesis), the meiotic phase, and the transformative phase (spermiogenesis) (Oatley and Brinster, 2006). In spermatogoniogenesis, the SSC undergo a series of mitotic divisions, one daughter differentiating germ cells divides into several clones to increase the total spermatozoa output. Cells recruited for spermatogenesis begin the process of mitotic phases, each cell dividing into daughter cells to form a chain of 4, 8, and then 16 Aal cells connected by intercellular bridges. The 16 Aal cells differentiate into A1 spermatogonia. This group of A1 spermatogonia are first referred to as differentiated spermatogonia. Multiple mitotic divisions of 16 cell chains A1 divide to make 32 A2 cells, another mitotic division yields 64 A3 cells, and these divide to give chains of 128 A4 cells. The A4 cells divide to form an intermediate cell type, a further division yields type B spermatogonia. At this sThere are 512 type B spermatogonia for every A1 cell that entered mitosis at this stage type B spermatogonia undergo mitosis into primary spermatocytes to produce 1024 cells. Many of these cells will

undergo apoptosis throughout this process, however, significantly reducing this number (Curtis and Amann, 1981).

Primary spermatocytes undergo two meiotic divisions. Signaling mechanism involving testosterone and other cytokines open up the tight junctions formed by the Sertoli cells, to facilitate the movement of primary spermatocytes into the immune-privileged, ad luminal area of the seminiferous tubule (Staub and Johnson, 2018). In the ad luminal compartment, primary spermatocytes enter in meiosis prophase 1 stage where there is a doubling of DNA, then an exchange of genetic material through crossing over occurs and the chromosomes migrate to opposite poles of the cell for cell division, resulting in two secondary spermatocytes which rapidly enter meiosis II division. This is followed by meiosis II which results in four haploid round spermatids that undergo spermiogenesis, a differentiation process that involves morphological changes into elongated fully mature spermatozoa (Fig 2.3).

Spermatozoa are spermiated into the lumen and then move to the epididymis for final maturation and storage (Russell *et al*., 1993). This process is maintained from puberty through most of the adult life under the regulated balance of SSC self-renewal and differentiation. The spermatogenesis process occurs continuously, thus the seminiferous epithelium is composed of different stages of germ cells at different stages of spermatogenesis. The duration of spermatogenesis is 47.7 days in the bucks (*Capra hircus)* (França, 1999).

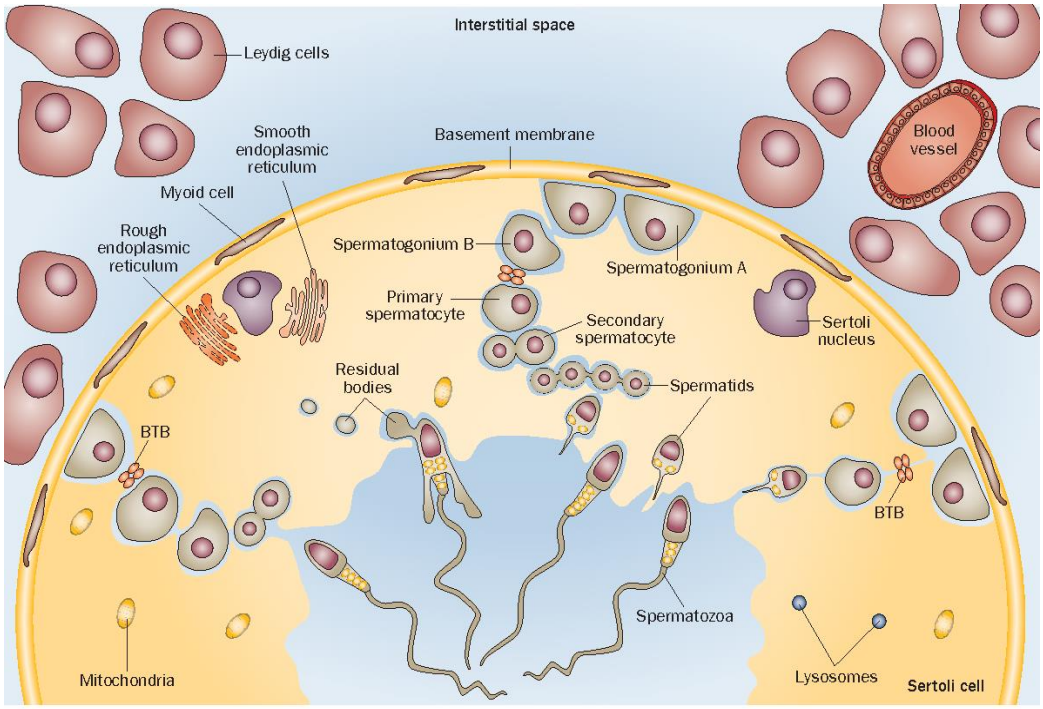


Figure 2. 3: Spermatogonia divison stages during spermatogenesis (Rato *et al.*, 2012)

2.7 Phenotypic characteristics of spermatogonial stem cells

Spermatogonial stem cells are a rare group of cells within the entire population of testicular cells. In the mice, it has been reported that there is only 1 SSC out of every 3000 (0.03%) testicular cells. This scarcity of the SSCs within the testis and the complexity of their identification is a bottleneck for the successful *in vitro* culture of pure SSC lines (Tegelenbosch and de Rooij, 1993). This notwithstanding, isolation of these cells and in vitro characterization is imperative for their use in reproductive technologies, animal transgenesis and genome editing. Many studies have examined ways of accomplishing this, mainly by identification of potential SSC markers (Kubota *et al.*, 2003; Oatley and Brinster, 2008; Bahadorani, 2011; Heidari *et al.*, 2012)**.** The study for SSC markers began with examination of the expression of other tissue-specific stem cell markers. This provided a basis for

isolation of an enriched SSC population although it was discovered that these surface markers are not unique to SSCs but can be expressed by other types of undifferentiated or differentiating spermatogonia and in some cases even somatic cells (Oatley and Brinster, 2008). As more investigation continues, there is a deeper understanding of SSC molecular markers unique to only these cells, being discovered every day (Savvulidi *et al*., 2019).

2.8 Cell Surface Markers

2.8.1 Thymocyte differentiation antigen 1 (THY 1)

THY1, also called CD90, is a glycoprotein present on the cell membrane cells: T lymphocytes, hematopoietic stem cells, and embryonic stem cells (Kubota *et al.*, 2003). This surface marker is present on a certain population of testicular cells in mice and upon transplantation analysis, it was shown that THY1+ cells were enriched for SSCs (Kubota *et al*., 2003). More recently, THY1 was reported to be a surface marker of SSCs in livestock such as goats (Bahadorani, 2011; Abbasi *et al.*, 2013; Wu *et al.*, 2013), in pigs (Zheng *et al.*, 2014), and bovine (Reding *et al*., 2010). Reding et al. (2010) showed via flow cytometric analysis, that a small sub-population of bull testicular cells were THY1+ and THY1+ cells were also positive for PLZF, a molecular SSC marker. When transplanted into immunodeficient nude mice, THY1+ cells had 6-fold greater colony formation than when non THY1 non-selected cells were used.

2.8.2 GDNF receptor complex- GFRα1

In vitro, GDNF has been confirmed to maintain SSC in an undifferentiated state (Lord and Oatley, 2017). It acts through a receptor complex consisting of the GDNF family receptor alpha 1 (GFRα1) (Kakiuchi *et al.*, 2018). If SSCs activities are influenced by this growth

factor, it is reasonable to test whether this receptor complex would be present on SSCs. There was no difference in transplantation analysis for colony formation of GFRα1+ enriched cells and unselected testicular cell population, an indication that the GDNF receptor complex may not be a valuable marker for SSC enrichment (Ebata *et al*., 2005). In neonatal porcine testis, GFRα1 was expressed in a sub-population of gonocytes, although GFRα1-negative cells were also present in gonocytes of the neonatal testis, and the proportion of GFRα1- positive cells was less than GFRa-1-negative cells (Lee *et al.,* 2013). In goats, a portion of THY 1 enriched SSC that were positive for GFRα1 was more than GFRα1 negative cells (Wu *et al.,* 2013). For a deeper understanding to have clarity if all undifferentiated spermatogonial express GFRα1, further investigations will be needed as some earlier studies have reported that Sertoli cells and spermatocytes also express this marker in rats (Fouchécourt *et al.*, 2006).

2.8.3 CD9 marker

A cluster of Differentiation (CD9) is a transmembrane protein involved in cell adhesion, migration, proliferation and fusion and is expressed in neuronal stem cell, hemopoietic stem cells, and embryonic stem cells (Oka *et al*., 2002). Expression of CD9 was demonstrated in rodent testis cells located at the basement membrane, which could be SSC. In another study, the selection of mice and rat testicular cells expressing the CD9 marker resulted in spermatogonial stem cell enrichment (Kanatsu-Shinohara *et al.*, 2005). Expression of CD9 marker has also been studied, where testicular cells were enriched for CD9+, thought to be high in SSC (Kaul *et al*., 2012). In Bovine testicular cells, SSC were selected through immunocytochemistry for CD9 and PLZF markers (Cai *et al.*, 2016). However, more studies are needed to confirm that testicular cells specifically SSC and not somatic cells express CD9.

2.8.4 C-KIT marker

This is a proto-oncogene factor that encodes for a tyrosine kinase receptor. C-KIT is expressed in gonocytes and other embryonic stem cells. It has been discovered that gonocytes are c-KIT positive during migration, lose this phenotype during the transition to an undifferentiated spermatogonial state, and then regained c-KIT expression upon transition into A1 differentiated spermatogonia (Kubota *et al.*, 2003). When using the c-KIT marker, an SSC-enriched cell isolate is obtained by eliminating cells that are positive for c-KIT (gonocytes and differentiated spermatogonia) (Kubota *et al.*, 2003). In goat testicular cells, c-KIT expression was minimal in undifferentiated spermatogonia compared to differentiated type A1 spermatogonia (Heidari *et al.*, 2012).

2.8.5 α6-and β1-integrin

Integrins are cell adhesion molecules and also make up the extracellular matrix in cell membranes. Integrins play roles in cell proliferation, attachment to the basement membrane, and act as cell signaling molecules. α6-intergrin and β1-integrin form a heterodimer, which serves as a receptor for laminin, a binding component of seminiferous tubule basement membranes. Since SSCs have been documented to bind preferentially on laminin, these molecules were thought to be possible surface markers of SSC. Selection of mice testicular cells for α6-and β1-integrin resulted in enrichment of SSCs and increased colonization following transplantation in comparison to unselected testicular cells (Kanatsu-Shinohara *et al*., 2004). In bovine testicular cells, it was impossible to enrich bovine type A spermatogonia using the α intergrins (de Barros *et al.*, 2012a).

2.9 Molecular Markers for SSC

2.9.1 Promyelocytic leukemia zinc finger protein (PLZF)

The transcription factor PLZF, also known as ZBTB16), is essential in the maintenance of mammalian SSC. Its expression is restricted to the spermatogonial stem cells (As, Apr, and Aal spermatogonia) in mice (Costoya *et al*., 2004) and bovine (Reding *et al.*, 2010, Anglin *et al.*, 2010, Crouse, 2012, Cai *et al.*, 2016, Oatley *et al.*, 2016). This marker was also conserved in sheep SSC (Borjigin *et al.*, 2010) and goats SSC (Abbasi *et al.*, 2013; Song *et al.*, 2013; Pramod and Mitra, 2014; Sharma *et al.*, 2020). PLZF expression in SSC has also been reported in porcine (Lee *et al*., 2013; Kim *et al.*, 2014b). PLZF transcription factor has therefore been shown to have a conserved expression in livestock species and is thus one of the most important markers for characterization of SSC in livestock species.

2.9.2 Ubiquitin carboxyl-terminal esterase L1 (UCHL1)

A commonly used marker in the enrichment of SSC from livestock species is UCHL1, also referred to as protein gene product 9.5 (PGP9.5). UCHL1 expression is found within the As, Apr, and Aal spermatogonia of livestock species, and a study with porcine SSC showed enrichment of UCHL1+ cells in PLZF+ populations (Luo *et al.*, 2009). This molecular marker has been demonstrated to be expressed in bovine SSC (Herrid *et al*., 2007; Suyatno *et al.*, 2018,), sheep SSC (Binsila *et al*., 2020), and goat SSC (Heidari *et al*., 2012; Heidari *et al.*, 2014; Shirazi *et al.*, 2014). However, UCHL 1 is considered a more general marker for Type A spermatogonia with PLZF being more specific to A single spermatogonia. To verify this, Reding et al. (2010) characterized this population in the bull, co-localization of PLZF

expression in some, but not all, UCHL1+ Spermatogonia, suggests that UCHL1 may be a more general marker of type A.

2.9.3 Dolichos biflorus agglutinin (DBA)

Expression of lectin DBA has not been documented in rodent SSC. DBA has been used to select for SSC in livestock species with no consistency in expression (Herrid *et al.*, 2007). Expression of DBA has been documented in porcine gonocytes although this expression is lost with age (Goel *et al.*, 2007). In bovine testes, DBA was expressed in gonocytes and type A spermatogonia (Herrid *et al*. 2007, Fujihara *et al.*, 2011). The expression profile of DBA is not specific to spermatogonial stem cells (Borjigin *et al*., 2010).

2.9.4 B-cell CLL/lymphoma 6 (BCL6B)

BCL6B, a transcription repressor has been documented as a molecular marker of SSCs (Oatley *et al*., 2006). BCL6B expression is conserved in the rodent's SSC. In an experiment where BCL6B was knocked out in mice SSCs, a high rate of apoptosis of the SSC was recorded (Oatley *et al.*, 2006). In bovine, Reding et al. (2010) reported expression of BCL6B in enriched THY1+ testicular cell population. More studies need to be done to verify the restricted expression of this marker in livestock species SSC.

2.9.5 Inhibitor of DNA binding 4 (ID 4) marker

Expression of ID4 has been demonstrated in spermatogonial stem cells. This factor plays a role fate decision of SSC. In a study by Oatley et al., (2011), ID4 was highly expressed by spermatogonial stem cells in mice. In bovine species, ID4 was identified as a marker for SSC. High levels of the ID4 gene were expressed in the selected germ cell population in

bovine, however, ID4 was not detected in the somatic cell feeder cell layer (Oatley *et al.*, 2016).

2.9.6 Lin 28A marker

Lin 28A is a group of transcription factors playing a role in mediating cell activities including cell proliferation and differentiation. Lin28A regulates the proliferation of undifferentiated spermatogonia (Oatley *et al.*, 2016). Recently in bovine SSC, Lin28A was reported to be co-expressed with PLZF (Oatley *et al.*, 2016).

2.10 Applications for SSC in livestock

In vitro culture of spermatogonial stem cells provides an opportunity to exploit and study mechanisms controlling self-renewal and the spermatogenesis process. In this era where gene editing using the CRISPR technology can be used to precisely make knock-in or knock-out gene mutations at a specific locus in the genome, established spermatogonial stem cell lines provide a unique platform for their utilization in gene editing. The SSCs can regenerate spermatogenesis when transplanted into germline ablated recipients, therefore enabling a male to carry sperm of donor-derived haplotype and disseminate the germplasm within the animal population (Savvulidi *et al.*, 2019). The *in vitro* culture and SSC applications will be discussed more in chapters four and five of the thesis.

CHAPTER THREE

3.0 A SYSTEMATIC REVIEW OF LITERATURE ON THE CULTURE OF SPERMATOGONIAL STEM CELLS AND THE USE OF SURROGATE SIRES AS A BREEDING TECHNOLOGY TO PROPAGATE SUPERIOR GENETICS IN LIVESTOCK PRODUCTION

3.1 Introduction

Spermatogenesis is the process through which spermatozoa are produced in males. The process is highly specialized and is dependent on the continuous actions of spermatogonial stem cells (SSCs). An equilibrium between self-renewal of SSC and the production of differentiating spermatogonia is key to sustaining optimal sperm production while preventing exhaustion of the stem cell reservoir. SSC fate is partly influenced by signaling mechanisms from growth factors secreted by somatic cells surrounding the SSC niche, most importantly Sertoli cells (de Rooij, 2015; Oatley and Brinster, 2012). SSCs have the unique potential to expand *in vitro* and form colonies of undifferentiated spermatogonia. These *in vitro* cultured SSCs when transplanted to testes of live recipient animals, re-establish spermatogenesis producing sperms of donor-derived haplotype (Oatley *et al*., 2016). The use of SSC to form gametes from specific sires provides an opportunity for genetic improvement in livestock. However, successful transplantation of SSC requires the establishment of robust and effective *in vitro* culture systems. Such systems will ensure the small number of SSC (Approximately 0.03% of total testicular cells) isolated from the testes can be multiplied to millions before transplantation is effected (Oatley and Brinster, 2012).

The lack of methodologies for long-term expansion of SSC in culture and effective methods for the preparation of ideal recipients to undergo transplantation of donor SSCs has limited exploitation as an alternative breeding technology in livestock production systems (Oatley *et al.*, 2016). Limited studies have documented protocols for long-term expansion of SSCs in livestock species with varying success (Aponte *et al*., 2006; Crouse, 2012; Oatley *et al*., 2016; Suyatno *et al*., 2018). The ultimate proof of the existence of SSCs in a culture dish is through transplantation and re-establishment of donor-derived spermatogenesis in the testes of the recipient animal (Ciccarelli *et al*., 2020). Additionally, successful SSC culture systems for livestock species will herald opportunities for the study of gene functions and exploration of gene editing methodologies for the *in vitro* cultured SSC.

The objective was to collate data on *in vitro* culture systems of spermatogonial stem cells in livestock, and specific SSC markers for identification and gene manipulation of these cells. Thereafter, document the standardized, workable, and reproducible *in vitro* culture conditions and feasible applications of SSCs in livestock production systems.

3.1 Materials and Methods

3.1.1 Data sources and search strategy

In line with PRISMA (Preferred Reporting Items for Systematic reviews and Meta-Analyses) guidelines (Page *et al.,* 2021), a systematic literature search was performed. Data search was done in three electronic databases: PubMed, ScienceDirect, and Google Scholar for research articles published between January 1990 and February 2021. A search syntax with various combinations of search terms such as spermatogonial stem cell terminologies, culture, and

livestock species was undertaken (Appendix 1). From the Pubmed database, a total of 6179 articles were exported to the Mendeley reference manager, the duplicates resolved and combined into one file and exported to *Rayyan QCRI software* for screening. The database search in science direct resulted in a total of 10,500 papers and 1500 papers from google scholar which were exported to the Mendeley reference manager.

3.1.2 Selection criteria and data extraction

Original research articles published in a peer-reviewed journal that reported on the culture of spermatogonia cells in at least one livestock species were included. Articles were excluded if (i) they were in a non-English language, (ii) the study of spermatogonia stem cells was in non-livestock species, and (iii) abstracts were not published as full manuscripts, (iv) they were non-experimental studies. Article searches and screening were performed by considering article titles and abstracts for inclusion according to the search criteria. Data extraction from studies was performed by the investigator who was a PhD student and independently checked by another independent reviewer using a customized checklist. All the articles from the 3 electronic databases were exported to the Mendeley reference manager. Duplicate articles were excluded and the resultant data file from each of the databases was exported to Rayyan systematic reviews software (https://www.rayyan.ai/) (Ouzzani *et al*., 2016) for screening.

3.1.3 Data analysis

For all the included studies, the data were categorized into the following groupings: (i) studies on *in vitro* culture of SSCs for short term ≤21 days or long-term culture ≥21 days; (ii) studies on SSC characterization using specific SSC and general pluripotent markers; (iii)

studies on SSC transplantation, methods of recipient preparation and fate of donor SSC; and (iv) studies on SSC gene manipulation methodologies. The quality of articles included in the review was assessed using the Cochrane handbook for systematic reviews version 6.2. Cochrane, 2021(Available from www.training.cochrane.org/handbook). Articles were evaluated based on methodological study design and grouped according to the following categories: (i) Good quality studies: methodology as clear and precise mainly on multiparameter enrichment procedures for SSC, confirmation of SSC markers through Real-Time PCR and immunochemistry and SSC transplantation (ii) Medium quality: Clear methodology on isolation, enrichment, and characterization of SSC through immunostaining only.

3.2 Results

3.2.1 Description of included studies

Of the 4786 distinct articles retrieved, 162 studies were reviewed (Fig. 3.1) and 93 studies met all inclusion criteria. The 93 studies were geographically diverse and included 13 countries. Geographical distribution was as follows Iran n=19, USA n= 17, China n=15, Australia n= 14, Korea n= 6, Japan n=1, United Aran Emirates n= 1, India n=6, Brazil n=3, Canada n= 4, Netherlands n= 5, Finland n=1, Switzerland n= 1. There was no study associated with spermatogonial stem cells in any species in Africa. Thirty-six studies focused on *in vitro* culture of SSC, 21 studies on identification of specific markers of SSC, 23 studies on transplantation of SSC, 6 studies on transfection, and 5 studies on donor-derived spermatogenesis. Categorization of studies based on the livestock species in which the study was conducted was as follows: bovine n=36, goats n=23, pigs n=17, sheep n=16, and camels

n=1. Record on the year of publication of the studies was as follows: the year 2016-2021, n= 28 studies; the year 2005-2015, n= 50 and the year 1990-2004, n= 14. The *in vitro* culture of SSC from livestock animals was first published in the year 1999. The early studies focused mainly on the isolation of a mixed germ-cell population including SSC and short-term culture of the cells (Dobrinski *et al*., 2000; Izadyar *et al.*, 2002; Oatley *et al.*, 2002; Dirami *et al.*, 1999). Since then, there have been striking advances in the standardization of protocols for isolation, purification, characterization, and culture of SSC. Furthermore, the SSC transplantation technology has been explored for its viability in the production and dissemination of superior male gametes in livestock production systems.

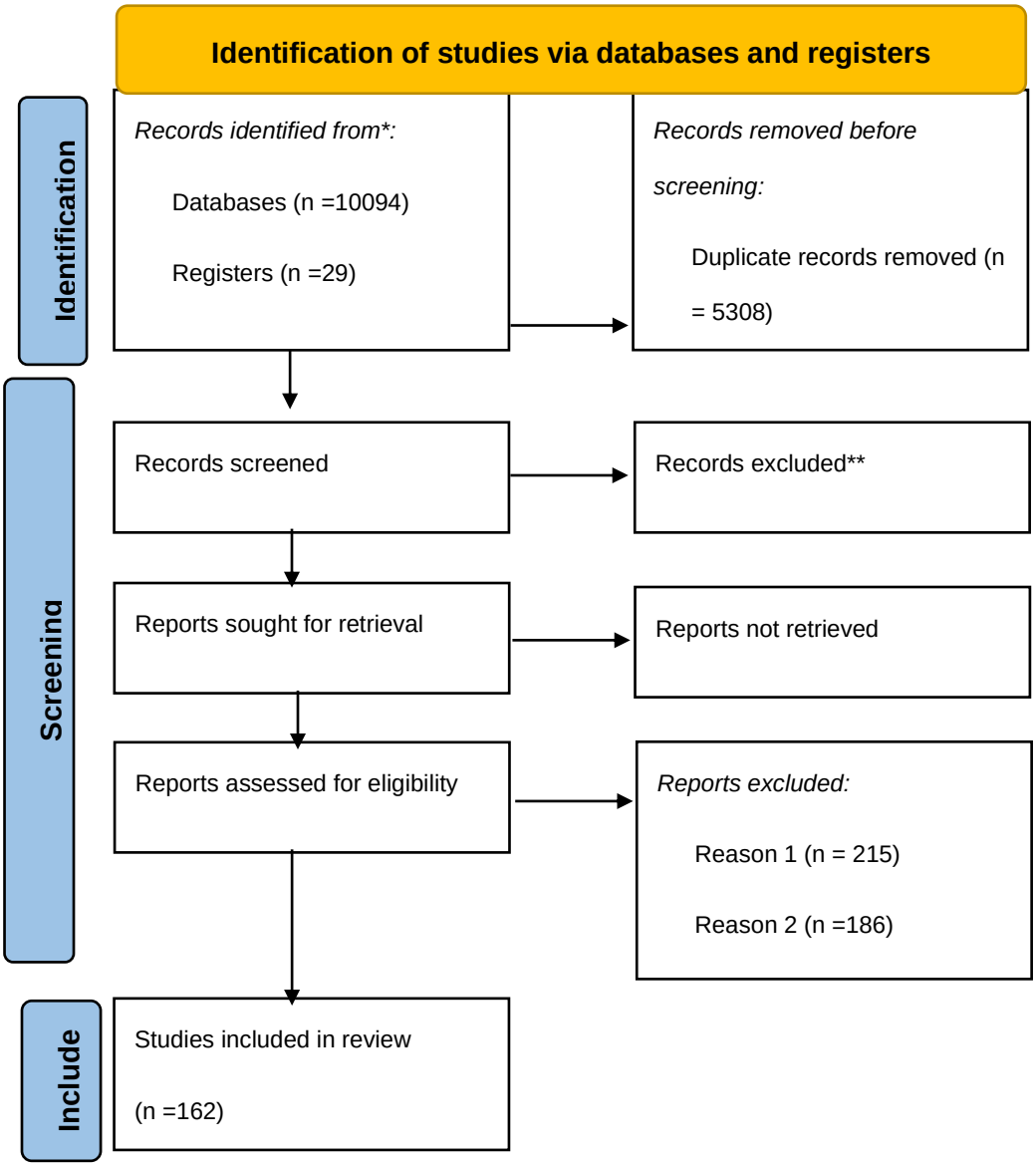


Figure 3. 1: PRISMA literature search results

Key:

Reason 1: Wrong population

Reason 2: Wrong study design

Reason 3: Wrong outcome

Reason 4: Not cells of interest

Reason 5: Non-English language

3.2.2 Long-term culture of SSC and the culture conditions required

The duration for which SSC were maintained in culture was categorized into 3: culture for 1-3 days (n=8), 5-18 days n=23, >21 days n=7. Of much interest was the long-term culture of the SSC for a period >21 days (Table 3.1). From the review findings, the longest period of SSC culture was 3 months on a feeder cell monolayer (Sandos inbred mouse (SIM)-derived 6-thioguanine- and ouabain-resistant cells (STO)) using a serum-free medium (Knock out serum replacement) (Suyatno *et al*., 2018). The SSC from immature and mature bovine testis stably expressed SSC markers during the culture period. Interestingly, SSC from mature testis required supplementation with the drug 6-bromoindirubin-3′-oxime (BIO) in culture. The drug, BIO activates the signaling pathway associated with SSC self-renewal. Additionally, bovine SSC from an immature testis were maintained in serum-free medium on bovine fetal fibroblast (BFF) feeder cells for 2 months (Oatley *et al*., 2016). However, when the bovine SSC were cultured on laminin-coated plates (feeder-free) in preconditioned serum-free medium, the cells could only persist in culture for 1 month. These SSC in culture expressed SSC-specific markers in cattle PLZF/ZBTB16 and LIN28 (Oatley *et al*., 2016). Lastly, bovine SSC were also cultured in serum-free medium on bovine somatic testicular cells feeder layer 3 for weeks (Crouse, 2012). Generally, from the findings of the review, long-term cultures of bovine SSC utilized serum-free medium on feeder cell layer with a cocktail of growth factors, which included: Glial cell line-derived neurotrophic factor (GDNF), Fibroblast growth factor 2(FGF2), Colony Stimulating Factor (CSF-1) and Stromal-Cell-Derived Factor (SDF-1).The long-term bovine SSC culture studies reported the detrimental effects of serum on SSC self-renewal and thus used a serum-free medium (Crouse, 2012;

Oatley *et al.,* 2016; Suyatno *et al*., 2018). On the contrary, in sheep and goats, the long-term culture medium of SSC was supplement fetal bovine serum (FBS) (Pramod and Mitra, 2014; Binsila *et al.,* 2020). The proliferation in culture was reported for extended periods, although the evidence of SSC undifferentiated status through marker expression cells expressed CDH1, UCHL1, GFRα1, PLZF, and ITGA6 in sheep and of PLZF, α6 integrin in goats was not sufficient to conclude that the cultures were indeed made up of undifferentiated SSC.

Table 3. 1: Reports of long-term culture of SSC in livestock

Culture period	Species	Age of donor	Culture Period	Growth factors	Medium used	Marker evaluation	Culture conditions
Suyanto *et al*.,2018	Bovine	3 months	3 months	LIF or GDNF	15% KSR and 1% FBS for 5 days n then 20% KSR	Dome-shaped ES cell-like colonies UCHL-1, DBA. PCR detection: NANOG, OCT4,SOX2	3 months in SFM in 5%CO2 37°C STO feeder cells
Oatley *et al.*, 2016	Bovine	4-5 months	2 months	GDNF, FGF,LIF	Stempro Serum free	Germ cell clumps PLZF, LIN28,GFRA1, ID4,NANOS2 markers	5%CO2,10%O2 at 35°C on BFF feeder for 2months 1 month on laminin-coated plates n preconditioned media
Crouse, 2012	Bovine	3-4 months	3 weeks	GDNF, FGF, SCF SDF	Stempro serum free	ZF	3-week BSC feeder layer better than BEF 37°c
Pramod and Mitra, 2014	Goat	3-4 months	2months	NONE	10%FBS on Sertoli cell layer	PLZF, α6 integrins	Sertoli feeder layer 37°C, 5%CO2
Binsila *et al*., 2020	Sheep	Prepubertal rams	36 days	GDNF,IGF,EGF	Stempro ,10% FBS	PLZF, ITGA, GFRα1	Laminin-coated plates feeder-free culture
Izadyar *et al.*, 2003	Bovine	5 months	3 months	none	2.5% FCS in MEM	DBA, colonies, Cells differentiated to spermatids	Sertoli feeder layer 5%CO2
Dobrinski *et al*., 2000	Boar, Bovine	6-week boar, 6-month bovine	1 month	none	DMEM	None. Cells transplanted to mice testes	STO feeder32 °C, 5%CO2
Aponte *et al*., 2006	bovine	4-6 months	25 days	None	MEM with 2.5% FCS and fungizone	Blob-like colonies Cells transplanted to mice testes	A Monolayer of Sertoli cell developed in the germ cell culture

Key for Table 3.1:

KSR: Knock out Serum Replacement
SFM: Serum Free Media, DMEM; Dulbecco's Modified Eagle Medium, MEM; Minimum essential media
FBS: Foetal Bovine Serum, FCS: Foetal Calf Serum
STO: Sandos inbred mouse (SIM)-derived 6-thioguanine- and ouabain-resistant (STO) cells
SDF: Stromal-Cell-Derived Factor; SCF- stem cell factor

BEF: Bovine embryonic fibroblast

3.2.3 Characterization of SSC in culture using specific spermatogonial stem cell markers in livestock

Markers used for SSC characterization in the review included: VASA, PLZF, THYI (CD9), UCH-LI (PGP9.5), αIntegrins, DBA, OCT 4, GFRα1, LIN28 and NANOG (Table 3.2). In all the studies (n=74) where SSC were cultured, the expression of more than one marker in SSC was evaluated for their identification in addition to the typical morphology of germ cell colonies. Ubiquitin carboxy-terminal hydrolase 1 (UCH-L1) also called Protein gene product (PGP9.5) was the most commonly used marker (n=47). Expression of promyelocytic leukaemia zinc finger transcription factor (PLZF) was evaluated in 21 studies and was also the main specific marker used for the identification of bovine, caprine, and porcine SSC. Thymocyte differentiation antigen 1 (THY1) expression was reported in SSC of 15 studies. Evaluation of LIN28 gene expression by SSC was conducted in only one study and was reported to be uniquely expressed (Oatley *et al.*, 2016). From the review, different studies used different markers for verification of SSC undifferentiated status, however, expression of PLZF as being confined to SSC was reported not only in rodents but also in livestock, as supported by recent studies in bovine (Crouse, 2012; Oatley *et al.*, 2016), in sheep (Binsila *et al.*, 2018; Borjigin *et al.*, 2010), in pigs (Kim *et al.*, 2013) and goats (Song *et al.*, 2013; Sharma *et al.*, 2020).

Table 3. 2: Reports on molecular markers used in SSC characterization in Livestock.

Markers	Bovine	Ovine	Porcine	Caprine	Camel	Total
VASA	Oatley *et al*., 2016, Kim *et al.*, 2014a, McMillan *et al*., 2013, n=3	Borjigin *et al*.,2012, Niu *et al*.,2015, Borjigin *et al*.,2010, Herrid *et al*.,2010 n=4	Kim *et al.*, 2013, Zhang, 2020 n=2	Jiang *et al*.,2014, Bahadorani *et al*.,2012, Wang *et al*.,2014 n=3	0	n =12
PLZF	Oatley *et al*. 2016, Reding *et al*., 2010, Crouse *et al*.,2011, Cai *et al*.,2016, Anglin *et al*.,2010, McMillan *et al.*,2013, n=6	Borjigin *et al*.,2012, Borjigin *et al*.,2010 n=2	Lee *et al*.,2016, Lee *et al*.,2014, Lee *et al*.,2019, Kim *et al.*, 2013, Zhang, 2020 n=5	Abbasi *et al*.,2013, Kumar *et al*.,2014, Ren *et al.*, 2020, Zhu *et al*.,2020, Bahadorani *et al*.,2012, Sharma *et al*.,2020, Abbasi *et al*.,2015, Song *et al*.,2013 n=8	0	n =20
THY 1	Tajik *et al*.,2017, Giassetti *et al*.,2012, Reding *et al*., 2010, Nasiri *et al*.,2012, Youssefi,*et al*.,2016 n=5	Binsila *et al.*, 2018, Kim *et al.*, 2013 n=2	N/A	Abbasi *et al*.,2013, Jiang *et al*.,2014, Ren *et al* 2020, Bahadorani *et al*.,2012,Kaul *et al.*, 2010, Sharma *et al*.,2020, Abbasi *et al*.,2015, Song *et al*.,2013 n=8	0	n =15
UCHL1 (PGP9.5)	Suyatno *et al.*, 2018, Giassetti *et al*.,2016, McMillan et al.,2013. n=3	Binsila *et al*, 2020, Binsila *et al.*, 2018, Chuan-ying *et al*.,2017 n=3	Lee *et al*.,2019, Kim *et al*.,2019b, Luo *et al*.,2009 n=3	Sharma *et al*.,2020, Wang et al.,2014, Song *et al*.,2013, Zeng *et al* 2012 n=4	0	n =13
OCT 4	Tajik et al.,2017, Nasiri et al.,2012 Kim et al.,2015, Jabarpour et al.,2017, n=4	Qasemi-Panahi *et al*.,2018 n=1		Wang *et al*.,2014 N=1	0	n =6
DBA	Suyatno *et al.*, 2018, Herrid *et al*.,2009, Izadyar *et al*,2002, Aponte *et al*.,2006, Kim *et al*.,2014a, Herrid *et al*.,2007, Redden *et al*.,2009, n=7		Zhang,2020 n=1	Bahadorani *et al*.,2012, Sharma *et al*.,2020, Song *et al*.,2013 n=3	0	n =11
PGP9.5 (UCHL1)	Kim *et al*., 2015, de Barros *et al*.,2012a, Herrid, *et al*.,2006, Kim *et al.*,2014a, Herrid *et al*.,2007, Redden *et al*.,2009, n=6	Moghaddam *et al*.,2016, Zandi *et al*.,2015, Rodriguez-Sosa *et al*.,2006, Borjigin *et al*.,2010, Herrid *et al*.,2010 n=5	Lee *et al*.,2016, Kim *et al*., 2014b, Lee *et al*.,2014, Luo *et al*.,2006, Kim *et al.*, 2013 n=5	Shirazi *et al*.,2015 Heidari *et al*.,2012, Shirazi *et al*., 2014, Heidari *et al.*, 2014 n=4	0	n =20
Gfr1	Suyatno *et al*., 2018, Kim *et al.*, 2015 de Barros *et al*.,2012a, Oatley *et al*.,2004, n=4,	Rasouli *et al*.,2020, Binsila *et al*, 2020 n=2	Chuan-ying *et al*.,2017 n=1	Zhu *et al*.,2020 n=1	0	n =8
LIN28	Oatley *et al*., 2016. n=1	n/a	n/a	n/a	0	n =1
NANOS2	Oatley *et al.*, 2016 n=1	n/a	n/a	n/a	0	n =1
CD9+	Cai et al.,2016 n=1	n/a	n/a	Kaul et al., 2012 n=1	0	n =2
C-KIT	Dirami *et al*.,1999 n=1	n/a	n/a	Heidari *et al*.,2012, Heidari *et al*., 2014 n=2	0	n =3

CXCR4	Giassetti *et al*.,2012, n=1	n/a	n/a	n/a	0	n =1
Markers	**Bovine**	**Ovine**	**Porcine**	**Caprine**	**Camel**	**Total**
Aintegrin	Giassetti *et al*.,2012 de Barros et al.,2012a, Giassetti *et al*.,2016, Kim *et al*., 2013 n=4	n/a	n/a	Pramod and Mitra .,2014 N=1	0	n =5
SSEA	Kim et al., 2013 n=1	n/a	n/a	n/a	0	n =1
CD49f	n/a	n/a	n/a	Wu *et al*.,2013, Jiang *et al*.,2014	0	n=2
NANOG	Kim *et al*.,2019b n=1	n/a	n/a	n/a	0	n=1

3.2.4 Gene manipulation of SSC (Transfection)

Transfection involves the methodologies of introducing foreign DNA or nucleic acids into host cells with integration in the cell genome. Transfection of SSC attempts were conducted in 11/93 studies (Table 3.3). The SSC have been commonly transfected using viral vectors for gene delivery across the host cell membrane into the cell cytosol. Optimization of the SSC transfection methods and efficiency using the enhanced green fluorescent protein (eGFP) was done in 7/11 studies. The purpose for using the eGFP was to optimize the transfection protocols specifically for SSC and transmission of the gene was confirmed by the presence of green fluorescence in the donor cell population under a fluorescent microscope or flow cytometry. The eGFP transfected cells were transplanted into the recipient testis and the animal was castrated after some time. Colonization of donor cells or donor-derived spermatogenesis was evaluated through the detection of fluorescent donor cells in seminiferous tubules of recipients (Rodriguez-Sosa *et al.*, 2009; Kim *et al.*, 2014b; Abbasi *et al.*, 2015). If donor SSC are transplanted into a compatible recipient, the donor-derived spermatogenesis is expected if the donor cells successfully colonized the seminiferous tubules (Honaramooz *et al.*, 2003). Detection of eGFP-expressing spermatozoa and eGFP-expressing embryos after *In vitro* fertilization using the transgenic semen was successful

(Zeng *et al.,* 2013; Kim *et al.*, 2014b). The findings indicated that transduced SSCs were able to colonize the recipient testis, initiate donor-derived spermatogenesis, and produce transgenic sperm, although quantification of percentages of transgenic donor sperm/DNA was not reported.

Lipofectamine transfection was carried out in 3/11 studies. In the first study, the eGFP gene was transfected successfully into bovine SSC (transfection rate of 37%) (Tajik *et al.,* 2017). In the second study, the *Enhancer of zeste homolog 2* (EZH*2)* gene was successfully knocked out in goat SSC using iRNAs against the gene(Cai *et al.,* 2020). In the third study, recombinant plasmid (pPLZF-IRES2-EGFP*)* and Lipofectamine reagent were effectively transfected into goat SSC to overexpress PLZF protein. Importantly, the findings conclusion reported achievement of desired transfection effect through use of liposomal carriers. The other method of transfection according to the review findings was nucleofection (2/11). Nucleofection was successfully used to deliver transcription activator-like effector nucleases (TALENS) targeting *Duchenne's muscular dystrophy* gene locus (DMD*)* into the porcine SSC nucleus (Tang *et al*., 2018). Insertions and deletion mutations were detected in up to 18% of transfected cells. A similar technique was also used to deliver a transgene construct harboring the human growth hormone gene (hGH) and a chicken beta-globin insulator (CBGI) sequence in goat SSC (Zeng *et al*., 2012). These transfected SSC were transplanted into recipient bucks. Genomic analysis of the recipients' semen revealed the presence of hGH and CBGI sequences in 31.3%±12.6% of ejaculates (Zeng *et al.,* 2012). Lastly, electroporation of SSC was reported in a single study, in which an eGFP plasmid was introduced into porcine SSC. The cells were cultured and evaluated for green fluorescence

reporting, the transfection efficiency >7.5%, and the 80% survival rates of cells (Park *et al*., 2019).

Table 3. 3: Methods and efficiency of transfection of livestock SSC

Author	Country	Species	Transfection method	Transgene	Transfection efficiency
Tajik *et al.*,2017	Iran	Bovine	lipofectamine	GFP	37% uptake of transgene
Kim et al., 2014b	Korea	Pig	Lentivirus vector	GFP	eGFP gene was detected in the donor-derived transgenic sperm and embryos after ICSI 33% eGFP-expressing sperm was produced by the 2 of 6 recipient pigs
Tang *et al*.,2018	Canada	Pig	Nucleofection	DMD gene construct TALENs DNA + eGFP	2.80% to 9% deletions mutations
Kim *et al*.,2014a	Korea	Bovine	Lenti virus vector	e-GFP	17% transduction efficiency
Abbasi *et al*.,2015	Iran	Goat	Lenti virus	eGFP	72% of enriched cells expressed eGFP gene
Rodriguez-Sosa *et al*.,2009	Canada	Sheep	Lenti virus	eGFP	Donor cells expressing eGFP detected in 0.2% Seminiferous tubules
Cai *et al*.,2020	China	Goat	Lipofectamine	siRNAs targeting EZH2 gene	EZH2 gene knockdown
Zeng *et al.*, 2013	USA	Pig	AAV vector, LV	eGFP	eGFP detected in 20% and 5.9% of recipients' ejaculates
Song *et al*.,2013	China	Goat	Lipofectamine	pPLZF-IRES2-EGFP	Overexpression of PLZF increased SCC survival and renewal
Zeng *et al.*,2012		Goat	Nucleofection	Transgene construct both hGH and CBGI.	31.3%±12.6% of ejaculates were positive for both hGH and CBGI sequences
Kim *et al*.,2019b	Korea	Pigs	Electroporation	eGFP	Transfection efficiency (>7.5%) and higher survival rates of cells (>80%)

3.2.5 Recipient preparation methods in livestock species and germ cell transplantation

Successful transplantation of SSC and production of semen of donor-derived genotype would enable utilization of this technology in natural livestock breeding systems and also gene editing platforms (Table 3.4). From the review findings, SSC transplantation was conducted in 4 studies in bovine, 4 in goats, 5 in sheep, 4 in pigs, and 1 in camels. Transplantation of testicular cells into livestock recipients began in 2002 (Honaramooz *et al.,* 2002; Honaramooz *et al.,* 2003; Izadyar *et al.,* 2003) although earlier reports had documented transplantation of livestock species SSC into mice recipient testes (Dobrinski *et al.,* 2000; Oatley *et al.,* 2002). The success of transplantation with resultant donor-derived spermatogenesis can only be achieved through endogenous depletion of the germ cell layer of the recipient animal. In the current review, ablation of the germline layer through irradiation was conducted in 5 studies, ablation through the use of chemo-toxic drug busulfan was carried out in 3 studies; use of Dolichos Biflorus agglutinin DBA in one study, and NANOS2 gene knockout (n=1). Busulfan germline ablated mice recipients were used in 5 studies for evaluation colonization of seminiferous tubules by labeled SSC. When transplantation was done using mice recipients or germline intact livestock recipients, the donor SSC were labelled with fluorescent markers such as Red linker dye (Kaul *et al.,* 2010) or transfected with eGFP prior to transplantation. The fluorescence enables the identification of donor cells or donor spermatozoa through fluorescent microscopy or flow cytometry. Transplantation of SSC into germline intact was conducted in earlier studies in boars, bucks and sheep (Honaramooz *et al.*, 2002; Honaramooz *et al.,* 2003; Herrid *et al.,* 2006; Rodriguez-Sosa *et al.*, 2009; Stockwell *et al.,* 2009; Kaul *et al*., 2010).

Exposing the testes to prescribed doses of irradiations destroys the germline layer. This method was used in a number of studies to prepare recipients and reported the presence of donor DNA or donor transgenes in the semen of recipients following transplantation of donor SSC (Izadyar *et al.,* 2003; Stockwell *et al.,* 2009; Herrid *et al.,* 2011; Zeng *et al.,* 2012; Stockwell *et al.,*2013). The use of irradiated recipients was an effective method of recipient preparation although the challenges included; (i) Use of high levels of irradiation, which would cause bone marrow depression and systemic toxicity, (ii) there was a decline in donor spermatozoa in semen ejaculates of recipients over time and quantification of donor DNA was a challenge due to low percentage in semen (Stockwell *et al.,* 2009). Treatment with the busulfan drug temporarily ablates the germline layer giving a narrow window for regeneration of donor-derived spermatogenesis after transplantation of SSC. In the three studies that prepared recipients using busulfan treatment, there were reports of donor-derived spermatogenesis through detection of donor DNA in the semen (Mikkola *et al.,* 2006) or eGFP expressing spermatozoa that was used for *in vitro* fertilization to produce eGFP expressing embryos (Zeng *et al.,* 2013; Kim *et al.,* 2014b). Although quantification of levels of donor DNA in the semen from the recipients was not carried out and the low levels f donor DNA was indicative of a low percentage of donor-derived spermatogenesis due to the presence of endogenous spermatogenesis.

Germline ablation through treatment with Dolichos Biflorus agglutinin (DBA) was conducted in camels in one study. Donor-derived DNA was detected in the ejaculates of DBA-treated recipients following transplantation of the testicular cells (Herrid *et al.,* 2019). To overcome the challenges associated with temporary ablation of the germline layer, recently genetically germline deficient recipients were generated (Ciccarelli *et al.,* 2020). The recipients were

generated through knockout of the *NANOS2* gene, which is responsible for germline development, hence the germline layer fails to develop but somatic cell support is fully developed and functional (Park *et al*., 2017; Ciccarelli *et al*., 2020). Transplantation of SSC into the *NANOS2* knockout boar, buck, and bull recipients resulted in regeneration of complete and continuous donor-derived spermatogenesis (Ciccarelli *et al.*, 2020).

Table 3. 4: Summary of studies that carried out the transplantation of SSC and donor-derived spermatogenesis

Author	Country	Species	Testis cells	Recipient species	Colonization of transplanted cells
Honaramooz *et al*.,2003	USA	Goat	Fresh	3-5 months Intact germline goats	Fluorescent cells in basement membran of ST of recipients for 12 weeks
Herrid *et al*., 2019	UAE	Camel	Fresh	DBA germline ablated camels	Microsatellite detection of donor DNA sperm
Shirazi *et al.*, 2015	Iran	Goat	Cultured >3 weeks	Busulfan treated mice	Labeled cells in mice seminiferous tubules
Rodriguez-Sosa *et al.*, 2009	Canada	Sheep	Fresh	Germline intact sheep	eGFP donor cells were in average of 0.2% of tubules after 2 months
Mikkola *et al*, 2006	Finland	Pig	Fresh	Busulfan treated (in feed) pigs with immotile short tail sperm defect	Donor-derived DNA in semen
Herrid *et al.*, 2006	Australia	Bovine	Fresh	Intact germline bulls	Fluorescent labeled cells in basement membrane of seminiferous tubules of recipients for 6 months
Izadyar *et al*., 2003	Netherlands	Bovine	Fresh	Irradiated bovine	Colonization determined by DBA staining
Stockwell *et al* 2009	Australia	Bovine	Fresh	Germline intact bovine	Presence of fluorescent cells in seminiferous tubules and spermatogenesis
Honaramooz *et al.*, 2002	USA	Pig	Fresh	Germline intact pigs	Fluorescent labelled cells in BM of ST recipients
Kaul *et al*., 2010	India	Goat	Fresh	Germline intact goats	The fluorescent cells were observed up to 12 weeks after transplantation
Honaramooz *et al., 2003*	USA	Goat	Fresh	Germline intact goats	Donor derived spermatogenesis
Oatley *et al*., 2002	USA	Bovine	Fresh, cultured cells	Busulfan treated mice	Fresh cells colonized seminiferous tubules cultured cells did not
Dobrinski *et al*., 2000	USA	Bovine	Cultured >3 weeks	Busulfan mice	Colonization of ST basement membran
Herrid *et al.*, 2010	Australia	Sheep	Fresh	Irradiated sheep	Donor DNA detected in ejaculate
Herrid *et al*., 2009	Australia	Sheep	Fresh	Irradiated sheep	Donor DNA detected in ejaculate
Kim *et al.*, 2014b	Korea	Pig	Fresh (pLV-TH-GFP) cells	In utero busulfan treated pig	colonies of transduced SSC in the recipients' testes.
Ciccareli *et al.*, 2020	USA	Boar, Goat, Bull	Fresh	NANOS2 knocks	Donor-derived spermatogenesis
Zeng *et al*., 2013	USA	Goat	(AAV), (LV)-Transduced SSC	Busulfan treated goats	EGFP transgene ranged from 0% to 54.8% for recipients of AAV. 0% to 25 for recipients of LV
Rodriguez-Sosa *et al.*, 2009	Canada	Sheep	Fresh	Non treated sheep	donor cells expressing eGFP detected i ST at 2 months
Abbasi *et al*., 2015	Iran	Goat	Fresh LV-EGFP transduced cells	Busulfan treated mice	transduced-goat SSCs colonized mice seminiferous tubules

Oatley *et al*., 2004	USA	Bovine	2 wk. culture in testes explant	Busulfan treated mice	Colonies of SSC in the seminiferous tubules of mice
Zeng *et al*., 2012	USA	Pig	Fresh transduced (human growth hormone - GH) SSC	Irradiation	Of 62 ejaculates, 63.9±17.3% were positive for hGH of ejaculates were positive for transgene hGH.
Stockwell *et al*., 2013	Australia	Ram	Fresh	Irradiated ram	Donor DNA detected in ejaculate

3.3 Discussion

The systematic review of literature compiled published information on *in vitro* culture systems and applications in livestock production from January 1990 –to February 2021. The main focus of the review was to have an overview of the current developments in SSC culture techniques, methods of recipient preparation, and intra-testicular transplantation methodologies in livestock. The emergence of precise gene editing technologies such as clustered regularly interspaced short palindromic repeats (CRISPR-Cas9) provides an opportunity for harnessing SSC potential as a transgene carrier, for *in vitro* gene manipulation and development of transgenic animals. With the scarcity of SSC in the total testicular cell population, *in vitro* long-term culture of the cells is required to amplify the few numbers of SSC freshly isolated to millions, which would be adequate for gene editing and transplantation (Oatley and Brinster, 2012). From the current review, the methodology for long-term culture is still not fully standardized and carried out frequently because it was reported only in 7 studies that reported different conditions. The long-term culture of SSCs involved either of the two strategies: first, by supplementation of culture medium with a cocktail of growth factors (GDNF, bFGF, LIF, SCF, SDF); second, by growing SSCs on a feeder cell layer or feeder cells free culture using preconditioned serum-free media.

Early studies utilized serum as one of the important components of a culture medium but published reports have shown that presence of serum in the medium enhances growth of somatic cells and inhibits SSC self-renewal (Crouse, 2012; Oatley *et al*., 2016; Suyatno *et al.*, 2018; Sharma *et al.*, 2020). Culture studies of goat SSC in media containing 10% FBS have been documented (Pramod and Mitra, 2014; Binsila *et al*., 2020), although the SSC colonies did not form the typical germ cell clumps as described by (Bahadorani *et al.*, 2015; Oatley *et*

al., 2016) and also transplantation into recipient testis was not conducted. Therefore, whether the SSC cultures that were grown for an extended period were undifferentiated cannot be conclusively reported. Recent studies for goat SSC have demonstrated the detrimental effects of serum on SSC self-renewal and the enhanced proliferation of somatic cells in an SSC culture medium with serum (Bahadorani *et al*., 2012; Sharma *et al*., 2020).The long-term culture of SSC has largely failed due to difficulties in providing all the conditions *in vitro* that mimic the *in vivo* spermatogonial stem cell niche, which has physical, mechanical and chemical support from the surrounding somatic cells and also the lack of unique markers for SSC identification in culture (Lord and Oatley, 2017). There has been a controversy on the unique markers used to identify SSCs due to their non-specificity to SSC cell type, with some of the markers being expressed by other germ cell subtypes and even somatic cells. Expression of common SSC markers used in most studies such as UCHL1, OCT4, SOX2, KLF4, and THY1 is not restricted to undifferentiated spermatogonia only (Giassetti *et al*., 2019; Oatley *et al*., 2016). Currently, the only unequivocal measure of SSC existence within a culture dish is through transplantation into germline ablated recipient testes to assess the capacity for re-establishment of spermatogenesis.

From the findings, none of the long-term culture studies conducted transplanted the cultured SSC to a recipient testis for assessment of donor-derived spermatogenesis, hence the absence of enough evidence for long-term maintenance of SSC in their undifferentiated status.

The conclusion from studies on the successful long-term culture of SSC and their maintenance in culture was based on findings from presumptive marker expression: PLZF, LIN28, NANOS2, and GFRα1 with high similarity of the cell clump morphology to bonafide mouse cultures of SSC (Crouse, 2012; Oatley *et al*., 2016; Zhang *et al*., 2017; Zhang, 2020). That notwithstanding, morphological characteristic of SSC germ cell clumps consisting of a

cluster of cells resembling a bunch of grapes with clear cell borders has been documented and similarity in morphology with livestock SSC have been reported (Oatley *et al*., 2016; Kubota and Brinster, 2017; Suyatno *et al*., 2018). This characteristic germ cell clumps of SSC in culture is an indication of the existence of undifferentiated spermatogonia stem cells although not a definitive measure of stem cell capacity (Kubota and Brinster, 2017).

With regards to culture conditions, the use of feeder cells for SSC cultures is still taking the lead as compared to feeder-free culture. Cultures of SSC in STO feeders performed better than Sertoli cell feeders or other somatic cells (fibroblast) feeder cells. Growth of SSC on feeders presents a major challenge in transplantation of SSC as they may interfere with colonization in the recipient testes (Oatley *et al*., 2016), thus there has to be a way to culture these cells feeder free. Preconditioned media on feeder cells and culture of cells in laminin-coated plates has demonstrated promising success for long-term maintenance of the SSC in culture (Binsila *et al.*, 2020; Oatley *et al*., 2016). Although the morphological characteristics and marker identification are promising findings towards standard conditions for long-term cultures of SSC in livestock species, the stem cell activity of the spermatogonial populations must be assessed by intra-testicular transplantation to recipient livestock species.

Transplantation of SSC and regeneration of donor-derived spermatogenesis is the definitive proof and potential utilization of the SSC cultures in livestock production. A number of hurdles have been experienced in translating the germ cell transplantation procedures to livestock species from rodents where it has successfully been achieved. The difficulty has been attributed to the low number of SSC within the heterogeneous germ cell population and

the lack of methods to prepare germline ablated males with a functional testicular somatic cell ultrastructure. Ablation of the endogenous germ cell layer avails empty niches for colonization of exogenous SSC, thus regeneration for donor derived spermatogenesis. Methodology of intra-testicular transplantation of the SSC through ultrasound guided rete testis injection technique is well standardized, but the success and sustainability of donor-derived spermatogenesis requires a male that is permanently/genetically germline ablated but with functional somatic cell structures as in the case with the gene-edited *NANOS2* gene knockouts published by Ciccarelli et al. (2020). Chemical and physical methods for inducing germ cell apoptosis have in the past been used to prepare recipients. However, there is gradual regeneration of recipient spermatogenesis, with the endogenous SSC occupying the stem cell niches and thus preventing effective colonization of the basement membrane by transplanted donor SSC, hence donor-derived spermatogenesis in such recipients becomes difficult to quantify.

Transplantation of SSC in livestock species into mice recipients does not result into donor-derived spermatogenesis due to different genetic signaling mechanisms, but the livestock SSC are able to colonize the mice seminiferous tubules as evidenced by immunohistochemistry experiments. Donor-derived spermatogenesis necessitates transplantation into same animal species as documented in pigs (Zeng *et al.*, 2013; Kim *et al.*, 2014b). The chemotoxic drug busulfan has detrimental effects on other fast dividing cells such as bone marrow cells, which results in untargeted systemic damage affecting various organs in the recipient animals. In addition, the somatic cell population is also destroyed, thus affecting the robustness of spermatogenesis (Giassetti *et al.*, 2019). Regardless of the route of administration of busulfan to recipient animals, whether done in feed or systemic

administration, in all studies that used this drug, the recipients showed resumption of endogenous spermatogenesis with the production of semen having the recipient's genotype. However, donor-derived spermatogenesis also occurred with low levels of donor DNA irregularly detected by microsatellite markers, but the levels declined with time (Mikkola *et al.*, 2006; Zeng *et al.*, 2013; Kim *et al.*, 2014b). In addition, the use of irradiation to deplete endogenous germ cells is also cytotoxic to the somatic cell population, testicular seminiferous tubular structure and the rest of the cells in the body, which may limit the capacity for donor SSC colonization. Irradiation causes temporary depletion of endogenous germ cells (Herrid *et al*., 2013). Dolichos biflorus agglutinin (DBA), which is a plant-based lectin with selective toxicity to bovine Type A spermatogonia also resulted in temporary germline ablation with detection of donor DNA in recipients'ejaculate although the authors were not able to quantify the amount of donor-derived sperm in the ejaculate (Herrid *et al*., 2019).

Notably, efforts have been made in using recipients with temporary germline ablation for transplantation with donor SSC but in most cases, the presence of both donor-derived and recipient endogenous spermatogenesis makes it difficult to quantify the intended donor-derived spermatogenesis conclusively. Generally, the methods that result in temporary germline ablation are not effective especially if the goal is to use them as alternative breeding technology in livestock production systems. To utilize the potential of germ cell technology in livestock species, several researchers have suggested genetic engineering of surrogates/recipients, which are genetically germline ablated but have intact testicular tubular structure and functional somatic cell support (Park *et al.*, 2017). This will avail SSC niches for occupation and colonization by exogenous donor SSC after transplantation. This would lead to 100% donor-derived spermatogenesis. From the findings in the review, SSC

transplantation recipients were successfully generated through gene editing to knockout the *NANOS2* gene. Complete donor-derived spermatogenesis was confirmed in the recipients (Ciccarelli *et al.*, 2020).

Precise gene editing technologies are growing at a faster rate and use of SSC is the new frontier for gene manipulation and dissemination of elite desirable genetics. The SSC populations are progenitor cells for spermatozoa, and hence it would be easier to spread the gene of interest if gene edited SSC are used in transplantation. *In vitro* gene manipulation of SSC and subsequent transplantation will be useful in the development of transgenic livestock. Successful attempts to generate transgenic animals by use of SSC have been made in rodents (Kim *et al.*, 2019a). So far none of the studies in which SSC transfection was carried out was aimed at the introduction of genes targeting disease resistance or production traits of interest, so this research area remains unexploited. The main focus of the studies was to determine the potential transfection efficiency of different methods using the eGFP reporter gene or other transgenes in a few cases, of which transfection success was varied in all of the studies with the most of them below 10%.

Although the findings are intriguing, there is still a lot of research to be conducted in this area as the most efficient method of viral transduction of SSC using viral vectors which have associated safety risks especially if the cells are going to be transplanted (Zeng *et al.*, 2012).

Other methods of introduction of exogenous genes into cells such as nucleofection (Tang *et al.*, 2018) and lipofectamine transfection (Tajik *et al.*, 2017; Cai *et al.*, 2020) methods have also been shown to have a great potential for exploitation of *in vitro* SSC gene manipulation. Lastly, prepubertal animals have been poised as the best donors for SSC since at this age most gonocytes have differentiated to spermatogonia, thus maximum harvesting of

mitotically active SSC. At this age, the seminiferous tubules are only made up of Sertoli cells and undifferentiated spermatogonia (Murta *et al*., 2010). Therefore the population of germ cells isolated from prepubertal testes is likely to contain a high population of SSC, which are mitotically active (Oatley *et al*., 2016).

CHAPTER FOUR

4.0 *IN VITRO* CULTURE OF KENYAN GOAT SPERMATOGONIAL STEM CELLS

4.1 Introduction

Spermatozoa are the male gametes formed through a series of mitotic and meiotic division from spermatogonial stem cells (SSC) also referred to as undifferentiated spermatogonia. Spermatogenesis begins at puberty and continues throughout a male's life, and this is dependent on continuous self-renewal of SSC to maintain a reservoir for the continuity of the process (de Rooij, 2015). The SSCs are unique adult stem cells that contribute genes to subsequent generations, making them a perfect target for genetic manipulations and development of transgenic animals through germ cell transplantation of gene edited SSC (Dobrinski, 2018) and also utilization in the surrogate sire breeding technology (Ciccarelli *et al.*, 2020). Brinster and Avarbock (1994) demonstrated in mice that SSC from a donor testis can proliferate *in vitro* and re-establish spermatogenesis when transplanted to a compatible recipient male.

This unique feature provides an opportunity for *in vitro* expansion and transplantation of SSC from elite sires to accelerate the spread of desirable genetics within livestock populations. Recently, SSC transplantation into genetically sterile males (*NANOS* gene knockout) with donor-derived spermatogenesis was reported in cattle, pigs, and goats (Ciccarelli *et al*., 2020). These new developments provide a clear pathway for the utilization of SSC and surrogate sire breeding technology in livestock production systems. Slow rates of genetic

gains in low middle-income countries (LMICs) represent a major constraint to the improvement of performance in smallholder livestock systems (Marshall *et al*., 2019) and modeling demonstrates the potential of SSC to accelerate the rate of genetic gains (Gottardo *et al.,* 2019). Therefore, SSC culture systems, expansion, and characterization in indigenous goats in sub-Saharan Africa need to be optimized to fast track exploitation of the technology. The potential to utilize SSC and SSC transplantation in livestock breeding systems and gene editing is huge, however, their scarcity within the testicular cell population presents a major bottleneck for their practical applications in these technologies. In mice, it has been documented that SSC consists of 0.03% of entire testicular cell populations and this is thought to be the case in all mammalian species including goats Tegelenbosch and de Rooij,1993; Oatley and Brinster, 2012). On that account, the development of efficient SSC *in vitro* culture systems for amplification of these cell populations in livestock species is crucial. The conditions for long-term maintenance of SSC in culture have been well developed, optimized, and reproduced in rodents, with the development of long-term serum-free and feeder-free culture systems (Kanatsu-Shinohara *et al.*, 2003, 2005, 2008, and 2011).

To date, these conditions have been used as a benchmark for the establishment of SSC culture conditions in livestock with almost all culture systems having been supplemented with serum and/or cultured over feeder cells. The main aim of the culture is to amplify the population of SSC while maintaining their undifferentiated stem cell status facilitated by growth factors added and components of the culture medium. Propagation of SSC *in vitro* is dependent on the provision of an artificial micro-environment similar to *in vivo* conditions. In vivo, the micro-environment also referred to as the SSC niche, provides structural, chemical, and biological cues that regulate the fate of the SSC by secretion of growth factors and adhesive

molecules (Kubota *et al*., 2004a; Kubota *et al.*, 2004b). Sertoli cells produce Glial cell line-derived neurotrophic factor (GDNF), which is essential in SSC self-renewal (Kubota *et al*., 2004b; Aponte *et al.*, 2006). Other factors essential for the maintenance of SSC in undifferentiated status include; bFGF (basic fibroblast growth factor), LIF (Leukemia inhibitory factor), EGF (Epidermal growth factor), CSF1 (Colony stimulating factor 1), and SDF (Stromal Derived Factor) in combination with GDNF in expansion of SSCs across different species (Kanatsu-Shinohara *et al.*, 2003; Aponte *et al.*, 2008; Oatley *et al.*, 2009; Oatley *et al.*, 2016; Yang *et al*., 2013). There are limited reports on long-term maintenance of goat undifferentiated spermatogonia *in vitro* especially under serum-free conditions since there is considerable evidence that serum has detrimental effects to SSC self-renewal and enhances proliferation of somatic cells within the SSC culture (Bahadorani *et al.*, 2012; Oatley *et al.*, 2016; Sharma *et al*., 2020).

The serum has been one of the main components of the cell medium although in the case of SSC, the undesirable effects have been documented. Consequently, the establishment of a serum-free culture system is considered a prerequisite to the establishment of long-term SSC culture. Although serum-free culture systems for rodent SSC have been standardized, there is a paucity of data for the conditions in livestock species including goats. Additionally, the use of feeder cell monolayers in the culture of SSC mimics the somatic cell structural support and also provides the growth factors and adhesive molecules produced *in vivo* by the Sertoli cells and Leydig cells. However, feeder cells are thought to have a negative impact on colonization efficiency following SSC transplantation and present a variable component that is difficult to standardize (Oatley *et al*., 2016). Thus, for efficient utilization of SSC in the surrogate sire's concept, feeder-free culture systems are likely to be required.

There have been no reports of SSC culture in livestock species in Africa, especially in goats, which are considered "climate smart" livestock in this area since they thrive well and adapt faster to the climate change effects where sub-Saharan Africa is being hit by effects of climate change (Bjornlund *et al*., 2020). Long-term *in vitro* propagation and sustenance of goat SSC on serum-free feeder-free cultures has not been documented. The current study carried out *in vitro* culture of Galla goat SSC in Serum-free feeder-free culture conditions for extended duration using a multiparameter approach. This was followed by evaluation for formation of typical germ cell clump colonies and expression of bona fide molecular markers for undifferentiated spermatogonia.

4.2 Materials and methods

4.2.1 Study area

The study was carried out at the ILRI Nairobi Campus in the reproductive platform laboratory. The ILRI campus is located on Naivasha Road

+near the Kabete National Polytechnic (cgspace.cgiar.org, 2019). The study was carried out from June 2018 to September 2021.

4.2.2 Study animals

Seventy Indigenous Galla goats or their crosses aged between 3-6 months were used as donors of spermatogonial stem cells. Optimization of the SSC culture conditions was done through various trials for 2 years before successful conditions were attained. The Galla breed was chosen because of its resilience in harsh climatic conditions, hence its suitability for arid and semi-arid pastoral lands of Kenya as well as its versatile utility as a milk and meat producing breed. The goat has also been documented as climate-smart livestock that thrives

well in the changing climatic conditions. The Galla goats were sourced from ILRI Kapiti farm and pastoralist Maasai livestock owners. All the animal procedures were carried out according to the approved guidelines by the Institutional Animal Care and Use Committees at International Livestock Research Institute in Kenya (IACUC Ref no: 2018-15) and the Faculty of Veterinary Medicine, University of Nairobi. (Ref: FVM BAUEC/2019/243)

4.2.3 Isolation of germ cells from the testicular parenchyma

Reagents and chemicals were purchased from different suppliers mainly Gibco (Grand Island) and Sigma Aldrich (USA).

4.3.1.1 Media preparation

a) Phosphate-buffered saline (PBS) Ca2+/Mg2+ free (1L) with antibiotics

Reagent	**Amount to add (for 1× solution)**	**Amount to add (for 10× stock)**
NaCl	8 g	80 g
KCl	0.2 g	2 g
Na_2HPO_4	1.44 g	14.4 g
KH_2PO_4	0.24 g	2.4 g
Gentamicin	500µl	

The amounts of various listed salts were weighed into a 1000ml sterile glass beaker. 1 L of double distilled MilliQ water was added to make either 10x or 1x PBS solution. The media was sterilized by autoclaving and stored in the refrigerator at 4^0C.

b) Collagenase Type IV (0.25 mgmL-1)

100ml of HBSS (Gibco 14175-079) was pipetted into 500ml sterile beaker and 25mg of Collagenase, Type IV (Gibco Cot. 17104019) was dissolved. The enzyme solution was filtered using 0.2µm filter to sterilize. The media were used within 4 hours after preparation.

c) 0.25% Trypsin/0.04% EDTA (100ml)

100ml of PBS Ca2+/Mg2+ free was pipetted into a 500ml sterile beaker and 0.25g of Trypsin (Gibco™ Catalog number: 27250018) and 200µl of 0.5M EDTA (PH8.0, in MilliQ) added. The media were filtered and used while still fresh**.**

d) DNase 1 (7mg/ml) 20 ml)

20 ml of HBSS (Gibco 14175-079) was measured into a 50ml falcon tube and 140 mg of DNase 1 Grade 11 (10104159001 ROCHE) was added. The enzyme solution was filtered to sterilize and used while still fresh.

e) Heat Inactivation of Fetal Bovine Serum (Gibco™ 16000044)

Foetal Bovine Serum (FBS) was stored frozen at -20°C until use. The FBS was heat-inactivated before use in cell culture to inactivate the complement system and other undetermined inhibitors of cell growth in culture. The frozen FBS was thawed at 37°C. The FBS in a bottle was immersed in a 56°C water bath for 30 minutes. After complete heat

inactivation, the serum was cooled immediately in an ice bath and aliquoted into smaller volumes for single use, and kept at -20°C until use.

f) 10% Foetal Bovine Serum /MEM α (20ml)

The media was prepared by adding 18ml of MEM α (Gibco 41061-029) and 2 ml of FBS in a sterile 50ml falcon tube. It was filtered to sterilize, aliquoted into 2ml tubes, and stored at 4°C.

g) 30% percoll gradient

30 ml percoll (Sigma p4937), 1% FBS, 1 ml Pen strep, and 10 ml PBS (8ml 10X PBS, 2 ml H20) were measured into a 50 ml tube. The percoll was not filtered and used freshly.

The materials needed during castration of the male goats included: Lidocaine hydrochloride, Dexamethasone, Amoxicillin solution, surgical blades, hemostatic forceps, chromic catgut suture number 2, needle holders, and scissors.

4.2.4 Testicular cells isolation through two-step enzymatic digestion

The prepubertal male goats were physically restrained on lateral recumbency and 2 ml of 2% lidocaine hydrochloride was injected subcutaneously into the scrotum to attain local anesthesia. Semi-open castration was done following a routine aseptic procedure (Yami, 2016). Scrotal skin incisions were made on the distal one-third of the lateral aspects to expose the testicles within the tunica vaginalis. Each testicle was exteriorized and the spermatic cord ligated with chromic catgut suture before being severed for removal of the testicle. After testicular removal, the incision was sprayed with betadine solution and 0.5ml of tetanus toxoid vaccine (Antivax limited) was administered intramuscularly, anti-inflammatory drug (dexamethasone) and amoxicillin (Betamox®) was administered to prevent post-castration wound infection. The testicles were sterilized in 70% ethanol and placed in a beaker with

Hank's Balanced Salt Solution (HBSS) supplemented with 100 IU mL-1 penicillin (Sigma-Aldrich, St. Louis, MO, USA), 50 mg mL-1 streptomycin (Sigma- Aldrich) or Dulbecco's modified Eagle's medium (DMEM Gibco™,Cot.11330032) with antibiotics and transported to the laboratory within 3 hours. In the laboratory, each testis was picked up with forceps, and dipped into 70% alcohol for sterilization in a sterile biosafety cabinet level 2 (Fig 4.1).

A two-step cell isolation process by Oatley et al. (2016) with minor modifications was used to isolate testicular cells from the goat testes. Briefly, testes were washed in HBSS and disentangled gently to expose seminiferous tubules with blunt-edged forceps (Fig 4.2). About 150-200mg of tissue was digested in 10 ml of 0.25mg/ml of collagenase Type IV enzyme and 0.5ml of 7 mg/ml DNase 1 in HBSS for 5-7 minutes in a water bath at 37°C. Elimination of the interstitial cells was done through gravity sedimentation of seminiferous tubules on ice and discarding of the supernatant and the process was repeated 5 times. This was then followed by incubation of seminiferous tubules with 8 ml of 0.25% Trypsin/0.04 EDTA and 2 ml of DNase 7 mg/mL in a water bath at 37 °C for 30 minutes. The trypsin reaction was terminated by the addition of 10% FBS/MEMα. The cell suspension was passed through 40 μm cell strainer (BD Biosciences, Durham, NC), washed in HBSS twice by using centrifugation and followed by enrichment for spermatogonia. The cell suspension from the enzymatic digestion consisted of different testicular cell types including Sertoli cells and spermatogonia with different sizes as observed under light microscopy. Total cell number and cell viability were determined after trypan blue staining. The cell suspension was mixed with 0.4 % trypan blue (1:1, v/v). The percentage of live (cells that did not take up trypan blue) and dead cells (percentage of cells that took up the blue dye into the cytoplasm) were determined using a hemocytometer. Cells were then enriched for SSC through a multi-parameter selection approach (Figure 4.3).

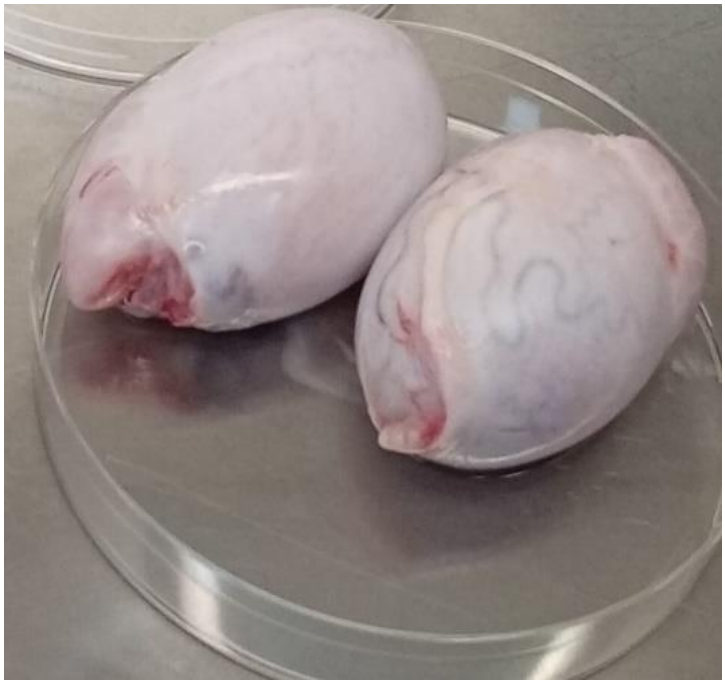


Figure 4. 1: Testicles from 5month old buck

Figure 4. 2: Disentangled testicular tissue ready for enzymatic digestion

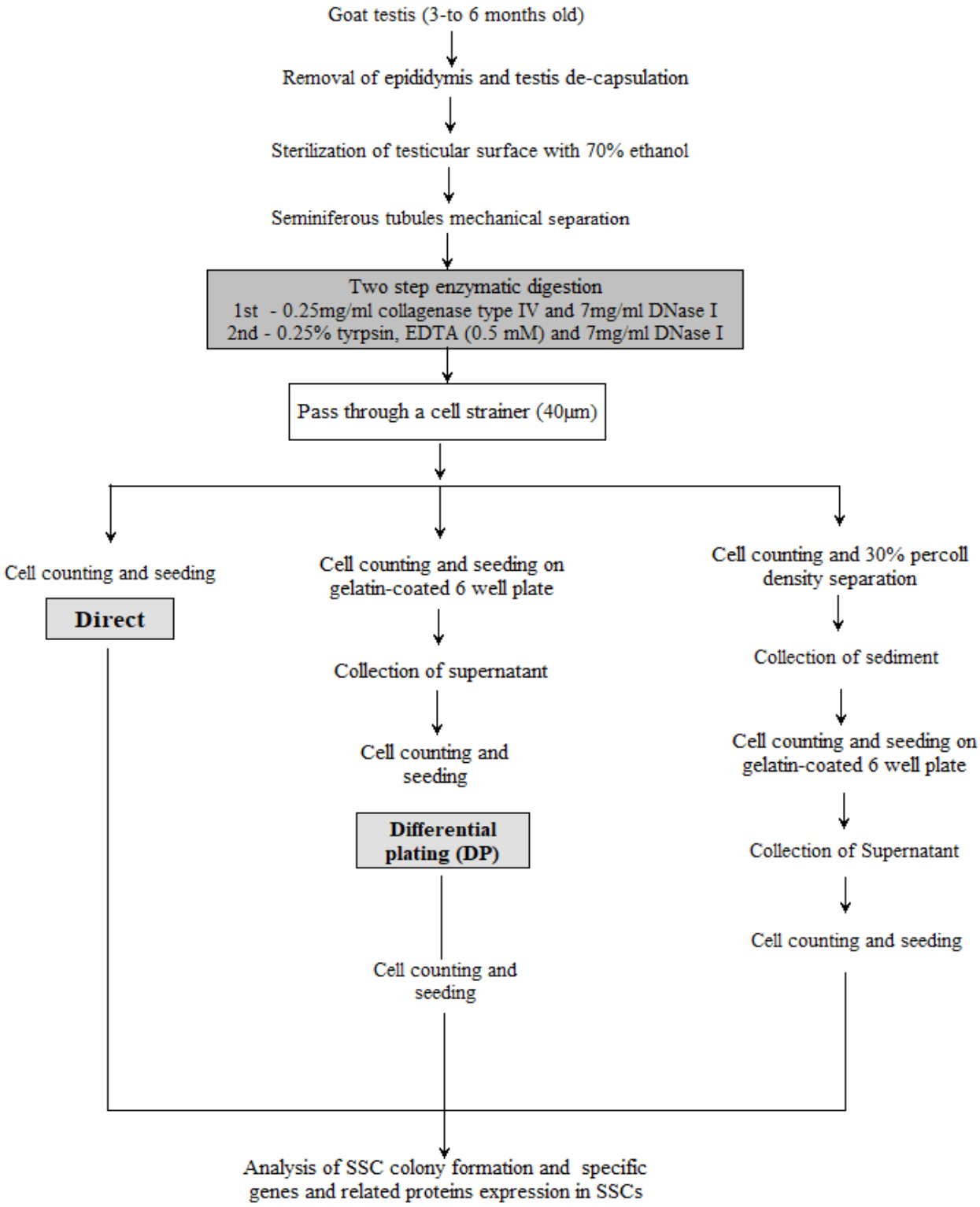


Figure 4. 3: Experimental design for SSC isolation, enrichment, and culture.

4.2.5 Multiparameter enrichment for spermatogonial stem cells

The multiparameter technique used for the enrichment of SSC included: Magnetic Activated Cell sorting (MACS) selection, percoll density gradient separation, and differential plating. The MACS selection was discontinued since the cells that passed through the magnetic field did not grow in the culture. Cell fractions obtained after enzymatic digestion were divided into 3 portions to test enrichment for SSC [cell fraction 1: direct seeding without enrichment, 2: enrichment through differential plating only, and 3: double enrichment through percoll gradient and differential plating (Fig.4.3)]. The double enriched portion had the highest number of PLZF positive cells and clear colonies with few somatic cells, hence the protocol was adopted for the study.

4.2.6 THY 1 surface marker Magnetic activated cell sorting (MACS)

The MACS protocol used was by Abbasi et al. (2013) with minor modifications. The testicular cells were selected for cell surface marker THY 1 antibody. This was a positive selection where the THY1 positive population was expected to have a higher percentage of undifferentiated spermatogonia (Abbasi *et al.*, 2013). The MACS column technology based on MACS microbeads, MACS columns, and MACS separators by Milteny Biotech Company was used. The cells were pelleted by centrifugation in 500µl of MACS buffer. The working solution for MACS buffer was prepared by diluting the MACS Bovine Serum Albumin (BSA) solution 1:20 with AutoMACS rinsing solution, which is part of the MACS kit provided by the manufacturer, Miltenyi Biotech). Aliquots of $1x10^7$ cells were re-suspended in 90 µl of MACS buffer and incubated with 10 µl mouse anti-mouse THY1 antibody (1:50; Abcam, Cambridge, MA Catalog no: 212 885) for 30 minutes at 4^oC with slow mixing every 5 minutes. Cells were washed 2 times with MACS buffer by centrifugation for 7 minutes at

600 x g, followed by incubation with anti-mouse IgM-conjugated MACs beads at the ratio of 20 μl per 10^7 cells at 4 °C for 20 minutes on a roller.

The cells were washed twice in MACS buffer and loaded on top of the MACS separation columns set on the MiniMACS magnetic station (Fig.4.4). The flow through containing unlabelled cells was collected in a sterile tube, and placed under the columns. The MACS columns were washed twice with MACS buffer and unlabeled cells were collected in the flow through. To collect the THY 1 positive labeled cells, a clean 15 ml tube was placed under the MACS columns and magnetically labelled cells were flushed out by firmly pushing in the plunger (Fig. 4.4). The THY1+ eluted cells were washed in a culture medium by centrifugation and the cell concentration and viability were determined by a hemocytometer. Control cells were incubated with MACS buffer alone. Typical yields of $1\text{-}2x10^5$ THY1+ cells from a starting population of $1x10^7$ cells were obtained. The THY 1 negative and Thy 1 positive cells were resuspended in culture medium with growth factors and seeded onto laminin-coated 96 well plates for comparison of SSC colony formation and proliferation. The cells had very low viability and growth rate and therefore MACS separation was discontinued.

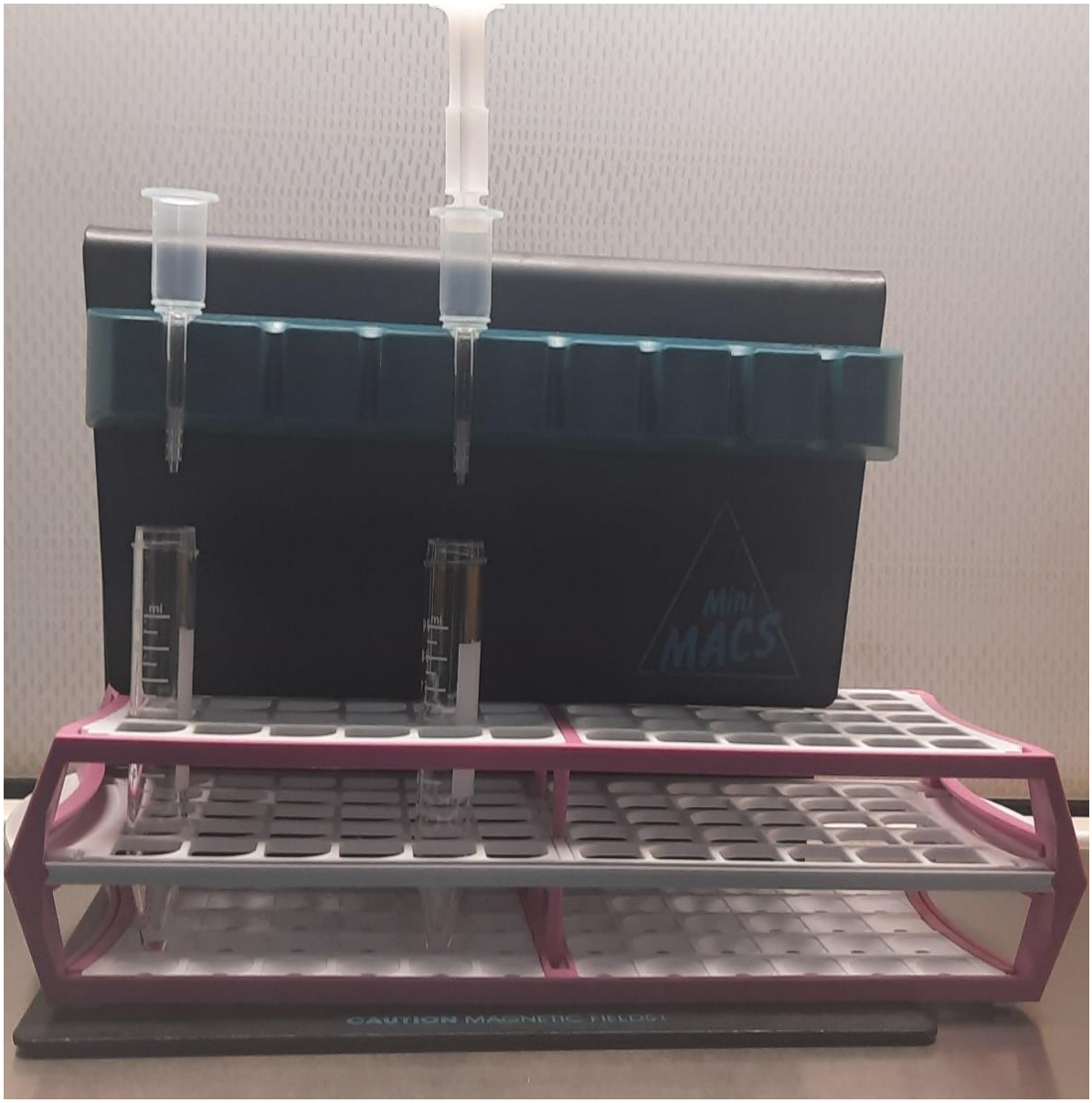


Figure 4. 4: Mini MACS Magnetic separator with the columns used for Magnetic activated cell sorting.

4.2.7 Enrichment of SSC through differential plating using gelatin-coated 6 well plates

Sertoli cells and other somatic cells within the testicular cell population have been shown to preferentially bind on gelatin-coated surfaces in culture plates, separating them from the floating germ cells. The cell suspension with aliquots of $1x10^7$ cells was washed by centrifugation at 600xg for 7 minutes at 4°C and seeded on 0.1% gelatin-(EmbryoMax® 0.1% Gelatin Solution)-coated on 6 well plates followed by incubation at 37°C overnight (24 hours.). Each well of the 6-well plates (Corning TC Multiple 6-well Plates, PS with Lid Cat No: 3516) were coated with 1 ml of 0.1% gelatin solution and incubated at 37°C for 30 minutes. The gelatin solution was discarded, and the wells were rinsed with HBSS. The cell suspension was added immediately before the wells dried off. The cells were incubated overnight, and spermatogonial stem cell rich portion of floating cells were harvested. The attached cells were also harvested and used to prepare the Sertoli cell feeder layer.

4.2.8 Percoll density gradient centrifugation and differential plating

A percoll gradient was prepared for the separation of the Sertoli cells and germ cells, which have different particle sizes and therefore settle at different rates of centrifugation. Aliquots of $2x10^6$ cells in 5 mL of HBSS were separated by percoll density gradient centrifugation. According to Oatley et al. (2016), a 30% percoll gradient was effective in the separation of Sertoli cells and other somatic cells from the germ cell rich portion, which pellets at the bottom of the 10 ml tube. A solution of 30% percoll gradient was prepared by pipetting 30% percoll isotonic solution (Sigma; catalog no: P4937), 1% FBS, 0.5% Penicillin (50 U/mL),streptomycin (50 µg/mL) and 10% 10X PBS. Two milliliters of the 30% percoll was pipetted into 10 ml test tubes and 5ml cell suspension slowly layered over 2ml of a 30% Percoll gradient followed by centrifugation at 600xg for 8 minutes at 4°C (Figure 4.5). The

supernatant was removed, and the pellets were washed, combined into one tube, and collected via centrifugation at 600 x g for 7 minutes in HBSS medium. The cells were resuspended in serum free medium (SFM) containing StemPro® 34 SFM, Minimal essential medium (MEM α) supplemented with 0.5% BSA (catalog no. A10008-01; Gibco) and StemPro nutrient supplement. Cells were seeded on gelatin-coated wells at $2x10^6$ cells/well of a 6-well plate and incubated at 37ºC in a 5 % CO_2 incubator overnight at 37 °C overnight (Fig. 4.5).

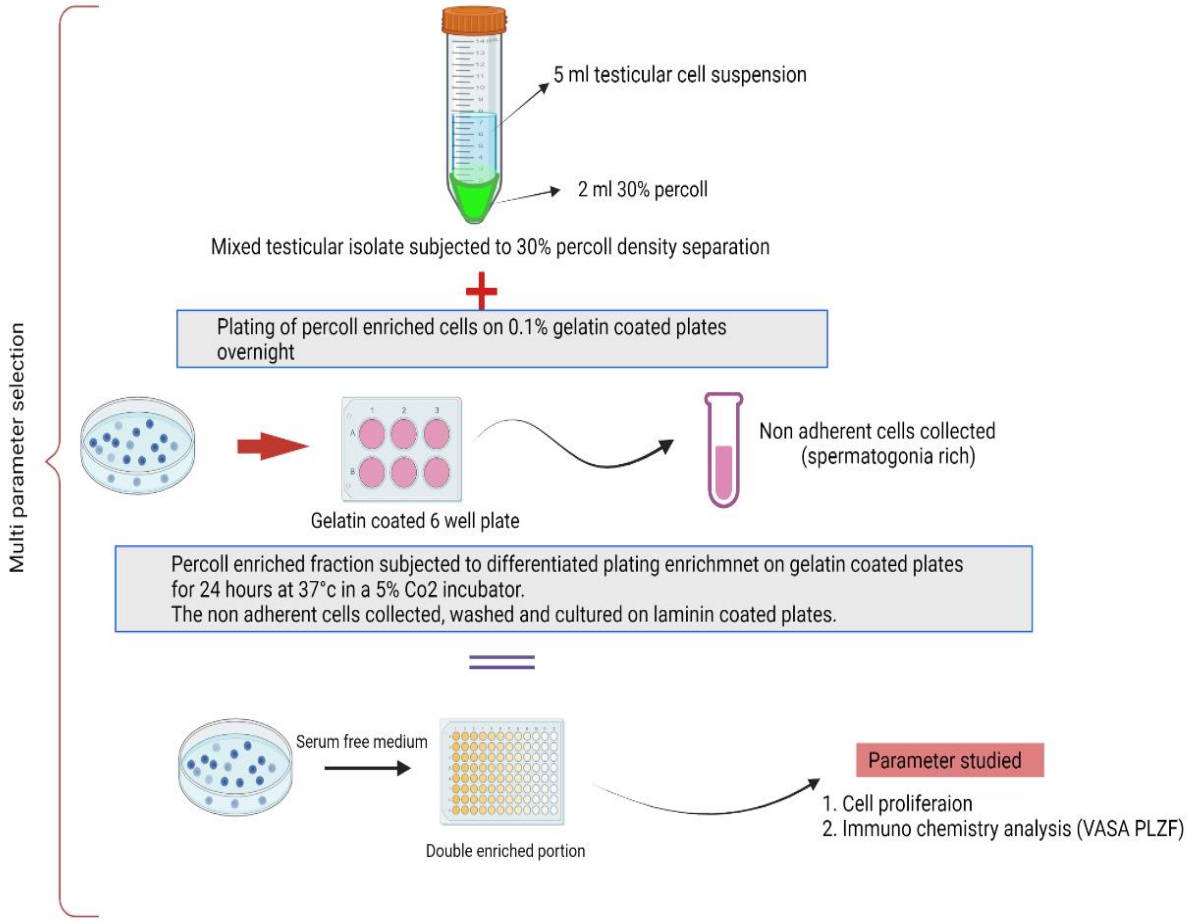


Figure 4.5: Enrichment protocol for spermatogonial stem cells isolated through enzymatic digestion.

4.2.9 Short-term (7 days) and long-term (>1 month) culture of spermatogonial stem cells

4.2.9.1. Feeder cell-free culture of goat SSC on laminin-coated plates

After overnight differential plating, the non-adherent population of spermatogonia cells were collected by gently swirling the plate and sucking out the suspension without disturbing the attached somatic cells. The non-adherent population was considered the spermatogonia enriched fraction and was pelleted by centrifugation at 600xg for 7 minutes at 4°C. The supernatant was discarded and cells were re-suspended in a serum-free culture medium. Different base medium conditions were tested using DMEM/F12 as the base medium, using StemPro^{TM} 34SFM, or MEMα (Fig 4.6 and Table 4.1). The cells were maintained in serum-free medium prepared as in Table 4.2 and supplemented with growth factors including recombinant human Glial cell line-derived neurotrophic factor (GDNF; 20 ng/mL of media; R&D Systems; Minneapolis, MN), recombinant human Fibroblast growth factor 2 (FGF2; 1 ug/mL of media; BD Biosciences; San Jose, CA), and Stromal derived factor 1 (SDF-1; 10 ng/mL of media; R&D Systems; Minneapolis, MN). Growth factors were reconstituted according to the manufacturer's instructions.

The cells at a concentration of 20,000/ 200µl per well, with the three different base media, were maintained on 96 well laminin-coated plates serum-free medium in incubators with an air atmosphere containing 5% CO_2 at 37°C, and media changed every alternate day. On the day of feeding the cells, 100µl of media was sucked out and 130µl of pre-warmed media was added. The serum-free medium was conditioned by incubating on goat fibroblast cells overnight. Briefly, the goat fibroblast culture medium was sucked out and the flask was washed with HBSS to remove traces of FBS, then serum-free media was added and incubated at 37 °C with 5% CO_2 overnight. The following day the medium was sucked out and filtered

using 0.2μm filter and then used for culturing of feeder-free SSC. The protocol was adapted from Oatley et al. (2016). The cells were passaged after 7-9 days.

StemPro^{TM} 34 SFM base medium had the highest colony development and thus continued to be used in the study. The cells were cultured for 45 days and monitored for an increase in cell numbers and colonies. The growth factors included: human forms GDNF, FGF2, SDF, and LIF. The colony growth was monitored, and images were taken using the Zeiss AXIO Vert. 1 inverted microscope (Fig 4.7) and the results were recorded. The number of colonies formed in the recipient testis is a direct indicator of the number of SSCs injected (Dobrinski *et al.*,1999). Due to this correlation, colony counting was done to determine the efficacy of different isolation methods or culture conditions. Increased colony formation after addition of a new growth factor demonstrated that the growth factor was beneficial to SSC maintenance and/or proliferation.

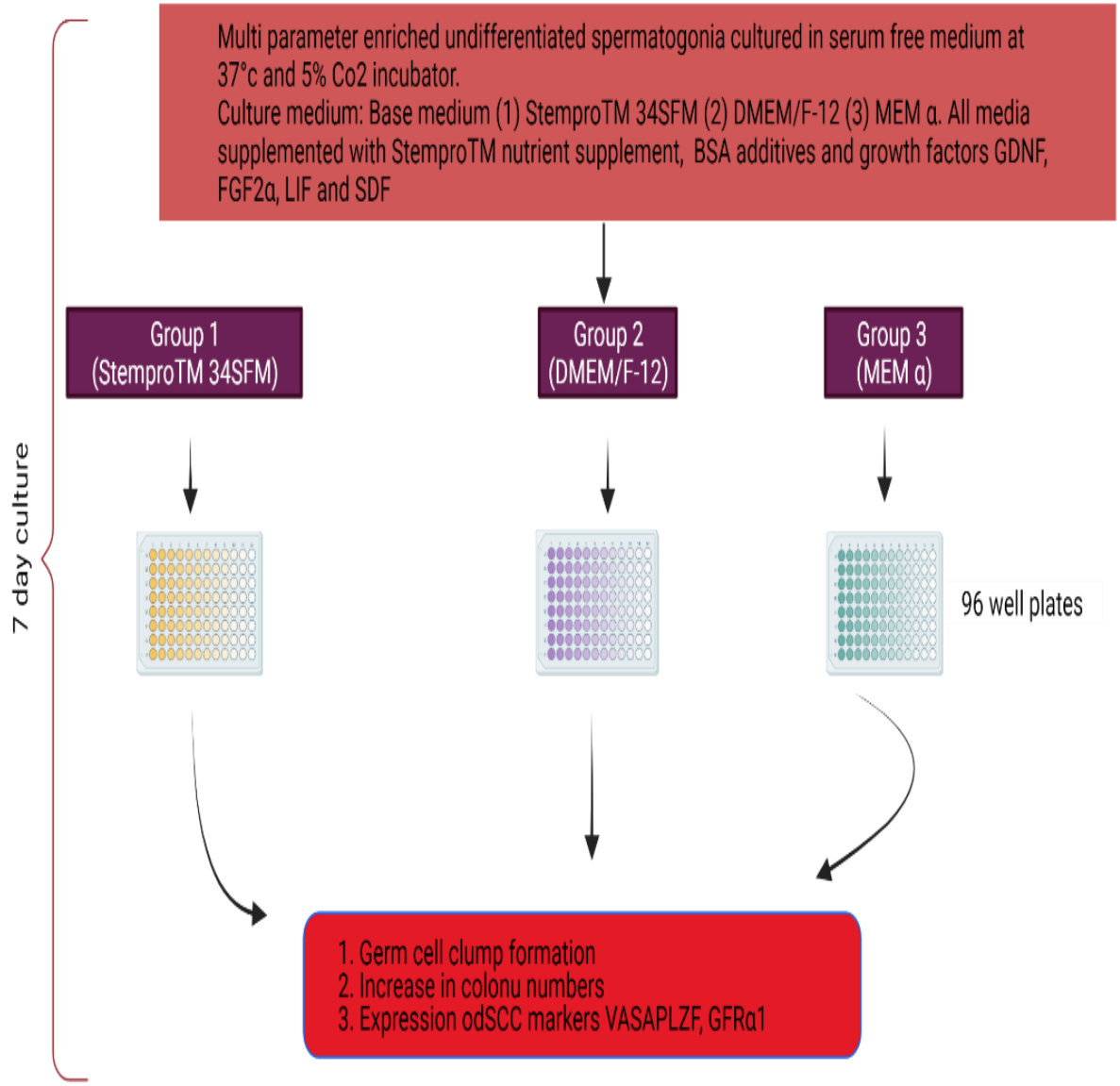


Figure 4.6: Diagrammatic representation of 7-day short-term goat SSC culture system.

Table 4.1: Previously published and current study base media and growth factors used to maintain undifferentiated SSC in culture and evaluate germ cell clump formation.

Conditions	Base medium	Protein source	Growth factor supplementation	Germ cell clump formation	Species	References
A	MEMα	StemProBSA	GDNF, FGF2, LIF	Yes	Cattle	(Oatley *et al*., 2016)
B	Knock out DMEM	Knock out serum replacer	GDNF	Yes	Goat	(Sharma *et al*., 2020)
D	StemPro-34 SFM	StemProBSA	GDNF, FGF2, LIF	Yes	Mouse, Rat	(Kanatsu-Shinohara *et al.*, 2005)
E	StemPro-34 SFM	StemPro BSA	GDNF, FGF2, LIF, SDF	Yes	Goat	Current study
F	MEMα	StemPro BSA	GDNF, FGF2, LIF, SDF	Yes	Goat	Current study
G	DMEM/F-12	StemPro BSA	GDNF, FGF2, LIF, SDF	Few	Goat	Current study

Table 4.2: Components of 100ml volume of culture medium.

Media	**Company Catalog Number**	**Required Concentration**	**Volume**
Stempro™-34 SFM (1X)	(Gibco: 10639011)	1X	95ml
Iron saturated Transferrin	Sigma T1283-	10mg/ml	1 ml
Sodium Selenite	Sigma S5261	0.003Molar	20µl
2- mercaptoethanol-	sigma M3148	100mMolar	200µl
Insulin	Life TECH. 12585-014	4mg/ml	640 µl
Putrescine Hydrochloride	Sigma P5780	16.1mg/ml	120 µl
MEM NEAA (100X) Solution	Gibco; 11140050	100X	1 ml
MEM) Vitamin solution	Gibco; 11120052	100X	1 ml
Glutamine	Gibco; 25030024),	100X	1ml
BSA Stempro	Gibco : A100081),	1X	2.4ml
Stempro®hESC supplement	(Gibco : A10006-01	1X	2 ml
Hepes solution	Sigma H0887	10mMolar	1 ml
Penicillin-Streptomycin	Gibco;15070-063	(5,000 U/mL)	0.5ml

MEM-Minimum essential medium, NEAA-Non-Essential Amino Acids

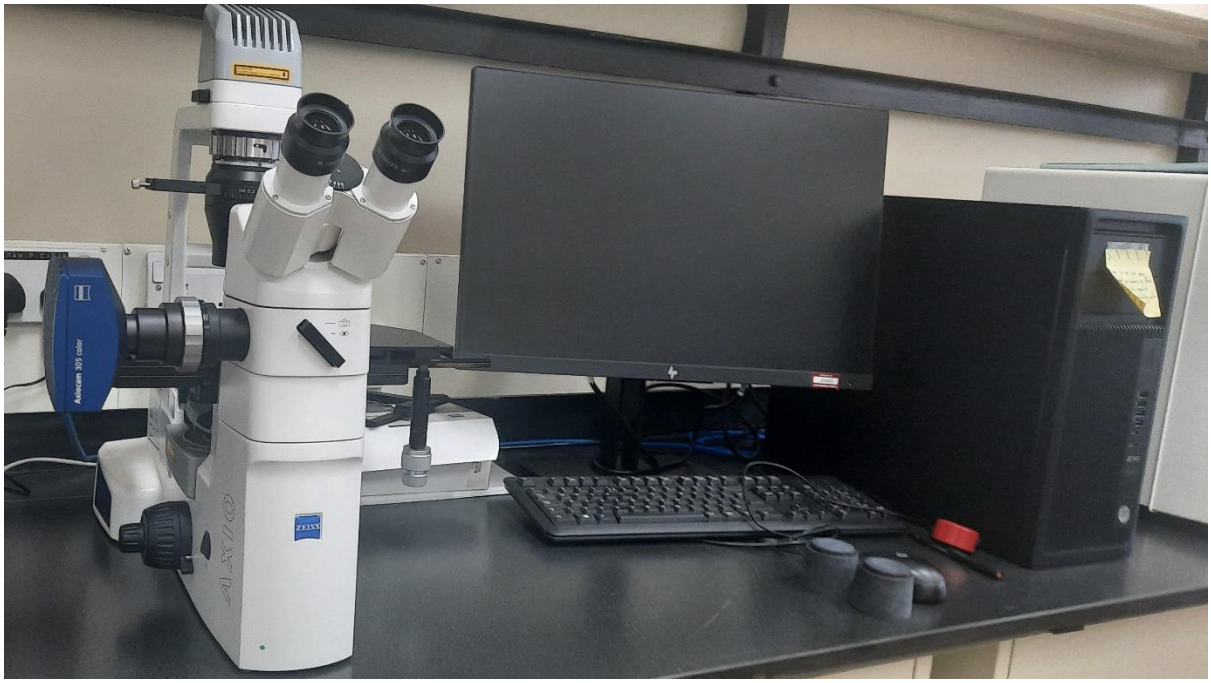


Figure 4.7: Inverted Zeiss AXIO 1 microscope used for imaging the SSC colonies.

4.2.11 Preparation of serum-free SSC culture media

4.2.11.1 Preparation of working solution for culture medium components

a) Iron saturated holo-transferrin (Sigma-Aldrich) 10mg/ml

10 ml of MEMα (Gibco 41061-029) was added to a sterile 20 ml falcon tube, followed by the addition of 100mg of Holo-Transferrin bovine(Sigma T1428-100MG). The medium was filtered to sterilize it, aliquoted into 0.12ml /tube, and stored at -20°C.

b) Sodium selenite (Sigma S5261) 3mM Solution (5.2mg/ml 100x)

100 ml of tissue culture grade water (sterile) was added into a tube followed by the addition of 5.2mg of Sodium selenite. The medium was filtered to sterilize it, followed by being aliquoted into 20 µl/tube and frozen at -20°C as storage.

c) Putrescine hydrochloride 100mM (Sigma; P7505) (16mg/ml)

A volume of 0.062 ml of tissue culture grade water (sterile) was added to a tube followed by addition of 1mg Putrescine hydrochloride. The medium was then filtered to sterilize it and aliquoted into 20 µl/tube before being frozen at -20°C as storage.

d) Mitomycin C Stock solution, 1mg/mL (Mitomycin C, 2-mg vial M4287)

A volume of 2 ml of HBSS was measured and dispensed into the mitomycin vial. The solution was sucked out and dispensed into a 5ml tube. The solution was stored at -20°C.

e) Freezing Medium (50ml)

40 ml of the SSC culture medium was added to a 50ml tube followed by addition of 5 ml dimethyl sulphoxide (DMSO). The medium was filtered and used while fresh.

f) GDNF Recombinant Human Protein (100µg/ml)

To prepare a stock solution, 10 µg of GDNF Recombinant Human Protein (R & D systems 212 GD) and 100 µl of Tissue culture grade water (sterile) were mixed in a sterile 0.5ml tube. The medium was aliquoted to 10 µl per tube and stored at -20°C. The growth factor solution was not filtered.

g) 0.1% BSA/PBS

10mg of BSA was weighed and placed in a tube with 10 ml sterile PBS. The solution was filtered and stored at 4°C.

h) GDNF Recombinant Human Protein (10 µg/ml)

To prepare working solution, 100 µl of GDNF (100 µg/ml) was added to 900 µl of 0.1% BSA/PBS in a sterile 2ml tube. The solution was aliquoted to 170 µl per tube and stored at -20°C. No filtration was done.

Growth factors: FGF (1ug/ml), SDF (10ug/ml), SCF (10ug/ml), LIF (10ug/ml) growth factors reconstituted similar to GDNF (10ug/ml).

i) 0.01%Poly- L-lysine (A-005-C - Merck Millipore) for coating cell culture plates

0.5 mL of 0.01% poly- L-lysine was added to each well in a 24-well plate. The solution was left on the wells for 5 minutes at room temperature, with dish lids on. The solution was discarded, and plates were dried at room temperature for at least 2 hours.

4.2.10 Establishment of goat somatic testicular cells feeder cell monolayer for culture of spermatogonial stem cells

The adherent testicular goat somatic cell (GSC) population consisting mainly Sertoli cells on the gelatin-coated plates was harvested by adding 0.5 ml of 0.25% Trypsin/0.04%EDTA and incubated for 1 minute, followed by addition of 10% FBS/DMEM to stop the trypsin reaction. The cell suspension was centrifuged at 800 rotations per minute (rpm) for 5 minutes. The cells were re-suspended in fresh culture medium containing 10 % (v/v) FBS in DMEM, essential amino acids (EAA), non-essential amino acids (NEAA), Glutamine, and antibiotic-antimycotic solution and incubated at 37 °C with 5% CO_2 incubator for 4

days.

The medium was changed every 3 days and cells passaged when confluent. On confluency, a portion of GSC were treated with 1 ug/ml of mitomycin by addition of 100µl of mitomycin to T25 culture flask and incubated for 3-4 hours in the incubator at 37 °C. After the incubation, the medium was sucked out and the wells were rinsed thrice with HBSS. To detach the somatic cells from the bottom of the flask, 1 ml of 0.025% Trypsin/0.04%EDTA was added to each well and incubated at 37°C for 1-2 minutes. To stop the trypsin reaction, 2 ml of 10%FBS/DMEM was added to each flask/well and the cell suspension was sucked out into 10 ml tubes for centrifugation at 800rpm for 5minutes. The supernatant was discarded, and cells re-suspended in a 5 ml 10%FBS/DMEM medium. The mitomycin-treated GSC were plated on a 0.1% gelatin pre-coated 24-well plates at a concentration of $1x10^5$ cells per well in 500µl of culture medium. Within 24-48 hours, the cells will form a monolayer at the bottom of the wells, and they are used as feeder layer for spermatogonial stem cells. Proliferation of SSC on the GSC feeder cell monolayer in serum-free medium was monitored for 60 days.

4.2.12 Passaging of spermatogonial stem cell cultures

The cultures were passaged when 70-80% confluent at around 7-9 days post-culture. The spermatogonial stem cells attach to the bottom of the laminin-coated plates or surface of feeder cells. The cells were dislodged by gently pipetting up and down across the wall and the edges and sucking out the floating cells. However, precautions were taken not to allow the pipette to touch the bottom of the plate (on laminin-coated plates) or the feeder cell monolayer. Confirmation was made under the microscope that the cells had been lifted off the bottom surface or the surface of feeder cells and were suspended in the medium. The

medium was pulled off into a 15 ml tube and washed through centrifugation at 600xg for 7 minutes at 4°C. The supernatant was discarded, and cells re-suspended in a serum-free culture medium and seeded on 96-well laminin-coated plates and on new GSC feeder cell monolayer. After the 2nd passage, a portion of cells were cryopreserved after every passage.

4.2.13 Cryopreservation of spermatogonial stem cells

A portion of SSC were cryopreserved for future recovery and use. The cells were cryopreserved after the second passage in their proliferative phase. The germ cell clumps were harvested, washed by centrifugation and counted as described during passaging. The cells were resuspended in 10% dimethyl sulphoxide (DMSO) in serum-free Stem pro-culture medium. The freezing medium was gently added and mixed with the cells. They were cryopreserved at a concentration of 1 million cells per vial. Each vial was barcoded for easy tracing of the sample. A volume of 1 ml of cell suspension was aliquoted into separate cryovials, which contained 1 ml of freezing medium and placed in freezing containers (Nalgene) with isopropanol overnight at −80°C. The next day, the vials were plunged into liquid nitrogen for long-term storage

4.2.14 Thawing SSCs

The barcoded vials were removed from liquid nitrogen storage and thawed for 60 seconds in a water bath at 37°C. The thawed cell suspension was sucked out and added into 3 ml of culture medium in a 15 ml conical tube. The cells were washed twice through centrifugation at 600g for 7 min at 4°C to remove traces of DMSO freezing reagent. The cells were re-suspended in a culture medium and plated on 96-well laminin-coated culture plates at a concentration of 40,000 per well, then incubated at 37°C with a humidified atmosphere of 5% CO_2 in the air. The cells were monitored for recovery and proliferation. Colony formation

started after one week post-cell recovery. The protocols for cryopreservation and thawing were adapted from a previous report (Oatley *et al*., 2004).

4.2.14 Establishment of Goat Foetal Fibroblast (GFF) cell line

4.2.15 Goat fetal fibroblasts medium Preparation

a) DMEM+antibiotics

The following chemicals were weighed and placed into a one-1L sterile beaker: 13.4g of DMEM powder (Gibco 12100-017)**;** 3.7g of NaHCO3**,** 0.06g of Penicillin (100 IU/ml); 0.1g Streptomycin (100 µg/ml). 1L of MilliQ water was added to dissolve the solutes. The medium was filtered for sterilization and stored at 4°C.

b) Goat Foetal Fibroblast (GFF) culture Medium (500 ml)

The medium was prepared by mixing the following reagents in a 1000 ml sterile beaker:
440ml of DMEM+antibiotics (prepared as above) ; 5 ml of MEM Non-essential amino acids (Gibco 11140-050); 5 ml of L-Glutamax (Gibco) and 50 ml of FBS.

c) Freezing Medium (50ml)

30ml of BEF Medium (prepared as above), 10ml of FBS, and 10ml of DMSO were pipetted into a sterile 50ml falcon tube. The medium was filtered and used freshly.

d) Mitomycin C Stock solution, 1ug/mL (Mitomycin C, 2-mg vial M4287)

2 ml of HBSS was measured and dispensed into the mitomycin vial. The solution was sucked out and dispended into a 5ml tube. The solution was stored at -20°C.

4.2.16 Isolation of GFFs from the embryos

From day 30 to day 55 fetuses were used (n=10). Ultrasonography was done to confirm pregnancy before animals were slaughtered. The pregnant goats were slaughtered at the ILRI slaughterhouse using the normal humane slaughter procedure. The abdominal cavity was opened and using a sterile knife, the peritoneal wall was cut open to expose the uterine horns and the cervical end was clamped. The uterine horns were removed and placed in a clean 2L beaker. The gravid uteri were taken to the laboratory in a sterile beaker. The uterus was placed on a sterile steel square plate in the biosafety hood. An incision was made in the uterus and the embryonic sac cut open with sterile scissors to release the fetus, which was lifted into a new 15-cm petri dish. The fetus was washed thrice with Ca_2+/Mg_2+ free PBS to clear off the blood and transferred to a new 15-cm petri dish and 2 ml of Ca_2+/Mg_2+ free PBS added to the dish to keep the fetus moist. The crump rump length was measured in centimeters using a tape measure straight distance from the occipitus to the distal end of the coccygeal. The age of the fetus was estimated through Y=2.49√ (crown rump length√0.0028). Y=Fetal age (Karen *et al.*, 2009). The head was cut off and the embryo was washed in a clean 15-cm petri dish with Ca_2+/Mg_2+ free PBS and transferred to the lid of a 15 cm Petri dish. The soft tissues were minced finely with a curved sterile iris scissor or surgical blade. Minced tissues were transferred to a 2×50 ml tube and 15 ml 0.25% Trypsin/0.04% EDTA and 2 ml of 7mg/ml of DNase 1 enzyme were added to each tube. The tissue with enzyme was incubated for 1 hour in a 37°C water-bath with occasional vortexing. After 1 hour, the 7 ml GFF medium containing FBS was added to each tube to stop the trypsin action. The tissue chunks were removed by sifting the tissue into a sterile beaker or 10 cm dish with steel sterile sifters. The sifted cell suspension was transferred into 2×50 ml tubes and centrifuged at 800 rpm for 10 minutes at room temperature and the supernatant was discarded. The cell suspension was

resuspended in 10 ml of GFF and passed through a 70μm cell strainer to remove debris followed by centrifugation at 800 rpm for 5 minutes. The supernatant was discarded and GFF medium was added (15 ml per T75 flask) to each tube and the cells dissociated by gently pipetting. One embryo could seed on 4×T75 flasks (final cell density 2×10^6 cells/ml). The flasks were incubated at 37°C with 5% CO_2. The cells were passaged at 70-80% confluency at a ratio of 1 flask: 3 flasks after 4 days. Protocol used was a modification from Mehrabani et al. (2016).

4.2.17 Passaging of GFF

The culture flasks containing GFF were removed from the incubator and the culture medium was sucked out. To remove traces of FBS, culture flasks were rinsed with 5 ml of PBS Ca_2+/Mg_2+ free. To detach the monolayer of fibroblasts from the bottom of the flask, 3 ml of 0.025% Trypsin/0.04%EDTA was added to each flask and the flasks were incubated at 37°C for 3 minutes. To stop the trypsin reaction, 6 ml of GFF medium was added to each flask and the cell suspension was transferred to a 50 ml tube for centrifugation at 800rpm for 5 min. The supernatant was discarded, and cells were re-suspended in 15 ml GFF medium (split 1flask:3flasks). The cells were counted, and viability was determined at each passage. The cells were dissociated by gently pipetting up and down, then 30 ml GFF medium was added, and cells were seeded on 3×T75 flasks, with a volume of 15 ml in each flask and incubated at 37°C, 5% CO_2. The medium was changed every 3 days. A fraction of the cells were treated with mitomycin similar to GSC (subsection 4.2.10) and cryopreserved and a portion was sub-cultured and used for SSC media preconditioning.

4.2.18 Cryopreservation and thawing of GFF

The cells were cryopreserved starting from the 3rd passage and each subsequent passage. Freezing medium was prepared freshly, and 1 ml pipetted into each cryovial. Similar to the steps done in passaging (subsection 4.2.14.3), the culture flasks were removed from the incubator and the culture medium sucked out. To remove traces of FBS, culture flasks were rinsed with 5 ml of PBS Ca2+/Mg2+ free and pipetted out. To detach the monolayer of fibroblasts from the bottom of the flask, 3 ml 0.025% Trypsin/0.04%EDTA was added to each flask, incubated at 37°C for 3 min and then 6 ml GFF medium was added each flask to stop trypsin action. The cell suspension was transferred to a 50 ml tube for centrifugation at 800 rpm for 5 min. The supernatant was then discarded, and cells were re-suspended in 15 ml freezing medium. The cells were counted, and viability was determined. One ml of cell suspension was added to the cryovials (final cell density of $2X10^6$cells/ml). Each vial was barcoded for easy tracing of the sample. Cryovials were put overnight at −80°C. The next day, the vials were plunged into liquid nitrogen for long-term storage.

During thawing, frozen cryovial was recovered from liquid nitrogen and put into a 37°C water-bath immediately until it completely thawed. The cell suspension was removed into 3 ml pre-warmed GFF medium in a sterile 10-cm tube and centrifuged at 800 rpm for 5 minutes at room temperature. The supernatant was discarded, and cells re-suspended in 15 ml GFF medium. The cells were pipetted gently to yield single cells and seeded on a T75 flask at 37°C with 5% CO_2. Protocols used were adapted from previous reports (Oatley *et al*., 2004; Bai *et al.,* 2012; Mehrabani *et al.,* 2016). After the 3rd passage, a portion of cells was seeded in 24-well plates at a concentration of $1.0x10^5$ cells and cultured for 8 days. The cells in at least three wells were counted every day and the cell numbers were plotted against time in

Graph-pad in Windows 10. Population doubling time was determined based on this curve.

4.2.19 Immunochemistry for SSC specific markers

The following working solutions were prepared: 1x Phosphate Buffered Saline (PBS), 0.1% or 0.3%Triton in PBS to make (PSBT); 0.5% Bovine Serum Albumin in PBST, and 10% Normal Goat serum in PBST. The 0.3% PBST was used as the washing buffer, 10% Normal Goat Serum in PBST was used as a blocking buffer, 0.5% Bovine Serum Albumin in PBST was used as antibody dilution buffer.

4.2.20 Immunocytochemical staining of cultured testicular cell populations

Freshly isolated and cultured SSC were detached from the bottom of the laminin-coated plate by gently pipetting. The detached suspended cells were collected in a sterile tube, centrifuged at 600xg for 7 minutes at 4°C, and the pelleted cells were resuspended in PBS. The cells were then cytospined (Shandon™ Cytospin 4™, Thermo Scientific) to make cytosmears on Poly-L-Lysine coated slides at a concentration of 1X10^5 cells per slide. The cytospin was used according to the manufacturer's instructions at a speed of 600xg for 5 minutes. The procedure was modified version adapted from previous reports (Reding *et al*., 2010). The slides were fixed in 0.5mL of 4% paraformaldehyde in PBS for 10 minutes at room temperature. After fixation, slides were washed thrice 0.5 ml of 1× PBS/0.1 % (v/v) Triton (PBST) for 5 minutes. The cells were permeabilized by treating with 0.5ml of 0.3 % (v/v) Triton-×100 for 15 min. Cells were blocked for nonspecific binding by incubation in 0.25ml of 10% Normal Goat Serum in PBST overnight at 4 °C. The cells were then washed 3 times with 1X PBS and incubated at 4°C overnight with 250µl per slide of primary antibodies diluted in 0.5% BSA and 0.1% Triton X-100 in PBS. Primary antibodies used were: DDX/ VASA (bs-3597R), PLZF (sc-28319), GFRα-1(sc-271546), NANOS2 (sc-393868). After

overnight incubation, the cells are washed in 1X PBS and stained with 250µl per slide of fluorescent dye–labelled secondary antibodies (dilution 1:1000) (Alexa flour ® 488 goat anti rabbit IgG and Alexa flour ® 488 Goat anti-mouse IgG) in 0.5% BSA/PBST and incubated for 1 hour at room temperature in the dark. After which the cells were washed with PBS and mounted with Prolong Antifade mounting medium with DAPI for nuclei staining to view under fluorescence microscopy (EVOS M5000 Thermo Fischer microscope). Analysis of images was done by (Celleste 5.0 software analyser) and the percentage of undifferentiated spermatogonia positive to the markers was estimated by counting the percentage of cells that were positive for a specific marker. PLZF was selected as a specific SSC marker in this study based on previous studies that revealed it to be a specific marker for undifferentiated spermatogonia in sheep (Binsila *et al.*, 2018) and goats (Bahadorani, 2011; Bahadorani *et al*., 2012; Pramod and Mitra, 2014). The percentage of cells positive for PLZF were counted in 10 different fields of view for each slide and divided by the total number of DAPI stained nuclei in the field of view. For the other markers, the slides were observed for at least 40% marker positive cells in a field view and images taken.

4.2.21 Immunohistochemical analysis of PLZF expression in the Goat testis

Goat testicular tissue pieces were sectioned into slices and fixed in 10% formalin overnight. Tissue was dehydrated, embedded in paraffin, sectioned at a thickness of 7µm, and adhered to glass slides. The sections were deparaffinized in xylene and rehydrated with descending series of gradient ethanol and water incubations. Antigen retrieval was done by boiling the slides in sodium citrate buffer (pH 6.0) for 20 minutes. Non-specific binding sites were blocked through overnight incubation in 10% normal goat serum/1% BSA in 0.2% Triton in PBS at 4°C. The next day, slides were washed in PBS and incubated overnight with the

primary antibody at 4°C (1:100 DDX/ VASA polyclonal antibody (bs-3597R), 1:100 PLZF antibody (sc-28319). The following day, sections were washed in PBS and then incubated with the secondary antibody for 2 hours at room temperature (Dilution factor 1:500). Afterwards, the tissue was washed in PBS and then a glass coverslip was mounted on the tissue section using an aqueous DAPI-containing medium. Slides were observed under fluorescent microscopy.

4.2.22 PCR analysis of gene expression levels of cell markers by SSC

4.2.22.1 RNA extraction from SSC colonies

The RNAeasy Minikit (Qiagen CA) was used. The following reagents were availed for the experiment: β-mercaptoethanol (β-ME:14.3 Molar), Sterile, RNAse-free pipette tips, Microcentrifuge 2 ml tubes, 96–100% ethanol, blunt needle, and syringe.

4.2.22.2 RNAeasy Mini kit RNA extraction protocol

The following reagents were contained in the RNAeasy kit (catalog. No. 74104 and 74106): Buffer RLT, 1.5ml, and 2ml collection tubes, RNeasy Mini spin column, Buffer RW1, Buffer RPE, RNase-free water, Quick-Start Protocol. The full names of the buffer's abbreviations were not given.

The Quick-Start Protocol in the kit RNeasy® Mini Kit was used for RNA extraction. Before starting the experiment, 10ul of β2-Mercaptoethanol was added per 1ml of RLT buffer. Four volumes of ethanol (96–100%) were added buffer RPE for a working solution. The reagents were removed from -20°C freezer and held for 30 minutes at room temperature. The feeder-free SSC colonies were harvested from laminin-coated plates and pelleted through

centrifugation at 600xg for 7 minutes. The cells were re-suspended in PBS and washed twice. The cells were counted and 1 x 10^7 cells, were used for RNA extraction. If the RNA extraction process did not start immediately, the cells were stored at-80°C. All the PBS was sucked out before adding the RLT buffer. The pellet was loosened by flicking the tube and 350µl volume of buffer RLT was added. The sample was homogenized by pipetting up and down and vortex twice. A volume of 350µl 70% ethanol was added to the lysate and mixed well by pipetting. The ethanol created conditions that promote selective binding of RNA to the RNeasy membrane. A volume of 700 µL of the sample, including any precipitate, was transferred to a RNeasy Mini spin column placed in a 2 ml collection tube. The lid was closed and centrifuged for 15s at ≥8000 x g. The flow-through was discarded. A volume of 700 µl Buffer RW1 was added to the RNeasy spin column, and the lid was closed and centrifuged for 15

s at ≥8000 x g. The flow-through was discarded followed by the addition of 500 µl of buffer RPE to the RNeasy spin column. The lid was closed and centrifuged for 15s at ≥8000 x g, followed by discarding the flow-through. Buffer RPE (500 µl) was added to the RNeasy spin column, the lid was closed and centrifuged for 2 min at ≥8000 x g. The RNeasy spin column was placed on a new 2 ml collection tube and centrifuged at 12000x g for 1 min to dry the membrane. The collection tube was discarded and the RNeasy spin column was placed on a new collection tube. A volume of 30 µl of RNase-free water was added directly to the spin column membrane. The lid was closed and centrifuged for 1 min at ≥8000 x g to elute the RNA into the collection tube. The second volume of 30µl of RNase-free water was added directly to the spin column membrane. The lid was closed and centrifuged for 1 min at ≥8000 x g to elute the RNA into the same collection tube used in the previous step. Total RNA binds

to the membrane and was eluted in RNAse-free water. All binding, washing, and elution steps were performed by centrifugation in a microcentrifuge (Eppendorf 5424R). cDNA was synthesized from the RNA extracted.

4.2.22.3 RNA extraction using Trizol™ Reagent

Materials required: Trizol™ Reagent kit(Catalog Numbers 15596026 and 15596018), heat block at 55–60°C, Isopropanol, Ethanol, 75%(in DEPC-treated water), RNase-free water of 0.5% SDS

Procedure

The cultured cells (5×10^6 cells) were pelleted by centrifugation and supernatant was discarded. A volume of 0.75 ml of Trizol™ Reagent was added to the pellet. The lysate was pipetted up and down several times to homogenize the cells. The lysate was incubated for 5 minutes for complete dissociation of the nucleoproteins complex, after which 0.2 ml of chloroform was added. The lid was closed, and the tube shaken vigorously for 25 seconds, then incubated further for 2–3 minutes at room temperature. The lysate was centrifuged for 15 minutes at 12,000 × g at 4°C. The mixture separated into a lower red phenol-chloroform, the interphase, and a colorless upper aqueous phase. The aqueous phase containing the RNA was transferred to a new tube by angling the tube at 45° and pipetting the solution out. A volume of 0.4 ml of 100% isopropanol was added to the aqueous phase and incubated for 10 minutes. The lysate was centrifuged for 10 minutes at 12,000 × g at 4°C. Total RNA precipitated to form a white gel-like pellet at the bottom of the tube and the supernatant was discarded. The pellet was resuspended in 1 ml of 75% ethanol, then vortexed for 15s, and centrifuged for 5 minutes at 7500 × g at 4°C. The supernatant was discarded and RNA pellet

was air-dried for 5 minutes. The RNA was re-suspended in 30 µL of RNase-free water by pipetting up and down and incubated on a heat block set at 55°C for 10 minutes. The extracted RNA was used for cDNA synthesis.

4.2.22.4 Superscript™ III First-Strand cDNA Synthesis System

The components of the Invitrogen (Catalog no.18080-051) contained the following: Oligo(dT)$_{20}$ (50 µM), 10X RT buffer, 0.1 M DTT, 25 mM magnesium chloride,10 mM dNTP mix, Superscript III RT (200 U/µl), RNaseOUT (40 U/µl), RNase H (2 U/µl), DEPC-treated water, protocol. The Superscript® III First-Strand Synthesis System for RT-PCR was optimized to synthesize first-strand cDNA from total RNA. The Superscript® III Reverse Transcriptase was the enzyme used to synthesize cDNA at a temperature range of 42–55°C, providing increased specificity, higher yields of cDNA, and more full-length product**.**

The components in the table below were mixed in a 0.5-ml tube

Component	**Amount**
RNA	5ug
50 µM oligo(dT)	1 µL
10 mM dNTP mix	1 µL
DEPC-treated water	5 ul

The tube with the mastermix components was incubated at 65°C for 5 minutes and cooled on ice for 1 minute. The cDNA Synthesis mix was prepared as in the Table below and the mix was added to the RNA/primer mixture tube.

Component	1 Reaction	6 Reactions
10X RT buffer	2 µL	12 µL
25 mM $MgCl_2$	4 µL	24 µL
0.1 M DTT	2 µL	12 µL
RNaseOUT™ (40 U/µL)	1 µL	6 µL
Superscript® III RT (200 U/µL)	1 µL	6 µL

A volume of 10 µl of cDNA Synthesis mix was added to each RNA/primer mixture (4 tubes). The contents of the tubes were mixed gently and centrifuged for 15s at 10000xg, followed by incubation for 50 minutes at 50°C. The tubes were further incubated at 85°C for 5 minutes and afterwards chilled on ice. The tubes were centrifuged for 15s at 10000xg and a volume of 1 µl of RNase H was added to each tube and incubated for 20 minutes at 37°C. The synthesized cDNA was quantified using NanoDrop One/One^C Spectrophotometer at 260 nm absorption (Thermofisher Scientific) according to the manufacturer's instructions. Briefly, sample type DNA was selected. The machine was blanked using 2 µl of milliQ water and the measure was confirmed to be 0 (no DNA), this was followed by the addition of 2µl of the sample on the reader stage and the DNA measurement shown on the screen. The sample measurement process was repeated for the rest of the samples. The concentration of DNA is shown in the table below.

DNA samples	Nucleic acid conc.	DNA purity 260/280nm
1	1104.6ng/µl	1.55
2	907.6ng/µl	1.52
3	870.3ng/µl	1.56
4	871.9ng/µl	1.54

4.2.22.5 Amplification of target cDNA using PCR

The cDNA samples were diluted to 100ng/ml by addition of RNAase-free water (2.2µl DNA template in 37.8µl RNAase-free water). The PCR components were mixed and briefly centrifuged (15s) before use. The 4 samples of cDNA template were used. Amounts for 10 reactions were prepared for ease of pipetting small volumes (Table 4.3). Each component for 1 reaction was added to the 96-well molecular microplate.

Table 4. 3: PCR reaction components for cDNA

Component	Amount (1 reaction)	Amount (10 reactions)
Master mix (Bioneer®)	5 µL	50 µL
Primer 1 forward	0.2 µL	2 µL
Primer 1 Reverse	0.2 µL	2 µL
cDNA sample	1.5 µL	30.5 µL
Bovine Serum Albumin	0.05 µL	15µL
Water	3.05 µL	0.5 µL

The following primers were used for the PCR reactions from Macrogen (HO00103002). The primers were reconstituted to 100pmol/ul by adding of 170ul Tris-EDTA (TE) buffer.

The goat primer sequences were obtained from Sharma et al. (2020). The thermal cycling conditions consisted of initial denaturation at 95 °C for 10 min, followed by 40 cycles of 30s at 95 °C, 30s at the appropriate annealing temperature (Table 4.4) and 30s at 72 °C, followed by 95 °C for 10s and melting curve. Gel electrophoresis was carried out to visualize the PCR product.

Table 4. 4: Primer sequences and annealing temperatures or PCR amplification

Gene	Annealing temperature	Primer sequence	Primer type
PLZF	58	GCAACAGCCAGCACTATACTC	Forward
		TACAGCAGGTCATCCAGGTC	Reverse
BCL6B	58	GCCACCACCTTTAATTTCTCAC	Forward
		GAAATCAGGCTTCCAGTCTC	Reverse
UCHL1	58	GATAAAGCACTTACCCTCAACC	Forward
		GCCTTAACTTACAGACACAAACC	Reverse

4.2.22.6 Gel electrophoresis using agarose gel

The 2% agarose gel in 1X Tris-acetate-EDTA (TAE) buffer was prepared by adding 3g of Agarose agar to 150ml of 1X TAE buffer. The gel was heated for 2 minutes in a microwave until the agar dissolved completely and the solution was clear. A gel red visualizing dye (3μL) was added to the gel and it was left to cool at room temperature for 5 minutes. The agarose gel was poured into a gel tray with the well comb in place and left to dry at room temperature for 30 minutes until the gel completely solidified. The loading buffer (1μl) was added to each well of the microplate containing the DNA template and control (mastermix without DNA template). The loading dye was a visible dye that helps with gel loading and allows gauging of how far the DNA has migrated. Once solidified, the gel comb was removed, and agarose was placed into the gel box (electrophoresis unit) (Fig 4.8). The gel box was filled with 1x Tris-acetate-EDTA (TAE) buffer until the gel is covered. A volume of 4μl molecular weight ladder (100 bp DNA, Biolabs Inc.) was loaded into the first lane of the gel. The same volume of the DNA template was loaded into the wells of the gel. The gel was run at 150 V, 500KA current, 150 watts of power for one and half hours until the dye line was approximately 75-80% of the way down the gel. The power was turned off and the electrodes were disconnected from the power source. The gel was removed from the gel box and read using a gel reader machine with UV light 9fusion, DNA fragments referred as bands were read. The size of the DNA ladder (100 bps) in the first lane was used as a guide to infer the size of the DNA in the sample lanes and the results recorded. Once the method for RNA extraction, cDNA synthesis, PCR was optimized, and the templates were used to run RT-PCR.

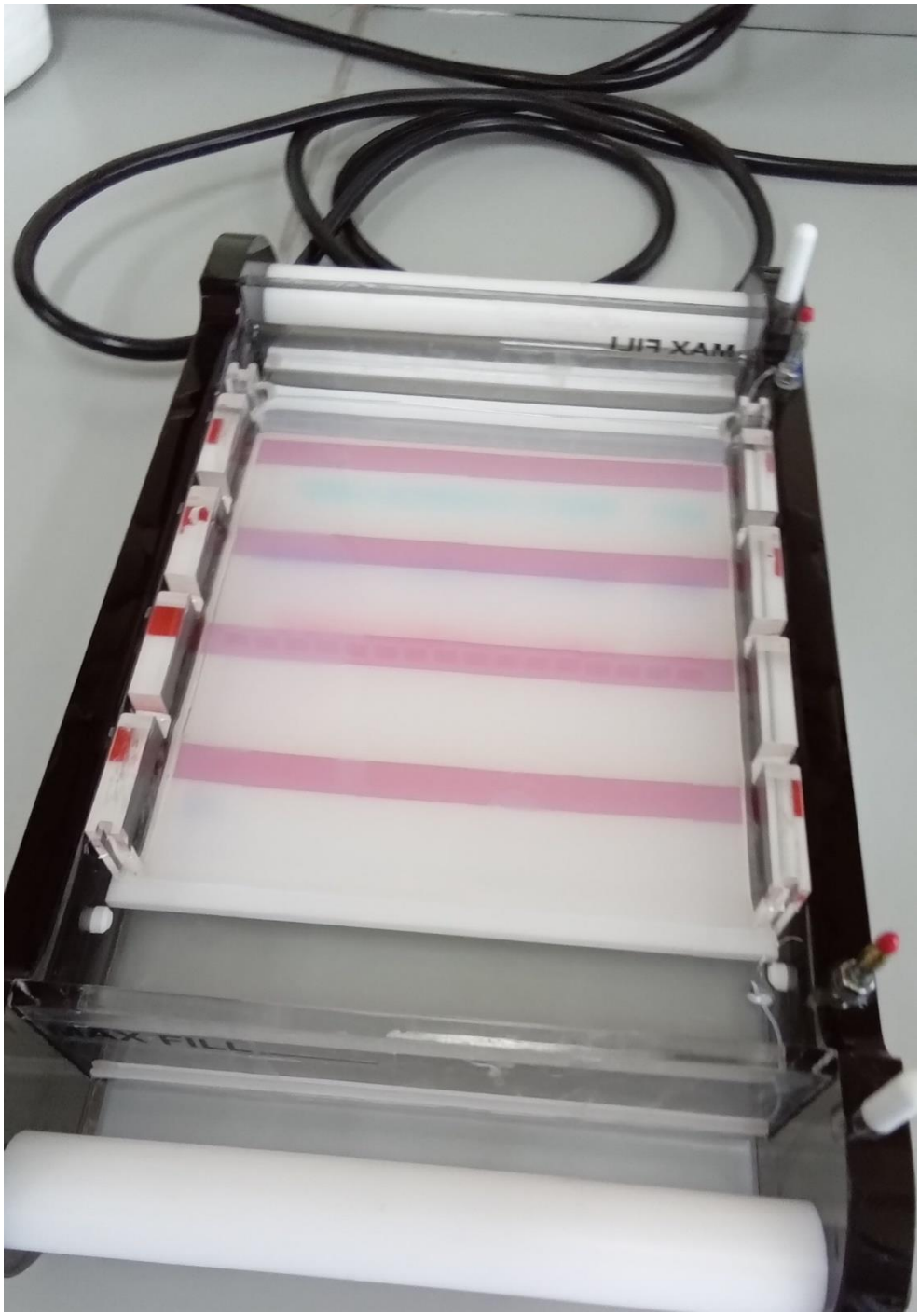


Figure 4.8: Gel box loaded with solidified 2% agarose gel.

4.2.22.7 Real time PCR for quantification of gene expression levels for SSC markers

PCR was performed on Quantstudio 5.0 PCR machine (Applied Biosystems) using of SYBR Green Master Mix (SYBR Green Master Mix (Maxima SYBR Green Maxima; Fermentas; Fisher Scientific). The PCR reaction conditions and goat primer sequences were obtained as presented previously (Sharma *et al.*, 2020). The thermal cycling conditions were similar to what was used in conventional PCR in section 4.2.16.4. The expression of GAPDH, THY 1, VASA, BCL6B, UCHL1, NANOS2 and PLZF genes in multiparameter-selected SSC and cultured SSC colonies was assessed. The primer sequences for genes used are indicated in the table below.

Table 4. 5: RT PCR annealing temperatures for SSC primer sequences.

Gene	Annealing temperature	Primer sequence	Primer type
1PLZF	58	GCAACAGCCAGCACTATACTC	Forward
		TACAGCAGGTCATCCAGGTC	Reverse
BCL6B	58	GCCACCACCTTTAATTTCTCAC	Forward
		GAAATCAGGCTTCCAGTCTC	Reverse
UCHL1	58	GATAAAGCACTTACCCTCAACC	Forward
		GCCTTAACTTACAGACACAAACC	Reverse
ID4	56	TGTCACTGAGTTTCATGTCTG	Forward
		AGAAAGTGTTCATTGCCAAGAG	Reverse
THY1	56	CTGACCCGTGATACAAAGAAGTG	Forward
		TGAAGTTGGACAGGTAGAGGA	Reverse
GAPDH	56	TCAAGAAGGTGGTGAAGCAG	Forward
		CCCAGCATCGAAGGTAGAAG	Reverse

The qPCR data was analyzed by using Quantstudio 3 and 5 data analysis software v1.5.2. Relative gene expression for each gene was calculated as a ratio to that of the target reference GAPDH using Delta Delta CT analysis (Livak and Schmittgen, 2001). PCR products were visualized by agarose gel electrophoresis. The volumes and amount of components for the PCR reaction were prepared as follows:

Component	**Amount (1reaction)**	**Amount (5 reactions)**
SYBR green Mastermix	5µL	1 µL
Primer 1 Forward	0.2 µL	1 µL
Primer 1 Reverse	0.2 µL	1µL
BSA	0.05 µL	0.25 µL
Water	3.05 µL	15.25 µL
Sample	1.5 µL	
Total volume per well	10 µL	

A volume of 10 µL of DNA/master mix was pipetted into 96-well 0.2ml microplate. The samples were done in triplicate for each of the 6 primers as shown in figure 4.9a. The amplification temperature settings for the RT-PCR Quantstudio 5 are shown in figure 4.9b below.

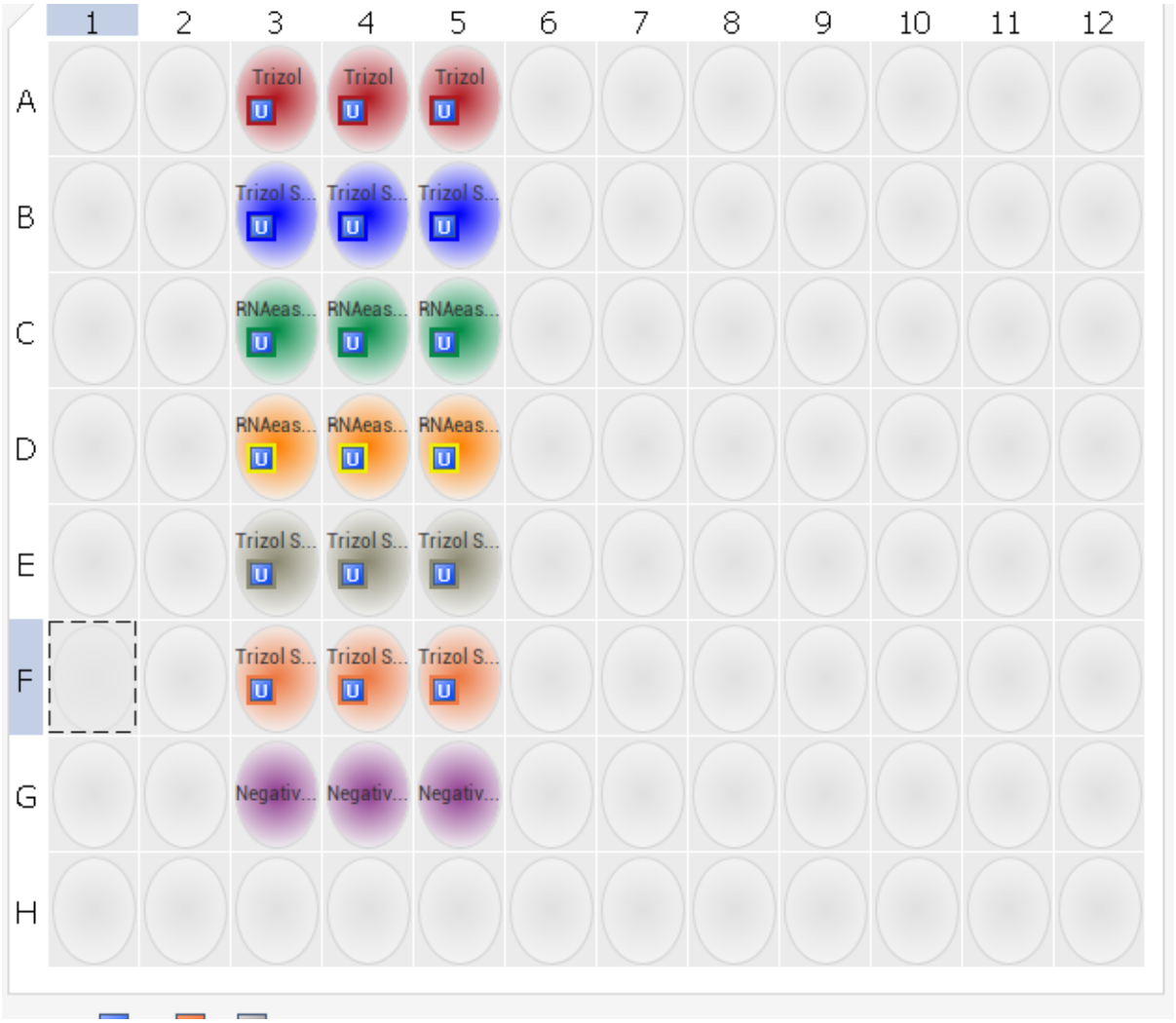


Figure 4. 9a: Sample and primer target arrangement in the 96 microwell plate.

Key for Figure 4.9a: A-PLZF, **B**-BCL6B, **C**-UCHL1; **D**-ID4; **E**-THY1, **F**-GAPDH; **G**-Negative control. **Trizol**- DNA template in which RNA was isolated using the Trizol reagent method. **RNAeasy**- DNA template in which RNA was isolated using the RNAeasy kit method

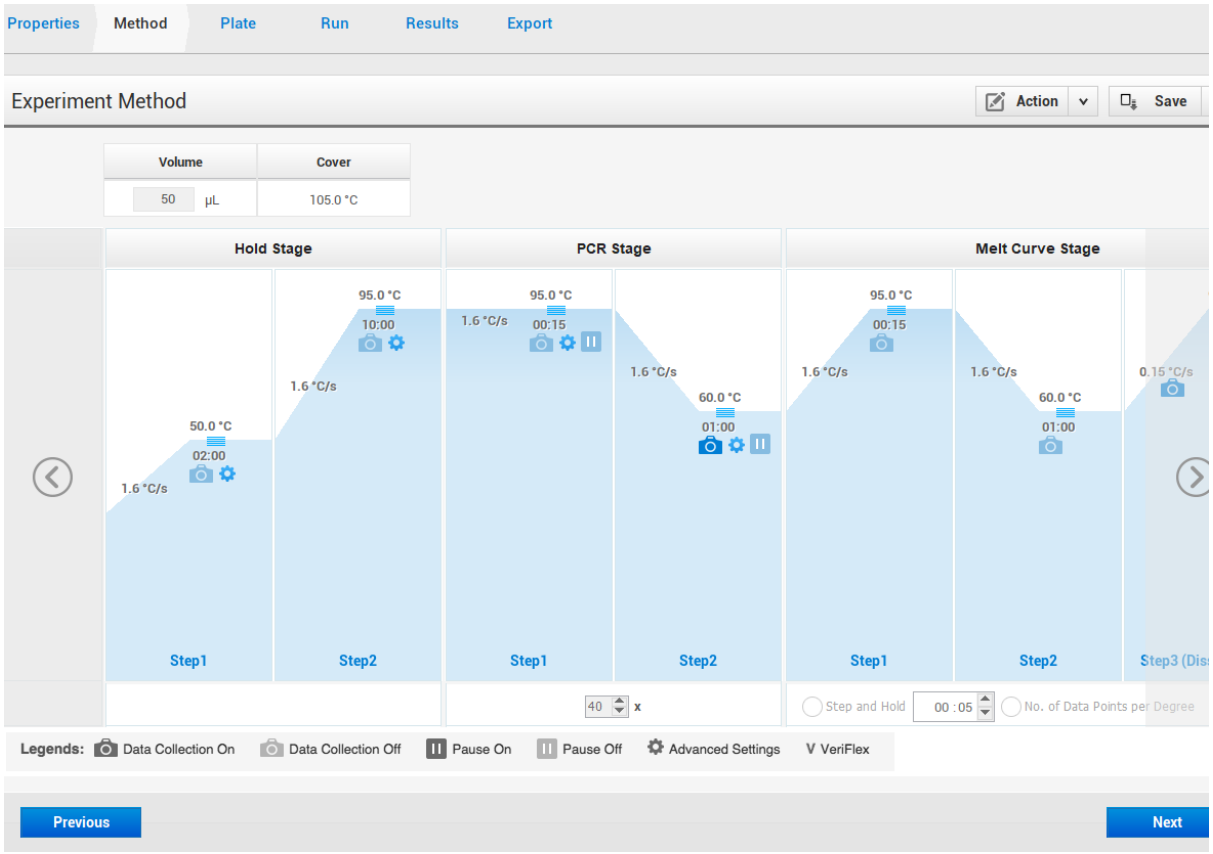


Figure 4. 9b: The amplification and annealing temperatures for the RT-PCR Quantstudio 5 reaction

4.2.23 Alkaline phosphatase test for pluripotency

Alkaline phosphatase activity is usually high in pluripotent proliferating cells and thus its activity was tested in cultured SSC (Sharma *et al.*, 2020)**.** Reagents required: Stem Alkaline phosphatase staining kit 11 (Stemgent 00-0055) was used. The kit contains solution A, B, and C, which are used to prepare the alkaline phosphatase substrate solution in a ratio of 1:1:1. In the study 1 ml: 1ml: 1 ml of solution A:B:C were mixed and used as the substrate solution. The kit also contains the alkaline phosphatase fix solution. Four percent paraformaldehyde was used to fix the cells and 0.1% triton in phosphate buffered saline (PBS) detergent was used as a lubricant for cell washing.

Procedure

The cultured SSC were gently detached from the culture plate by gently pipetting and the suspended cells were collected in 1.5ml tubes. The cells were washed by centrifuged at 1200rpm for 5 minutes. The cells adhered to slides through cytospining. The cells on the slides were fixed in 0.5ml 4% paraformaldehyde for 10 minutes. The fixative was washed off thrice using PBS, each wash lasting 5 minutes. The slides were thereafter incubated for 2-5 minutes in 0.5ml fixing solution from the kit. The cells were washed thrice with PBS and the freshly prepared alkaline phosphatase substrate was added and the cells were incubated in the dark for 30 minutes. The cells were closely monitored for development of colour change and the reaction was stopped by the washing of the substrate with PBS. The cells were covered with 1X PBS and observed under light microscope for the brown-red stain, which was indicated positive for Alkaline phosphatase expression and lack of stain indicated the as absence of alkaline phosphatase activity.

4.2.24 Cell Concentration and viability evaluation

The cell concentrations obtained from testicular isolation and the different enrichment steps per gram of testis were estimated using a hemocytometer. Marker expression through immunochemistry were estimated by calculating the percentage of positive cells divided by total cell nuclei stained by DAPI dye. Trypan blue (0.04%) exclusion criteria was used to determine the percentage of dead cells (with blue colour) vs live cells (not take up blue colour). The cells were observed at ×400 magnification and the percentage of viability was calculated.

4.3 Statistical analyses

The results were expressed as mean ± SD. The statistical significance between the mean values was determined by one-way analysis of variance (ANOVA) (Student Newman Keuls Method) in Excel. A value of $P \leq 0.05$ was determinant for statistical significance.

4.4 Results

4.4.1 Isolation and enrichment of spermatogonia for prepubertal goat testes

Isolation of undifferentiated spermatogonia population from a heterogeneous population of testicular cells was successfully done through two-step enzymatic digestion. The enzymatic digestion in collagenase Type IV enzyme for 5 minutes and trypsin enzyme for 30-35 minutes ensured a fine digestion of seminiferous tubules obtained from 1st digestion (Fig. 4.10 and Fig 4.11) and maintained a high viability of testicular cells (77.4±1.19%). The multiparameter selection criteria resulted in a subpopulation of testicular cells that were enriched for undifferentiated spermatogonia suitable for starting up the germ cell culture (Fig 4.12). A total population of $16.10 \pm 1.4 \times 10^6$ SSC enriched cells was isolated per gram of

testicular tissue. The double enrichment criteria were effective in producing cells in cell suspension in which a higher percentage of cells exhibited the SSC markers on immunocytochemistry. Typical yields of 2.7-3.2 $x10^6$ multiparameter enriched spermatogonia cells were isolated from 200 mg of testicular tissue.

Morphologically, SSCs were observed as round or oval cells, with a large nucleus and scanty cytoplasm, some of the cells being seen in clusters of 2-6 cells (Fig. 4.13). The multiparameter selection yielded cell cultures with higher colony formation, cells of uniform size and few somatic cells. PLZF staining revealed that the multiparameter selection yielded a population with a mean of 69.20%± 1.0 undifferentiated spermatogonia cells (PLZF positive), which was significantly higher ($P<0.05$) than the 6.85%± 0.36 in the non-selected population (Fig. 4.14). A single isolation protocol of only differential plating resulted in 25.62%± 1.76 cells expressing PLZF marker (Fig. 4.14). Gene expression of the SSC markers in the enriched undifferentiated spermatogonia cell population was further confirmed by RT-PCR. The enrichment criteria used in the study yielded a goat testicular cell population that is viable and contains a high proportion of undifferentiated spermatogonia.

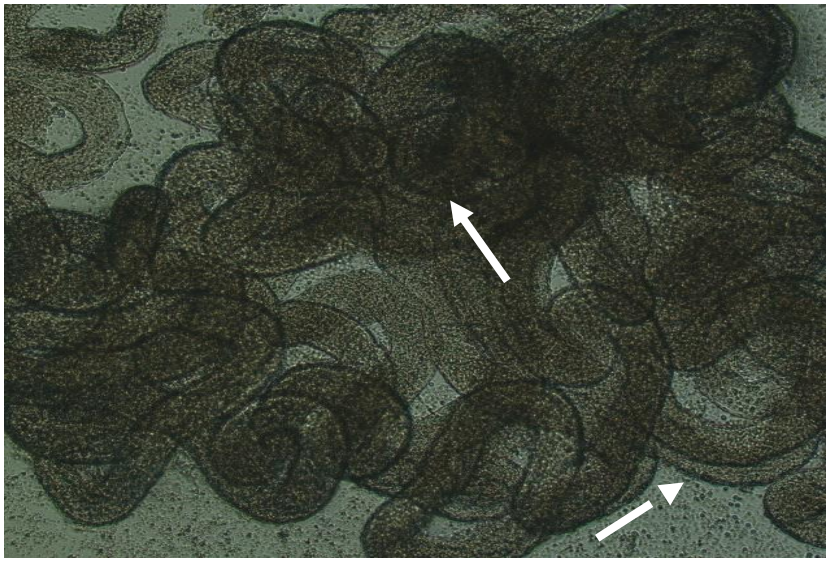


Figure 4. 10: Seminiferous tubules after digestion in collagenase type IV. (white arrow). Interstitial cells (dashed arrow) (Magnification factor x50).

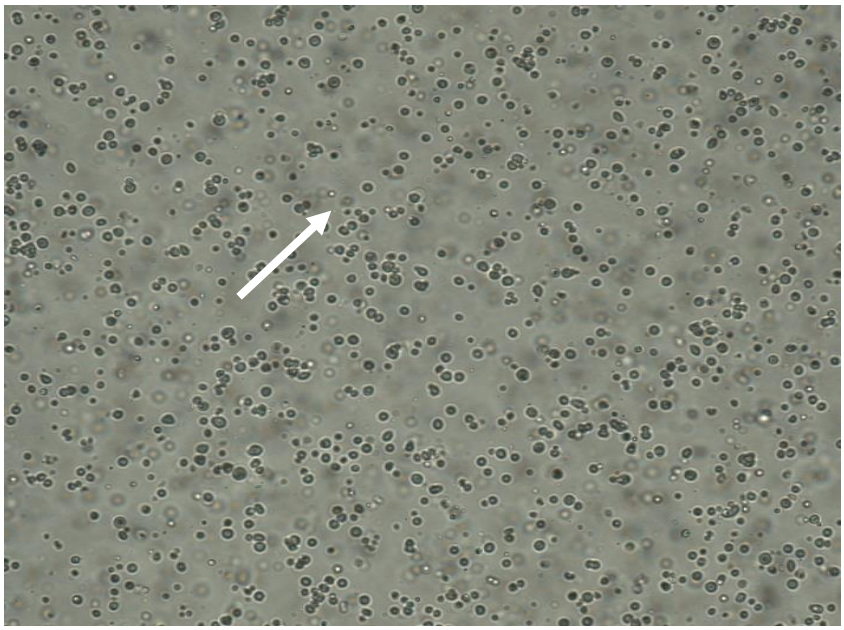


Figure 4. 11: Mixed testicular cell population after trypsin digestion.

(Magnification factor x100).

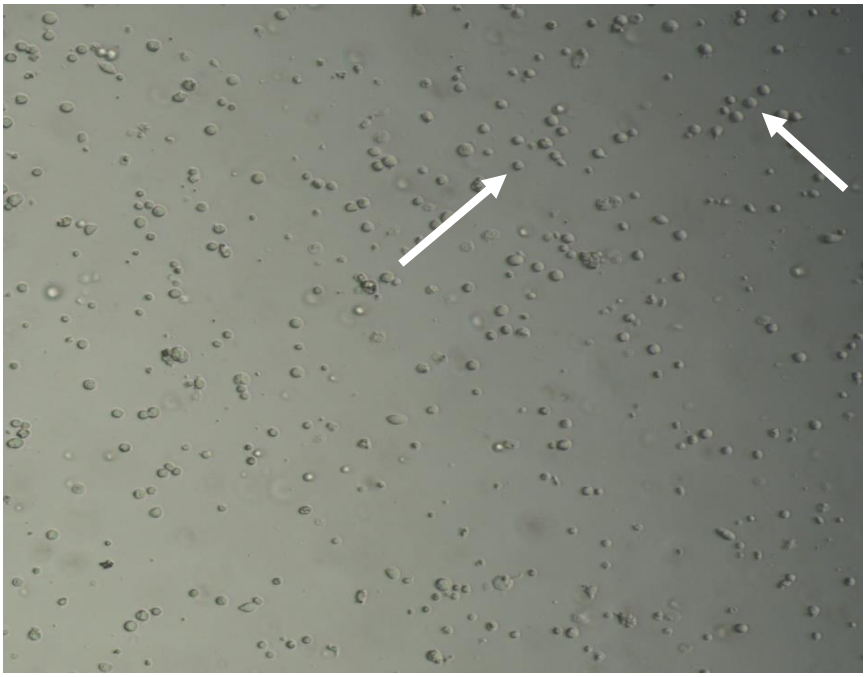


Figure 4. 12: Undifferentiated spermatogonia in the initial isolate following double enrichment. Represented by a white arrow (Magnification factor x100).

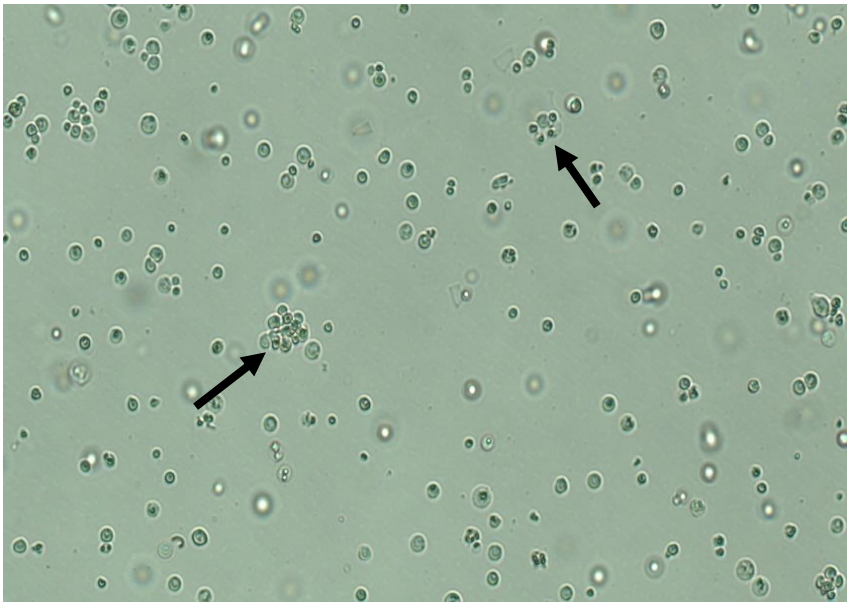


Figure 4. 13: Cell clusters of undifferentiated spermatogonia in the fresh cell isolate(arrows) (Magnification factor x100)

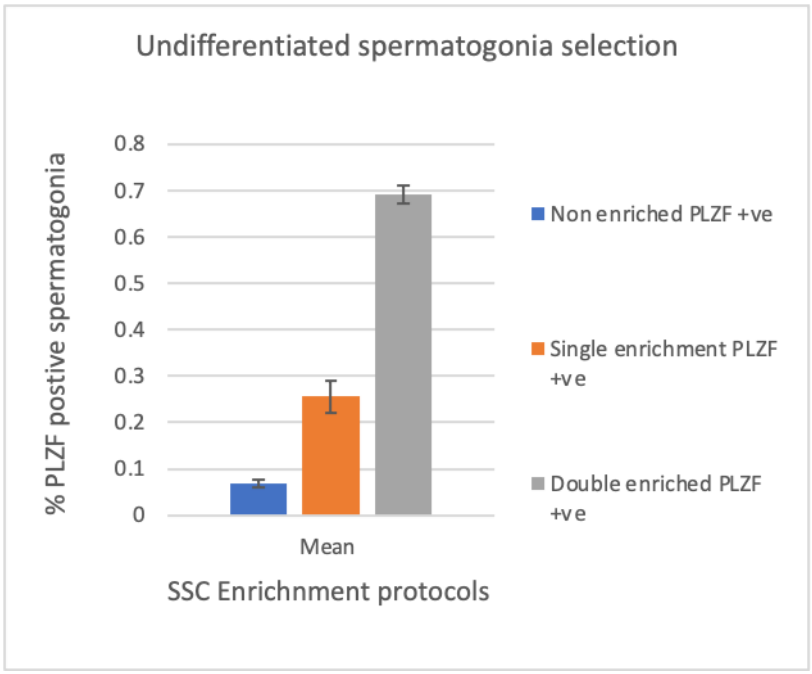


Figure 4. 14: Percentage of PLZF positive cells in non-enriched cell isolate, single procedure of enrichment and multiparameter selection.

4.4.2 Germ cell colony formation and evaluation

After the goat SSC were cultured on laminin-coated plates with serum-free medium, germ cell colonies appeared on day 3 after initial plating as round cells in clusters of 3 or 4 cells. The colonies increased progressively in numbers during culture with a cluster getting to have as many as 14 cells (Fig. 4.15, 4.16, and 4.17). Increase in colonies in laminin-coated plates in preconditioned medium declined after 45 days, while culture of SSC on goat testicular somatic cells feeder layer enhanced proliferation of SSC for up to 60 days. After 7 days of culture, the cell colony populations were significantly higher than on day 0 ($p<0.05$).

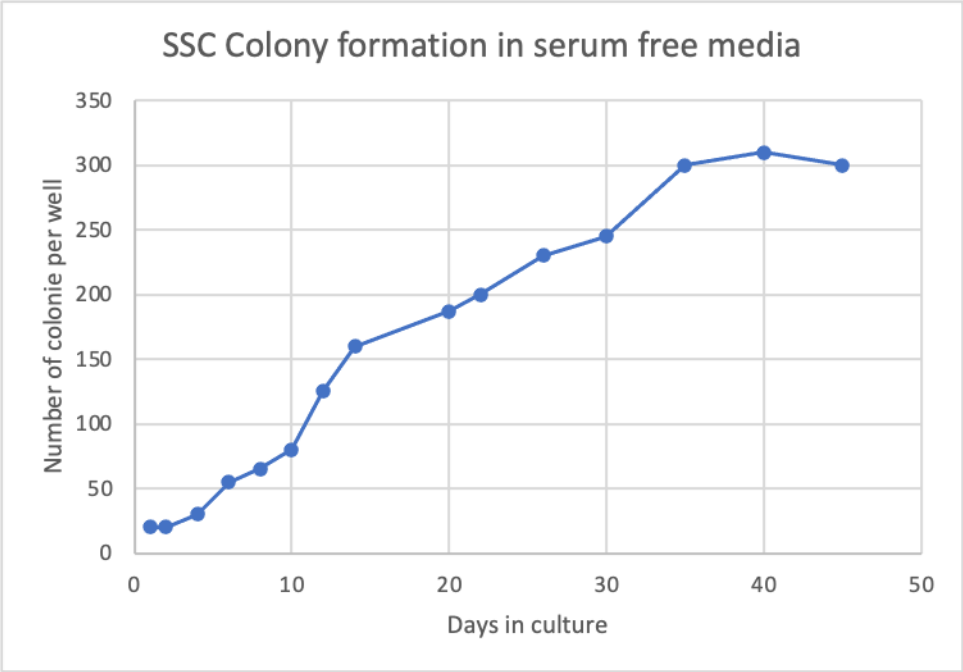


Figure 4. 15: Colony numbers in serum-free feeder culture over the 45-day culture period.

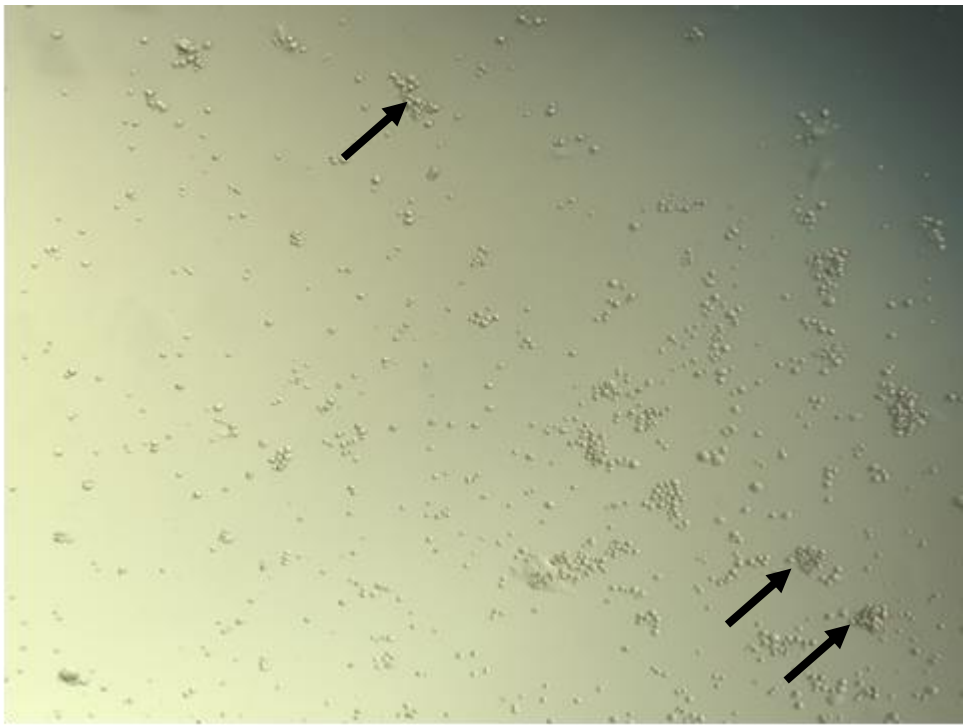


Figure 4. 16: Feeder-free cell culture germ cell clumps (arrow) ((Magnification factor x100)

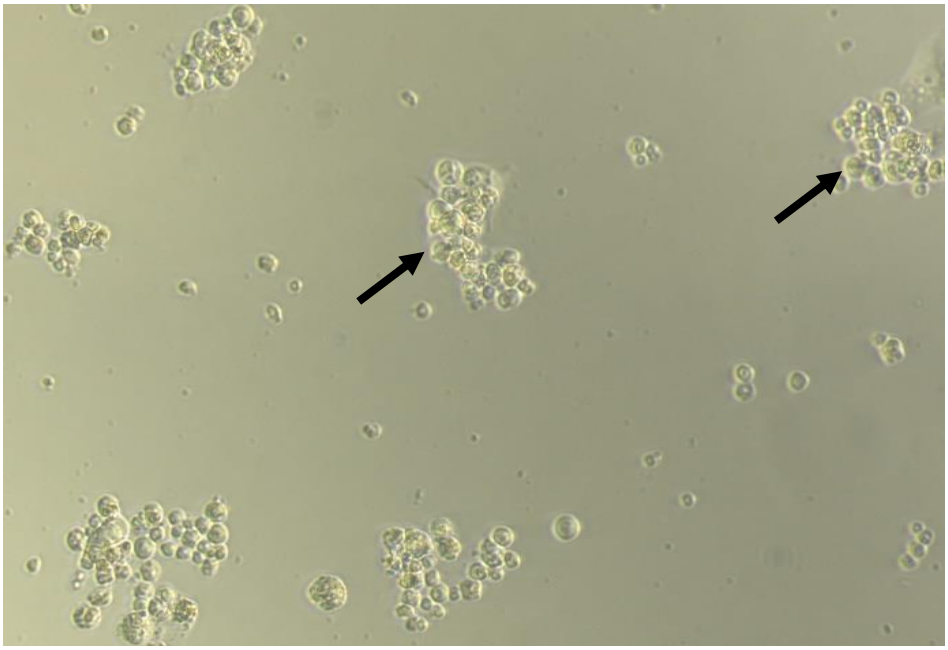


Figure 4. 17: Feeder-free cell culture germ cell clumps (arrow) ((Magnification factor x50)

4.4.4 Serum-free culture of SSC

Typical germ cell colonies were maintained in the serum-free medium and the SSCs began to form cell clusters on day 2-3 and colonies were visible on day 4. In the serum-containing cultures, there was an enhancement of somatic cell proliferation, which suppressed the growth of SSC germ cell colonies. These serum-containing cultures were observed to have morphology of tightly packed cell spheres that resembled somatic cell outgrowths surrounded by SSC (Fig 4.18, 4.19 and 4.20). Thus, serum supplementation was discontinued, and the study protocol was changed to use a serum-free medium only. The serum-free media formulations contained BSA and StemPro as the serum replacement, and all media were supplemented with recombinant human forms of growth factors GDNF, FGF2, LIF, and SDF. All the cultures were maintained at 37°C and an atmosphere of 5% CO_2 according to what was documented in cattle (Suyatno *et al*., 2018). Germ cell clump formation was noted in the three media formulations (DMEM/ F12, MEMα and Stempro 34 SFM). Colony formation appeared on the 4^{th} day of culture in MEMα medium and Stempro SFM, but appeared after one week of culture in DMEM/F12 medium. The increase in number of cells was recorded for 7 days in culture (Fig 4.21, 4.22 and 4.23). Stempro 34^{TM} SFM had the highest number of germ cell colonies and the highest increase in cell numbers. This medium was noted to favorably support goat SSC growth in culture and therefore subsequent experiments were done using it. Under the serum-free culture conditions similar to what was reported previously, there was the formation of germ cell clumps with morphology resembling those in rodent undifferentiated culture (Oatley *et al*., 2016).

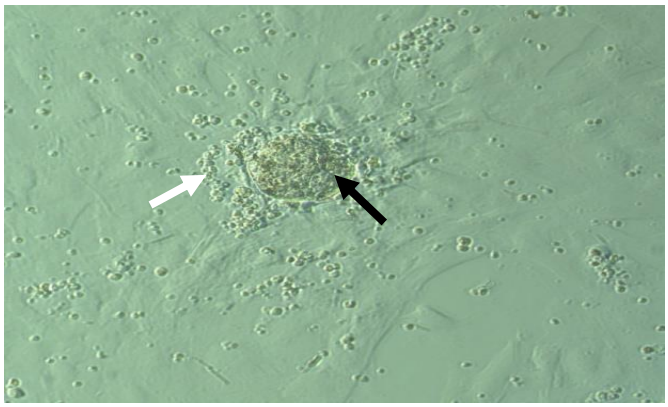


Figure 4. 18: Tight spheres of somatic cells (black arrow) surrounded by germ cells (white arrow) seen in 10% FBS supplemented culture medium ((Magnification factor x100).

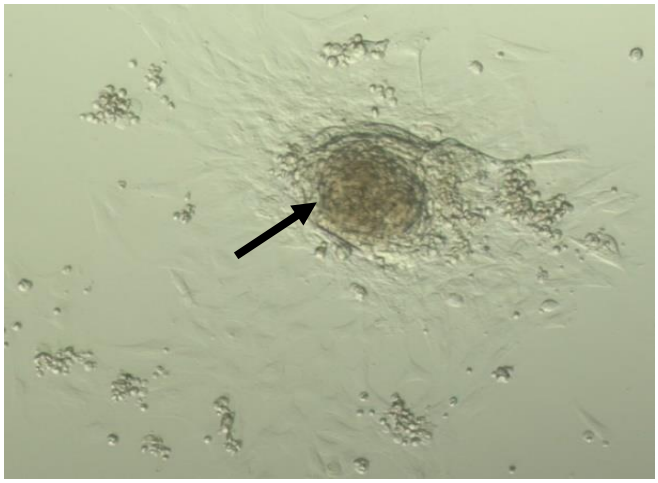
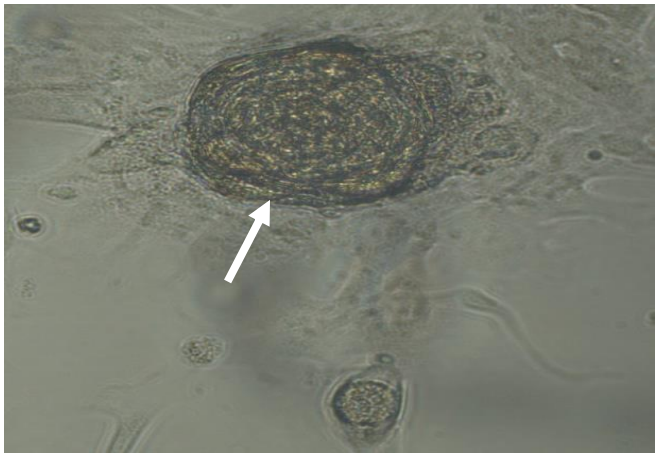


Figure 4. 19: Tightly packed somatic cell spheres in an SSC culture supplemented with FBS ((Magnification factor x100)



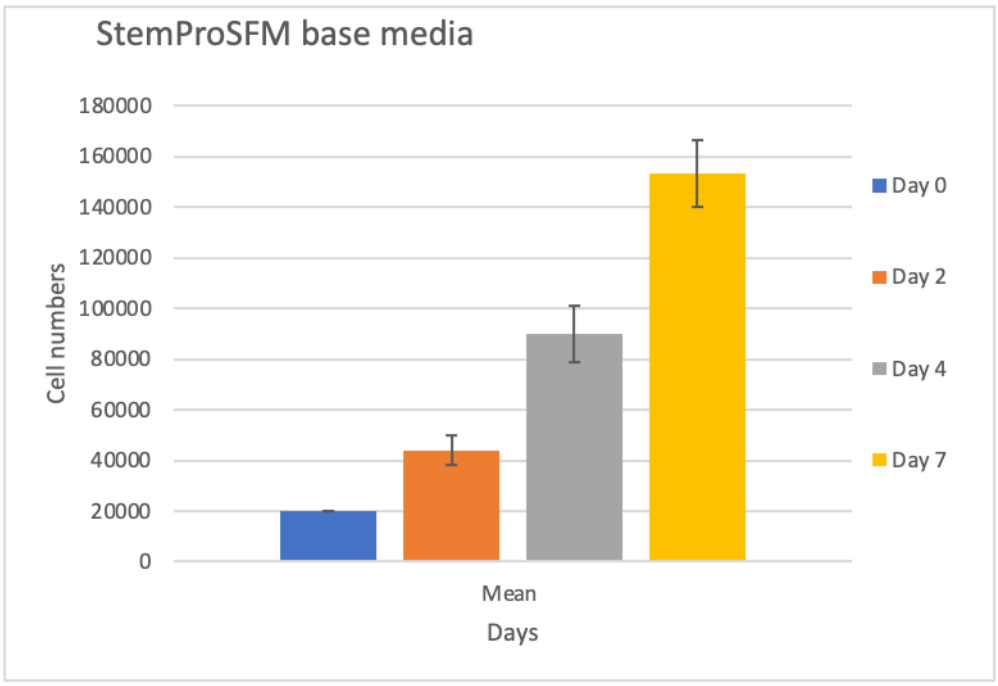


Figure 4. 20: Tight spheres of goat testicular somatic cell culture (x100)

Figure 4. 21: SSC colony growth on StemPro34 SFM base medium.

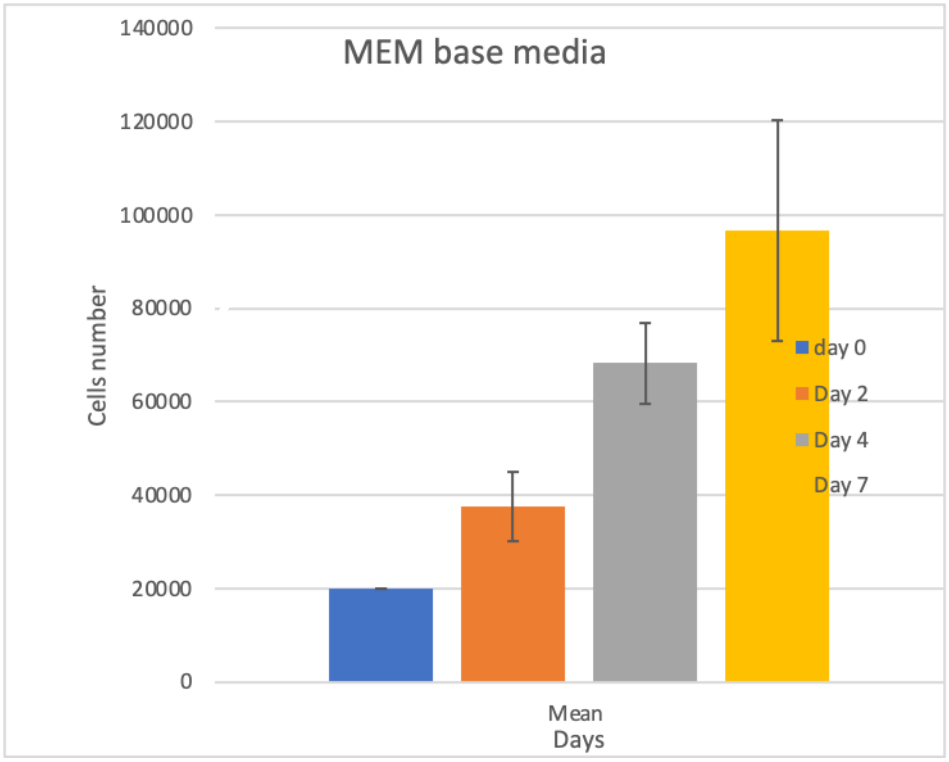


Figure 4. 22: SSC colony growth on MEMα base medium.

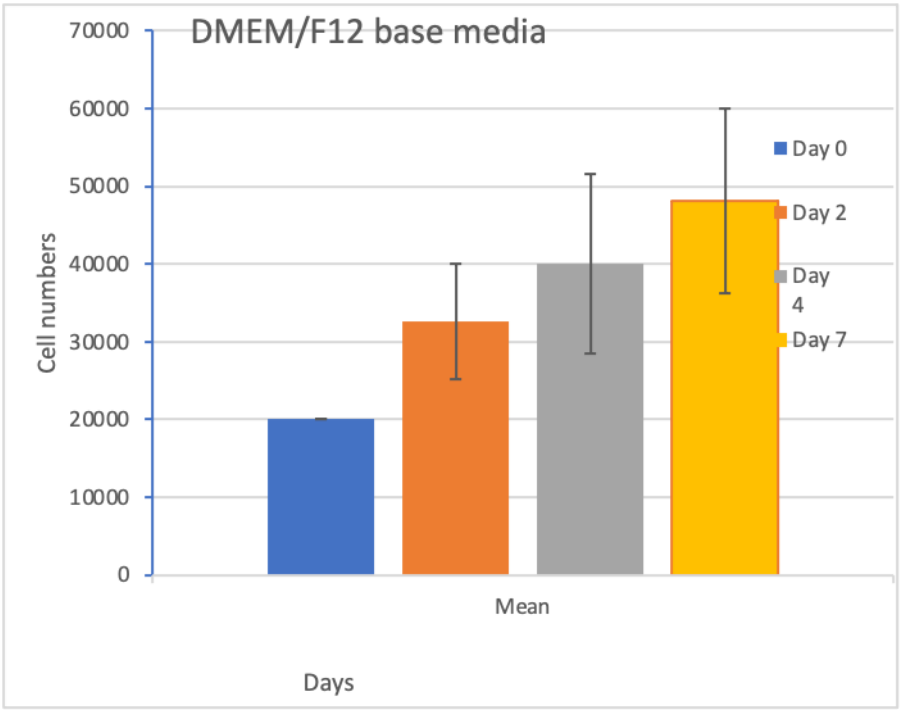


Figure 4. 23: SSC colony growth on DMEM/F12 base medium.

4.4.5 Long term feeder free germ cell culture

In the current study, spermatogonial stem cells were cultured on feeder-free on laminin-coated cell culture plates. Goat fetal fibroblast cell lines were established first and used to precondition the SSC culture medium for 24 hours before being used for culture. Preconditioning of the culture medium on goat foetal fibroblasts was to enrich the medium with growth factors secreted by these somatic cells. The enriched cell population of SSC was plated on laminin-coated plates after enrichment with the preconditioned serum-free medium. A cocktail of human forms of growth factors (GDNF, LIF, FGF2α, and SDF) was added to the preconditioned medium before use, which resulted in formation of germ cell clumps (Fig. 4.24 and Fig. 4.25). All the cultures (10/10) showed typical germ cell clumps, which appeared from day 3-4 of culture and increased in size as well as number of cell clusters. The germ cell clumps resembled those previously reported in rodent SSC and other livestock species (Fig. 4.26) as well as the expression of germ-cell-specific markers and pluripotent stem cell markers. The germ cell clumps persisted for 45 days and then the numbers started to decline. In comparison, the germ cell clumps on co-cultured goat testicular somatic cells were maintained for 60 days (Fig.4.27 and Fig 4.28). Immunocytochemistry of the feeder-free germ cell clumps revealed stable expression of PLZF, an indicator of the undifferentiated state of the spermatogonia.

Figure 4. 24: Goat SSC germ cell clumps on Serum-free feeder-free long-term culture (arrows) ((Magnification factor x50).

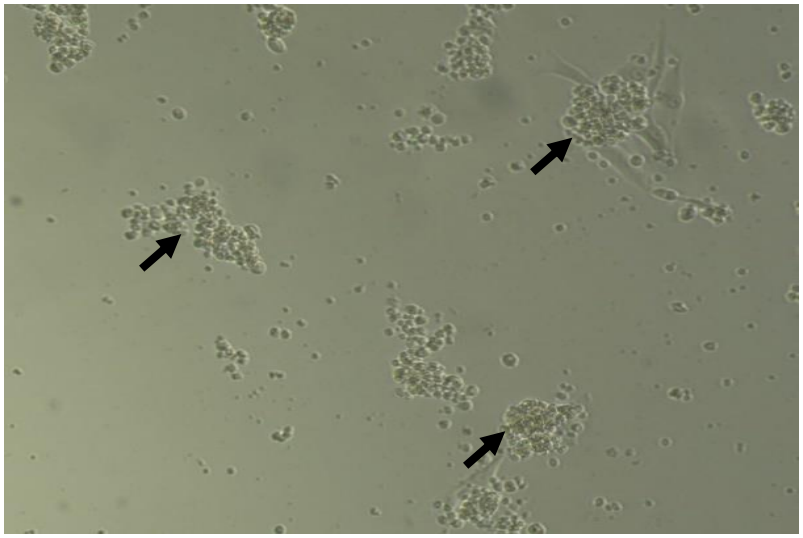
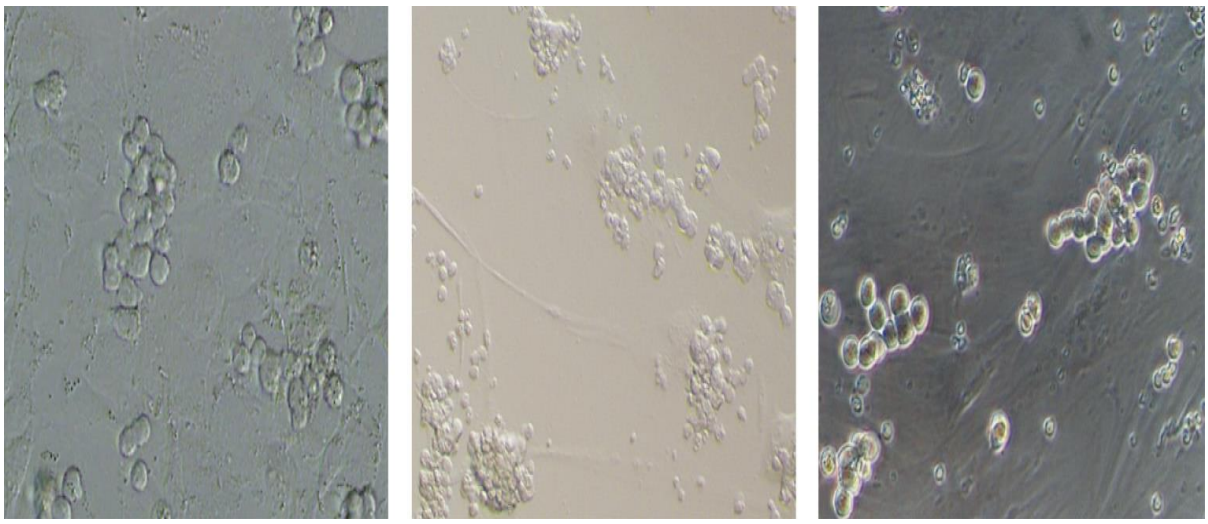


Figure 4. 25: Goat SSC germ cell clumps on Serum-free feeder-free long-term culture (arrows) (Magnification factor x100).

Published reports of pig, cattle and mouse SSC (Oatley *et al*., 2016) Images of cell clumps in culture well that develop from undifferentiated spermatogonia were isolated from testicular tissue of mice (a), pigs (b), and cattle (c). Clump morphology is indicative of an undifferentiated spermatogonial state, with the grape-like appearance being conserved among mammalian species (Fig.4.26).



a) Mice **b) Pigs** **c) Cattle**

Figure 4. 26:Previously published images of primary culture of mammalian undifferentiated spermatogonia (Oatley *et al* 2016). Image in Mice (a), in pig (b) and in cattle (c) (x100).

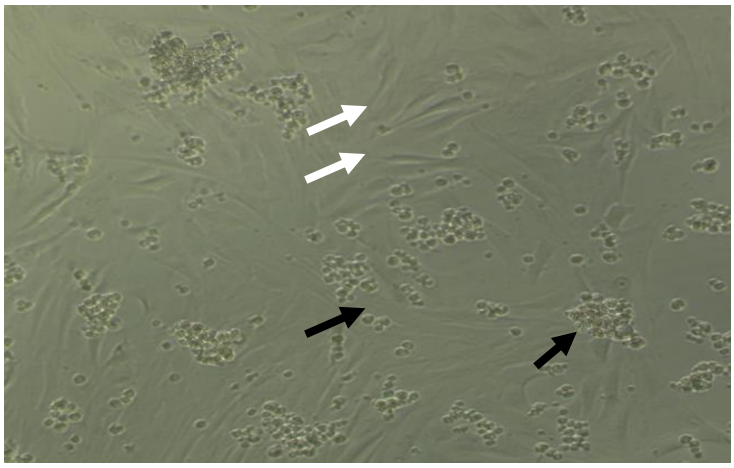


Figure 4. 27: Long-term culture of SSC clumps (black arrows) cultured on goat testicular somatic cells feeder layer (white arrow) ((Magnification factor X100).

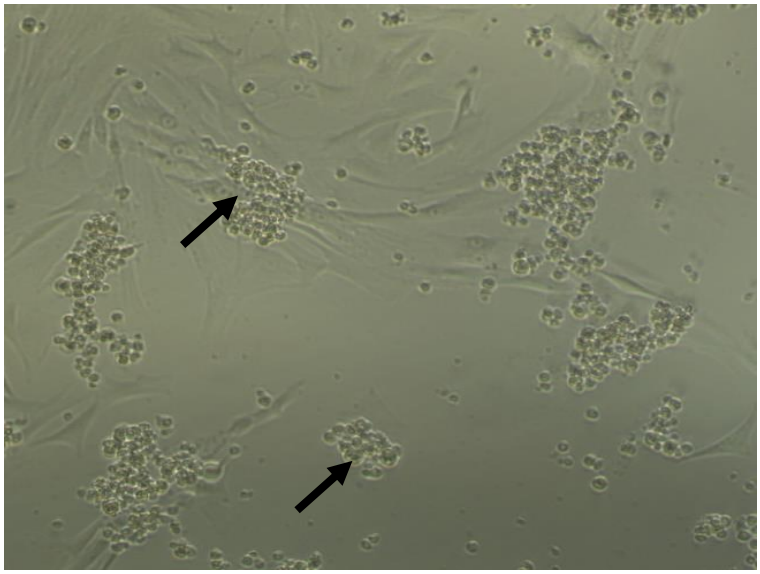
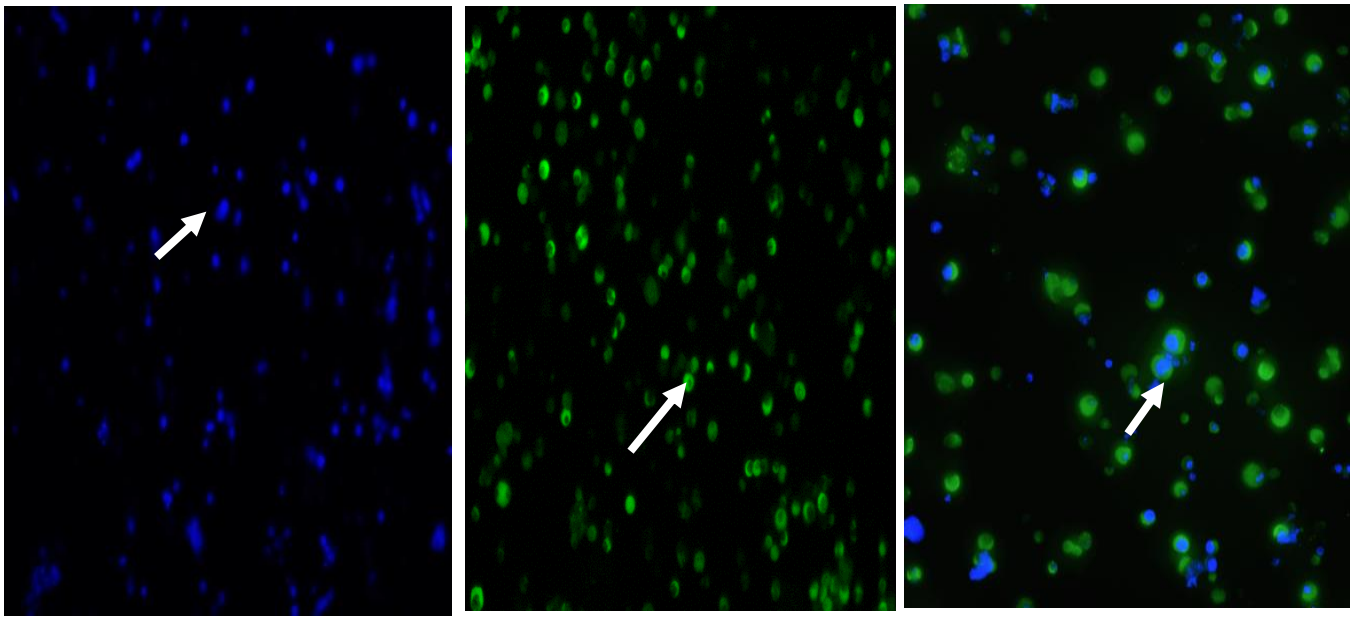


Figure 4. 28: Long-term culture of SSC clumps with larger colonies (arrows), cultured on goat testicular somatic cells feeder layer (Magnification factor x100).

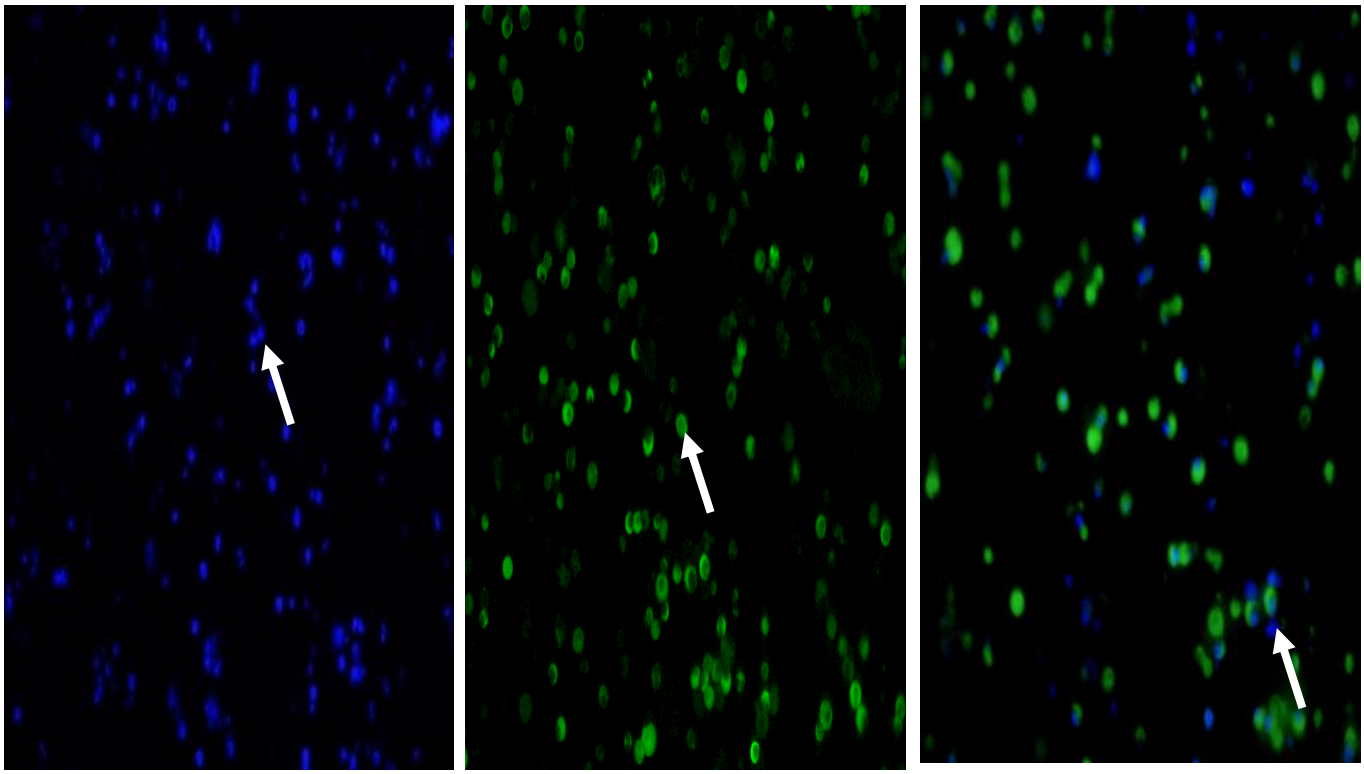
4.4.6 Characterization of SSC colonies through marker analysis

Single enriched procedure of differential plating on gelatin-coated plates resulted in about 25.62%± 1.76 cell population of PLZF staining cells, while the double enriched portion had 69.20%±1.0%, which presented a significant difference ($p<0.05$) between them. Expression of VASA was seen in almost all colonies or germ cell clumps (Fig. 4.29 A, B and C, Figure 4.30 A, B and C). On average, 89.2% ±0.7 of cells were VASA positive. There was a 10-fold increase in the population of PLZF staining cells in the multiparameter selected testicular cell population. Of great importance is that immunostaining of the feeder-free cultured germ cell clumps stably expressed PLZF indicating maintenance of an undifferentiated spermatogonial phenotype (Fig. 4.31 A, B and C; Fig. 4.32 A, B and C). The expression of other SSC related markers (NANOS2, GFRα1) confirmed their bonafide spermatogonial identity (Fig. 4.33 A, B, C and Fig.4.34 A, B, and C respectively). The control slides were stained with secondary antibodies only without primary antibodies (Fig 4.35). The germ cell colonies also exhibited a high alkaline phosphatase activity (Fig. 4.36).



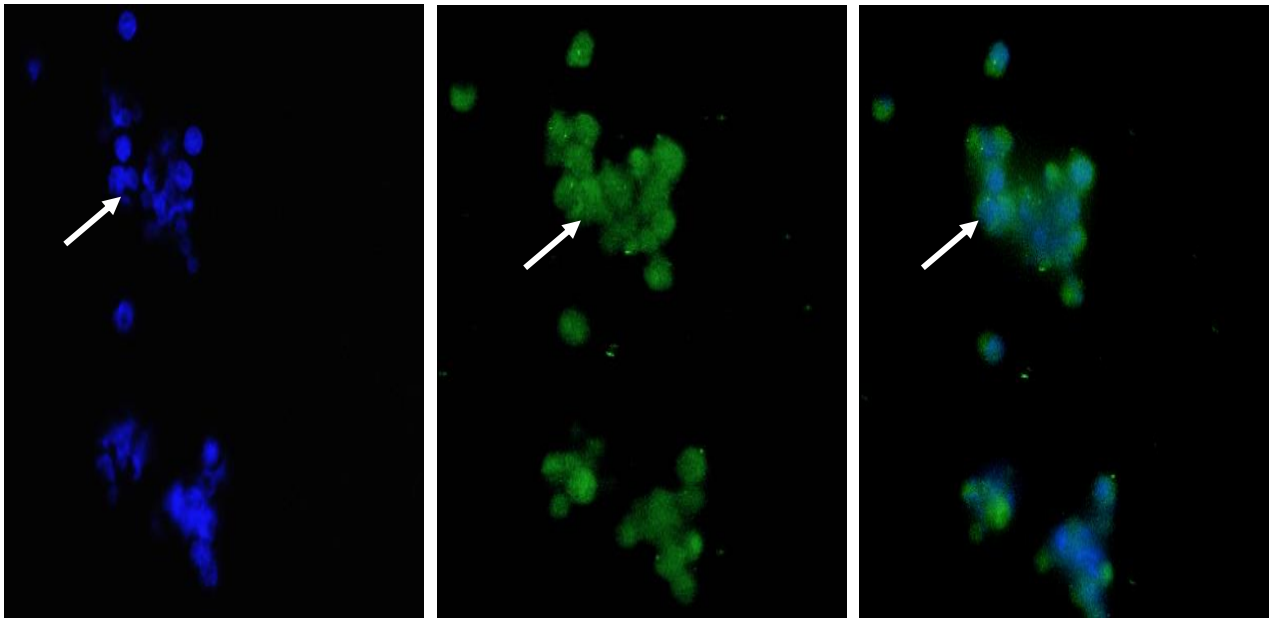
A **B** **C**

Figure 4. 29: Immunocytostaining of cultured spermatogonial stem cells Magnification factor x100, Scale bar= 20µm. A- DAPI (blue) cell nucleus staining of the cultured spermatogonia (arrow). B- Cytoplasmic staining cultures spermatogonia by VASA antibody (arrow). C- The merge of cytoplasmic VASA and DAPI nucleus staining of spermatogonial stem cells (arrow).



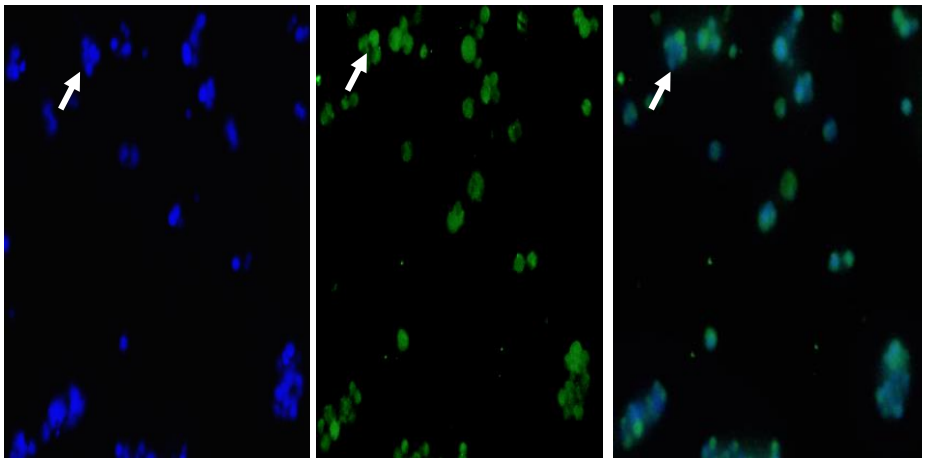
A **B** **C**

Figure 4. 30: Immunocytostaining of cultured spermatogonial stem cells. **A**- DAPI (blue) cell nucleus staining of the cultured spermatogonia (arrow). **B**- Cytoplasmic staining cultures spermatogonia by VASA antibody (arrow). **C**- The merge of cytoplasmic VASA and DAPI nucleus staining of spermatogonial stem cells (arrow). Magnification factor X100, Scale bar= 20µm.



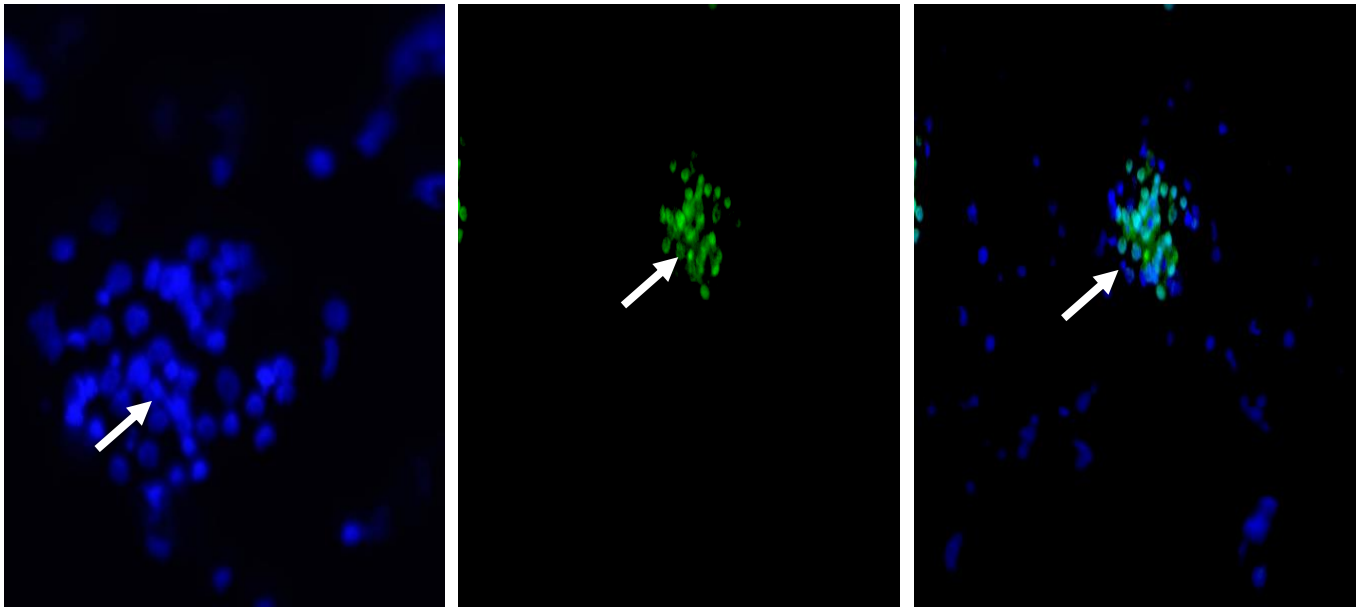
A **B** **C**

Figure 4. 31: Spermatogonial germ cell clumps staining with PLZF and DAPI. A- DAPI (blue) cell staining of the cultured spermatogonial stem cell clumps (arrow). B- staining of the cultured spermatogonial stem cell clumps by PLZF antibody (arrow). C- The merge of PLZF and DAPI staining of spermatogonial stem cells (arrow). Magnification factor x100, Scale bar= 20µm



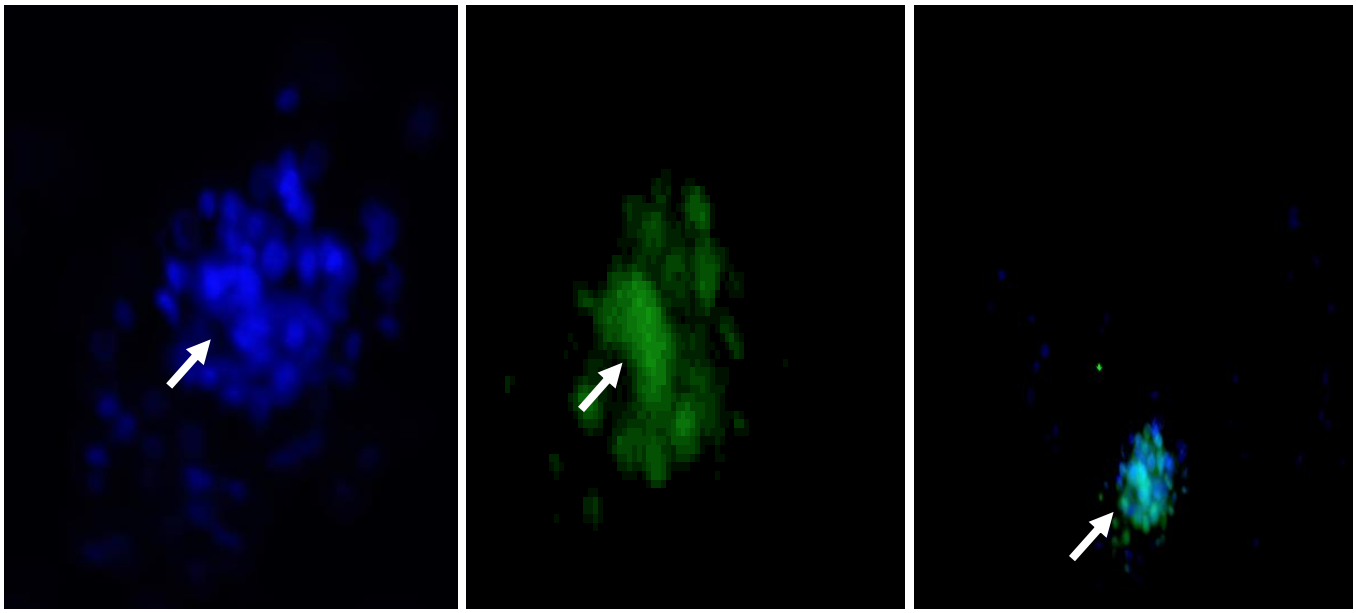
A **B** **C**

Figure 4. 32: Spermatogonial germ cell clumps staining with PLZF and DAPI. A- DAPI (blue) cell staining of the cultured spermatogonial stem cell clumps (arrow). B- staining of the cultured spermatogonial stem cell clumps by PLZF antibody (arrow). C- The merge of PLZF and DAPI staining of spermatogonial stem cells (arrow). Magnification factor x100, Scale bar= 20µm



A **B** **C**

Figure 4. 33: Spermatogonial germ cell clumps staining with NANOS2 and DAPI. A- DAPI (blue) cell staining of the cultured spermatogonial stem cell clumps (arrow) .B- staining of the cultured spermatogonial stem cell clumps by NANOS2 antibody (arrow). C- The merge of NANOS2 and DAPI staining of spermatogonial stem cells (arrow) Magnification factor x100, Scale bar= 20µm.



A B C

Figure 4. 34: Spermatogonial germ cell clumps staining with GFRα1 and DAPI. A- DAPI (blue) cell staining of the cultured spermatogonial stem cell clumps (arrow). B- staining of the cultured spermatogonial stem cell clumps by GFRα1 antibody (arrow). C- The merge of GFRα1 and DAPI staining of spermatogonial stem cells (arrow) Magnification factor x100, Scale bar= 20µm.

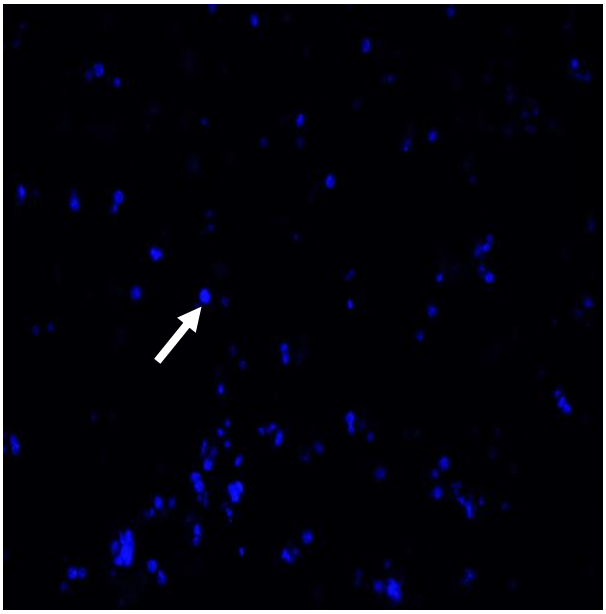


Figure 4. 35: DAPI staining of spermatogonial stem cells that had been counter-stained with secondary antibody only (control).Magnification factor x100, Scale bar= 20µm.

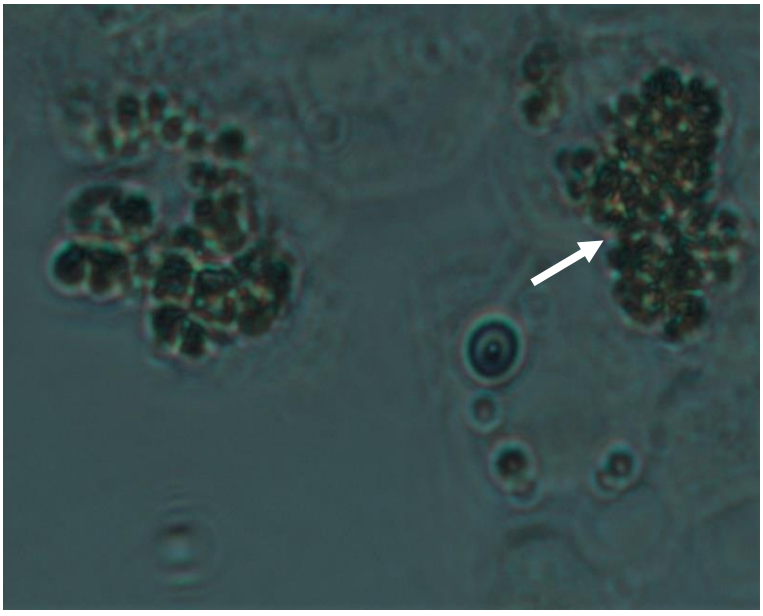


Figure 4. 36: Alkaline phosphatase staining of undifferentiated spermatogonia (arrow)Magnification factor x100, Scale bar= 20µm.

4.4.7 Relative gene expression determination of SSC markers through qPCR

Two different methods were used for mRNA extraction (RNAeasy and Trizol). The samples from both methods were used in cDNA synthesis and PCR for amplification and gel electrophoresis was carried out to visualize the PCR product. Three SSC genes amplification was evaluated PLZF, BCL6B and UCHL (Fig. 4.37). There was no difference in band intensity between the expression of the genes in mRNA extracted using RNAeasy or Trizol methods; therefore, both templates were used in RT PCR. The expression of genes for markers associated with SSC in mammalian species SSC; PLZF, BCL6B, UCHL, ID4, THY 1 were found to be expressed in goat cultured germ cell clumps after 1 month as shown by the RT-PCR amplification plots Fig.4.38, Fig. 4.39 and Fig.4.40. Relative gene expression levels were determined using the $Ct = Ct_{\text{target}} - Ct_{\text{internal reference}}$. GAPDH was used as an internal reference gene in the experiment. All the 5 genes tested were expressed in the SSC colonies with THY I and BCL6B having more mRNA relative abundance (Fig 4.41).

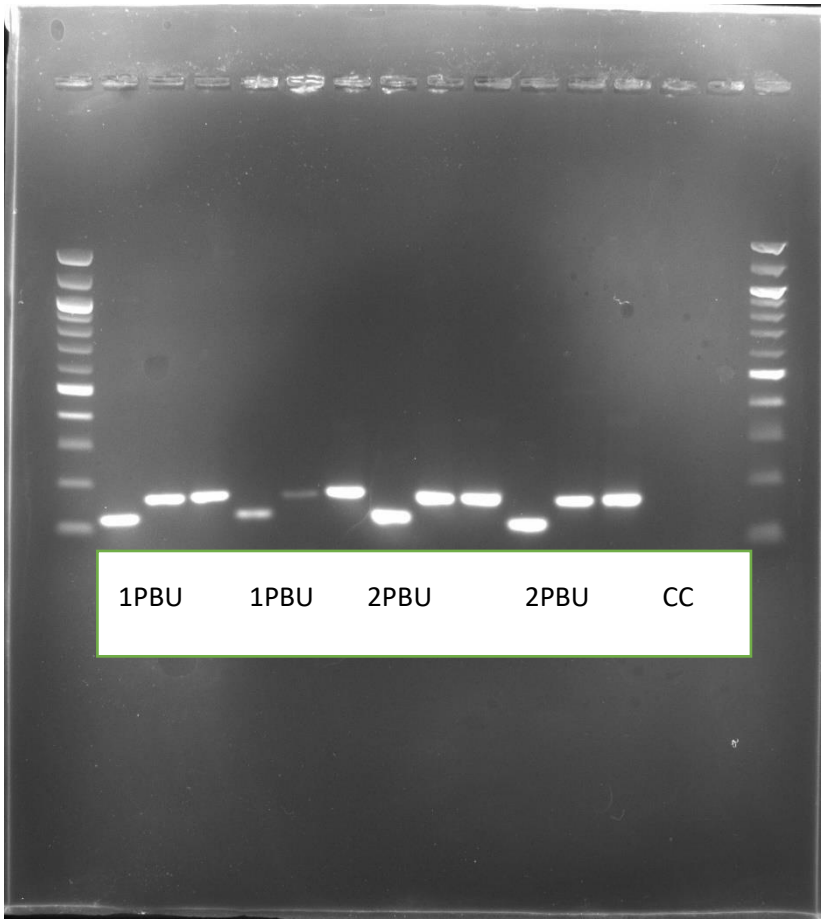


Figure 4. 37: PCR amplification for genes P(PLZF), (B)BCL6B and U(UCHL1) in SSC colonies on the 30th day of culture.

Key for Figure 4.37

The mRNA was extracted using Trizol reagent in samples (1) and RNAeasy minikit in samples (2). The negative controls did not amplify and is labelled as CC.

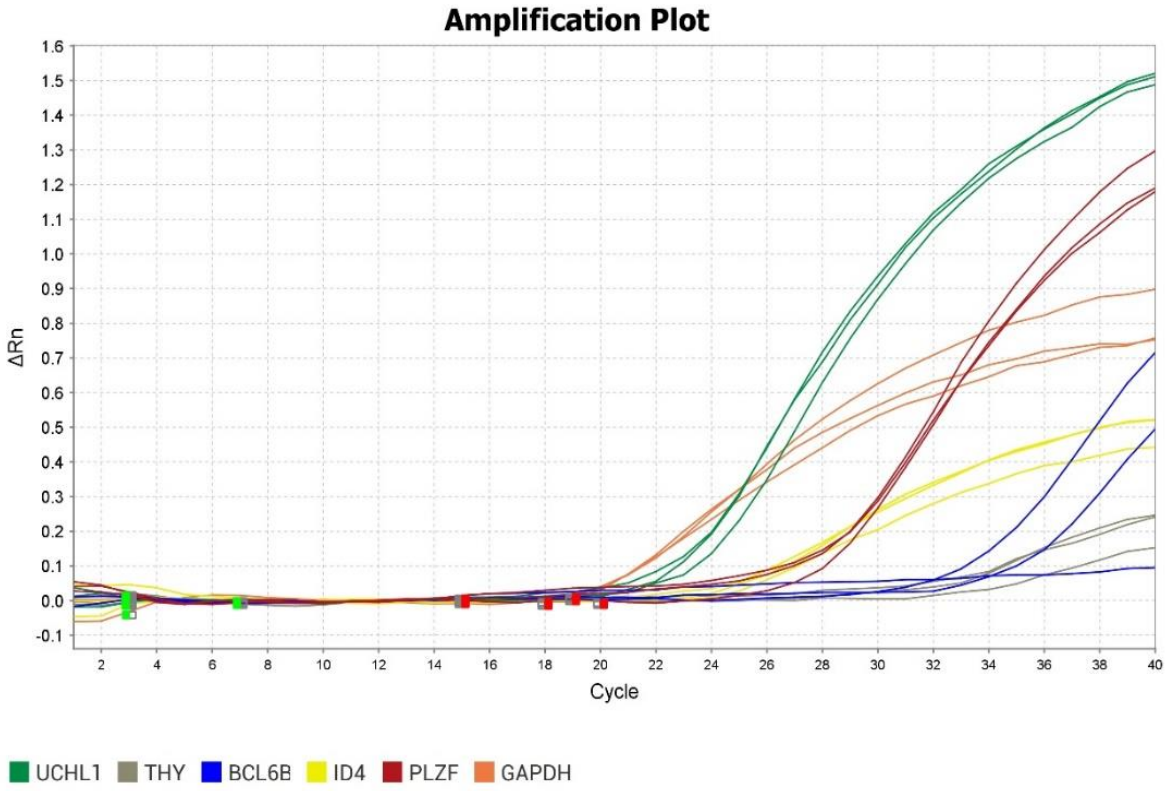


Figure 4. 38: RT PCR amplification plots for gene expression of associated markers and GAPDH (internal reference gene) in 1-month cultured spermatogonial stem cell colonies.

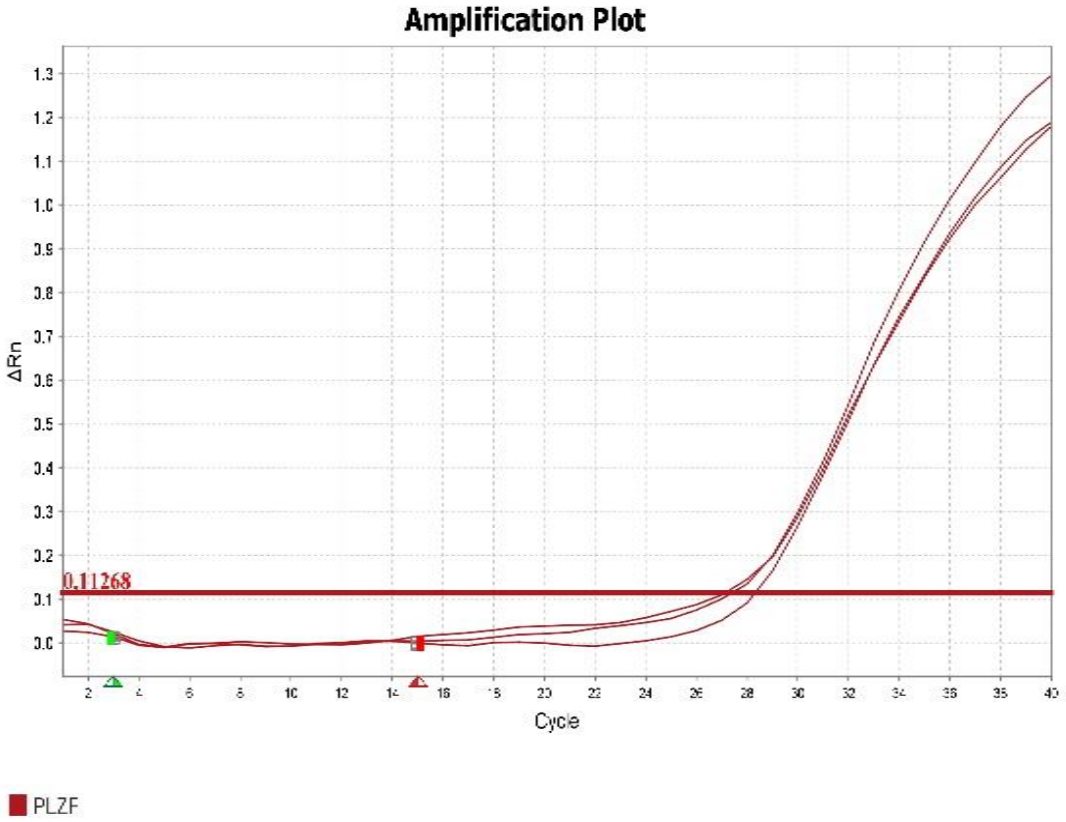


Figure 4.39: RT PCR amplification plots for gene expression of PLZF gene in 1 month cultured spermatogonial stem cells.

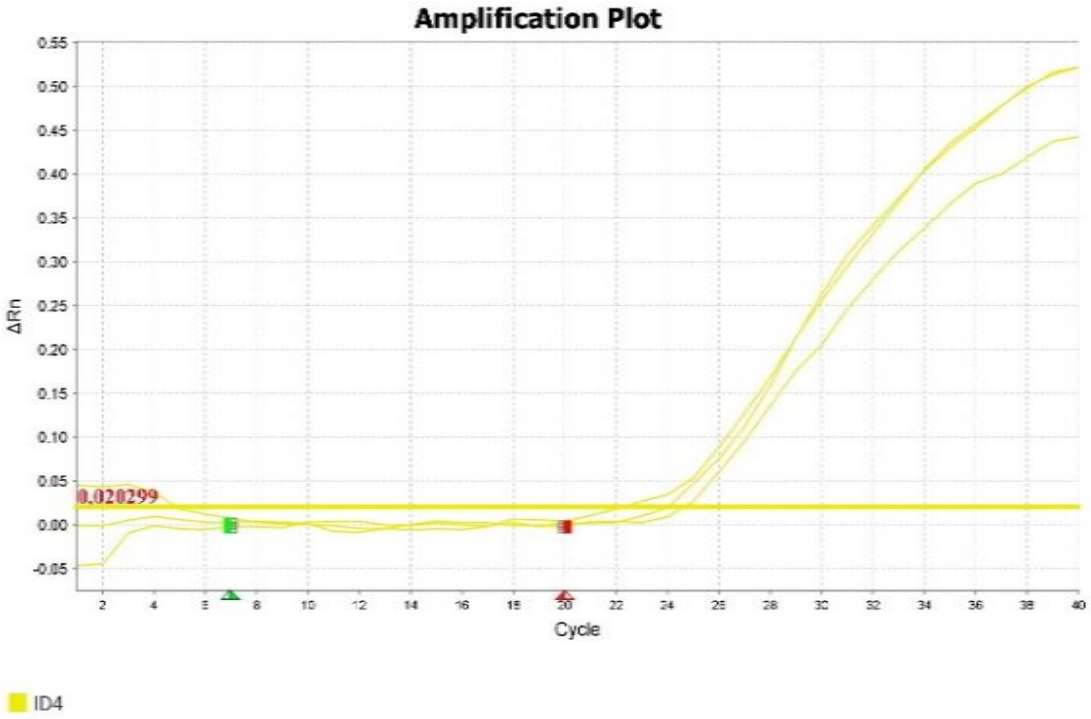


Figure 4.40: RT PCR amplification plots for gene expression of ID4 gene in 1 month cultured spermatogonial stem cell colonies.

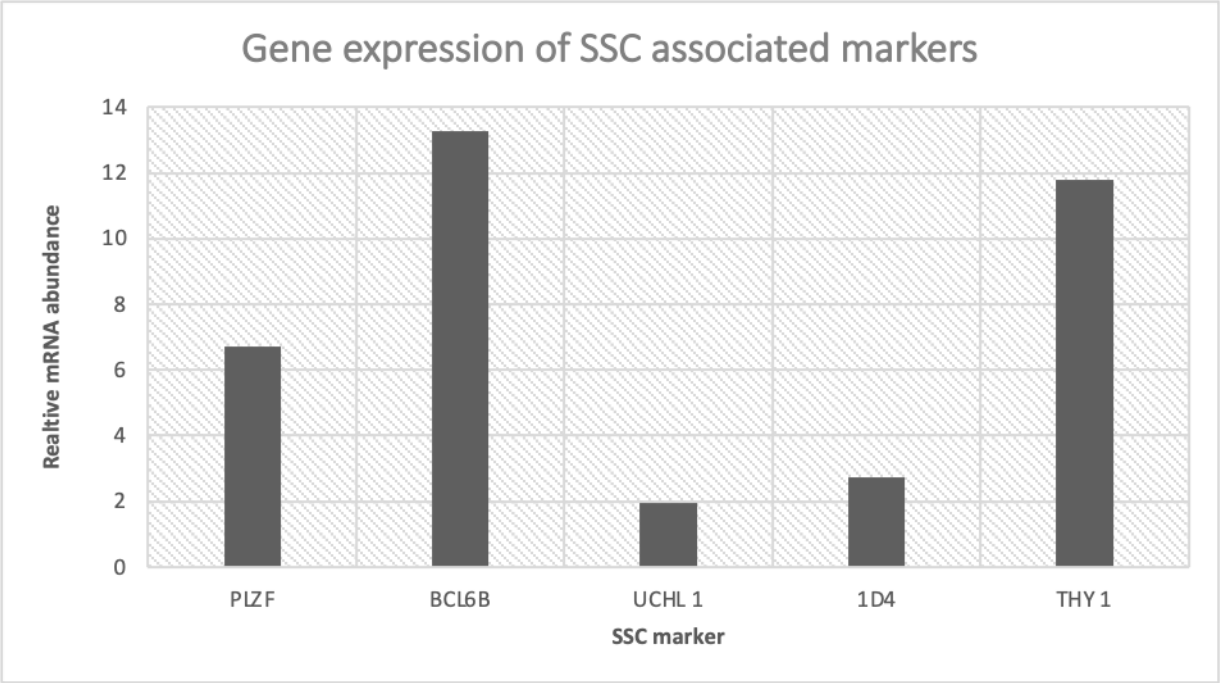
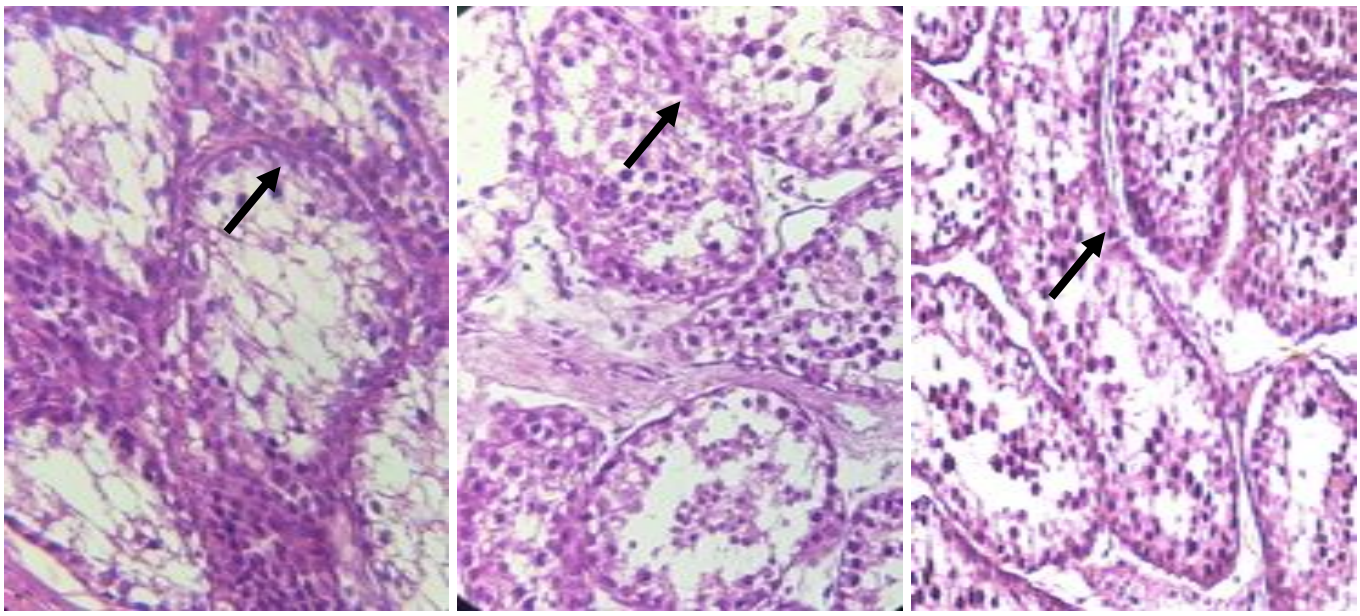


Figure 4. 41: Relative quantitative expression of spermatogonial stem cell markers and self-renewal related genes on the 30th day of goat spermatogonial stem cell culture.

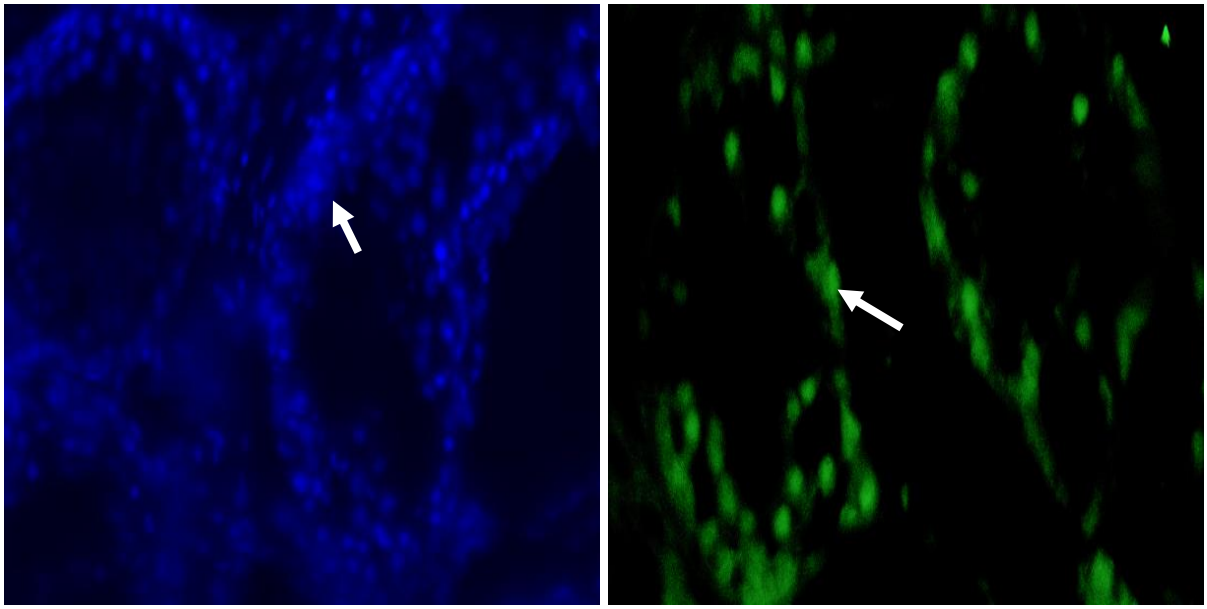
4.4.8 Histochemical analysis of testicular tissue

Histological sections of prepubertal goat testicular tissue stained with haematoxylin and eosin morphologically appeared as two layers of cells from the basement membrane to the tubules, presumably different stages of spermatogonia (Fig. 4.42 A, B, and C). PLZF staining was confined to the nuclei and cytoplasm of spermatogonia in prepubertal testis (Fig. 4.43 A and B) (Reding *et al*., 2010). In the prepubertal testis, PLZF-positive spermatogonia cells were randomly distributed in the central area of the tubule, mainly as single cells and occasionally as two or three aligned cells. The PLZF-stained cells were negative for somatic cell marker vimentin. About 58% (62/106) of the cells within 10 microscope slide views of the seminiferous tubules were PLZF positive.



A **B** **C**

Figure 4. 42: A, B and C -Histological cross-sections of the seminiferous tubules of different prepubertal bucks stained with Hematoxylin and Eosin with various sizes of spermatogonia (arrows) Magnification factor x100.



A **B**

Figure 4. 43: Images of cross-sectional seminiferous tubules stained for spermatogonial stem cell-marker. A -DAPI (blue) staining for a cross-section of seminiferous tubules (arrow). B -PLZF staining for cross-section of seminiferous tubules (arrow) Magnification factor x100.

4.4.9 Goat fetal Fibroblast cell culture results

4.4.9.1 Fibroblasts morphological characteristics

After 2-3 days of culture, fibroblast like cells were attached to the bottom of the flask (Fig. 4.44). On days 4-5, there was a confluent monolayer of cells with filamentous processes attached on the bottom of the flask (Fig.4.45 A and B). The cells had fibroblast-like characteristics; fusiform cell morphology with a central oval nucleus, turgor vitalis cytoplasm, and fibroblast-like filaments radiating from the cell nucleus (Fig.4.45 B).

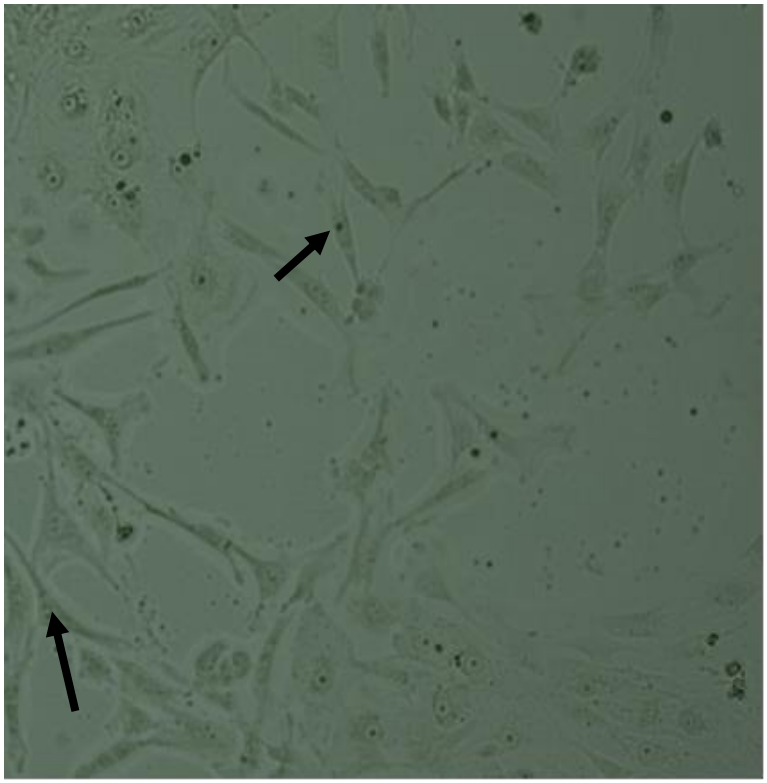
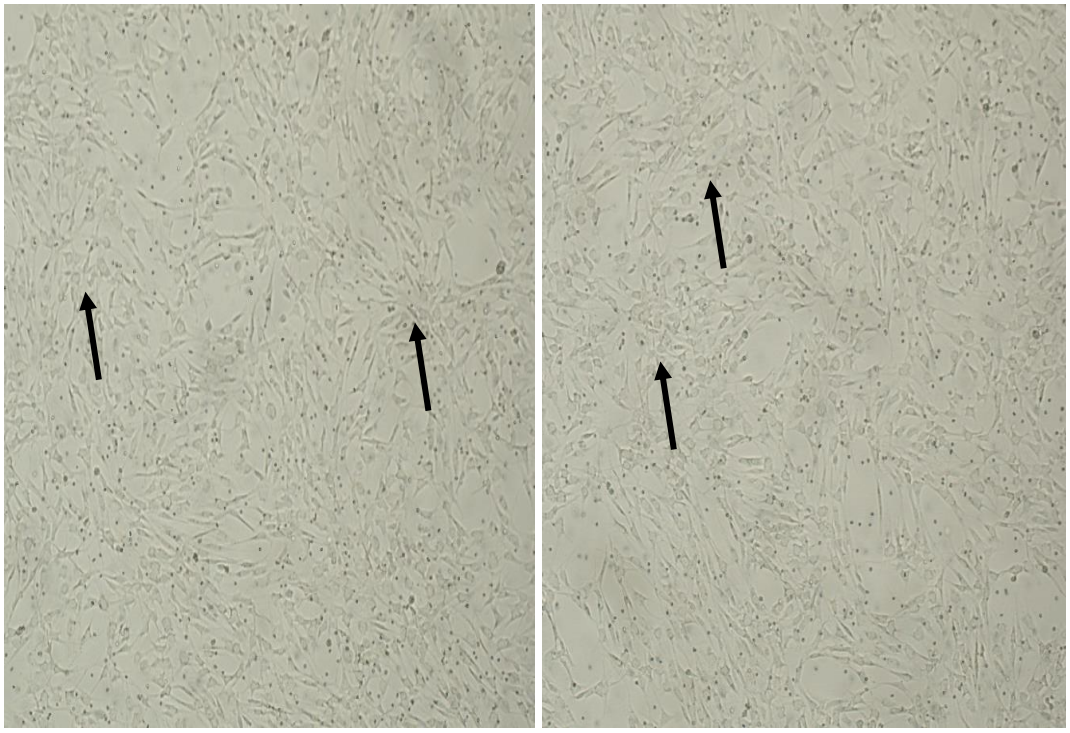


Figure 4. 44: Goat fibroblast cells after 2 days in culture following isolation (30-40% confluent) (arrow) Magnification factor x50.



A **B**

Figure 4. 45: A -90% confluent goat fibroblast cell culture. The filamentous cell processes from adjacent cells are in contact to form a monolayer of cells (arrows). B - Confluent goat fibroblast cell culture. The filamentous cell processes from adjacent cells are in contact to form a monolayer of cells(arrows) Magnification factor x250.

4.4.9.2 Goat foetal fibroblasts growth curve analysis and cell viability

When cells were counted on each passage and viability (trypan blue exclusion) determined by a cell counter machine, the viability was above 85% in all passages before freezing (Fig. 4.46). The fibroblast counts plotted after culture for 8 days, showed a typical S-shaped growth curve (Fig 4.47). The latent phase was 24 hours, which was due to the effect of trypsin. This was followed by an exponential growth phase for 6 days and then a stationary phase. There was no significant differences between the cell concentrations in each day ($p>0.01$).

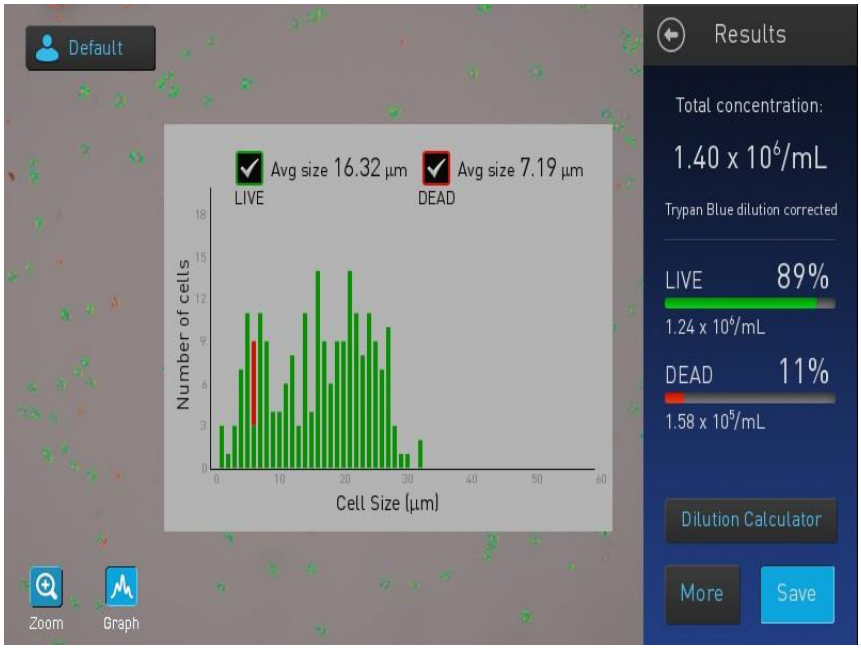


Figure 4. 46: Image of automatic cell counter results for male fibroblast cell line 3rd passage diluted in 30 ml GFF with a cell concentration of 1.40 X10^6 cell/ml *30 = 42X10^6 cells.

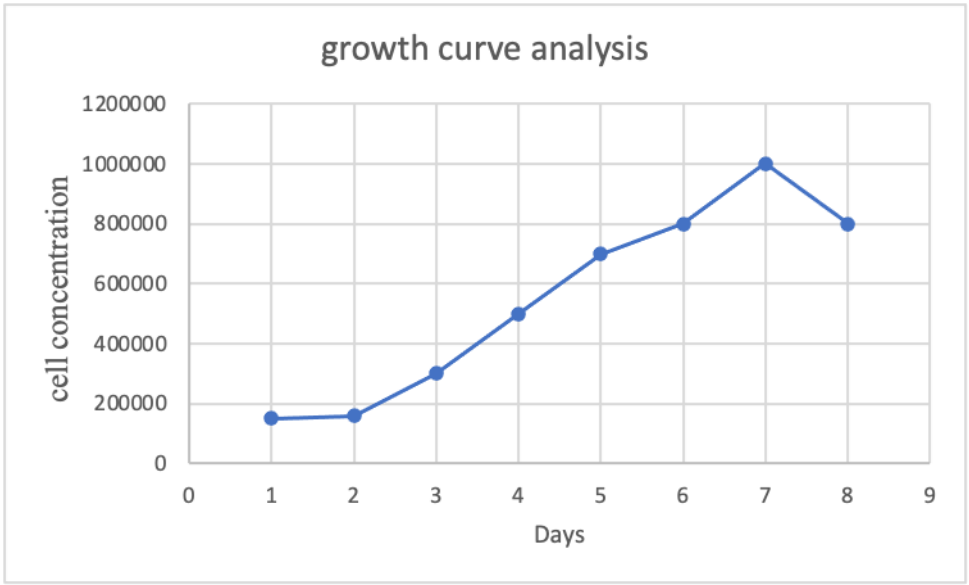


Figure 4. 47: Growth curve analysis of fibroblast cells from 4th passage.

4.5 Discussion

Reproductive biotechnologies have been used to disseminate desirable germplasm obtained through selective breeding in livestock. Superior germplasm has conventionally been spread by use of various breeding methods such as natural bull service, Artificial insemination (AI), combination of AI and bull service and to a limited extent multiple ovulation and embryo transfer (Murage and Ilatsia, 2011). In Sub-Saharan Africa, the uptake of reproductive technologies in livestock breeding has been slow owing to various reasons including financial constraints, farmer attitudes and poor infrastructure among others. These methods and technologies apart from natural breeding have hardly been adopted in the goat in Sub-Saharan Africa. In Africa, the goat is one of the main livestock kept by pastoralist communities in the arid and semi-arid lands (ASALs) where financial scarcity is rife and infrastructure that would enable reproductive technologies to be practiced in these lands is extremely poor or dilapidated. Hence the only methods and technologies that would attain some success in the goats in the ASALs are those that would promote transmission of superior genetics through natural breeding. Transfection and transplantation of spermatogonial stem cells (SSC) from donor bucks with superior traits to recipient bucks or surrogate sires would create an alternative method of disseminating desirable superior gametes from the donors. Prospects of success in long-term culture and propagation of Spermatogonial stem cells with possibilities of their transfection using superior donor genes and subsequent transplantation in recipient males that develop donor-derived spermatogenesis renders potential for reproductive improvement in goats and other livestock. Spermatogonial stem cell transplantation has been reported in goats over the last decade with

varying success of donor-derived spermatogenesis, which is highly dependent on the method used for germ line ablation ranging from use of germline intact males (Honaramooz *et al*., 2003) to irradiated males (Zeng *et al.*, 2012). Complete donor-derived spermatogenesis was achieved by using NANOS2 knockout males that did not have the endogenous germline layer and thus were not capable of their own spermatogenesis (Ciccarelli *et al*., 2020). Exploitation of the germ cell transplantation has been limited by several factors mainly the low population of SSC isolated from the testicular cell population and lack of standardized feeder-free and serum-free culture systems that promote goat SSC propagation. Adaption of these technologies in Sub-Saharan Africa necessitates the development of culture systems for expansion of SSC from indigenous goats before SSC transplantation can be considered as an alternative breeding technology.

From the documented low percentage of SSC in the heterogenous testicular cell population in mice (Oatley and Brinster, 2012), it is essential to have a culture medium that would enhance SSC component to boost their numbers while suppressing the other cellular components. The current study successfully isolated Galla goat SSC and for the first time developed a cocktail of serum-free feeder-free culture medium that enhanced and supported *in vitro* SSC culture for significantly longer period than previously reported. This was made possible by a multiparameter enrichment protocol essential for reducing somatic cells and other testicular cell types with subsequent development of a robust SSC culture. The multiparameter selection approach involved several washing steps during enzymatic digestion to remove interstitial cells. The isolated testicular cells were overlaid on a percoll density gradient in which upon centrifugation the population of spermatogonia pelleted at the bottom of the tube. Previously, percoll density centrifugation has been employed to successfully yield an enriched SSC component in the goat (Heidari *et al*., 2014), bovine (Oatley, 2010) and sheep

(Rodriguez-Sosa *et al.*, 2006). Various (20%, 28%, 30% and 32%) gradients of percoll in PBS have been used with good results (Heidari *et al.*, 2014; Oatley *et al.*, 2016). The purified testicular cell population attained in the current study as a result of using a 30% percoll density separation gradient, concurs with previous findings by Oatley et al. (2016) who first used the same percoll gradient and effectively purified undifferentiated spermatogonia. The subjection of percoll enriched cell fraction to a second enrichment protocol with differential plating on 0.1% gelatin-coated plate for 24 hours followed by cellular incubation, caused the somatic cell attachment to the bottom of the culture plate due to their anchorage dependence, which enabled the fast adherent velocity of the Sertoli and Leydig cells to be removed from cell suspension. This made it additionally easier to collect the floating spermatogonial germ cells from the culture as previously found (Park *et al.*, 2014; Oatley *et al.*, 2016) especially after incubating the testicular cells overnight in the culture medium at 37°C with 5% CO_2. The results of the study revealed that a combination of percoll density gradient with gelatin differential plating yielded an enriched population of SSC compared to a single purification through differential plating only. From the findings in the current study, enrichment only through differential plating did not sufficiently get rid of somatic cells and on PLZF immunostaining, the positive cells were fewer.

The serum-free medium (Stempro 34 SFM, StemPro nutrient supplement, BSA, additives and growth factors) used to successfully culture goat spermatogonial stem cells in the current study, was constituted by benchmarking on previous long-term serum-free SSC culture system previously established in rodents. These previous rodent SSC culture media had multiple components including: StemPro-34 SFM, with StemPro nutrient Supplement, insulin, saturated iron-transferrin, putrescine hydrochloride, sodium selenite, Stempro-BSA, L-glutamine, 2-mercaptoethanol, MEM Vitamin Solution, MEM non-essential amino acid

solution, penicillin-streptomycin, and Hepes buffer. For successful culture, the medium was supplemented by a combination of human forms of growth factors, which included bFGF, LIF, SDF, and GDNF (Kanatsu-Shinohara *et al*., 2011; Oatley *et al*., 2016). Apart from the goat, these media combinations were used as a benchmark for establishing serum-free SSC culture in other mammalian species for cattle and sheep with minor modifications on the components and concentration. The SSC cultures in the serum-free medium were morphologically similar to the ones described in other livestock species (Oatley *et al.*, 2016; Giassetti *et al*., 2019).

Similar reports have been published on the use of serum-free medium to culture goat SSC. One of these is a study by Sharma et al. (2020) in which putative SSC were cultured on a Sertoli cell feeder layer on Knockout DMEM medium supplemented with 10% KO-DMEM and 10% Knock Serum replacement Medium (KOSR). The author reported expression of PLZF SSC specific marker and other related pluripotent markers, the relative expression of ID4, THY1, BCL6B and UCHL1. In another study by Bahadorani et al. (2012) goat SSC were cultured short-term on feeder-free cultures with inclusion of different serum concentrations (1%, 5%, 10%), which reported better marker-expressing SSC colonies with serum concentrations of 1%, while higher concentrations (5%,10%,15% FBS) resulted in excess somatic cell proliferation. The current study further confirms that the use of serum in SSC culture inhibits self-renewal and causes the proliferation of somatic cells.

Unequivocally, the *in-vitro* culture of SSC on feeder cell layer and serum is likely to affect SSC self-renewal ability by enhancing their differentiation that is probably induced by undetermined components within the serum as well as promoting excessive somatic cells that

also suppress propagation and survival of germ cells (Kanatsu-Shinohara *et al.*, 2005 and 2011; Bahadorani *et al.*, 2012; Oatley *et al.*, 2016; Sharma *et al.*, 2020).

However, the use of feeder-free media to culture goat SSC has not been fully explored. This is necessary, for culture of SSC earmarked for transplantation into the testes of recipient males particularly due to the negative suppressive impact of feeders on colony-forming ability of SSC efficiency feeders as a result of unknown variable components that are difficult to standardize in the feeders (Oatley *et al.*, 2016; Sahare *et al.*, 2016). Nevertheless, somatic cells provide the required structural and chemical support to maintain SSC in undifferentiated state both *in vivo* and *in vitro* (Kanatsu-Shinohara *et al*., 2011), hence the use of feeder cell layers with Sertoli cells or fibroblasts in culturing of goat SSC previously (Pramod and Mitra, 2014; Sharma *et al.*, 2020). In the current study, development and culturing of isolated goat SSC on laminin-coated plates with the success of maintaining the cultured cells for 45 days, was based on the previous reports, which stated that stem cells have been shown to preferentially bind on laminin, an extracellular matrix coating successfully utilized in feeder-free culture of SSC in rodents, cattle and sheep respectively (Binsila *et al.*, 2020; Kanatsu-Shinohara *et al.*, 2005; Oatley *et al.*, 2016).

Feeder-free serum-free SSC cultures have been documented in cattle where SSC were cultured on laminin-coated plates in serum-free MEMα base medium with Stempro nutrient supplement and BSA combined with other additives having recombinant human forms of GDNF, FGF2, and LIF (Oatley *et al.*, 2016). In another study, cattle SSC were cultured in 15% KSR supplement on poly-L-lysine-coated plates for 2 months. The feeder-free serum-free cultured SSC in the current study formed germ-cell clumps/clusters on laminin-coated plates with germ-cell colonies increasing in size and numbers for 40 days but started to

decline thereafter. The germ cell clumps were similar in morphology and molecular characterization to the SSC clumps that were cultured on goat testicular somatic feeder cells in the current study. This was in concurrence with the findings when feeder-free and serum-free media were used in cattle (Oatley *et al*., 2016) and in sheep (Binsila *et al*., 2020). To further provide the feeder-free SSC with factors produced by the somatic cells surrounding the SSC stem cell niche *in vivo*, the Stempro culture medium was preconditioned by incubating on goat foetal fibroblast cells overnight. Followed by the use of this conditioned medium for SSC culture. The media preconditioning technique was adapted from Oatley et al. (2016). The author reported that preconditioning culture medium on foetal fibroblasts, drastically improved the survival and proliferation of SSC on feeder-free systems. In the current study, goat fibroblast cell lines were established from fetal tissues and proliferated to form a confluent monolayer. The proliferation rate and morphology of the cell lines was typical of fibroblast cells as reported previously (Mehrabani *et al*., 2016).

The expression of the germ-cell clumps through VASA staining marker and PLZF staining marker that indicates specificity for SSC was a clear proof of maintenance of undifferentiated status. This is comparable to what was reported in bovine where feeder-free SSC cultures were established on laminin-coated plates and maintained undifferentiated status verified through the expression of SSC-specific markers and genes (Oatley *et al*., 2016). Notwithstanding, in the current study goat SSC cultured on goat somatic cells feeder layer were maintained in culture for 60 days, which was longer than 45 days for feeder-free cultures. The findings confirm that feeder cells still provide essential chemical and structural support to the SSC. Therefore, there is still a need for more research to identify other factors that may be incorporated into feeder-free cultures to increase the longevity of expansion of

SSC. The extended periods of maintenance of SSC feeder cell layer in serum-free media has been reported (Oatley *et al.*, 2016; Suyatno *et al*., 2018).

The verified conserved marker for SSC that confirms their undifferentiated state is PLZF in cattle (Reding *et al*., 2010; Oatley *et al.*, 2016), and sheep (Binsila *et al*., 2020) and in goats (Bahadorani *et al.*, 2012; Pramod and Mitra, 2014). This evaluation of the successful SSC culture is done by morphological observation with stem cell markers through immunostaining of SSC, histological sections of seminiferous tubules or polymerase chain reaction (PCR). Various germ-cell markers including VASA, THY1 and PLZF have been used in the sheep, goats, cattle and rodents (Bahadorani *et al.*, 2012). In the current study, the colonies were evaluated for VASA, PLZF, NANOS2, and GFRα1 amongst other related SSC markers. Similar results were replicated in the current study with the use of immunochemistry, which revealed that majority of the germ cell clumps expressed PLZF SSC specific marker, but almost all the germ cell clumps expressed the germ cell marker VASA. The expression of genes for PLZF, BCL6B, UCHL, ID4, and THY 1 markers-associated with SSC in mammalian species was further confirmed by qPCR. The relative gene expression levels in the SSC cultures for one month were similar to what was reported for mRNA abundance expression in other studies *(*Binsila *et al*., 2018). The relative mRNA abundance of PLZF, and the SSC surface marker THY 1 in the current study, were higher than those reported in sheep SSC (Binsila *et al.*, 2020). The relative mRNA expression for SSC markers in the current study was determined on the 30th day of culture for SSC as follows: PLZF (6.7) ID 4 (2.7), BCL6B (13), UCHL1 (1.9), THY 1(11.79). These relative gene expression levels were comparable to gene expression levels previously reported for goat SSC on the 15th day of culture: PLZF (13) ID 4 (1.8), BCL6B (8), UCHL1 (2.5), THY 1(1.5) (Sharma *et al*., 2020).

These current findings indicate the formation of undifferentiated SSC clumps that express SSC markers as previously reported.

The morphological appearance of the cultured germ-cell clump colonies with cells loosely attached to one another in the current study, was similar to the one reported previously in the mice with an appearance of a bunch of grapes that is characteristic for undifferentiated spermatogonia (Kanatsu-Shinohara *et al*., 2005, 2014). This SSC morphological appearance is thought to be similar across mammalian species (Oatley *et al.*, 2016).

Development of long-term culture conditions for SSC is inevitable since this is the only way in which the small number of isolated SSC can be propagated to millions, which can be applied for the development of transgenic animals and utilized successfully in SSC transplantation using surrogate males. Comparatively, the rodents are much ahead of livestock species regarding standardized conditions for long-term maintenance of SSCs *in vitro*. The SSCs were maintained under growth factor defined serum-free feeder-free culture conditions for up to 6 weeks (4th passage). Previous long-term culture of goat SSC was 4 weeks on a Sertoli cell feeder layer (Sharma *et al*., 2020) and 2 weeks for SSCs cultured in serum-supplemented basal media (Heidari *et al*., 2012).

It was paramount in the current study to establish primary cultures of SSC using undifferentiated spermatogonia. This was made possible by isolating testicular cells from 3-6 months prepubertal goats whose majority of gonocytes at that age have transformed into undifferentiated Type A spermatogonia that possess regenerative capacity, which is the fundamental factor of SSCs (Curtis and Amann, 1981; Murta *et al*., 2010). Although

gonocytes and undifferentiated spermatogonia have similar biochemical characteristics, the success was facilitated by having donor goats with a higher population of undifferentiated spermatogonia than gonocytes. This is the reason for the *in vitro* culture that resulted in germ-cell clumps with a high number of undifferentiated spermatogonia evidenced by PLZF marker expressing cell clumps. Despite the morphological characteristics and marker identification being a promising finding towards standard conditions for long-term cultures of SSC in livestock species, the stem cell activity of the spermatogonial populations (ability to regenerate spermatogenesis) must be assessed by intra-testicular transplantation to recipients before conclusions are made on *in vitro* maintenance of SSC.

Research has reported transplantation of SSC using surrogate sires that are genetically sterile (not having endogenous germ cell layer, but have functional somatic cells) in goats, cattle and pigs with evidence of re-establishment of donor-derived spermatogenesis (Ciccarelli *et al.*, 2020). Further studies that include transplantation of the germ-cell clumps are pertinent to ascertain whether the cells maintained in culture using the conditions reported in the study, would have SSC that can regenerate spermatogenesis in the recipient. This will be possible once the technologies for generating ideal recipient males are established (Ciccarelli *et al.*, 2020).

In conclusion, this is the first documented report of the serum-free feeder-free culture of goat SSC as well as culture of livestock SSC in Africa. The *in vitro* culture conditions established in this study can be modified further to develop robust long-term culture conditions for goat spermatogonial stem cells.

CHAPTER FIVE

5.0 TRANSFECTION OF SPERMATOGONIAL STEM CELLS

5.1 Introduction

The introduction of foreign nucleic acids into cells to incorporate the new DNA sequences into the host cells genome is called transfection. Spermatogonial stem cells are a unique adult type of stem cells that transfer genetic materials from one generation to the next through the production of spermatozoa, which are the male gametes. Therefore, SSC are a perfect target for genetic manipulation to disseminate transgenic genes within animal populations (Miao, 2011). When the foreign DNA is incorporated into the animal genome, the sequence becomes more detectable in more cells and is transmitted to next generation to produce transgenic animals. The science of transfection can be applied to study the mechanisms for modulation and regulation of gene function, signaling mechanisms in spermatogenesis process and the production of transgenic animals with specific traits (Miao, 2011; Kim *et al.*, 2019a). The establishment of an efficient gene transfection system in livestock animal cells will be a great milestone towards investigating how gene transfer functions and reproduction of transgenic animals with improved production traits as well as the capability to produce pharmaceutical proteins (Niu and Liang, 2008). For stability of the transfection, the foreign nucleic acids are tagged with a marker gene for selection. The transgenes are integrated into the host genome and transferred to daughter cells during cell division. Production of transgenic animals through modification of the male germline and transplantation of transfected germline stem cells is gradually gaining momentum and may in the future be the most important application of *in vitro* cultured spermatogonial stem cells (Shirazi *et al.*, 2015). Spermatogonial stem cells are the precursor cells that produce spermatozoa, which are the male gametes that

transmit genetic information to the off-springs through fertilization of the female gametes. The SSC are capable of continuous self-renewal to produce daughter differentiating cells that proceed with spermatogenesis and maintain a reservoir pool of SSC for the future. Brinster (2002) postulated that in rodents one single SSC can produce 4096 spermatozoa. Intra-testicular transplantation of SSC carrying desired transgenes, will most likely result in production of transgenic spermatozoa if the SSC successfully re-establish spermatogenesis in the recipient testis (Zeng *et al*., 2013). The use of transgenic spermatozoa in natural mating or artificial insemination will be a faster way of disseminating the transgenes within the population (Tajik *et al.,* 2017). Transfection of genes into the germline shortens the generational interval required for the production of transgenic animals and the realization of genetic gains in genetic improvement programs (Niakan *et al.,* 2016). Additionally, when the transfected SSC proliferate and self-renew, the foreign gene is duplicated and retained for longer periods in the stem cells. This foreign gene may then be duplicated with the proliferation of SSCs and retained for long in stem cells. The unique characteristics of SSC for *in vitro* proliferation and the ability to re-establish spermatogenesis in recipient testes, make them a perfect target for gene manipulations.

Transfection techniques have been used for many decades for gene transfer into host cells by utilizing special carrier molecules that bypass the host cell membrane to deliver the foreign genes intracytoplasmic or through electric shock (electroporation) that creates small pores on the host cell membrane through which the genes are delivered into the cell. Viral vectors have been genetically engineered and used as vehicles to deliver foreign DNA into cells (Zeng *et al.,* 2013). These methods have varying shortcomings that vary from the techniques being too expensive for laboratories in low income countries, to some methods having low cell viability

as well as low/transfection efficiency (Whitelaw *et al*., 2008). Non-viral methods such as the use of liposomal or polycationic complexes carrier and electroporation are commonly used for the introduction of nucleic acids bound to plasmid vectors into mammalian cells (Niakan *et al.*, 2016).

The viruses used for viral-mediated gene transfer include retrovirus, lentiviruses and adeno/herpes virus. Transfection efficiency using viral vectors is high and it results in a stable transgene expression. The viral-DNA is integrated into the host genome and becomes part of the cell; integrated viral-DNA replicates as the host cells do. Lentivirus has been the most common viral vector used to deliver foreign nucleic acids such as the enhanced green fluorescent protein (eGFP), a reporter gene used in experimental transient expression of genes. The eGFP gene has been used as a marker in animal cells for gene expression. The eGFP is used as a genetic encoded fluorescent marker (Kumar, 2016). In transfection experiments, eGFP has been used in the optimization of the protocols. The SSC have been previously transfected using lentivirus as reported in bovine and porcine (Zeng *et al.*, 2013; Kim *et al.*, 2014b). In the goat and sheep SSC both lentivirus and adeno associated virus has been used to transfect eGFP into goat SSC (Abbasi *et al.*, 2015); sheep SSC (Rodriguez-Sosa *et al.,* 2009). Despite the success reports of efficient viral transduction, use of viral vectors have a risk of viral gene transcription and insertion of these genes into the host genome (Whitelaw *et al*., 2008). Therefore, if the transgenes are to be delivered to livestock species then optimization of transfection through non-viral methods is inevitable. Non-viral vectors, such as plasmids that can be transported into cells via liposomes or electroporation are available. These are gene carriers that can be used *in vivo* safely. The best targets for non-viral gene transfer are cells with potential for efficient proliferation and self-renewal such as embryonic and adult stem cells (Lai *et al*., 2008). Non-viral methods, use of liposome carriers

(lipofection), and use of electric charge (electroporation) have been used to transfer the foreign DNA into testicular cells and SSC in bovine, porcine and sheep (Niakan *et al.*, 2016; Tajik *et al.*, 2017; Kim *et al.*, 2019b). The use of liposome carriers is an easy technique and efficient gene transfer method both *in vivo* and *in vitro*. Lipofection utilizes the lipofectamine reagent made up of liposome carriers that is mixed into a tube with plasmid DNA to form a DNA/lipid complex. The complex is pipetted into the cells in culture and based on electrostatic interaction, the DNA is carried across the cell membrane into the cytoplasm. The DNA/lipid complex is put into the cell culture wells and the liposome will transport foreign DNA into the cells. However, the exact mechanism of action of liposome carriers is still unclear (Tajik *et al.*, 2017). The transfection efficiency of the plasmid DNA-liposome carrier depends on the mixing ratio of the two and the cell proliferation status (Garrett *et al*.,1999). Bovine SSC were successfully transfected through use of lipofectamine with a transgene uptake of > 37% (Tajik *et al.*, 2017). Gene knockout was also performed in goat SSC by using lipofectamine to deliver siRNAs targeting the EZH2 gene, where the knockout was confirmed through PCR (Cai *et al*., 2020). In another study, there was successful delivery of pPLZF-IRES2-EGFP or PLZF siRNA gene construct to study effect of overexpression of PLZF gene in SSC through liposome carriers (Song *et al.*, 2015). There is limited data on the transfection of foreign genes into goat SSC cytoplasm through use of liposomal carriers.

Electroporation using electric charges has been commonly used to transfect the slowly dividing stem cells. The cells are exposed to high intensity electric field pulses for a short period resulting in a disturbance in the membrane integrity, thus creating temporary pores on the cell membrane. Exogenous foreign DNA molecules diffuse into the cytoplasm or nucleus

through the pores formed (Niakan *et al*., 2016; Rae and Levis, 2002). The cell viability after electroporation is dependent on the choice of efficient electroporation parameters (voltage, duration, number, amplitude and length of pulses). The parameters cause changes in the cell membrane permeability. When they are too high for the cell type, the pores created on the cell membrane will be irreversible and would result in cell death. Optimal parameters will result in reversible cell-membrane permeability; hence the cell survive post-electroporation. The efficiency of this technique is largely dependent on selection of optimal electroporation parameters on the electroporator machine and not on the size and nature of a vector. Electroporation can be used to transfect a large number of cells within a short period (Gehl, 2003).

Successful reports of electroporation of sheep testicular cells (Niakan *et al.*, 2016) and porcine spermatogonial stem cells have been reported (Kim *et al.*, 2019b). Low cell recovery post electroporation have been reported (Kim *et al.*, 2019b). Transfection parameters vary from one cell type to another. Nucleofection an electroporation-based transfection method has been documented for the introduction of genes into goat undifferentiated spermatogonia (Zeng *et al*., 2012). Nucleofection allows the transfer of large DNA molecules into the cell nucleus. This technique also allows transfection of non-dividing cells, although low efficiency of transfection and cell recovery has been reported. There is limited data on goat SSC transfection parameters and efficiency using non-viral methods such as lipofection and electroporation. In the current study, the two non-viral methods were used to transfect eGFP gene into an enriched and proliferating SSC, and the transfection efficiency is documented.

5.2 Materials and methods

5.2.1 Lipofectamine transfection of Spermatogonial stem cells

Reagents required included: Lipofectamine® 2000 Reagent (Invitrogen: Catalog no: 11668-019) or Lipofectamine™, Stem Transfection Reagent (Invitrogen: Catalog no: STEM00015), Plasmid DNA (Nepa Gene plasmid DNA (pCMV-EGFP) (5μg/μl in a tube), DMEM (Dulbecco's Minimum Essential Medium) without antibiotics and Opti-MEM medium.

Gene transfer for enhanced green fluorescent protein (pCMV-EGFP) to the spermatogonial stem cells was evaluated using two lipofectamine reagents: Lipofectamine™ Stem Transfection Reagent and Lipofectamine® 2000 DNA Transfection Reagent. The concentrations and volume of the lipofectamine used were derived from the manufacturer's protocol of pluripotent cells and not specifically SSC. The protocol by Tajik et al. (2017) for lipofectamine transfection of bovine SSC was modified and applied in the study. The SSC were cultured up to day 4 when the germ cell clumps had formed and also the cells were highly proliferative at this stage as seen from SSC colony evaluation results in chapter 4. The DNA to liposome ratio and cell density were varied to identify parameters that yield a high transfection efficiency.

5.2.1.1 Experiment 1: Lipofectamine stem cell transfection reagent

For control wells, spermatogonial cells were co-cultured with DNA without electroporation or lipofectamine reagents. Spermatogonial stem cells were isolated from goat testes and cultured in serum-free culture medium for 4 days for the germ cell clumps to start forming in a 24-well plate with a concentration of 0.5-2 X10^5 cells/well or 96-well plate 20,000 cells

per well in 200µl of culture medium. A commercially available DNA plasmid with CMV promoter and Green Fluorescent Protein (GFP) was used. A day before transfection, the cells were plated at a concentration of 0.5-2 x 10^5 cells in 500 µl of serum-free growth medium without antibiotics. The SSC clumps were mechanically broken down through pipetting up and down. Prior to transfection, the cells were detached from the surface bottom by gentle pipetting and washed through by centrifugation 600xg 7 minutes at 4°C, then re-suspended in DMEM medium without antibiotics and serum and seeded on 24-or 96-well culture plates. Various lipofectamine concentrations and DNA were prepared as in Tables 5.1 and 5.2. DMEM medium was used to dilute both the lipofectamine reagent and DNA. The diluted lipofectamine was added to the diluted DNA in a sterile micro-tube and incubated for 5 min at 37°C. The lipofectamine-DNA complex was added to each well and mixed gently by rocking the plate back and forth. The cells were incubated at 37°C in a 5% CO_2 incubator for 48 hours prior to testing for transgene expression. The medium was changed after 24 hours by removing DMEM and adding serum-free culture medium. To determine gene transfer in SSCs, the cells were examined under an inverted fluorescent microscope (EVOS M5000, Thermo Fischer Scientific) using UV radiation of wavelengths 460-500 nm (blue filter). Successful gene transfer was manifested as green fluorescence.

5.2.1.1 Concentration of lipofectamine and DNA used in lipofection

Prior to transfection the SSC were counted and plated at concentrations of 0.5-2x 10^5 cells in 24-well plates or 1.1.5-1x10^4 cells per well in 96-well plates. All the experiments were done in quadruplets and samples pooled for fluorescence microscopy on cytosmears.

Table 5. 1: Experimental design for lipofection with Lipofectamine™ Stem Reagent.

Procedure	**Component**	**Test wells a 24-well plate**				
Dilute Lipofectamine™ stem reagent in DMEM medium	DMEM	**1**	**2**	**3**	**4**	**5**
		25µl×4	25µl×4	25µl×4	25µl×4	25µl×4
	Lipofectamine™ stem reagent	1 µl×4	2µl×4	3µl×4	4µl×4	5µl×4
Dilute DNA in DMEM medium	DNA	5ug×4	2.5ug×4	2.5ug×4	5ug×4	5ug×4
	DMEM	25µl×4	25µl×4	25µl×4	25µl×4	25µl×4
Add diluted DNA to diluted Lipofectamine and incubate for 10 minutes at room temperature						
Add DNA-lipid complex to cells	DNA-lipid complex per well	50µl	50 µl	50 µl	50 µl	50 µl
	Final DNA used per well	5ug	2.5ug	2.5ug	5ug	5ug
	Final lipofectamine per well	1 µl	2µl	3 µl	4µl	5µl

NB: For the 96-well plates the total volume of DNA-lipid complex was 10µl per well.

5.2.1.2 Experiment 2: Using Lipofectamine 2000 ™ Reagent

The second experiment was carried out using lipofectamine 2000 and the dilution volumes were as shown below in table 5.2.

Table 5. 2: Experimental design for lipofection with Lipofectamine™ 2000 Reagent.

Procedure	**Component**	**Test wells a 24 well plate**				
Dilute Lipofectamine™ 2000 reagent in DMEM medium	DMEM	**1**	**2**	**3**	**4**	**5**
		25µL×4	25µL×4	25µL×4	25µL×4	25µL×4
	Lipofectamine™2000 reagent	3 µL×4	4µL×4	4µL×4	5µL×4	5µL×4
Dilute DNA in DMEM medium	DNA	5ug×4	2 ug×4	2 ug×4	2.5ug×4	5ug×4
	DMEM	25µL×4	25µL×4	25µL×4	25µL×4	25µL×4
Add diluted DNA to diluted Lipofectamine and incubate for 5 minutes at room temperature						
Add DNA-lipid complex to the cell	DNA-lipid complex per well	50µL	50 µL	50 µL	50 µL	50µL
	Final DNA used per well	5ug	2 ug	2ug	2.5ug	5ug
	Final Lipofectamine per well	3 µL	4µL	4 µL	5µL	5µL

NB: 0.5-2 × 10^5cells per well

5.2.2 Electroporation of spermatogonial stem cells with eGFP

5.2.2.1 Materials and Reagents

1. Nepa 21 Electroporator machine with cuvettes 0.04mm gap.
2. Plasmid DNA (Nepa Gene plasmid DNA (pCMV- pCAGGS-EGFP) (5µg/µl in a tube).
3. Opti-MEM medium.

5.2.2.2 Procedure for electroporation

The 3–4-day cultured SSCs were harvested from the culture plate by gentle pipetting, followed by washing twice by centrifugation at 600g for 7 minutes. The cells were counted and re-suspended in ice-cold OPTI-MEM buffer without serum or antibiotic at a concentration of 1×10^6 cells/ in 98 ul. A concentration of 5µg of enhanced green fluorescent plasmid (pEGFP-N1, Clontech, Japan) was added to the tube that had cells and mixed gently without forming. The OPTI-MEM buffer with cells was pipetted into 0.4 mm gap electroporation cuvettes (Nepagene, Japan). The cuvette was inserted into the shocking chamber of an electroporator machine, and the electroporation parameters were set (Table 5.3). A guide of optimized parameters for electroporation of mouse embryonic stem cells was adapted as advised by the manufacturer of the electroporator. The start button was pressed and immediately after the burst, the cuvettes were placed at 4˚C for 3 minutes. After electroporation, the cells were immediately put in a pre-warmed culture medium on the plate and the cells were allowed to recover before evaluation 48 hours later. Forty-eight hours after electroporation, the cells were checked using a fluorescent microscope (EVOS M5000) with an excitation wavelength of 450-490 nm and emission wavelength of 515 nm, at a magnification of ×100 and ×400, to examine for gene expression. The cells were examined for eGFP gene expression as in lipofection reaction.

Table 5. 3: Electroporation parameters for spermatogonial stem cells.

Set Parameters												
Poring Pulse							**Transfer Pulse**					
#	voltage	Length (ms)	Interval (Ms)	No.	Decay rate	Polarity	voltage	Length (ms)	Interval (ms)	No.	Decay rate (%)	Polarity
1	*Control (with cells and DNA but no electroporation)*											
2	125	5	50	2	10	+	20	50	50	5	40	+/-
3	115	5	50	2	10	+	20	50	50	5	40	+/-
4	150	5	50	2	10	+	20	50	50	5	40	+/-
5	100	5	50	2	10	+	20	50	50	5	40	+/-
6	100	2.5	50	2	10	+	10	50	50	5	40	+/-

Key: ms- Milliseconds

5.2.3 Evaluation of eGFP expression

The eGFP positive cells as a percentage of total cells counted in the microscope field were detected using a fluorescent microscope (EVOS M5000 Thermo Fisher scientific) 48 hours after transfection. The cells were harvested by gently pipetting and re-suspending in ice-cold antibiotic- and ca/mg-free PBS. Cells were counted and attached on coated slides through cytospinning at a concentration of 30×10^4 cells per slide. Cells were counted according to their green fluorescence of eGFP gene. In addition, the mean fluorescent intensity of SSC was evaluated by examination of at least 10 microscope fields for quantitative expression of eGFP gene. Cell images were analyzed by Celleste 5.0 image analysis software.

5.3 Results

5.3.1 pCMV- eGFP gene transfer to the spermatogonial stem cell colonies

All the spermatogonial stem cells isolated through multiparameter selection had ≥80% viability rate prior to transfection. Observation of green fluorescence in the transfected cultures on a fluorescent microscope was an indicator eGFP gene transfer to the spermatogonial colonies (Fig. 5.1 A and B, Fig. 5.3 A and B). In the current study, there was a significant difference ($P<0.05$) between SSC colonies incubated with DNA plasmid alone without liposome carriers (0.6% GFP expression) and those incubated with liposome carriers and eGFP plasmid (18-25% GFP expression) (Table 5.4, 5.5 and Fig.5.2). There was a correlation between the concentration of lipofectamine reagent and transfection efficiency, whereby low volumes of lipofectamine such as 2µl resulted in low a transfection rate even when the concentration of DNA was above 3µg (Table 5.4 and 5.5). In addition, using concentrations of more than 5µg DNA also resulted in low transfection efficiency. When a

volume of 4µl was used for both reagents with a concentration of 2-2.5µg DNA, Stem Cell Lipofectamine reagent had 25.25% of SSC colonies expressing eGFP gene, while for Lipofectamine 2000, 22.25% of the colonies manifested presence of eGFP. The transfection efficiency between the two reagents was not significantly different ($p>0.05$). Viability of the cells averaged 55%±0.21 in all the cultures that were transfected through lipofectamine carrier molecules, which was lower than the initial 80% cell viability (Tables 5.4 and 5.5). Concentrations above 5µg or volumes above 5µl were avoided in the study to avert cytotoxicity from foreign constructs as cautioned in the manufacturer's protocol.

It was possible to transfer eGFP and transiently express this gene in goat SSC using specific electroporation parameters (Table 5.6). Green fluorescence on fluorescent microscopy of the electroporated cultures represented eGFP gene transfer to the spermatogonial colonies (Fig. 5.3A and B). The most and least level eGFP expression of SSC cells was detected in 100 V/2.5 milliseconds (15%±0.54) and 150 V/5milliseconds groups (2±0.20%) respectively (Table 5.6). Lowering the voltage to 100V reduced the overall percentage of cell death and increased the percentage of eGFP expressing colonies from 2%±0.20 to 15%±0.54. Use of high electroporation voltage of 150V resulted in the lowest cell viability, the culture had a lot of cell debris and dead cells (Fig.5.4).

Lipofectamine transfection had a higher percentage of SSC colonies expressing eGFP gene than electroporation (Fig. 5.5). Hence lipofectamine was more effective in eGFP gene transfer into SSC than electroporation. The cell viability was higher in lipofectamine transfection with a mean of 55%±0.21 than in electroporation which had a mean of 38%±0.14.

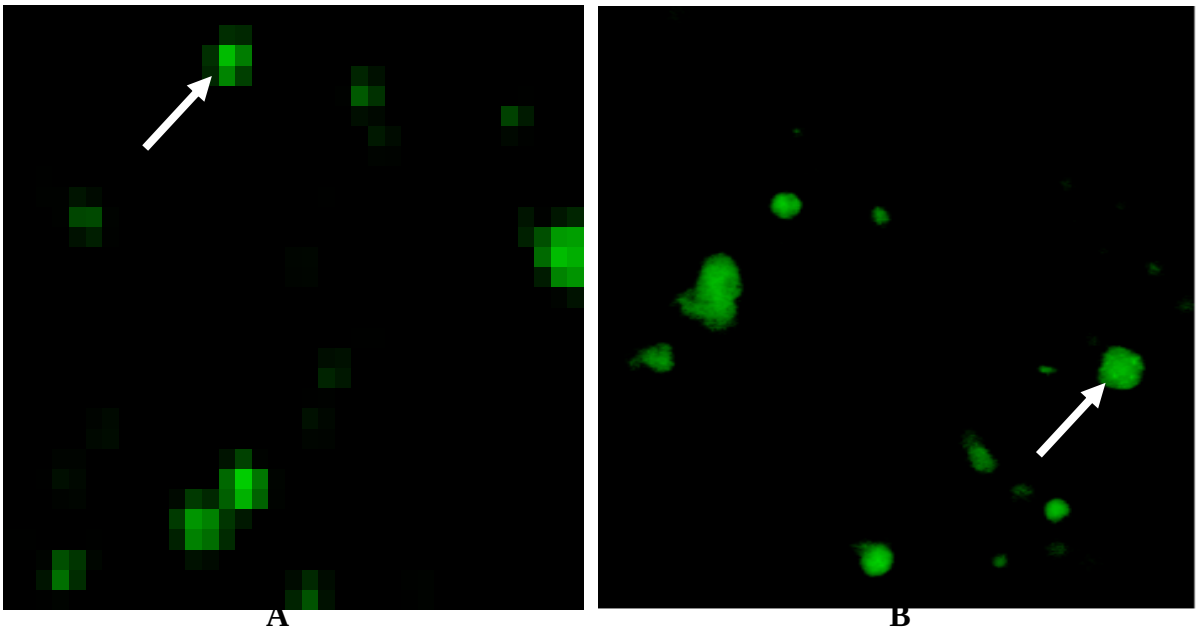


Figure 5. 1: A -Green fluorescence indicates eGFP gene transfer to the spermatogonial stem cells using lipofectamine stem reagent liposome carrier (arrows). B -Green fluorescence indicates eGFP gene transfer to the spermatogonial stem cells using lipofectamine 2000 reagent liposome carrier (arrow) (Magnification x100).

Table 5. 4: Summary of lipofectamine transfection results using Lipofectamine stem ™ reagent.

Experiment	Lipofectamine stem ™ Reagent volume	DNA plasmid	Percentage (%) colonies expressing eGFP after 48 hours	Percentage (%) cell viability after electroporation
Expt 1	1ul	5ug	5.50±0.52	52
Expt 2	2 µL	2.5ug	13.75±0.88	55
Expt 3	3 µL	5ug	18.0±0.24	60
Expt 4	4 µL	2.5ug	25.25±0.85	52
Control	0	5 ug	0.6±0.12	72

Table 5. 5: Summary of lipofectamine transfection results using Lipofectamine 2000 ™ Reagent.

Experiment	Lipofectamine 2000™ Reagent volume	Concentration of DNA plasmid	Percentage (%) colonies expressing eGFP after 48 hours	Percentage (%) cell viability after transfection
Expt 1	3 µL	5µg	4.50±0.32	60
Expt 2	4 µL	2µg	22.25±1.73	55
Expt 3	4 µL	2µg	20.50±0.77	48
Expt 4	5 µL	3µg	11.75±0.67	60
Expt 5	5 µL	5µg	4.75±0.24	50
Control	0	5µg	0	78

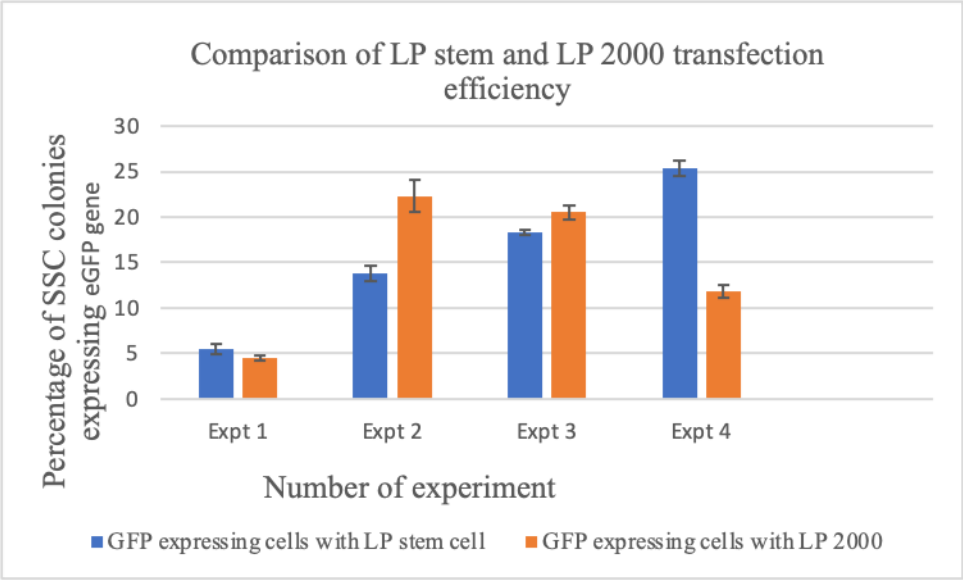
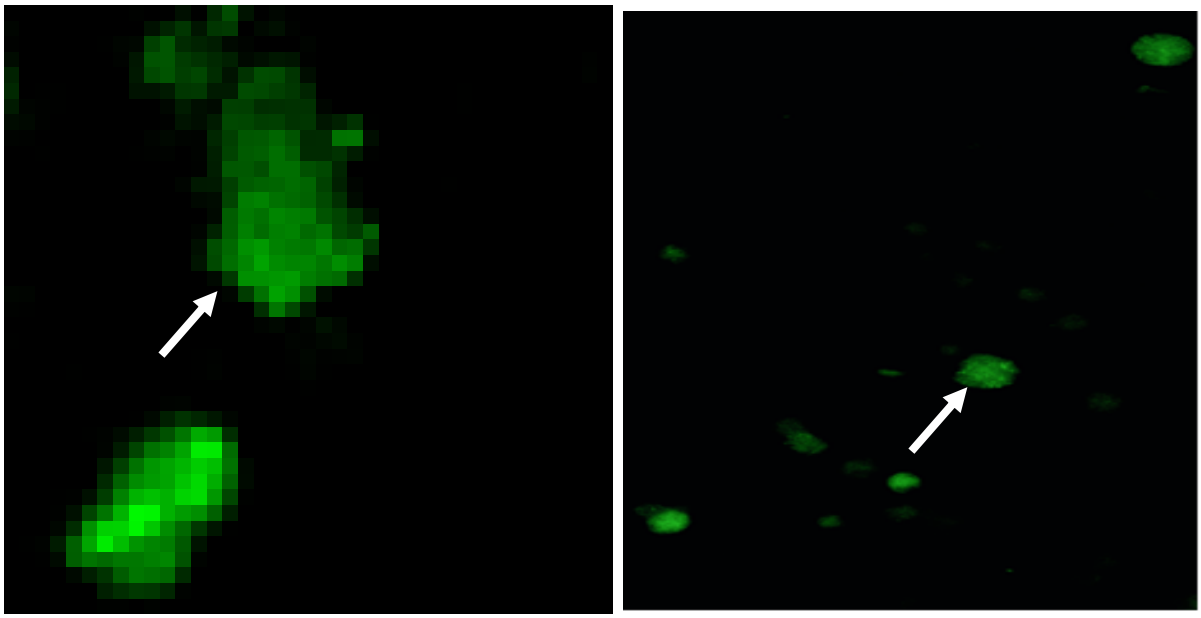


Figure 5. 2: Percentage of eGFP-expressing spermatogonial stem cell colonies following lipofection with Lipofectamine™ stem reagent (LP stem) and with Lipofectamine™2000 reagent (LP 2000).



A **B**

Figure 5.3: A and B Green fluorescence indicating eGFP gene transfer to the spermatogonial cells using following electroporation (arrow) (Magnification x100).

Table 5. 6: Percentage of Spermatogonial stem cell colonies expressing eGFP gene after electroporation.

Set Parameters															
	Poring Pulse						Transfer Pulse								
#	Voltage	Length (ms)	Interval (ms)	Number	Decay Rate (%)	Polarity	Voltage	Length (ms)	Interval (ms)	Number	Decay Rate (%)	Polarity	kΩ	% eGFP expressing SSC colonies	% Cell viability after electroporation
1	*Control (with cells and DNA but no electroporation)*														83
2	125	5	50	2	10	+	20	50	50	5	40	+/-	0.030	3.25±0.31	38
3	115	5	50	2	10	+	20	50	50	5	40	+/-	0.030	4.25±0.24	30
4	150	5	50	2	10	+	20	50	50	5	40	+/-	0.035	2±0.20	25
5	100	5	50	2	10	+	20	50	50	5	40	+/-	0.035	11.25±0.74	45
6	100	2.5	50	2	10	+	10	50	50	5	40	+/-	0.037	15±0.54	51

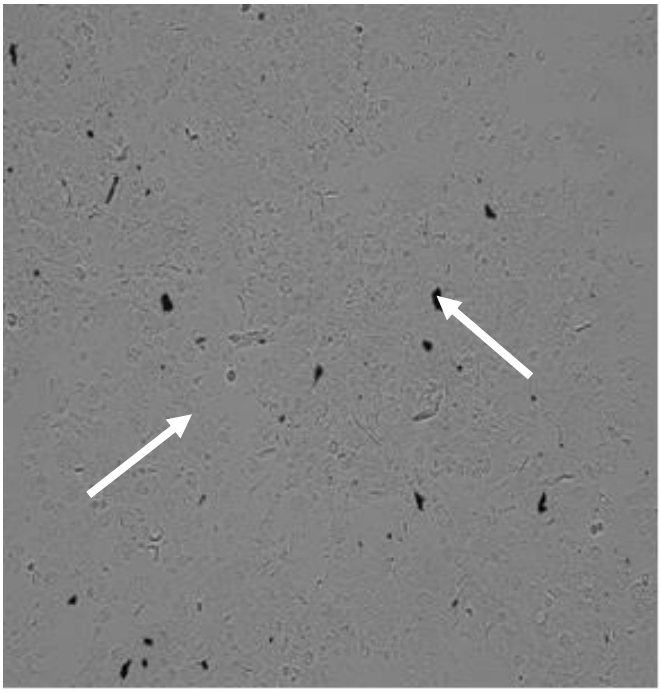


Figure 5. 4: Cell debris and dead cells (arrows) following electroporation with high voltage (x50).

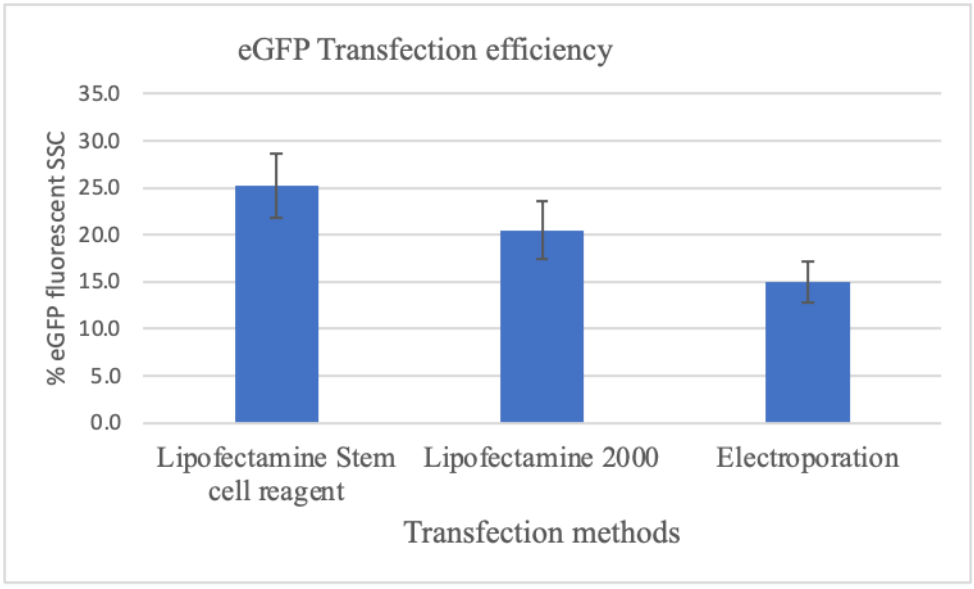


Figure 5.5: Comparison of Spermatogonial stem cell transfection efficiency between using lipofectamine and electroporation.

5.4 Discussion

Genetic manipulation of cells is commonly done through transfection. Transfection using viral vectors, lipid carrier molecules and electroporation have been experimentally done on testicular cells in an attempt to optimize and increase transfection efficiency (Zeng *et al.*, 2013; Niakan *et al.,* 2016; Tajik *et al.*, 2017; Kim *et al.,* 2019b). Safety concerns have been raised in transfection of livestock cells using viral vectors, hence non-viral methods such as electroporation and lipofection have been poised as alternatives especially when the transfected cells are aimed for transplanting into live recipients (Whitelaw *et al*., 2008). In the current study, foreign DNA (pCMV-eGFP) was introduced into the cytosol of enriched and cultured goat SSC through the use of the recommended electroporation and lipofectamine reagents, followed by evaluation of transfection efficiency for both as proof of their effectiveness. The two lipofectamine transfection reagents used were Lipofectamine® 2000 DNA and Lipofectamine™ Stem Reagent, Invitrogen as suitable carrier molecules to transfer foreign eGFP gene to the SSCs. The spermatogonial stem cells were transfected when at the peak logarithmic growth phase, which was shown in a previous report to be the most appropriate stage for foreign gene uptake (Tajik *et al.,* 2017). The negligible (0.6%) uptake of eGFP plasmid in one of the SSC cultures incubated with only eGFP without lipid carrier, was similar to previous findings in which 1.6% uptake of eGFP plasmid by SSC was observed when transfection was done on day 4 of culture without the use of the lipofectamine 2000 reagent, despite the SSCs being in the peak logarithmic growth phase (Tajik *et al*., 2017). Similar findings have also shown a lack of uptake of DNA in rodent spermatozoa when lipid carriers were not used (Yonezawa *et al*., 2002). The findings of the current study further confirm that use of liposomal carriers facilitates the movement of foreign genes across the cell membranes of the host cell as well as incorporation into the host cell genome.

Although the use of Lipofectamine™ Stem Reagent carrier resulted in a higher percentage of SSC colonies expressing eGFP gene than Lipofectamine™ 2000, the difference in transfection efficiency was not statistically significant. The Lipofectamine™ Stem Reagent is specifically optimized for lipofection of stem cells and in this study, it was expected that the transfection efficiency using this reagent would be significantly higher than lipofectamine 2000. However, this was not the case in the current study. The lipofectamine to DNA ratio contributed immensely to the eGFP uptake and viability of the SSCs, but the transfection efficiency was lower than the 37% ± 6.5 reported in bovine SSC with the use of Lipofectamine™ 2000 carrier (Tajik *et al.*, 2017). Comparatively, Low lipofection transfection efficiency of< 1.5% and survival rates of < 80.0% were reported with porcine SSC (Kim *et al.*, 2019b). The weakening of the SSC membrane resulting from chemical stimulation may contribute to the low survival rates. The high transfection efficiency found when using a DNA concentration of 2.5ug with 4ul Lipofectamine™ 2000 and 2 ug of DNA with 4ul Lipofectamine™ Stem Reagent liposome carriers, was similar to lipofection of bovine SSC with 2ug of DNA and 4 ul of Lipofectamine™ 2000 that was reported to produce transfection efficiency of 37%% ± 6.5 (Tajik *et al.*, 2017).

Electroporation has been reported to be an efficient non-viral transfection method used for introduction of foreign genes into various cell types including stem cells (Cukjati *et al.*, 2007; Niakan *et al.*, 2016). The efficiency of electroporation can be modified through adjustment of electrical stimulation parameters mainly: voltage, length of the pulse application, and electric pulse frequency (Guo *et al*., 2012). Electric stimulation causes the formation of small pores on the cell membrane to microseconds of cell polarization, that allow large gene constructs and molecules to enter the cell cytosol through simple diffusion. The efficiency of

electroporation is lower than the one obtained by use of lipofectamine or the use of viral vectors, because of irreversible damage to the cell membranes that occurs in some cells (Kim *et al.,* 2019b). However, the simplicity buffer free usage and the easy use of electroporation necessitates optimization of its parameters for electroporator machines such as NEPAGENE electroporator which is easy to use and does not require use of buffer solutions.

The current study purposed to optimize electroporation parameters for transfection of foreign genes in spermatogonial stem cells, in this case, the pCMV-eGFP gene using NEPAGE electroporator for the first time. The highest transfection efficiency of 15% with a viability of 50% achieved after electroporation of $1x10^6$ spermatogonial stem cells with 5 µg DNA of eGFP plasmid, in the current study, was consistent with the previously reported findings that obtained a transfection efficiency of 25.3% ± 2.4% with a cell viability rate of 78.5% after electroporation of 1×10^6 goat testicular cells with 2 µg DNA using PLUS BTX® electroporation cuvette and electroporator, USA and 2 µg of pEGFP-N1 plasmid (Niakan *et al.*, 2016). Similarly, transfection efficiency > 7.5% and survival rates > 80% for porcine spermatogonial stem cells using a Gene Pulser electroporator Cuvette (Bio-Rad, Richmond, CA) was previously reported (Kim *et al.*, 2019b).

Another consistent finding was reported in cock spermatogonial stem cells in which 10^6 cells were efficiently electroporated with 1-1.6 µg of plasmid (Trefil, 2014). The total voltage and the length of the pulse were the most important parameters for modification in electroporation, which was similar to the current study that varied these parameters and obtained varying results, but a voltage of 100V applied for 2.5 milliseconds gave the highest electroporation efficiency. These results revealed that high voltages applied for long periods resulted in cell death, which was similar to the findings of Niakan et al. (2016). Cell viability is an important parameter when selecting gene delivery methods.

Similar results were reported in electroporation of mouse SSC with a transfection positivity of 20.3% with 8.7% viability rate (Kanatsu-shinohara *et al*., 2005). Conversely, some studies have reported very high viability of SSC at > 80% following electroporation but a lower transfection efficiency of 7.5% (Kim *et al.*, 2019b). Another study obtained 29.37% of positive cells with a 69.86% viability rate after electroporation transfection of cock SSC (Yu *et al.*, 2010). In the current study, the viability of cells drastically decreased with electroporation from 80% to 25%, with some eGFP expressing cells that survived showing unusual morphological changes in the shape of the cell membrane and nuclei fragmentation suggesting apoptosis concurred with previous findings after electroporation of sheep testicular cells (Niakan *et al.*, 2016). The author suggested that apoptosis was caused by irreversible changes that occur in membrane permeability due the electroporation process, causing the inflow of ions and molecules into the cells and leading to cell bursting. A similar suggestion was made that electroporation induced cell death in hematopoietic stem cells, attributing the death to the uptake of ions through the pores created on the cell membrane (Li *et al.*, 2001).

The promoter used with the eGFP plasmid has also been shown to contribute to the transfection efficiency (Zeng, 2003). However, high eGFP expression has been reported by using cytomegalovirus (CMV) promoter in GFP gene delivery which was used in the current study (Niakan *et al.*, 2016). Despite the promising results for successful electroporation of goat SSC and the possibility of utilization of the technology for production of transgenic goat SSC for their transplantation, the electroporation parameters used did not result in high transfection efficiency. Therefore, it is important to evaluate the use of transfection enhancing reagents such as dimethyl sulfoxide and alkyl methyl sulfoxide in the buffer solution and further manipulate the poring pulse and transfer pulse parameters for improved reversible cell

membrane permeability**.** Additionally, more optimization is required for electroporation since there are no published reports on SSC transfection using the NEPAGENE super electroporator that has several parameters to be set and optimized.

For decades, several researchers have invested in the development of transgenic animals with specifically targeted traits using different cell types such as in somatic cell nuclear transfer. This notwithstanding, large-scale production of transgenic livestock has been limited by the low efficiency of production of transgenic offspring and non-random integration of the transgene. Multiplication of these transgenic founder populations is a challenge. With culture systems of SSC in livestock having been optimized, these cells can be used for genetic modification and transplanted into the surrogate males for production of transgenic semen. Additionally, lipofectamine and electroporation gene transfer systems may be optimized further to make them favorable for the delivery of transgenes into SSC.

In conclusion, transfection of goat SSC with eGFP expression was accomplished through lipofection and electroporation. The parameters used in the study will play a role in the establishment of a highly efficient transfection system for goat SSC. However, more research needs to be done to increase the transfection efficiency, while maintaining cell viability and the undifferentiated status of the stem cells.

CHAPTER SIX

6.0 INTRA-RETE TESTIS SPERMATOGONIAL STEM CELL TRANSPLANTATION IN PREPUBERTAL MALES

6.1 Introduction

Spermatogonial stem cells (SSC) fit into stem cell category if they are capable of re-establishing spermatogenesis when introduced into a compatible live animal testis. Therefore, the existence of the SSC population within a culture of testicular cells can satisfactorily be confirmed through transplantation and colonization of the seminiferous tubules (Izadyar *et al.*, 2003; Honaramooz, *et al.*, 2003). The SSC transplantation technique and successful colonization of donor SSC in the recipient seminiferous tubules were first developed in mice (Brinster and Avarbock, 1994). The transplantation technique created a way of evaluating the maintenance of stem-cell capability of SSC within *in vitro* culture conditions.

Successful colonization of the seminiferous tubules by donor SSC requires the following: selection of prepubertal donor from which the testicular cells will be obtained, and the donor cells must be labelled with a marker such as green fluorescent protein (eGFP) to enable identification of donor-derived spermatozoa or SSC colonies (Oatley and Brinster, 2006). Furthermore, a key aspect of successful SSC transplantation is the use of recipient males lacking an endogenous germline layer but having functional somatic cells within the seminiferous tubules. The endogenous germline line layer that harbors spermatogonia stem cells must be eliminated for donor SSCs to successfully access the empty SSC stem cell niches. If this is not done effectively, donor SSCs will be largely blocked from reaching the basement membrane by endogenous germ cells occupying stem cell niches (Izadyar *et al.*,

2003). As discussed in chapter 3, the elimination of endogenous the germline layer can be accomplished through testicular irradiation or treatment with chemotherapeutic drug busulfan or gene editing to knock out the NANOS2 gene that regulates survival and development of the germline layer in mammals.

Transplantation of SSC into germline intact recipients will result in low or no levels of donor DNA in the recipient's semen due to competition between the endogenous and exogenous SSC. Despite this shortcoming, this is one of the ways of confirmation that the cells in a culture dish are capable of colonization of the basement membrane in the seminiferous tubules. The use of intact germline recipients necessitates that the transplanted cells are marked with fluorescent markers (eGFP) or dyes that are used to detect the presence of fluorescent cells in the recipient's seminiferous tubules or semen for a period of time. As documented in the systematic review in chapter three, several authors carried out preliminary studies to evaluate the methodology of transplantation and donor SSC colonization efficiency. Testicular irradiation destroys endogenous germ cells but is associated with other side effects especially bone marrow suppression, which could easily lead to the death of recipients (Herrid *et al.,* 2009; Stockwell *et al.,* 2013). Treatment with the chemotherapeutic drug busulfan temporarily ablates the germline layer at lower doses, hence the germline layer regenerates gradually after a few months. At higher doses, busulfan causes systemic effects followed by death (Mikkola *et al.,* 2006). Endogenous spermatogenesis was found to regenerate after 30 weeks following busulfan treatment (Zeng *et al.,* 2013; Kim *et al.,* 2014b). Recently, Dolichos Biflorus agglutinin (DBA) treatment was used to ablate the germline in camels prior to transplantation and donor-derived DNA was detected in the ejaculates of recipients following transplantation of the testicular cells (Herrid *et al*., 2019).

However, these methods are reversible and the host endogenous germ cell layer regenerates, thus ceasing the donor-derived spermatogenesis. Researchers have come up with genetically modified surrogate pigs and goats, which do not have a germ cell layer due to knocking out the NANOS2 gene that controls their development (Park *et al*., 2017; Ciccarelli *et al.*, 2020). In the current study, germline intact bucks were used for the transplantation of an enriched population of spermatogonial stem cells. Similar studies exist where transplantation with donor-cell colonization of the recipient's epithelium was done experimentally in goats (Honaramooz *et al.*, 2003; Kaul *et al*., 2010) and sheep (Rodriguez-Sosa *et al.*, 2009). The current study evaluated *in vivo* transfer of eGFP transfected SSC into prepubertal bucks through intra-rete testis injection and the persistence of eGFP positive cells traced through fluorescent microscopy following castration of the recipient testes.

6.2 Materials and methods

6.2.1 Ultrasound guided transplantation of spermatogonial stem cells suspension in intact testes of goats

6.2.1.1 Materials and reagents

The materials and reagents used in this process included: eGFP plasmid, DMEM media, FBS, trypan blue stain, an intravenous catheter(20-gauge × 1-1/4″; Surflo; Terumo Medical Co., Elkton, MD), intravenous infusion set, Lidocaine Hydrochloride, 10 ml syringes. Prepubertal (6-7 months) recipient bucks, SSC, and B-mode ultrasound scanner (REF.: 23500/1005).

6.2.1.2 Donor testis cell preparation

Enriched spermatogonial stem cells were collected after overnight plating and washed by centrifugation at 600xg for 7 min at 4°C. The floating cells, which were the SSC-rich portion

were counted and assessed for viability by trypan blue exclusion. The cells were subjected to lipofectamine transfection in 6-well plates (15µL lipofectamine stem reagent in 150µL of DMEM and 14µg of DNA in 150µl DMEM). The concentration of the lipofectamine and eGFP used was extrapolated from the results obtained in Chapter 5 table 5.4. A cell population of 20 $x10^6$ cells were used for transplantation into each of the recipient testis (n=5). Other cells that were cultured for 4 days prior to lipofection were used for transplantation. After 24-hour overnight transfection, the cells were used for transplantation. The culture plates were removed from the incubator and cells recovered by gently pipetting and DMEM media was refreshed. The cell suspension was kept on ice until transplantation within 2 hours. The eGFP was used to allow for the identification of donor-injected cells in the recipient testis during evaluation for colonization (Rodriguez-Sosa *et al.*, 2009; Zeng *et al.*, 2013).

6.2.2 Ultrasound guided transplantation of germ cells in the seminiferous tubules of recipient goats

The transplantation protocol was derived with minor modifications from (Honaramooz *et al.*, 2003; Kaul *et al.*, 2010)

6.2.2.1 *Ex vivo* germ cell transfer

Abattoir-derived goat testes (n=40) were collected and transported to the laboratory. Two different techniques of injection were tested; extra rete testis injection (Rodriguez-Sosa *et al.*, 2009) and intra mediastinum testis injection (Kaul *et al.*, 2010). For the intra rete testis injection, an intravenous catheter (20-gauge × 1-1/4″; Surflo; Terumo Medical Co., Elkton, MD) was inserted through the cauda epididymis and testicular parenchyma into the rete testis (Fig. 6.1). The position of the catheter was monitored by ultrasound, and its position was

adjusted to ensure correct positioning in the centre of the mediastinum testis. The catheter was inserted, followed by removing the steel needle and a syringe containing the dye solution connected to the catheter. In the pre-pubertal goats, the mediastinum containing rete testis is axial and centrally located and was visualized by ultrasound scanning. After the injection, testes were bisected, and the distribution pattern of the dye was recorded through a digital camera (Fig. 6.3). Below is a diagram of the catheter position and injection point as described by (Honaramooz *et al.*, 2003).

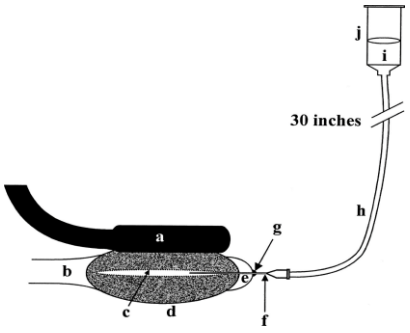


Figure 6. 1: Introduction of donor cell suspension into a recipient testis.

Key to Figure 6.1: a Ultrasound transducer; **b**, spermatic cord; **c**, rete testis; **d**, testis; e, cauda epididymis; **f**, catheter; **g**, tissue glue; **h**, infusion tubing; **i**, cell suspension; **j**, reservoir

For the extra rete testis injection: the head of the epididymis was identified and an incision to expose the head was done. The head of the epididymis was partially separated from the tunica albuginea by blunt dissection, and injection into the extra-testicular rete testis was done. A 20G intravenous catheter was placed about 2-3 centimeters into the extra-testicular rete and 5 ml of trypan blue was injected slowly using a 10ml syringe connected to the catheter.

6.2.2.2 *In vivo* SSC transfer

The ultrasound scan-guided SSC intra rete testis injection technique used to introduce trypan blue dye in the abattoir-derived goat testis was extrapolated *in vivo* for pre-pubertal germline

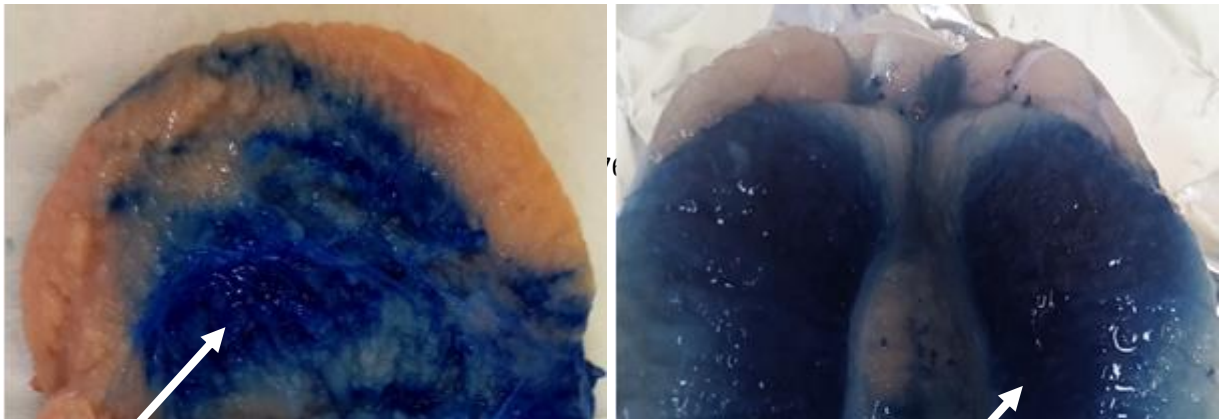
intact goats that served as recipients for donor cells. The extra rete testis was invasive and produced less filling of seminiferous tubules. It was therefore discontinued. Five prepubertal indigenous goats aged 7 months were examined for any lesions on the scrotum and scrotal diameter was determined. The animals were physically restrained in the field-based conditions and local anesthesia using 1ml of lidocaine hydrochloride around the spermatic cord and scrotal skin was administered. The prepared testicular cells were injected into the left testis only and the right testis served as a control for each buck. The whole procedure was ultrasound- guided using a 7.5 MHz linear transducer, attached to a B-mode scanner allowing high resolution (Minitube). The position of the mediastinum was scanned through longitudinal and transverse scans due to its higher echogenicity. A 20G catheter was inserted through the cauda epididymis into the rete testis and directed gradually to ensure the positioning of the needle in the centre of the rete testis. When the catheter was in position, the stylet was removed and a syringe containing 5ml cell suspension was connected to the catheter. The cell solution was gently released into the rete testis through hydrostatic pressure. The change in echogenicity of the rete testis was seen to change from white to black as the fluid flowed in. A total of $20x10^6$ cells in 5 ml solution cells were injected into each testis of the recipients. One week after transplantation, the recipients were castrated, and the pair of testes were collected. To examine for the presence of fluorescent-labeled cells, unstained histological cross-sections were prepared (described in section chapter 4.2.1.5.), and squash smears were prepared on slides for examination under fluorescent microscope. Testicular tissue samples from both the control testicles and transplanted testicles were fixed in 10% formalin overnight and processed for histology. Images of dispersed seminiferous tubules from squash preparations and histological sections were documented by using a fluorescent microscope (EVOS M5000) and an Inverted light microscope (Zeiss AXIO 1).

Five histological cross-sections from each of the testis were evaluated microscopically for evidence of tissue damage and inflammatory response. Scoring of lesions according to Rodriguez-Sosa et al. (2009), was as follows: (i) Normal testes- no apparent abnormality seen; (ii) mild damage- presence of vacuolation in some of the Sertoli cells and loss of germ cells, (iii) moderate- when vacuolation was present throughout the Sertoli cell layer and few germ cells remained and (iv) severe- when there was vacuolation and maximal attenuation of the Sertoli layer. The damage from each testicular cross-section was calculated and expressed as a percentage.

6.3 Results

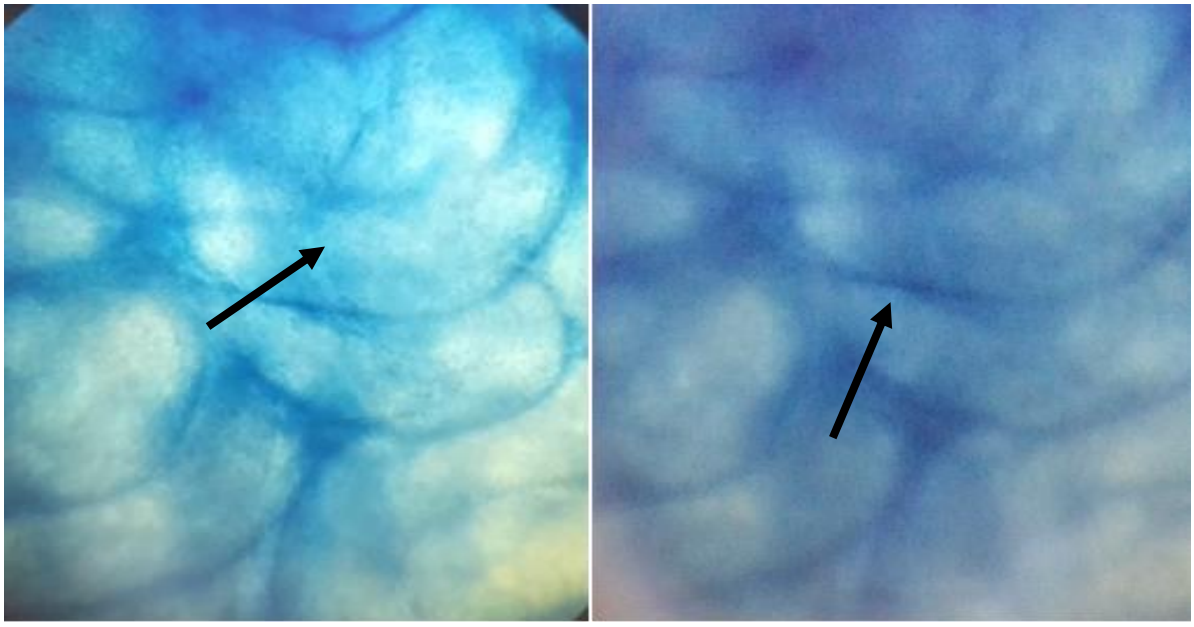
6.3.1 Intra rete testis injection of SSC suspension

The ultrasound guided injection into the mediastinum rete produced diffuse filling and distribution of the dye within the seminiferous tubules in 30/40 (75%) goat testes (Fig 6.2A and B). Ultrasound-guided injection of 5ml of trypan blue dye into the mediastinum rete testis resulted in filling of the seminiferous tubules (Fig. 6.3 A and B). Injection into extra rete testis involved deflection of the head of epididymis from the parietal tunica (Fig 6.4) and resulted in the distribution of the dye only in a small area of the tubules (Fig 6.5), but the procedure was invasive with the likelihood of developing adhesions. The intra-rete testis technique was adopted as the procedure for *in vivo* transplantation. The mediastinum testis was identified through ultrasonography (Fig.6.6) and the needle location was confirmed to be inside the mediastinum testis before the cell suspension was released into the testis (Fig.6.7).



A **B**

Figure 6. 2: **A** and **B**-Trypan blue spread widely within the tubules as a result of Intra rete testis injection (arrow).



A **B**

Figure 6. 3: A and B -Light microscopy image of seminiferous tubules with trypan blue staining following Intra mediastinum rete testis injection (arrow).

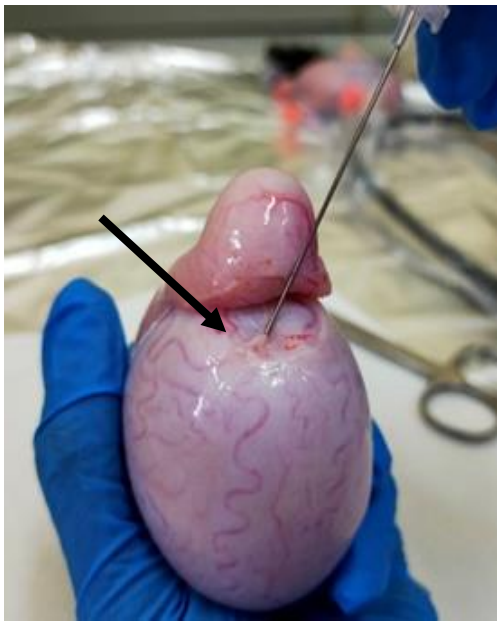


Figure 6. 4: Deflection of the head of epididymis and injection into extra rete testis in the ex vivo injection of trypan blue dye (arrow).

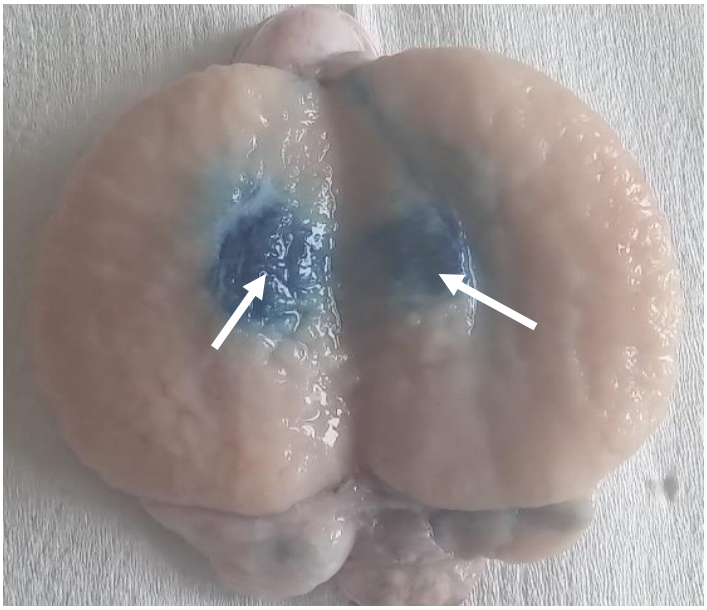


Figure 6. 5: Small area of distribution of trypan blue following extra rete injection in the ex vivo trypan blue injection (arrow).

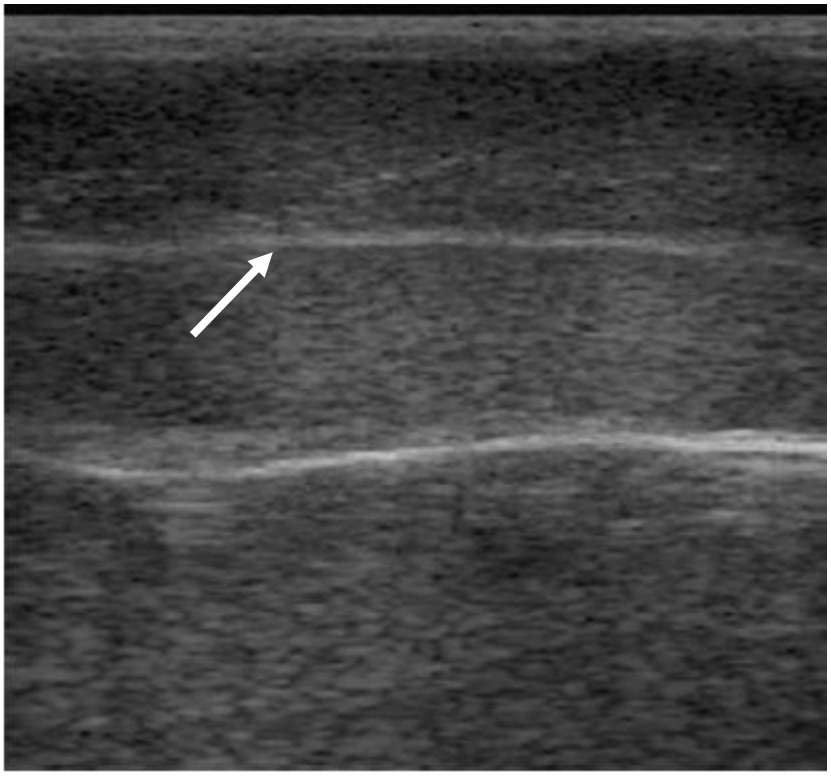


Figure 6. 6: Ultrasonography of the goat mediastinum testis (arrow)

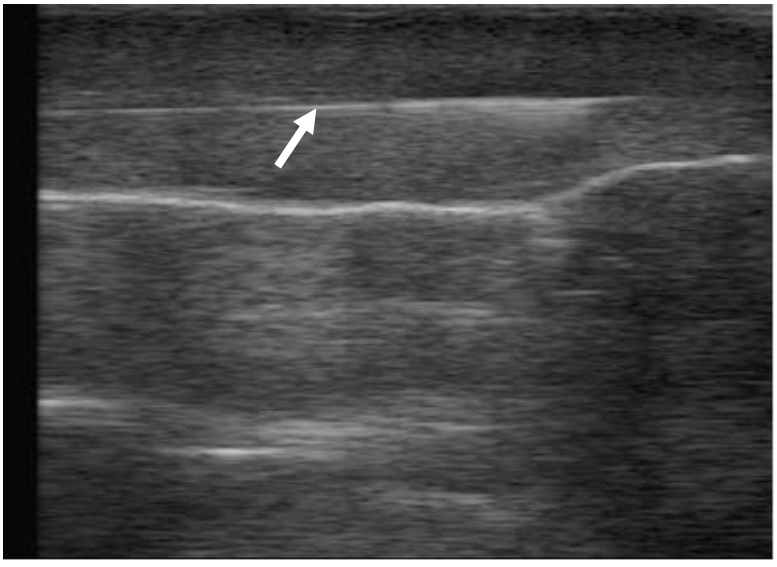


Figure 6. 7: The needle (hyperechoic) inside the mediastinum testes (arrow).

6.3.2 Gross pathological findings

The recipient goats were apparently healthy with no signs of inflammation or infection and without detectable local, systemic immunologic signs or any other reactions r 7 days post-transplantation. The animals had normal body temperature and were feeding well (Fig 6.8). All the testes manipulated were found to move freely in the scrotum, there was no adhesion between the scrotal skin and the parietal tunica vaginalis at the injection site or cauda epididymis**.** One week after transplantation, there was no difference in the scrotal diameter between the injected testis and the control**.** The testicular parenchyma appeared normal on macroscopic examination (Fig. 6.9). The tissue damage from histological cross-sections of the seminiferous tubules of recipient testes was categorized as mild, moderate, or severe. There was little to no difference between the recipient and the control testes in three of the animals, with the seminiferous tubules having normal histological structure (Fig. 6.10A and B). In 2 goats, there were a few tubules with occasional epithelial vacuolization on histological cross-sections (Fig 6.11A and B). The lesions were scored according to Rodriguez-Sosa et al. (2009). There was no testis whose seminiferous tubules had any severe inflammatory lesions.

Figure 6. 8: Healthy-looking recipients after transplantation with spermatogonial stem cells.

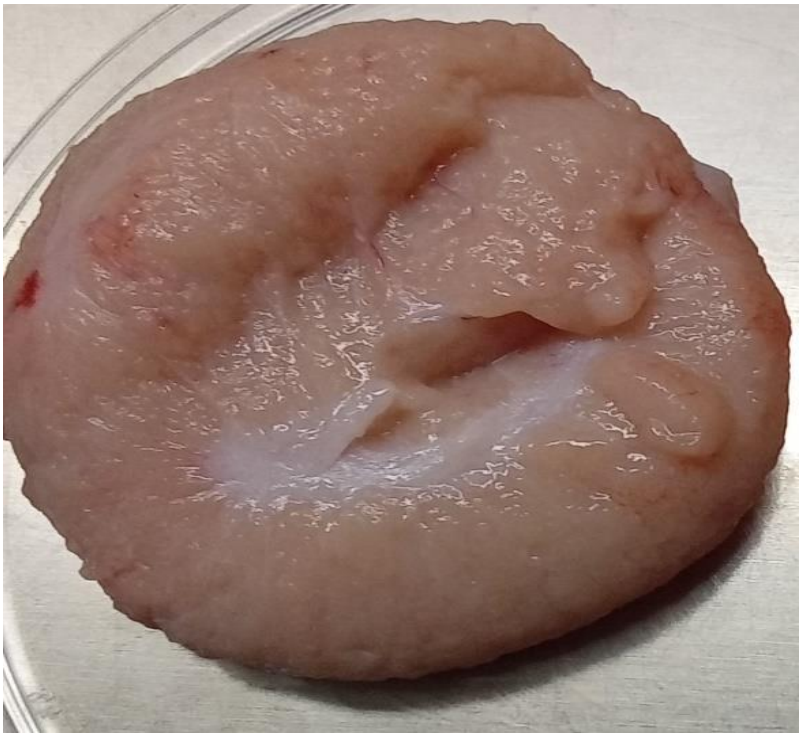
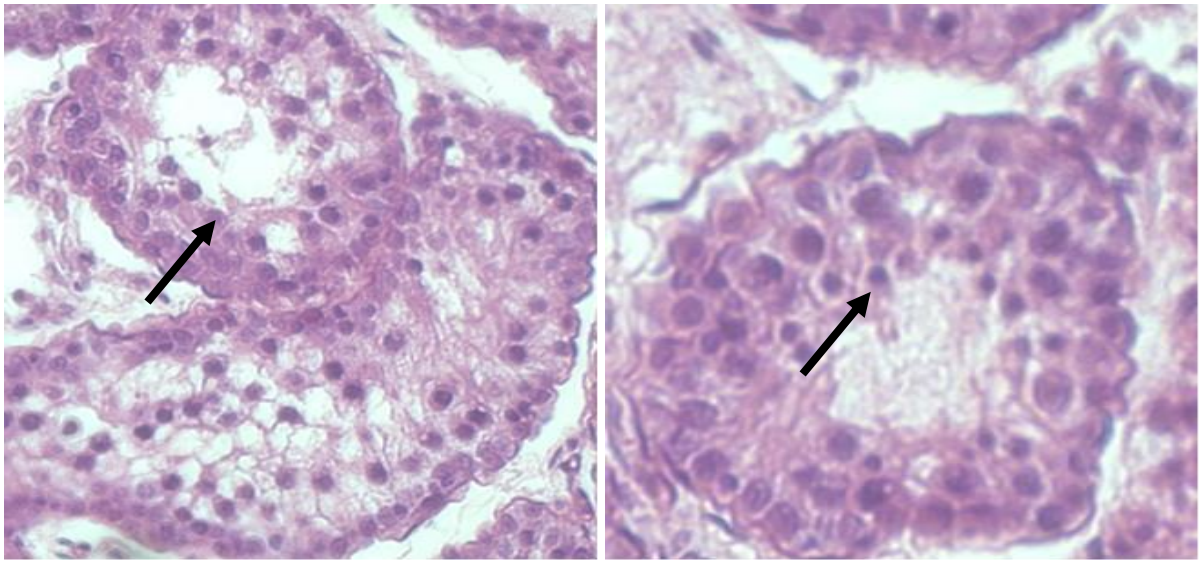
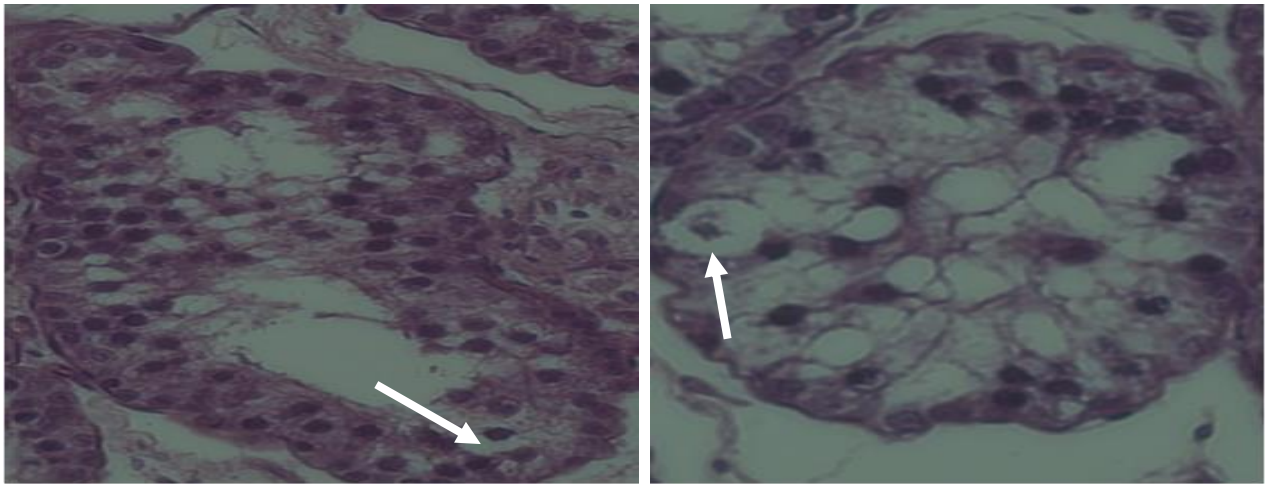


Figure 6. 9: Normal appearance of goat testicular parenchyma after transplanting with Spermatogonial stem cells.



A **B**

Figure 6. 10: A and B -Normal structure of seminiferous tubules with intact Sertoli cell and germ cell layer in the control testis (arrows) (Magnification factor x50).



A **B**

Figure 6. 11: A and B -Vacuoles (arrows) within the seminiferous tubule epithelium in the histological cross-sections from recipient testis that were transplanted with spermatogonial stem cells (Magnification factor x50).

6.3.3 Transplanted SSC fate

Squash smears were prepared for examination under a fluorescent microscope to ascertain presence of green fluorescing cells. It was impossible to pick out fluorescent cells from squash smears as the image was blurred and the entire tubules could not be visualized under the fluorescent microscope (EVOS M5000). However, on light microscopy, an image of seminiferous tubules in the squash smears was seen (Fig. 6.12). Histological sections of transplanted testis were examined in white field and green, fluorescent filter on the fluorescent microscope where fluorescent cells were observed within the seminiferous tubule epithelium (6.13 A and B). Donor cell-expressing eGFP were identified in some transplanted testes at 5% of the seminiferous tubules and in others at 15% of the seminiferous tubules in recipient testis of all the animals (n=5), which had undergone transplantation (Table 6.1). No fluorescent cells were observed in the control testes for each of the animals. Collectively, the findings demonstrate that SSC intra-rete injections were successful in transferring donor germ cells into the seminiferous tubules of recipient testes.

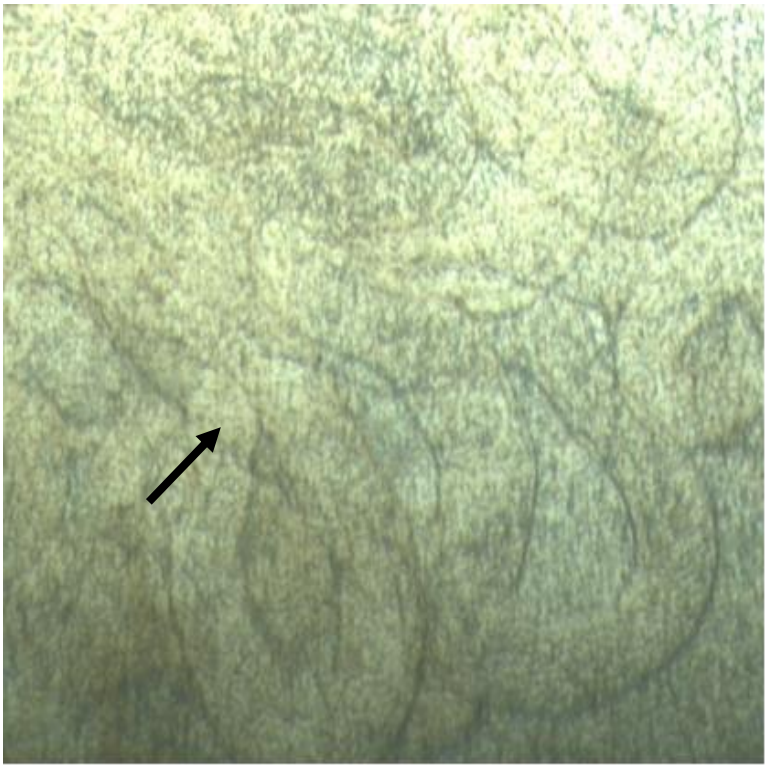
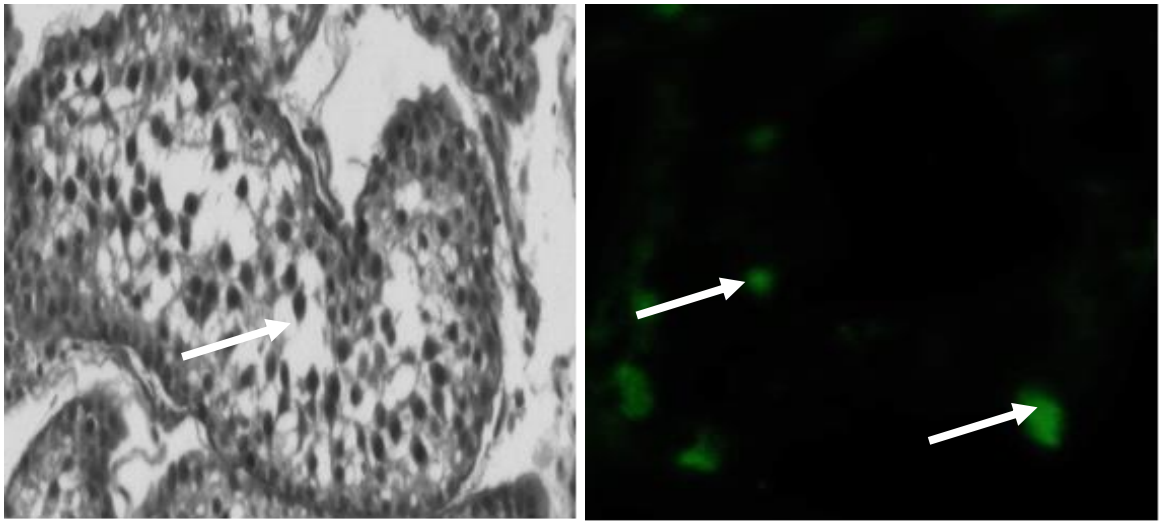


Figure 6. 12: Seminiferous tubules from squash preparations of SSC-transplanted testis as observed under light inverted microscopy (arrow) (Magnification factor x50).



A **B**

Figure 6. 13: A -White field image of the seminiferous tubule cross-section from SSC-recipient testis. B -eGFP green, fluorescent cells within the seminiferous tubules under fluorescent field microscopy (arrows) (Magnification factor x100).

Table 6. 1: Percentage of fluorescent cells within seminiferous tubules

Animal number	Total number of tubules counted	Number of tubules with fluorescent cells	Percentage (%) of tubules with fluorescent cells
1	40	2	5
2	38	3	7.8
3	48	7	14.58
4	50	4	8
5	38	4	10.52

6.4 Discussion

The stem cell capacity of SSC is evaluated by their unique ability to migrate to the basement membrane of the seminiferous tubules with subsequent colonization of the SSC niches within these tubules (Brinster and Avarbock, 1994; Honaramooz *et al.*, 2003; Yoshida *et al.*, 2006). Therefore, successful transplantation to compatible recipient males and migration into the stem cell niche within the seminiferous tubules is considered a proof of successful functionality of *in vitro* cultured SSC in the *in vivo* environment (Lord and Oatley, 2018). In the current study, the isolated SSC were transfected with eGFP and transplanted into mediastinum testis of germ line intact prepubertal indigenous Galla goats. This was the stepping stone to evaluating the characteristics and ability of SCC colonization of the propagation testicular niche in the recipient goats. This is the first report of the transplantation of enriched, cultured, and eGFP-expressing SSCs in recipient goats in Africa.

Successful transplantation of transfected SSCs *in vitro* cultures with molecular expression of markers is the prerequisite for the production of transgenic semen or goats. In this study, goat SSC were cultured and characterized through the molecular expression of markers as documented in other studies (Shirazi *et al.*, 2014; Sharma *et al.*, 2020). The SSC were transduced with an eGFP plasmid and the presence of fluorescent donor cells was evaluated in the recipient testes after castration one week post-transplantation. The evidence of donor cells expressing eGFP identified in an average of 5-15 % seminiferous tubules in the recipient male goats in the current study and some of the fluorescent cells showing contact with the basal membrane as well as morphological similarities with germ cells, is in concurrence with the findings of Brinster and Avarbock (1994) in mice. These authors reported that after germ-

cell transplantation in mice, only 10% of the seminiferous tubules were colonized with donor germ cells. Others reported 10–35% of the seminiferous tubules in germline intact recipient goats colonized by donor-spermatogonial stem cells after transplantation (Honaramooz *et al.*, 2003). The current results compare favorably with the findings in sheep where donor SSC were transduced with a Lentivirus vector bound to eGFP and transplanted in recipient testes, after which an average of 0.2% of the recipient seminiferous tubules were observed to have donor cells expressing eGFP (Rodriguez-Sosa *et al.,* 2009). The results of the current study supported by previous findings from other researchers suggest the potential establishment of recipient males with donor-derived spermatogenesis if transfected germ cells can persist in the seminiferous tubules of the recipients. This could be made possible with improvement for spermatogonial stem cell *in vitro* culture, transfection and transplantation as well as using germline ablated males.

Various techniques for testicular transplantation have been reported with varying success and varying effects that may damage the recipient's testis (Honaramo *et al.*, 2003; Rodriguez-Sosa *et al.*, 2009; Kaul *et al*., 2010). In the current study, the limited spread of the trypan blue dye to a small area of the longitudinal section of the testis after the extra testicular rete testis injection ex vivo method was found to be comparable to the findings of a previous study that reported a 22% filling of seminiferous tubules following ex vivo injection into ram testes (Rodriguez-Sosa *et al.*, 2009). This previous report documented the occurrence of adhesions between the head of the epididymis and the parietal tunica vaginalis due to blunt dissection separation of the head of the epididymis from parietal tunica vaginalis with suturing back. This method was decided against in the current study for *in vivo* injection due to its invasiveness and the risk of developing adhesions resulting from blunt dissection separation of the head of epididymis from the parietal tunica vaginalis with suturing back subsequently.

Comparatively, the intra mediastinum rete testis injection method was preferred in the current study owing to showing a more widespread distribution of trypan blue dye in the seminiferous tubules and devoid of risk of testicular damage. The adoption of this method and extrapolation for ultrasound-guided *in vivo* transplantation was in line with benchmarking made from previous research in goats that used the mediastinum rete testis technique for SSC (Honaramooz *et al.*, 2003) and used successfully with minor modifications by others (Kaul *et al.*, 2010; Zeng *et al.*, 2012). Testicular tissue damage following transplantation has been reported although the lesions are minor and do not interfere with colonization of the seminiferous tubules by donor SSC. Results of histological examination in the current study were contrary to the previous findings by Rodriguez-Sosa et al. (2009), which indicated the occurrence of severe lesions in some of the seminiferous tubules one month after transplantation, which is attributed to the surgical blunt dissection of the head of the epididymis from the tunics and suturing that followed injection into the extra testicular rete. In the current study, inflammatory lesions were mild with no detectable indication of severe local or systemic signs of immunologic reactions to the transplantations. Mild reaction was evidenced by the formation of vacuoles within the seminiferous epithelium, which was an expected response to the introduction of foreign material. However, this reaction clears with time (Honaramooz *et al.*, 2003; Zeng *et al.*, 2012; Ciccarelli *et al.*, 2020).

Rejection of donor SSC cells has not been reported in the livestock since the haploid spermatocytes are shielded by the blood-testis barrier from destruction by the body's immune system. It had been shown that there is no difference in the colonization of seminiferous tubules with SSC in autologous and heterologous recipient testis after their transplantation (Honaramooz *et al.*, 2003; Izadyar *et al.*, 2003). Colonization of the seminiferous tubules by transplanted SSC with evidence of donor-derived spermatogenesis following transplantation,

occurs in germline ablated recipients owing to the absence of endogenous germ layer in these recipients and the SSC niches are empty, hence easily occupied by the transplanted exogenous SSC. Genetically edited surrogate bucks devoid of germ line layer but having a functional somatic cell structure have been developed and they are potentially ideal as surrogate sires (Ciccarelli *et al.*, 2020), to achieve full utilization of transfected spermatogonial stem cells for transplantation as a breeding technology to produce of transgenic animals. The findings in the study demonstrate that intra mediastinum testis transplantation technique was successfully done without causing tissue damage to the recipient animals and the persistence of eGFP transfected cells are great steps towards proof of concept that the cultured Kenyan goat SSC possess the ability to colonize the seminiferous tubules of the compatible recipients.

CHAPTER SEVEN

7.0 GENERAL DISCUSSION

The unique characteristics of spermatogonial stem cells can be utilized in gene editing to develop transgenic spermatozoa for breeding. The SSC can be genetically edited to incorporate transgenes that impart resistance to specific animal diseases and to promote certain production traits. Transplantation of the genetically edited SSC to germline-ablated males would eventually result in donor-derived spermatogenesis that would produce spermatozoa with donor-haplotype, subsequently targeting this semen for use in breeding to disseminate desirable genetics to animal populations (Ciccarelli *et al.,* 2020). The focus of the current study was to establish *in vitro* culture method that would maintain and expand the population of undifferentiated spermatogonia referred to as spermatogonial stem cells (SSCs) for the Indigenous Galla goats in Kenya. This study managed to establish goat SSC lines that were characterized by morphological features and the ability to express molecular markers and which were successfully transfected with eGFP plasmid as proof of their potential to allow gene manipulation.

Furthermore, transplantation of the gene-edited SSCs to germline intact prepubertal indigenous bucks was the culmination of the process. Ability to manipulate SSCs would be essential in fostering their application in surrogate sire technology for developing transgenic animals. The surrogate sire breeding concept entails the development of males that are genetically deficient of the germ cell layer, which are devoid of endogenous spermatogenesis,

but when transplanted with a donor spermatogonial stem cell population, the donor germ cells re-establish donor-derived spermatogenesis that result in donor-haplotype semen (Gottardo *et al.*, 2019).

Sub-Saharan Africa has huge populations of indigenous goats in pastoralist arid and semi-arid areas where reproductive technologies have not been adopted due to infrastructural and cultural limitations. The low genetic value of indigenous goats can be genetically improved through the use of SSC transplantation to surrogate breeding sires that can eventually be incorporated into community livestock breeding systems.

The establishment of stable undifferentiated goat spermatogonia *in vitro* culture system to propagate and boost the number of SSCs while maintaining their stem cell ability, will open new research areas for *in vivo* SSC regulatory mechanisms that can be exploited for application in animal reproduction (Oatley *et al.,* 2016). Furthermore, transplanting SSCs from genetically superior bucks into recipient bucks would accelerate genetic gains through natural breeding using the recipient bucks as well as using the resulting superior semen for artificial insemination (Gottardo *et al*., 2019). In cases where the transplanted SSC have been edited with transgenes of superior production or disease-resistant traits, the production of multiple transgenic animals can be increased to a large scale through mating surrogate sires with females within the breeding systems.

More research on SSC has been done in rodents than in other species. One of the research outputs in rodents is the maintenance of their SSCs for extended periods in serum-free feeder-free media, which has been used as the benchmark and serves as the foundation for continuous improvement and optimization of the conditions to support culture of SSCs from

livestock in general (Kanatsu-Shinohara *et al.*, 2014). Previous studies showed that a spermatogonial stem cell-enriched fraction in goat testicular cells was maintained on Sertoli cell-feeder layer for 4 weeks in serum-free culture medium (Sharma *et al.*, 2020). Maintenance of goat spermatogonial stem cell colonies that are morphologically similar to those reported for rodent cultures in serum-free feeder-free culture for extended periods has not been reported previously. However, the current study has demonstrated that multi-parameter selection of SSC from a population of testicular cells followed by culture in StemPro^{TM}34 serum-free medium and supplemented with Stempro nutrients, growth factors GDNF, FGF2, SDF-1, LIF and maintained laminin-coated plates supported the formation of SSC germ cell clumps that were similar in morphology to rodent SSC cultures for up to 45 days. The extended proliferation of SSC in culture was speculated to be due to the cocktail of growth factors used especially the addition of SDF in combination with the other factors suggested to be essential in maintenance of SSC *in vitro* (Yang *et al.*, 2013). The use of the stem cells specific Stempro medium and Stempro supplement also provided a conducive environment for the proliferation of SSC. As earlier reported in bovine SSC culture by Oatley et al. (2016), the preconditioning medium on fetal fibroblasts as done in the current study also ensured that the culture medium contains other undetermined factors produced by the somatic cells that are essential for SSC proliferation.

In the determination of suitable culture for goat SSC by trials of different culture media, parameters that were considered important were the evaluation of the proliferation of SSC colonies, formation of germ cell clumps, increase in the number of SSC clumps, and PLZF immunocytochemical analysis. The media that supported the formation of more colonies in expressing PLZF marker was considered suitable for SSC culture. PLZF protein required for

mammalian SSC self-renewal has its expression restricted to As, Apr, and Aal spermatogonia, which are considered part of the SSC population (Costoya *et al.*, 2004). Expression of PLZF is conserved across mammalian species as well as in livestock and has been reported to be expressed by a sub-population of undifferentiated spermatogonia within seminiferous tubules of pre-pubertal bucks (Bahadorani, 2011; Pramod and Mitra, 2014; Bahadorani *et al.*, 2015). In this study, it was shown that PLZF expression was localized specifically to the SSC in the pre-pubertal goat testis, hence concluded as an effective marker for identifying and evaluating enrichment of goat SSC.

The repeated trials of culture conditions for propagation and maintenance of SSCs *in vitro* by varying culture medium components and conditions, was essential for developing a guidance protocol for use throughout the study. Although previous studies have reported that FBS (Fetal Bovine Serum) is essential for the culture and development of germ cell clumps of SSC (Pramod and Mitra, 2014), Conversely, the current study has shown the use of FBS in SSC cultures to enhance proliferation of somatic cells with the formation of tight spheres typical of somatic cell outgrowths. The current study corroborated similar findings when serum was used in the medium leading to enhancement of somatic cell propagation that led to detrimental effects on SSC proliferation (Oatley *et al.*, 2016; Sharma *et al.*, 2020). This was the reason for the decision to omit FBS in SSC culture media in the current study, but instead incorporated other cell protein sources such as BSA (Bovine Serum Albumin) as supplements. Secondly, Magnetic-activated cell sorting (MACS) isolation with the cell surface marker THY1 has been found to efficiently enrich testicular isolated cells for undifferentiated spermatogonia (Abbasi *et al.*, 2013). Contrary to this finding, the current study showed that the THY1+ cells from the goat testicular cells had low viability after being

passed through the magnetic field and they could not survive for more than one week in the culture. Similar findings of reduced viability for THY 1 positive MACS isolated SSC was reported in bovine SSC (Reding *et al*., 2010). This led to the use of other enrichment protocols and the omission of the MACS selection.

Previously, a multi-parameter approach to isolate testicular cells, involving differential plating and a discontinuous Percoll density gradient separation was documented (Oatley *et al.,* 2016; Binsila *et al.,* 2020). The current study adopted similar enrichment protocol since single enrichment protocol with cells passed only through differential plating and failed to completely get rid of somatic cells from the enriched spermatogonial stem cells. The proof of SSC cultures successfully enriched for undifferentiated spermatogonia was by marker analysis through immunocytochemistry, immunohistochemistry, and qPCR. This study further supports the concept that multiparameter selection is an effective methodology for isolating an enriched spermatogonial stem cell population from the testes of prepubertal goats. Hence, the reason for the adoption of this isolation technique in all the cultures in the study as a method for isolating viable goat testicular cells enriched for spermatogonial stem cells.

The use of StemPro®-34 SFM supplemented with Stempro nutrient supplement, additives, and growth factors in the experiments was adopted from previous reports by Crouse (2012) and Binsila *et al.* (2020). In the current study, a suitable base culture medium for goat SSC was established through testing of DMEM/F-12, MEMα media, and StemPro®-34 SFM a commercial media used for mice embryonic stem cell cultures, and also confirmed to support

SSC cultures in bovine. StemPro®-34 SFM was the only medium that supported the maintenance of goat undifferentiated spermatogonia for 45 days.

Goat SSC cultures have previously been established on Sertoli cell feeder layer that supported the maintenance of the cells for one month (Sharma *et al.*, 2020). In the initial stages of the study, goat SSC in serum-free StemPro®-34 SFM medium were maintained on Sertoli cell feeders for over 2 months. The germ cell clumps formed had similar morphology to rodent SSC colonies and also the feeder-free goat SSC. In the current study, feeder cells were referred to as goat testicular somatic cells (GSC) being generally a heterogenous population of somatic cells with majorly being Sertoli cells. Despite the support of germ cell growth, the feeder cells have been associated with negative effects on donor-cell colonization efficiency in the recipient especially when SSC have to be used for transplantation (Oatley *et al.*, 2016).

Laminin, which is a natural component of extracellular matrix in the basement membrane of the seminiferous tubules and on which SSCs preferentially bind, led to the decision of using culture plates coated at the bottom surface with laminin matrix to enhance proliferation of SSC. In addition, preconditioning SSC feeder-free medium culture by overnight incubation in goat fetal fibroblasts followed previous recommendations for the medium to possess factors secreted by fibroblasts that are essential for survival of SSC where feed cells are not used (Oatley *et al.*, 2016). The laminin-coated plates confirmed support for by propagation of SCCs by typical germ cell clumps that exhibited morphological similarities to those previously reported in mice and their expression of PLZF molecular marker both through immunocytochemistry and RT-PCR (Kanatsu-Shinohara *et al.*, 2014).

Within the testicular SSC stem cell niche, the cells are mingled with the somatic cell microenvironment where there is the production of growth factor milieu and other cytokines that influence SSC fate decisions of self-renewal or differentiation. Identification and use of these growth factors is essential for the development of an in vitro system that supports maintenance of SSC. Sertoli cells produce GDNF, which is a growth factor crucial for SSC self-renewal and their maintenance *in vitro* (Kubota, *et al*., 2004; Crouse, 2012; Bahadorani *et al.*, 2015; Sharma *et al.*, 2020). Moreover, several studies have shown that supplementation with FGF2, LIF in addition to GDNF, further enhances self-renewal proliferation of rodent SSCs (Crouse, 2012; Bahadorani *et al.*, 2015 Sharma *et al.*, 2020). Supplementation of the growth media with SDF also plays a role in the maintenance of the stem status of SSC *in vitro* (Crouse, 2012). From the available literature, it was indicated that human forms of growth factors GDNF, FGF2, LIF, and SDF are required to supplement goat SSC cultures in vitro, which gave reason for their use throughout the current study. The cocktail of growth factors was capable of enhancing proliferation of SSC and maintaining them in the undifferentiated form *in vitro* for over a month as evidenced by the expression of SSC-specific marker PLZF throughout the culture period. This cocktail of growth factors played a significant role in long-term culture of goat SSC in the current study.

It was postulated that any conditions or factors that enhance in vitro SSC propagation within the 40 days of feeder-free serum-free culture could promote long-term culture. This concept was adopted from the conditions for the long-term culture of bovine SSC (Oatley *et al.*, 2016). The current study identified a suitable feeder-free serum-free medium and a cocktail of growth factors that enhance the survival of spermatogonial stem cells over 45days. However, after the 45 days the cells diminished from the culture, which suggested that one or

more essential components required for self-renewal of cultured SSC on feeder-free systems were missing or decreasing from the culture system. Conversely, the co-cultured goat SSC were maintained on a goat somatic cells feeder for 60 days. The lacking component in the feeder-free systems would probably be an additional growth factor such as colony-stimulating factor 1 (CSF-1), which has been shown to enhance self-renewal of spermatogonial stem cells (Oatley *et al.*, 2009). Another factor that could be possibly added to the SSC culture medium is the Insulin growth factor (IGF-1), which was previously reported to enhance SSC self-renewal in goats (Bahadorani *et al.*, 2015). Further research needs to be done to confirm if the addition of these factors would prolong the survival and self-renewal of goat SSC *in vitro.*

The current study has significantly contributed to the knowledge of culture conditions for goat SSC on feeder-free and serum-free medium. This is also the first report on SSC culture in livestock species in Africa. The findings from the study will be useful in the next steps of developing surrogate sires and transplantation of the *in vitro* expanded goat SSC to the surrogates with a focus on subsequent achievement of production of transgenic animals.

Continues self-renewal of SSC *in vitro* and *in vivo* makes them the best targets for gene manipulation of the germline. Gene editing of SSC would result in permanent genetic modification of the germline due to the stem cell ability to continuously self-renew. If these genetically edited SSC were transplanted and donor-derived haplotype semen is produced, the spermatozoa would contain the gene of interest (de Barros *et al.*, 2012b). Previously, genetically modified animals were produced by pronuclear microinjection (the injection of foreign DNA into the pronucleus of a fertilized oocyte) (Gordon *et al.*, 1980) and somatic cell

nuclear transfer (Yu *et al.,* 2016). Unfortunately, the two methods are technically challenging, such that only a few full term transgenic offsprings have been produced and have a high percentage of mosaic mutant progeny, which can be avoided by direct gene editing on germ line stem cells. Additionally, once the genetically edited SSC are transplanted and spermatogenesis occurs in vivo, sperms with undesirable mutations will be eliminated in the process, hence the functional transgenic spermatozoa ejaculated will not have lethal or undesirable mutations (Zeng *et al.,* 2012). Of importance is the fact that once functional transgenic sperms are formed, the production and multiplication of the founder population of genetically modified animals will be faster compared to cloning or cell-based technology. Thus, the approach of the genetic modification of livestock through transplantation of genetically altered SSCs is currently being considered to complement the pronuclear microinjection and somatic cell nuclear transfer for the production of transgenic farm animals (Zeng *et al.,* 2013). It is important that different transfection procedures for SSC be optimized in preparation for the utilization of SSC in transgenesis.

Previously lipofectamine reagent was used to deliver siRNAs targeting the EZH2 gene, where the knockout was confirmed through PCR (Cai *et al.*, 2020). Additionally, gene knock-in was also successfully done using lipofectamine and pPLZF-IRES2-EGFP or PLZF siRNA gene construct (Song *et al.*, 2015). The two studies did not indicate the gene transfection efficiency but the success in gene knockout and knock-in is evidence that the gene transfer did occur. However, in the current study, transfection protocols of SSC were evaluated by delivering eGFP plasmid to the cell cytosol through liposomal carriers and electroporation. The findings of the current study demonstrated that it is possible to transfer a foreign gene into SSC through using lipofectamine 2000 and lipofectamine stem cell reagent. Further

optimization of this technique is required to increase the transfection efficiency as well as the cell viability. Additionally, transfection enhancer reagents can be incorporated in the process.

Electroporation of enriched and cultured goat SSC to deliver the eGFP was a fast and easy procedure as it involved mixing the cells with OPTI-MEM buffer and the GFP plasmid and electroporation in cuvettes, which took less than 2 minutes with one million cells per cuvette being electroporated per reaction. This makes it possible to electroporate millions of cells within a short time. The Nepagene super electroporator that was used in the current study had an advantage over other electroporators because it did not require the use of electroporation buffers except for low reduced serum medium (OPTI-MEM). The parameters that were used for electroporation were adapted from optimized mice embryonic stem cell electroporation parameters. Although the electroporation efficiency was low, the results are a step forward to developing electroporation protocol for enriched and cultured SSC. Further significant variables for increasing the transfection efficiency such as DNA quality, plasmid size, and use of transfection enhancing reagents should be evaluated in the future. Cell growth factors, cell density, and log phase of the growth curve have an important influence on successful transfection of cells.

Successful colonization of the recipient testis with SSCs following transplantation requires utilization of germline ablated males which do not possess their own germline layer but have a somatic cell framework that can support spermatogenesis. However, an attempt to optimize of transplantation technique using the available germline intact indigenous goats in Kenya was done to test for migration of the SSCs from mediastinum testis lumen into seminiferous

tubules. The presence of eGFP expressing cells following transplantation in prepubertal bucks indicated that ultrasound-guided transplantation of donor cells was successful, although the ability of these cells to persist long enough to colonize seminiferous tubules and have donor-derived genotype was not evaluated. But it was speculated that this would not have occurred due to competition from endogenous SSCs. Consequently, the study has made a step towards unlocking the potential for SSC transplantation technology by establishing a long-term culture of goat SSC, transfection, and transplantation protocols.

CHAPTER EIGHT

8.0 CONCLUSIONS AND RECOMMENDATIONS

8.1 Conclusions

From the findings of the study, the main objectives which goat spermatogonial stem cell culture, transfection and transplantation were achieved. The following conclusions were drawn from the study

1) The goat spermatogonial stem cell *in vitro* culture transfection and transplantation done in the current study is the first one in Kenya and Africa. It gives hope for the potential exploitation of this new method as a future reproductive technology for improvement and dissemination of goat genetics.
2) Prepubertal bucks aged 3-6 months are the most suitable donors from which to isolate spermatogonial stem cells because the seminiferous tubules at this age contain only spermatogonia and Sertoli cells. This makes it possible to isolate a pure culture of spermatogonial stem cells that are mitotically active for *in vitro* propagation
3) Two-step enzymatic digestion using collagenase Type IV (0.025mg/ml) and trypsin (0.25%, 0.04 EDTA) is adequate for isolation of testicular cells with retained cell viability rather than the use of 3-step enzymatic digestion. The more the number of enzymes the lower the retained cell viability.
4) Multiparameter enrichment protocol with a medium that is supplemented with a cocktail of growth factors yields a testicular cell fraction with a higher percentage of spermatogonial stem cells that are capable of forming typical germ cell clumps,

compared to single enrichment protocols that result in a cell fraction with a high percentage of somatic cells that outgrow SSC.

5) Stempro®-34 SFM serum-free medium is the most suitable medium for the culture of goat spermatogonial stem cells especially when supplemented with additives, Stempro nutrient supplement and suitable growth factors. The medium supports the proliferation of SSC germ cell clumps for more than one month in serum-free feeder-free cultures.

6) A cocktail of growth factors (GDNF, LIF, FGF2, SDF) is essential for mimicking the *in vivo* microenvironment provided to SCC by the somatic cells/mixture of chemicals. The growth factors support the survival of SSC in culture for more than 1 month and play a critical role in SSC self-renewal.

7) Spermatogonial stem cells proliferate *in vitro* in feeder-free systems on laminin-coated plates for more than one month successfully maintaining their stem ability as verified through the expression of SSC-specific markers. Preconditioning the culture medium on goat fetal fibroblasts increases the nutrient supply suitable for the *in vitro* cultured SSC.

8) Sertoli feeder cells can support survival of SSC in culture for over 60 days. This is speculated to be due to the chemical, physiological and structural support provided by somatic cells that provide an SSC niche similar to the actual *in vivo* conditions.

9) PLZF is a specific marker for SSC in goats being expressed by undifferentiated spermatogonia in the testes as confirmed through immunocytochemistry, immunohistochemistry, and qPCR.

10) Gene expression in cells can efficiently be evaluated through the use of the reporter gene GFP plasmid bound to the CMV promoter.

11) Germline intact males are inappropriate recipients for SSC transplantation owing to the presence of endogenous SSCs, which make colonization by donor-SSC ineffective for re-establishment of donor-derived spermatogenesis, hence the suitability for use of germline ablated males.

8.2 Recommendations

For full realization of the potential of SSC culture and transplantation in Kenya, the following recommendations are made from this study:

1) Collaboration between resourceful Research Institutions with Kenyan Universities doing research in animals to enable young graduates and scientists to foster fruitful interest in these advanced reproductive biotechnologies for improvement of animal breeding.

2) Government financial support for research Institutions should be enhanced for availability of research funds, equipment/facility acquisition and researchers' capacity building for the field of reproductive technologies.

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APPENDICES

Appendix 1: Search terms for the electronic databases

In PubMed, the queries for relevant keywords and the subject headings were used to generate 3 subsets of references, which comprised: “spermatogonial stem cells" OR "undifferentiated spermatogonia "OR "male germline stem cells" OR "undifferentiated Type A spermatogonia" OR "spermatogonial stem cells markers" Filters: from 1990 – 2021 yielded 2067 papers. The second subset comprised: "spermatogonial stem cells" OR "undifferentiated spermatogonia" OR "male germline stem cells" OR "spermatogonial stem cell transplantation" OR "donor-derived spermatogenesis" OR "spermatogonial stem cell transfection" Filters: from 1990 – 2021 yielded 2021 papers. The 3rd subset: "spermatogonial stem cells" OR "undifferentiated spermatogonia" OR "male germline stem cells" OR "spermatogonial stem cell transplantation" OR "donor-derived spermatogenesis" yielded 2091 papers. Limiting all outputs by species: NOT Human Not Mice. The addition of the term livestock did not yield required results and so it was omitted.

ScienceDirect electronic database has a word limit of the search terms to a maximum only 8 Boolean operators. To include all the livestock species and spermatogonial stem cell words in the original syntax from PubMed, a series of 5 search terms syntax were developed and all the papers extracted pooled into one. The search Boolean for ScienceDirect search engine included the following terms: "spermatogonial stem cells" OR "undifferentiated spermatogonia" OR "male germline stem cells" OR "spermatogonial stem cell transplantation" OR ("donor-derived spermatogenesis" OR ("spermatogonial stem cell transfection". “Undifferentiated spermatogonia” AND culture AND (livestock OR cattle OR SHEEP OR goats OR Bovine OR Pigs OR camels). Similar terms were used for google

scholar and 300 papers were picked up from each of the search terms input (a total of 1500 papers)