ISOLATION, CULTURE AND NEUROGENIC DIFFERENTIATION OF HUMAN DENTAL STEM CELLS

By

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Of

Master of Science in Medicine

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DECLARATION

I, Nyota Masumbuko Kahamba declare that this Dissertation is my own work. It is being submitted for the Degree of Master in Science in Medicine at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

NYOTA MASUMBUKO

15th day of

June 2016 in PARKTOWN

DEDICATION

To Almighty God

To my beloved family

And

In memory of my mother

Mamerthe Masumbuko

1945-1993

PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS RESEARCH PROJECT

1. Masumbuko Kahamba N, Meer S, Ihunwo A. Isolation, Culture and Neurogenic differentiation of Human Dental Stem Cells. Poster presentation. *92th International Congress of IADR.* Cape Town. June 2014.

2. Masumbuko Kahamba N, Meer S, Ihunwo A. Isolation, Culture and Neurogenic differentiation of Human Dental Stem Cells. Poster presentation. *12th International Symposium of the Society of Neuroscientist of Africa.* Durban. March 2015.
   Best IBRO Alumni poster presentation.

3. Masumbuko Kahamba N, Meer S, Ihunwo A. Neurogenic differentiation of Human Dental Stem Pulp Cells. Oral presentation. *92th International Congress of IADR.* Pretoria. September 2015.

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ABSTRACT

Dental stem cells (DSCs) have been identified in teeth and their supporting tissues. They represent an exclusive source of adult stem cells, easily isolated and manipulated for tissue repair and regeneration. This research project evaluated the neurogenic potential of the dental pulp stem cells (DPSCs) and stem cells from the pulp of human exfoliated deciduous teeth (SHEDs) in a South African cohort.

Sixty non-carious permanent and deciduous teeth were extracted from healthy patients aged between 18 and 30 years and 5 and 10 years, at the University of the Witwatersrand's Oral Health Clinic in Johannesburg Charlotte Maxeke Academic Hospital, South Africa. The cells, isolated from the extracted pulp tissue were cultured, counted and then phenotyped by flow cytometry analysis. The cells were further expanded in a neural induction medium and immunocytochemistry analysis for Ki-67, doublecortin (DCX) and nestin were performed.

Large colonies of both DPSCs and SHEDS were harvested from the extracted pulp tissues and positively cultured. Flow cytometry analysis confirmed the presence of $CD44^+$ and $CD29^+$ cells as well as the known mesenchymal stem cell markers CD90 and CD105. Both DPSCs and SHEDs demonstrated successful proliferation and neural differentiation. This study confirmed that DPSCs and SHEDs are highly proliferative human adult stem cells that exhibit a neurogenic potential that may contribute in the treatment of neurological disorders.

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INTRODUCTION

1.1. Background to the study

1.1.1. Stem cells

Stem cells are defined by two fundamental criteria, the ability of continuous self-renewal through indefinite mitotic division and the capacity to give rise to specialised cell lineages. They are primarily classified into embryonic or adult/postnatal type (Minguel *et al.* 2001). During the past decades, stem cells have been extensively explored and their unique biological properties have generated a great amount of interest for therapeutic use in the field of regenerative medicine (Caplan 2005).

1.1.1.1. Embryonic stem cells

Embryonic stem cells are derived from the embryo and they consist of the morula's totipotent cells in early intrauterine life. They are therefore capable of reproducing all cell types of the three germ layers of the embryo, including extra-embryonic structures such as the placenta (Mummery *et al.* 2010). They are also capable of propagating indefinitely in an undifferentiated state (Vazin *et al.* 2010). However, this totipotency persists in the embryo for approximately four days only after fertilisation. As differentiation proceeds and the fertilised egg is gradually implanted into the endometrium of the uterine wall, the totipotent cells are replaced by the inner mass cells of the blastocyst that have lost their competence to give rise to an entire organism and evolve into pluripotent cells: although they retain the potential to develop into all tissues of the embryo and the adult, they are no longer able to form extra-embryonic structures. Later in development, the stem cells in the foetus become multipotent and are able to give rise to all cell lineages.

1.1.1.2. Adult stem cells

Properties of adult stem cells

Adult stem cells exhibit a lower regeneration potential. They are multipotent, or unipotent when they differentiate into one cell type only, and they have been isolated from the umbilical cord as well as numerous postnatal tissue including bone marrow, brain, skin, hair follicles, skeletal muscle, teeth and periodontium (Turksen *et al.* 2004). The most extensively studied and best known adult stem cell is the bone marrow stem cell (BMSC), in which we distinguish two categories: the haematopoietic stem cells (HSCs) that differentiate into blood cells, and the bone marrow stromal cells (BMSCs). The latter forms the supporting tissue of the haematopoietic stem cells and consists of a mixed population of mesenchymal stem cells (MSCs) that generate bone, cartilage, adipose and fibrous connective tissues, when they are grown in specific *in vitro* conditions (Colter *et al.* 2000; Huang *et al.* 2009). Furthermore, various types of cells are observed in this heterogeneous population, with regard to the degree of cell differentiation (Gronthos *et al.* 1999). In rodents, bone marrow stem cells have a neurogenic potential as they are able to differentiate into both astrocytes and neurons when transplanted into the mouse brain (Kopen *et al.* 1999). However, this neurogenic potential is weaker compared to that of the known neural stem cells in the human subgranular zone of the dentate gyrus of the hippocampus and the subventricular zone in the lateral ventricle (Song *et al.* 2007).

Mesenchymal stem cells isolated from other sources constitute a good replacement to the bone marrow stem cells as they are obtained in an easier manner. Besides, they display similar morphologic and immunophenotypic characteristics and they tend to divide more rapidly and form more colonies (Bianco *et al.* 2001; Kern *et al.* 2006). They therefore represent a better alternative for many clinical applications. These cells maintain their

phenotype after each cell division. The cells further away from the inductive tissue retain their original stem cell properties. In contrast, those close to the inductive structure lose their competence and become fully differentiated (Mummery *et al.* 2010).

Clinical applications and limitations of adult stem cells

Adult stem cells are currently and successfully used as a source of specific cell types for regenerative tissue engineering approaches and clinical applications. A prominent clinical application in stem cells research has been bone marrow transplants (Jiang *et al.* 2002; Ide *et al.* 2010; Hunt 2011). However, the isolation of postnatal stem cells has limitations in many instances; especially with regard to collecting sufficient cells from each patient, as a result of variations in age, health condition and cell function. Furthermore, research on human mesenchymal stem cells and their isolation from postnatal organs raises sharp ethical, religious and political issues. Moreover, there are several difficult dilemmas, including consent of individuals to donate materials for research purposes.

1.1.1.3. Dental stem cells

Dental stem cells are mesenchymal stem cells that have been identified from the teeth and their supportive tissues or periodontium (Gronthos *et al.* 2000; Miura *et al.* 2003; Seo *et al.* 2004; Morsczeck *et al.* 2005; Sonoyama *et al.* 2008). These cells are adult mesenchymal stem cells that exhibit lower regeneration potential than the bone marrow mesenchymal stem cells. However, they have the capacity to give rise to distinct cell lineages (Huang *et al.* 2009) and originate from specialised tissues in which continuous remodelling, as in bone, does not occur, thus their differentiation potential is therefore restricted.

Five types of dental stem cells (DSCs) have been identified and characterised from human teeth and their periodontium: the dental pulp stem cells (DPSCs), the stem cells from the pulp of human exfoliated deciduous teeth (SHEDs), the stem cells of the periodontal ligament (PDLSCs), the stem cells of the apical papilla (SCAP) and the stem cells of the human dental follicle (DFSCs) (Huang *et al.* 2009). The dental pulp stem cells (DPSCs) and the stem cells from the pulp of human exfoliated deciduous teeth (SHEDs) are derived from the dental pulp of the permanent and deciduous teeth respectively (Gronthos *et al.* 2000; Miura *et al.* 2003). The periodontal ligament stem cells (PDLSCs) have been isolated from the periodontal ligament of permanent teeth (Seo *et al.* 2004). Stem cells of the apical papilla have been observed in the apical end of the dental papilla of the developing apex of the permanent teeth (Sonoyama *et al.* 2008). The dental follicle progenitor cells have been isolated from the dental follicles of impacted third molars (Morsczeck *et al.* 2005).

1.2. Overall objectives of the study

Most of the experiments in the literature have proven that the human dental stem cells, in addition to their odontogenic potential, demonstrate very broad differentiation capabilities. They are able to give rise to adipogenic, chondrogenic, osteogenic, myogenic and neurogenic cells. Furthermore, it has been established that dental stem cells display a specific profile of cluster of differentiation markers (CD) similar to those of mesenchymal stem cells. They positively follow the requirements decreed by the International Society for Cellular Therapy that unequivocally define the phenotype of mesenchymal stem cells: they must express the specific cluster of differentiation markers (CD) on their cell surface (CD73, CD90 and CD105) and they must be deprived of the common haematopoietic

markers, CD14, CD45 and CD34 (Dominici *et al.* 2006; Karaoz *et al.* 2010; Azouna *et al.* 2012).

The potential of dental stem cells to differentiate into neurologic tissues in specific induction media has been thoroughly researched (Arthur *et al.* 2008; Ulmer *et al.* 2010). Several studies have been performed in the Asian and European populations and have reported the successful isolation, proliferation, characterisation as well as the neurodegenerative properties of dental stem cells when transplanted into the injured central nervous system of adult rats or mice (Arthur *et al.* 2008; Sakai *et al.* 2012). These cells then are a valuable source of adult stem cells for regenerative medicine as they are easily isolated in a non-invasive manner and easily manipulated to obtain various multipotent cell colonies. These colonies can later, not only regenerate or repair lost dental tissues, but also other cell lineages damaged by disease, especially neurogenic cells.

Moreover, because of the contribution of the neural crest cells that originate from the neural tube prior to its closure (Baroffio *et al.*1991; Le Douarin *et al.* 2012), the dental tissues-derived stem cells tend to exhibit characteristics similar to neural crest cells. Consequently, due to the high prevalence of neurological disorders and the difficulties to harvest neural stem cells from the brain, establishing the neurogenic differentiation of dental stem cells could form a credible basis for clinical application.

This previous research supports the use of human dental stem cells as a unique source of clinical material for regeneration such as neuroregeneration therapies. Since no such study has been undertaken in South Africa, focusing on such a project will upgrade all

information on the tooth-derived cells and will significantly contribute to the future therapies for neurological disorders.

1.3. Literature review

1.3.1. Historical background of stem cells

The term "stem cell" emerged in the late nineteen century, when several investigations on the evolutionary basis of all plants and animals were increasingly conducted; it became gradually established in the field of embryology and cytology, although with some discrepancies in its definition (Maehle, 2011). In the early twentieth century, the idea of stem cells appeared to become reinforced in the haematological research of Artur Pappenheim (1870-1916) with his hypothesis of the presence of a multipotent stem cell in different forms of leukaemia (Brown *et al.* 2006).

However, the term "stem cell" was first considered for scientific use by the Russian histologist Alexander Maximow (1874-1928), at the congress of the Haematological Society in Berlin in 1909. He discovered the theory of haematopoiesis and proposed the notion of the origin and further differentiation of the various types of blood cells, from a common haematopoietic precursor, both during embryonic development and in the adult life of mammals (Konstantinov 2000). The existence of haematopoietic stem cells was formerly postulated and the "stem cell" name was then attributed to various cells that have the ability to differentiate into specific types of somatic or germ cells in the body (Konstantinov, 2000). Following this, several research projects were developed and many reports on the different properties of stem cells in embryology, haematology and histology were released.

1.3.2. Emergence of stem cell biology

Before World War I, the pluripotency of stem cells and germ cells was also gradually associated with the onset of neoplasms, and the theory of Julius Cohnheim (1839-1894) resurfaced. This theory was initially presented in 1877 and stated that, with a sufficient blood supply, stem cells could grow in an uncontrolled manner to form tumours and cancer (Cooper, 2009). In a similar manner, Theodore Boveri (1862-1915) in 1914 postulated that the malignant transformation of a tumour was due to lack of expression of mechanisms normally present in cells (Harris 2008). In the same period, embryonic tissue isolation and culture was developed and increasingly studied outside the embryo, leading to the modern approach of stem cell research (Maienschein 2009). In the early 1960s McCulloch *et al* further contributed to modern stem cell research in examining the effects of radiation on haematopoiesis in the bone marrow and introduced the term "colony forming unit" (CFU) to describe cell colonies deriving from a single haematopoietic stem cell (Till *et al.* 1961; Becker *et al.* 1963). This experiment represented the first quantitative assay that was performed for blood stem cells.

1.3.3. Experiments on stem cells

Further studies based on Maximow's fundamental research on haematopoiesis were then conducted. In the 60s, Alexander Friedenstein (1924-1998) and his co-workers discovered in the bone marrow of rodents, the co-existence of haematopoietic and adult non-haematopoietic cells. The function of the latter cells was initially defined as the supporting cells of haematopoiesis. However, further examinations revealed that they had a clonogenic potential and could also give rise to skeletal tissue derivatives namely, fibroblastic, reticular, adipocytic and osteogenic. They appeared to be a functionally distinct type of stem cells that occurred with the haematopoietic stem cells in the bone

marrow and were therefore labelled as the "bone marrow stromal stem cells" (BMSSCs) since they arise from the complex array of supporting structures in the bone marrow (Friedenstein *et al.* 1968).

In the 70s, Friedenstein demonstrated that these spindle-shaped precursors' cells were adherent to plastic culture dishes: they gathered in foci of two to four cells and, after a dormancy period of two to four days they became highly proliferative. As the daughter cells appeared to self-replicate from a single fibroblast-like cell, Friedenstein termed them the colony forming unit-fibroblast (CFU-F). He later established that when cultured in the appropriate conditions, the cells were able to regenerate the bone matrix and its micro-environment (Friedenstein *et al.* 1966; Friedenstein *et al.* 1974) *in vitro* and *in vivo* after their re-transplantation into laboratory animals (Friedenstein *et al.* 1980).

Extending his initial work to human bone marrow, Friedenstein was later able to show that the percentage of floating haematopoietic cells (30%) was less important than that of the adherent bone marrow stromal stem cells (Friedenstein *et al.* 1976). In addition, these replicating colonies were maintained in the primary culture for two to three weeks (Friedenstein *et al.* 1976). The type of differentiation that they underwent (fibroblasts, osteoblasts, chondroblasts, adipocytes or myoblasts) depended on the nature of the supplement added to the culture medium. For instance, in the presence of dexamethasone, 1, 25-dihydroxyvitamin D3, or cytokines such as BMP-2, the osteogenic, chondrogenic and adipogenic differentiation prevailed. On the other hand, in response to 5-azacytidine and amphotericin B or amphotericin B alone, the preferential differentiation was towards the myoblasts (Friedenstein *et al.* 1976; Owen *et al.* 1988). In that same era, Friedenstein

isolated mesenchymal stem cells from the stroma of the spleen and thymus (Friedenstein *et al.* 1972).

Later in 1991, Caplan and his co-workers showed that the transplantation of bone marrow in other anatomical sites of mice resulted not only in the elimination of the haematopoietic cells, but also the active division and differentiation of the stromal cells. This was followed by the development of ectopic bone and cartilage and he used the term "bone marrow mesenchymal stem cells" as the source of progenitor cells for mesenchymal tissues (Caplan 1991). He also confirmed that the precursor' cells isolated from the chick and the mice embryos preferentially exhibited chondrogenic or osteogenic potential, depending on the different culture conditions *in vitro*, which corroborated Friedenstein's findings (Caplan *et al.* 1991; Bruder *et al.* 1997).

Many experiments were further conducted on the adult bone marrow mesenchymal stem cells and additional differentiation such as ligament, tendon and stroma were successfully achieved (Caplan 1991; Bergman 1995). The characterisation of the bone marrow mesenchymal stem cells was accomplished later by Pittenger and his co-workers (1999). Cells harvested from the bone marrow of the iliac crest were culture-expanded and phenotyped by their ability to proliferate, their consistent expression of specific cell surface proteins and their consistent differentiation into multiple mesenchymal lineages under specific *in vitro* conditions (Pittenger *et al.*1999).

Following these studies, mesenchymal stem cells were further isolated in a variety of sources other than the bone marrow; these included the placenta and the umbilical cord in the foetus and mainly periosteum, muscle, adipose tissue, hair follicles, brain and the

cornea stroma (Mareschi *et al.* 2001; Reynolds *et al.* 1992). Gronthos and his co-workers searched for mesenchymal stem cells in the human teeth and their supportive tissue and identified a population of multipotent stem cells. These cells displayed the same characteristics as the mesenchymal stem cells and were therefore labelled dental stem cells (DSCs) (Gronthos *et al.* 2000).

1.3.4. Experiments on dental stem cells

Recently, postnatal stem cells have been isolated from the teeth and their supporting tissues. Like the bone marrow stromal stem cells and mesenchymal stem cells from other sources, they are multipotent cells able to give rise to distinct cell lineages (Huang *et al.* 2009) with, however, a restricted differentiation potential. Nevertheless, their discovery has brought a significant breakthrough in stem cell research and regenerative medicine. In order to ensure a successful use of dental tissues in tissue engineering, a sound understanding of tooth development and tooth structure is required.

1.3.5. Tooth development

Odontogenesis is determined in the embryo by a complex sequence of reciprocal inductions between the oral ectoderm and the neural crest-derived mesenchymal cells, which also contribute to craniofacial development. Tooth development begins in the fifth week of intrauterine life as a result of the reciprocal interactions between the oral ectoderm that will generate the enamel organ, and the underlying mesenchymal cells that will give rise to the dental papilla (Chiego 2014). In addition to these two categories of cells, the neural crest cells that originate from the neural tube prior to its closure, also contribute to tooth development (Baroffio *et al.* 1991; Le Douarin *et al.* 2012). The neural crest cells are arranged in a flattened irregular mass that will form several structures including the

ganglion cells, the neurolemma sheath of the peripheral nerves, the leptomeninges, the pigments cells, and the suprarenal medulla (Moore *et al.* 2013). In addition, the neural crest cells migrate into the craniofacial complex and teeth at an early stage of development and invade and expand the mesenchyme, thus later referred to as ectomesenchyme (Nanci 2008).

Each developing tooth grows as an anatomical distinct unit but the basic developmental process is similar for all teeth (Chiego 2014). The first sign of tooth formation is the thickening of the oral ectoderm into the primary epithelial band that will further develop into dental and vestibular laminae (Nanci 2008). The latter will evolve into the vestibule of the oral cavity in the adult, while each tooth germ will appear from the former through successive stages: the bud, cap and bell stages. The bud stage corresponds to the first incursion of the dental lamina into the underlying ectomesenchyme. During the cap stage, the dental lamina condenses and forms the enamel organ giving rise to enamel, while the ectomesenchymal cells converge into the dental papilla that will synthesize the dentine and pulp complex. Both the enamel organ and the dental papilla are surrounded by the dental follicle which will later generate the periodontium or supporting tissues of the tooth. In the bell stage, morphodifferentiation and histodifferentiation occur, resulting in the crown configuration and the hard tissues production (Fig. 1). This is followed by the root and periodontium development in the late bell stage of odontogenesis. Subsequently, the completed tooth crown erupts into the oral cavity, and root formation and cementogenesis continue until a functional tooth and its supporting structures are fully developed (Nanci 2008; Chiego 2014). Since the completion of tooth formation in all deciduous and permanent teeth continues in postnatal life, it becomes evident that diverse stem cell niches are still present in the human teeth and their supporting tissues.

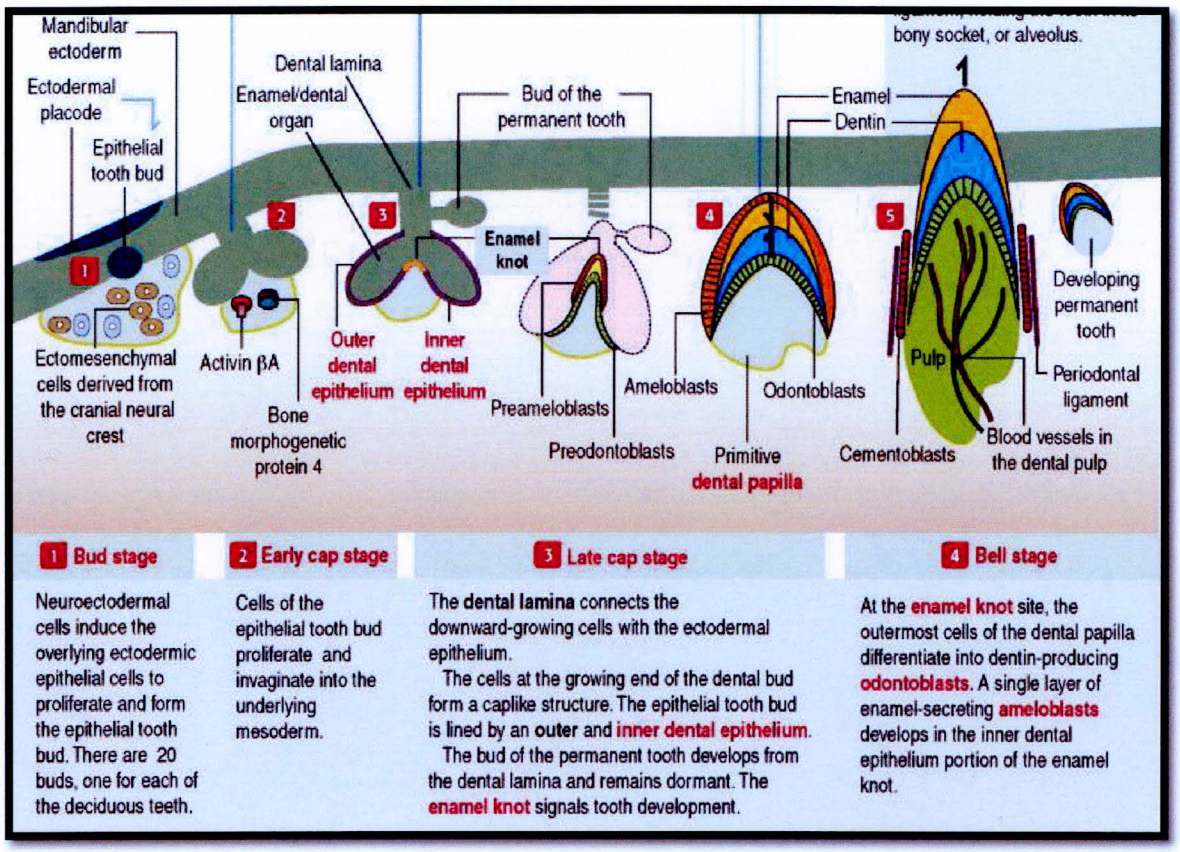


Figure 1: Representative diagrams of the stages of tooth development.

Kierszenbaum 2012

1.3.6. Categories of dental stem cells

As mentioned previously, five types of human dental stem cells (DSCs) have been identified and characterised from human teeth and their supporting tissues: the dental pulp stem cells (DPSCs), the stem cells from the pulp of human exfoliated deciduous teeth (SHEDs), the stem cells of the periodontal ligament (PDLSCs), the stem cells of the apical papilla (SCAP) and the stem cells of the human dental follicle (DFSCs).

The literature extensively discusses how these progenitor cells exhibit the characteristics of umbilical cord and postnatal mesenchymal stem cells. They have the potential for self-

renewal and they demonstrate very broad differentiation capabilities. In addition to their odontogenic potential, they are able to give rise to adipogenic, chondrogenic, osteogenic, myogenic and neurogenic cells. It has also been found that they express specific mesenchymal stem cell markers or clusters of differentiation (CD) such as CD73, CD90, CD105 and CD146; on the other hand, they lack haematopoietic stem cells markers such as CD14, CD45 and CD34 (Karaoz *et al.* 2010; Azouna *et al.* 2012). Furthermore, because of the contribution of the neural crest cells that originate from the neural tube prior to its closure, the dental tissues-derived stem cells tend to exhibit characteristics similar to neural crest cells. They are therefore a useful alternative for cell replacement in the treatment of tissues known to contain neural stem cells. The first type of DSCs was isolated from the dental pulp tissue and termed adult dental pulp stem cells (DPSCs) (Gronthos *et al* 2000; Huang *et al* 2009).

1.3.6.1. Dental pulp stem cells (DPSCs)

The general structure of an adult tooth consists of both hard and soft tissues arranged in the crown and the root(s). The dental pulp is the only soft tissue in the tooth and it supports the dentine which forms the bulk of the tooth (Nanci 2008). It can be divided into two parts: the coronal pulp or pulp chamber, located in the crown, and the radicular pulp that extends from the cervical region or anatomical neck of the tooth, to the apex of the root (Nanci 2008; Chiego 2014). The pulp tissue can be further subdivided histologically into four distinct zones consisting of the odontoblastic layer at the periphery, a cell-free zone of Weil, a cell-rich zone and lastly, the pulp core containing the principal cells of the pulp (Nanci 2008). The cell-free and cell-rich zones are prominent within the coronal pulp.

The dental pulp is a heterogeneous tissue that contains odontoblasts, fibroblasts, undifferentiated ectomesenchymal cells, nerves, vessels and immunocompetent cells (Fig. 2) (Nanci 2008).

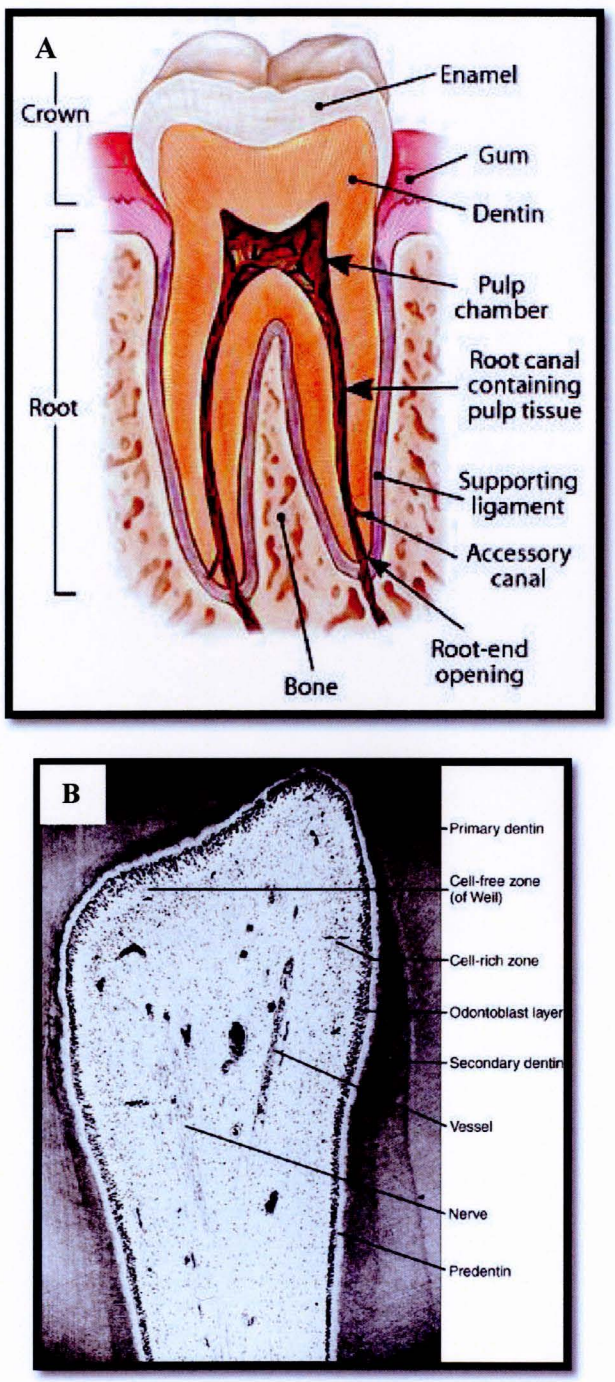


Figure 2: Representative diagram (**A**) and photomicrograph (**B**) illustrating tooth morphology and the histological zones of the dental pulp.

https://www.google.co.za/search?q=tooth+morphology+images (**A**) Nanci 2008 (**B**)

The dental pulp has five primary functions including nutritive, protective, inductive, formative and lastly reparative, through the production of tertiary or reactionary dentine (Nanci 2008; Chiego 2014). Indeed, in the adult tooth, progenitor cells are still present in the odontoblastic layer, and in the event of mechanical or inflammatory stimuli they are able to differentiate into odontoblast-like cells that secrete tertiary or reparative dentine. Previous studies have demonstrated that these dental pulp derived stem cells (DPSCs) are also able to generate diverse cell types, including neural and glial stem cells (Gronthos *et al.* 2000; Huang *et al.* 2009).

Several reviews have confirmed the expression of specific mesenchymal stem cells markers such as STRO-1, CD146, CD90 and CD105, CD90 (Miura *et al.* 2003), and bone markers including bone sialoprotein, alkaline phosphatase and osteocalcin (Gronthos *et al.*, 2002). Moreover, the expression of specific markers of neural precursors' and glial cells, nestin and glial fibrillary acid protein (GFAP) respectively, has also been noted in dental pulp stem cells (Gronthos *et al.* 2002). For all these reasons, the dental pulp is an interesting source of mesenchymal stem cells because of the large amount of cells identified and easily isolated in a non-invasive manner.

1.3.6.2. Stem cells from the pulp of human exfoliated deciduous teeth (SHEDs)

Evidence of dental stem cells is shown not only within the pulp tissue of permanent teeth, but also in the pulp tissue of deciduous teeth and other dental tissues such as the periodontal ligament, the dental apical papilla and the dental follicle, as confirmed by several studies (Miura *et al.* 2003; Huang *et al.* 2009). In humans, the transition from the deciduous or primary dentition to the permanent or secondary dentition is a unique and

dynamic process in which the development and eruption of permanent teeth coincide with the resorption of roots or exfoliation of the deciduous teeth.

The stem cells isolated from the pulp of exfoliated deciduous teeth (SHEDs) (Fig. 3) display an extensive aptitude to proliferation and differentiation into multiple cell lineages similar to umbilical cord stem cells. They have also been termed "immature DPSCs" (IDPSCs) (Kerkis *et al.* 2006) and they exhibit a high proliferative capacity analogous to that of neural stem cells as suggested by the numerous and adherent aggregates of sphere-like clusters seen in culture dishes (Miura *et al.* 2003). It has also been shown that after *in vivo* transplantation, in a mouse brain, they are able to induce bone, dentin and express similar neural markers to that of neural stem cells (Codega *et al.* 2014).

Moreover, SHEDs can be isolated and successfully cultured and expanded *in vitro*. They thereby may represent a unique pool of stem cells to use for potential clinical applications (Kadar *et al.* 2009). Like the DPSCs, they express the early mesenchymal stem markers STRO-1 and CD146 (Gronthos *et al.* 2000) as well as CD29 and CD30, more recently identified on SHEDs (Karaoz *et al.* 2010).

1.3.6.3. Stem cells of the periodontal ligament (PDLSCs)

Another category of dental stem cells is defined by the stem cells isolated from the periodontal ligament of permanent teeth (PDLSCs) (Seo *at al.* 2004; Coura *et al.* 2008). The periodontal ligament (PDL) is a specialised soft connective tissue situated between the cementum covering the root of the tooth and the inner plate of the alveolar bone housing the tooth (Fig. 4) (Nanci 2008). It invests the tooth and anchors it to the jawbone and helps it to withstand natural masticatory forces. It occupies the periodontal space and is

composed of type I collagen fibres, several cells types and intercellular substance (Chiego 2014). It has been observed by McCulloch (1995) and later by Isaka *et al.* (2001) that the periodontal ligament comprises a heterogeneous population of periodontal progenitor cells that primarily maintain the homeostasis, repair and regeneration of the periodontal tissue. They are also capable of developing into adipocyte, periodontal fibroblast, tendinoblasts as well as cementoblast and osteoblast precursors (Seo *et al.* 2004). They have been successfully isolated, cultured and characterised in mice and humans, and it has been established that they express bone-related markers such as alkaline phosphatase and bone sialo protein.

Furthermore, periodontal ligament stem cells expanded in a specific inductive medium, express neural markers such as nestin and neural crest cell markers (Coura *et al.* 2008). In addition, it has been shown that their cell surface displays the mesenchymal stem cell markers STRO-1 and CD146 that are also identified in bone marrow mesenchymal stem cells and dental pulp stem cells (Seo *et al.* 2004). Again, these experiments show that the periodontal niche of dental stem cells represents a potentially valuable source of clinical material for tissue repair and regeneration (Seo *et al.* 2004).

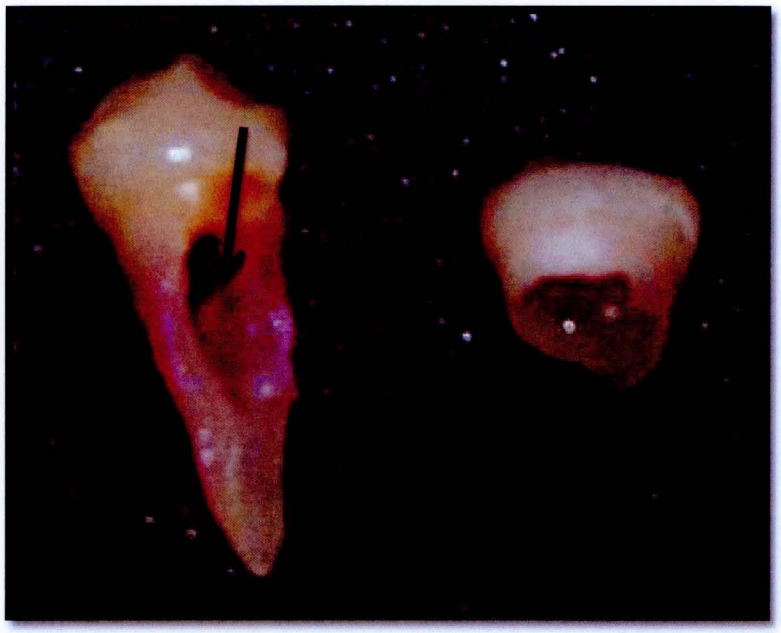


Figure 3: Representative photograph of the pulp tissue extracted from one of the exfoliated deciduous teeth in the study sample.

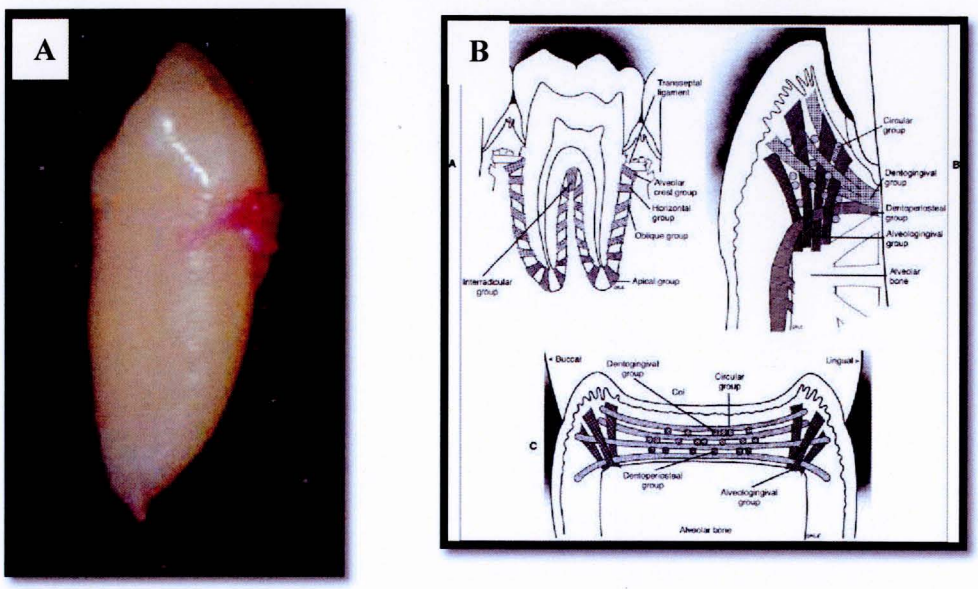


Figure 4: Representative photograph of the periodontal ligament of a tooth in the study sample (**A**) and diagrammatic representation of the periodontal ligament (**B**).

Nanci 2008

1.3.6.4. Stem cells of the apical papilla (SCAP)

Stem cells of the apical papilla are another type of dental stem cells that have been isolated from the apical end of the dental papilla of the developing tooth apex (Fig. 5) (Sonoyama *et al* 2006; Sonoyama *et al* 2008). The formation of the root during the late bell stage of tooth development is determined by the epithelial root sheath of Hertwig, a double layer of epithelial cells originating from the enamel organ at the point where enamel secretion ends (Fig. 5) (Nanci 2008). The epithelial root sheath lengthens and serves as a template for the future root. It forms the epithelial diaphragm, an area at the proliferating and bent termination of the root sheath (Nanci 2008; Chiego 2014). The epithelial diaphragm surrounds the highly dividing cells of the apical opening of the dental pulp (Nanci 2008; Chiego 2014). These cells, which aggregate at the apical end of the developing root to form the apical papilla, contain ectomesenchymal cells that are histologically distinct from the dental pulp and represent a unique source of mesenchymal stem cells (Fig. 5) (Fig. 6) (Huang *et al.* 2009). They have been successfully expanded *in vitro* and have successfully undergone odontogenic, adipogenic and neurogenic differentiation demonstrated by the expression of bone and neural markers in the appropriate induction medium (Sonoyama *et al.* 2008). The mesenchymal stem cells marker STRO-1 has also been identified on the cell surface (Lei *et al.* 2011).

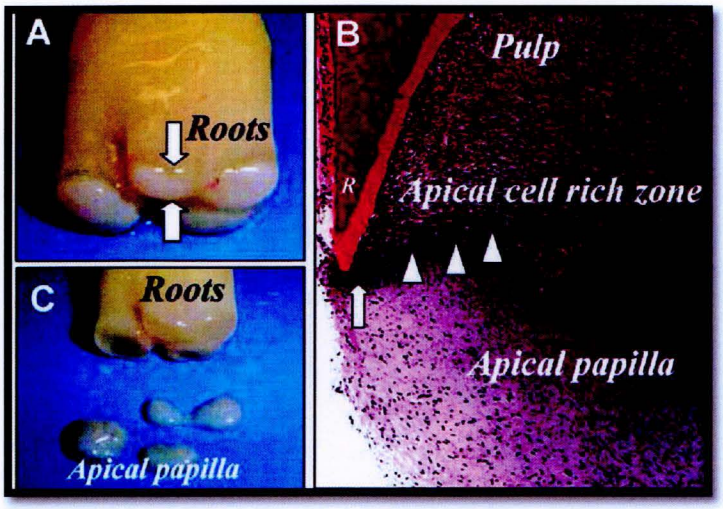


Figure 5: Representative photographs (**A** and **C**) and photomicrograph (**B**) of the apical papilla of the developing permanent tooth.

Huang et al. 2009

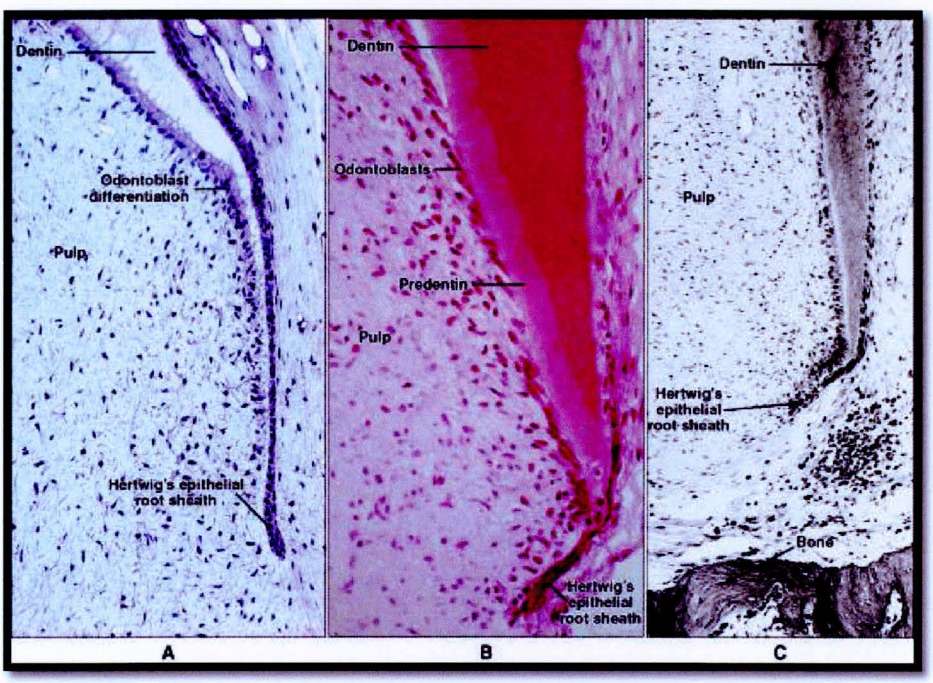


Figure 6: Representative photomicrograph of root formation steps in a permanent tooth showing the apical diaphragm.

Nanci 2008

1.3.6.5. Stem cells of the human dental follicle (DFSCs)

The dental follicle or dental sac is the loose and vascular ectomesenchymal tissue that encapsulates the dental papilla and the enamel organ of the developing tooth from the cap stage of tooth development (Nanci 2008). It is composed of a heterogeneous layer of ectomesenchymal cells (Fig. 7) that have two important functions in odontogenesis: namely, the development of the periodontium (Morsczeck *et al.* 2005) and the co-ordination of tooth eruption (Nanci 2008). The periodontium is composed of the periodontal ligament, the alveolar bone, the cementum covering the root of the tooth and the lamina propria of the gingiva facing the tooth (Nanci 2008).

The dental follicle progenitor cells have been isolated from the dental follicles of impacted third molars and, in expanded cultures, they initiate the formation of adherent colonies that are also competent to differentiate into adipogenic, osteogenic and neural cell lineages (Arthur *et al.* 2008; Huang *et al.* 2009; Kadar *et al.* 2009; Lei *et al.* 2011). Like the other dental stem cells, the *in vitro* characterisation of dental follicles stem cells has reported the expression of the mesenchymal stem cells marker STRO-1, bone markers including osteocalcin, bone sialoprotein and the neural progenitor cell marker nestin and Notch-1.

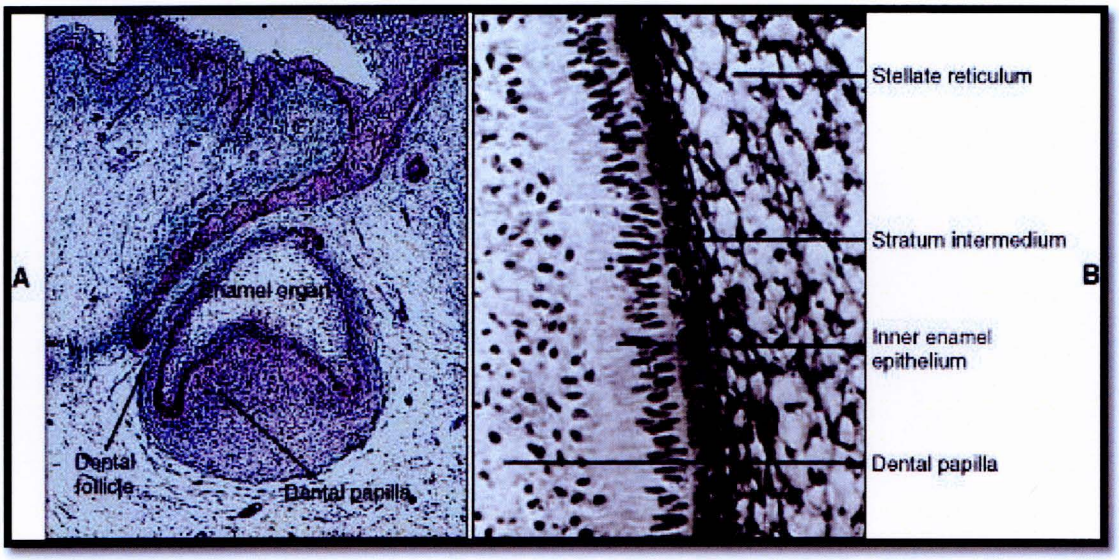


Figure 7: Representative light microscopic pictures of the dental follicle of the developing permanent tooth.

Nanci 2008

The five types of dental stem cells thus form a valuable source of adult stem cells that are easily accessible in a non-traumatic manner in the human. Indeed, the deciduous teeth are naturally lost when they are replaced by the secondary dentition in the genetically regulated event of dental exfoliation (Nanci 2008). This provides a source for the stem cells isolated from the pulp of human exfoliated deciduous teeth (SHEDs). Besides, healthy permanent teeth, such as maxillary and mandibular premolars, are often extracted for orthodontic treatment purposes. These teeth are the source of dental pulp stem cells (DPSCs) as well as stem cells from the apical papilla (SCAP), as at the time of tooth extraction, the root(s) maturation has not yet fully occurred. Furthermore, impacted maxillary and mandibular third molars that could be associated with pathological conditions are often surgically removed without detrimental health effects. These samples provide dental pulp stem cells as well as dental follicle stem cells (DFSCs). Finally, the periodontal ligament attaching the cementum of the root of the tooth to the alveolar bone is cut off in the extraction of all teeth and therefore supplies the periodontal stem cells (PDLSCs).

It is then evident that human dental stem cells can easily be manipulated to obtain various multipotent cell colonies that can, not only regenerate or repair lost dental tissues, but also other cell lineages damaged in disease, especially neurogenic cells. Indeed, the dental stem cells all demonstrate the ability to neural differentiation in a specific induction medium and can therefore be used in regenerative medicine (Arthur *et al.* 2008; Ulmer *et al.* 2010). Currently, the increase of nervous system disorders, especially neurodegenerative diseases in the elderly population, has expanded. They lead to the degeneration of neural cells in the central nervous system, followed by malfunction. To repair the damaged function, pharmacology therapy is used but is also limited in many instances. The use of neural

stem cells would be the ideal treatment of these conditions. However, the location of the neural stem cells in the subventricular zone in the lateral ventricle and subgranular zone of the dentate gyrus of the hippocampus of the adult, render the harvesting of the cells impossible. The use of the dental stem cells would be an advantageous, reliable and non-invasive alternative for tissue engineering.

As previously stated, several studies performed on dental stem cells in the Asian and European populations have demonstrated their successful isolation, proliferation and, characterisation as well as their neuroregenerative properties when they are transplanted into the injured central nervous system of adult rats or mice (Sakai *et al.* 2012). This earlier research advocates the use of human dental stem cells as an exclusive pool of clinical material for tissue regeneration such as neuroregeneration therapies. To date, no investigation of the neurogenic differentiation of human dental stem cells in South Africa has been undertaken. Focusing on such a study in the South African population could therefore upgrade the information in the tooth-derived stem cells in order to significantly contribute to the treatment of neurological disorders.

1.4. Objectives of the study

1.4.1. Main objective

The aim of the study was to evaluate the neurogenic potential of two categories of dental stem cells: namely, the dental pulp stem cells (DPSCs) and the stem cells from the pulp of exfoliated deciduous teeth (SHEDs) in a South African population.

1.4.2. Specific objectives

In order to achieve this aim, the following objectives were defined:

1. The isolation and culture of DPSCs and SHEDs from permanent and deciduous teeth respectively.
2. The characterisation of DPSCs and SHEDs using flow cytometry analysis.
3. The induction of neurogenic differentiation of DPSCs and SHEDs.
4. The assessment of the proliferation of the differentiated DPSCs and SHEDs using Ki-67.
5. The assessment of the maturation of the differentiated DPSCs and SHEDs *in vitro* by using Doublecortin (DCX: neural markers for immature neurons) and Nestin (neural marker for mature neurons).

CHAPTER 2

MATERIAL AND METHODOLOGY

2.1. Ethics

The Human Research Ethics Committee (Medical) of the University of the Witwatersrand approved the protocol design and procedures for this study (Clearance Certificate Number M120506) (Appendix A). Permission was also obtained from the Chief Executive Officer of the School of Oral Health Sciences of the University of the Witwatersrand for the teeth collection and their use for the research project.

2.2. Study sample

Thirty impacted and semi-impacted maxillary and mandibular molars were extracted from healthy adults aged between 18 and 28 years. In addition, thirty deciduous teeth were removed from healthy patients aged between 7 and 10 years, who had required dental extraction under general anaesthesia. The teeth were obtained following informed consent from patients and parents visiting the Wits Oral Health Clinic, Charlotte Maxeke Johannesburg Academic Hospital.

A week prior to the extraction, patients were instructed to pre-treat their teeth with an antimicrobial prophylaxis mouth rinse of 0.2% chlorhexidine gluconate, twice a day after brushing, in order to reduce the microbial flora of the oral cavity (Miura *et al.* 2003; Morsczeck *et al.* 2009; Gronthos *et al.* 2011; Tirino *et al.* 2011). Chlorhexidine is a form of bis-biguanide used as a gluconate salt. It is a positively-charged molecule that adheres to the negatively-charged sites on the bacteria and fungi cell wall. It impairs the integrity of the plasma membrane which results in leakage of cell contents and cell death

(McDonnell *et al.* 1999). It is therefore a broad-spectrum antimicrobial active against a wide range of Gram-positive and Gram-negative bacteria, fungi, facultative anaerobes and aerobes (Okino *et al.* 2004). Furthermore, it has a quicker kill time rate than other antimicrobials (McDonnell *et al.* 1999).

2.3. Isolation and culture of DPSCs and SHEDs

2.3.1. Tooth extraction

On the day of the surgical procedure, aseptic techniques were followed throughout. The teeth were disinfected with a 0.2% chlorhexidine gel for two minutes, before the extraction. Following local anaesthesia of the area, the teeth were extracted using a sterile tooth forceps. The extracted teeth were washed with a solution of 0.2% chlorhexidine gluconate for two minutes, the designated time needed to kill the *Candida albicans* in the specimens (Patel *et al.* 2008). The teeth were then washed with distilled water to neutralise the action of the chlorhexidine and stop the antimicrobial effect on healthy tissue. The dental specimens were then deposited in a sterile solution of Hanks Balanced Salt Solution (HBSS) (Lonza, Verviers, Belgium) to be transported to the laboratory.

2.3.2. Pulp tissue extraction

Firstly, a periodontal scaler was used to remove all debris of the periodontal ligament (PDL) on the roots. The teeth were then wiped with 70% alcohol and then circumferentially split into two halves, at the level of the cementoenamel junction, with a sterile tungsten fissure bur mounted on a sterile dental hand piece (Fig. 8). This was performed under irrigation to avoid heating of the dental tissues. The pulp tissue was then carefully isolated with a sterile excavator and an endodontic file and minced in a sterile petri dish with a sterile scalpel surgical blade. It is worth noting that to protect the pulp tissue from drying out it was kept wet at all times with a solution of 0.1M phosphate

buffered saline (PBS) (Appendix C) (Fig. 9). The squashed tissue was then immersed into a digestive solution of 3mg/mL of collagenase type I (Millipore, Temecula CA, USA) and 4mg/mL of dispase (Sigma-Aldrich, St Louis Missouri, USA) (Appendix C) at 37°C, in a water bath and under gentle shaking for 1 hour and 30 minutes to facilitate pulp tissue dissolution.

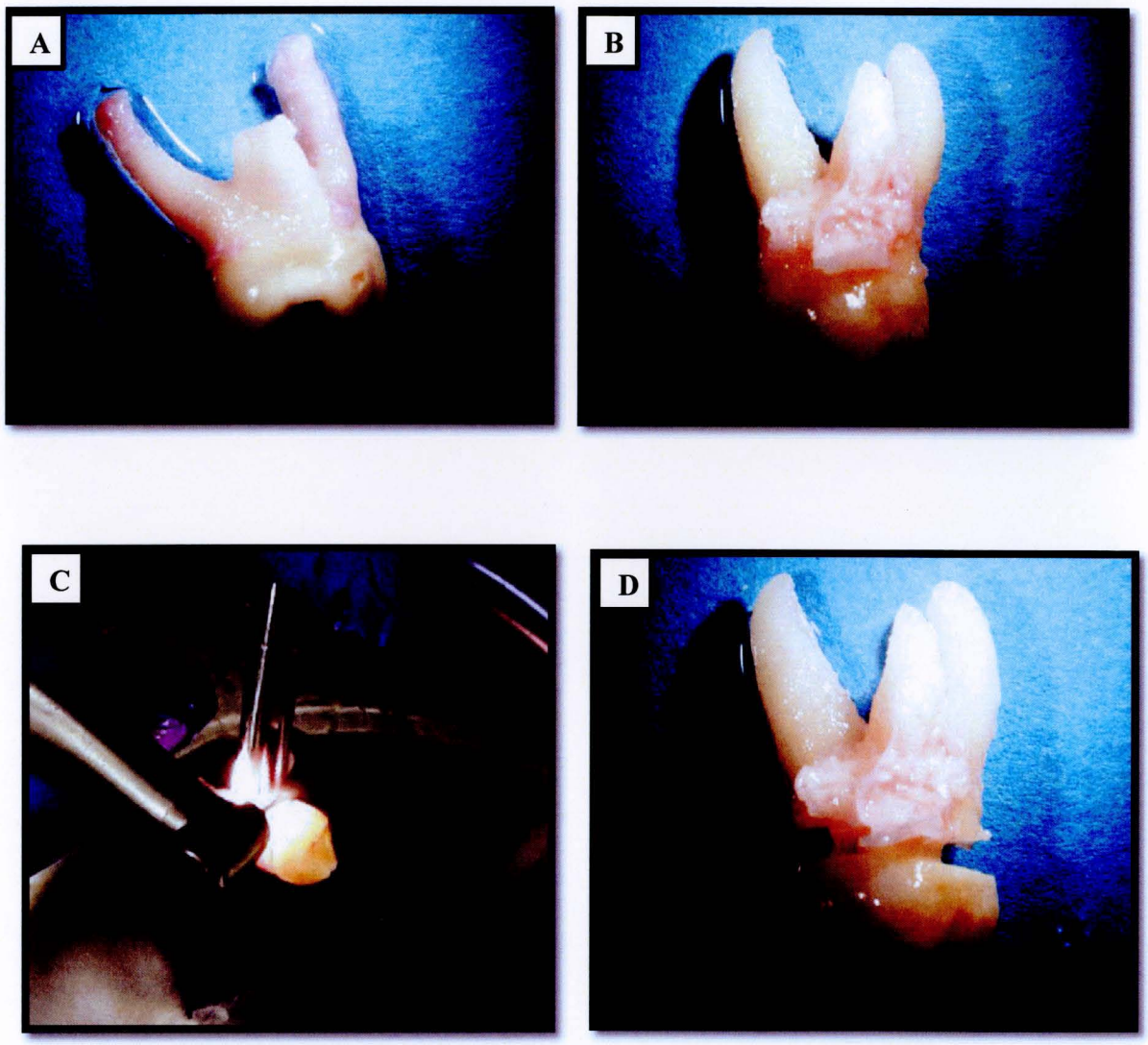


Figure 8: Representative photographs of teeth from the study sample: extracted deciduous tooth (**A**), permanent tooth (**B**), extracted deciduous tooth spilt using the bur(**C**) and permanent tooth cut at the cementoenamel junction (**D**).

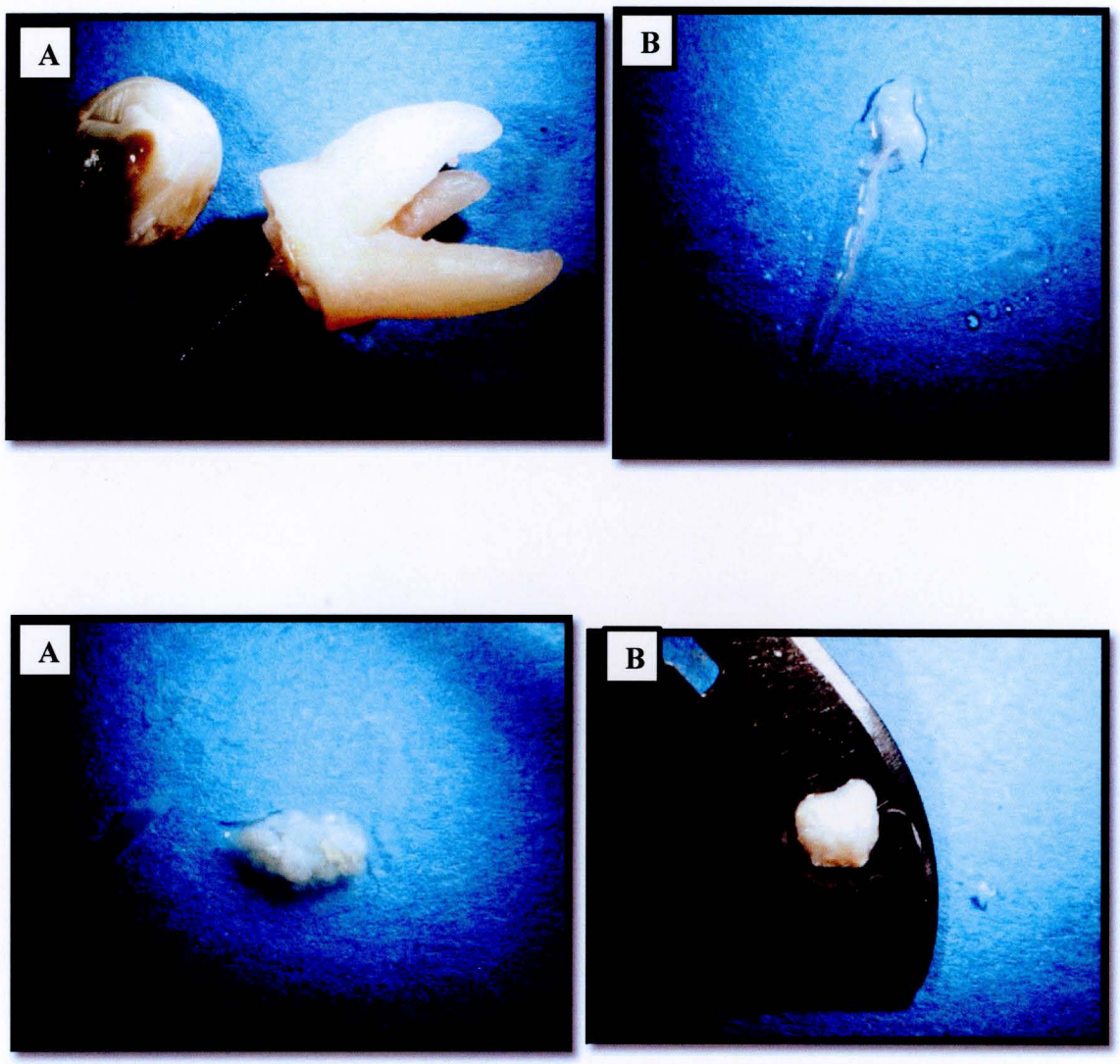


Figure 9: Representative photographs of the dental pulp tissue extraction with an endodontic file (**A**), the entire extracted dental pulp tissue (**B**), the minced dental pulp tissue, in a petri dish (**C**) and collected on the sterile scalpel surgical blade (**D**).

2.3.3. Pulp tissue culture

After the enzymatic digestion, the cell suspension was centrifuged in PBS at 400 x g for 10 minutes, filtered with 70 µm Falcon cell strainers and resuspended in a clonogenic medium composed of alpha-Modified Eagle's Medium (Lonza, Verviers, Belgium), supplemented with the following: 20% Foetal Bovine Serum (FBS) (Lonza, South Africa) 100 µl ascorbic acid 2-phosphate (Sigma-Aldrich, St Louis Missouri, USA), 2 mM L-glutamine (Lonza, Verviers, Belgium), 100 U/ml penicillin, and 100 µg/ml streptomycin (Lonza, Walkerville, USA), 250 µg/ml fungizone (Flow Laboratories, UK) and 5×10^{-5} M β-mercaptoethanol (Calbiochem, Darmstadt, Germany) (Appendix C). The cells were then cultured in 25 ml Nunc® flasks at 37°C in a 5% CO_2 humidified incubator (Heraeus Hera Cell 150). At this stage, the cells were presumed to be at passage 0 (P0).

After 3 days of culture, the non-adherent cells and the remnants of pulp tissue were washed with PBS and a fresh clonogenic growth medium was added. At days 7, 14 and 22 of culture, the primary expanded cells were enzymatically treated and passaged using Accutase (Sigma-Aldrich, St Louis Missouri, USA) in phosphate buffered saline. The use of accutase was preferred to trypsin-EDTA in order to avoid the occurrence of auto fluorescence of the cells during the flow cytometric analysis. The cultured medium was poured out of the flask, replaced by PBS to wash the cells twice. The cells were then incubated for 3 minutes in 2 ml of accutase.

Afterwards, the cells were rinsed twice with PBS and then centrifuged at 1200 rpm for 5 minutes. The cell pellet was resuspended in a wash buffer (PBS with 1% FBS) to quench the enzyme and filtered through a 70µm cell strainer and one group of the cell colonies were stained with trypan blue (BDH Chemical, England) to assess cell viability. This test

is based on the principle that viable cells possess intact membranes that are not permeable to trypan blue dye. Dead cells on the other hand, do have perforated cell membranes that incorporate trypan blue. 50 µl of cell suspension was incubated with 1 ml trypan blue solution for 1 minute at room temperature. Thereafter, viable and dead cells were counted using a haemocytometer chamber (Bright-Line® American Optical Company) under a light microscope at ×10 magnification. A cell count grid was used and the number of unstained cells in the five indicated blocks was obtained and averaged. The number of cells per millimetre was first ascertained and then the total percentage of viable cells was obtained using the following formula (Appendix B):

$$\text{Number of cells/ml} = \text{average value} \times \text{dilution factor} \times 10^4$$

$$\text{Total percentage of viable cells} = \text{number of viable cells (/ml)/ number of dead cells (/ml)} \times 100$$

The second group of expanded cells was stained for the flow cytometry analysis and the last group was transferred into a 75ml culture flask and replaced into the incubator to allow the cells to further expand and proliferate. This enzymatic detaching procedure was performed at days 7, 14 and 21 and the cells were considered to be at passage 1 (P1), passage 2 (P2) and passage 3 (P3) respectively.

2.4. Flow cytometry analysis

2.4.1. Flow cytometry requirements on mesenchymal stem cells

In 2006, the International Society for Cellular Therapy (ISCT) proposed the term "multipotent mesenchymal stromal cells" and postulated a set of standards to define human mesenchymal stem cells for both laboratory-based scientific investigations and for pre-

clinical studies (Dominici *et al.* 2006). It was decreed that in order to unequivocally establish the phenotype of mesenchymal stem cells, three minimum criteria ought to be met: the adherence to plastic culture dishes, the multipotent differentiation potential and the expression of specific surface antigens or clusters of differentiation (CD) (Dominici *et al.* 2006). These markers which define the mesenchymal stem cells surface and their internal molecules are CD73, CD90 and CD105, as measured by flow cytometry (Dominici *et al.* 2006). Furthermore, the cells must lack the expression of specific haematopoietic stem cell markers such as CD14, CD34 and CD45 (Dominici *et al.* 2006). Additionally, it has been demonstrated that dental stem cells also display a specific markers profile similar to those of mesenchymal stem cells (Gronthos *et al.* 2002; Foudah *et al.* 2013).

2.4.2. Antibodies selection and gating strategy

In an attempt to verify the mesenchymal stem cell identify and to ascertain the purity of the isolated cells, a negative and a positive selection was adopted for this multicolour flow cytometry experiment. A specific gating strategy was developed to evaluate the phenotype of culture-expanded cells obtained from the dental pulp of both deciduous and permanent teeth. To this end, the analysis panel had to include, in the negative selection, CD45 and CD14 in order to positively exclude cells of haematopoietic and monocytic lineage respectively. This allowed the positive discrimination of the haematopoietic stem cells, as the remaining unstained cells represented the population to gate in order to identify the cells of interest. This gating strategy also ought to include, in the positive selection, the known mesenchymal stem cell markers: CD90 was thus selected to stain for non-haematopoietic lineage, followed by CD29 and CD44 to select for dental stem cell lineage. Finally, CD105 was chosen for the $CD44^+$ and $CD29^+$ cells to ascertain further mesenchymal stem cell lineage.

For this 6-colour flow cytometry analysis, the BD™ Biosciences LSRFortessa flow cytometer was used. Flow cytometers operate by focussing a laser beam onto a sample of cells that are hydrodynamically focused into a unicellular flow. The cells are individually detected and illuminated as they pass through the laser beam and the signal from each interrogated cell is directed to the selected optical detectors. Photomultiplier tubes then detect the amount of forward and side scatter from each cell. The size of the cell will determine the quantity of light that is scattered in the forward direction and the internal complexity and granularity of the cell will determine the side scatter. Any fluorescent molecules such as the conjugated fluorochrome antibody that is present in the cell or on the cell membrane will fluoresce, as the cell is illuminated by the laser beam. The intensity of emitted light depends on the peak absorbance wavelength determined by the nature on the fluorochrome antibody.

It is important to state that throughout this flow cytometry analysis, monoclonal antibodies were preferred as, since they originate from a single clone, they are more specific and exhibit low cross-reactivity. Furthermore, for each antibody, titration was performed to determine the optimal antibody concentration as well as the position of the negatively stained population on the axis (Appendix E). Finally, the specific compensation for the procedure was conducted to set the lowest voltage that will give the primary fluorescence signals in each channel (Appendix F). This was always performed prior to the data collection with the flow cytometer in order to avoid any fluorescence spill-over across channels.

2.4.3. Sample preparation for flow cytometry

At each designated enzymatic passage (P1, P2 and P3) the flow cytometry analysis was performed with the semi-confluent and confluent stages observed at P2 and P3. The number of cells that were routinely recovered was approximately 1 x 10^5 per tooth. The cells treated with accutase were rinsed twice in 0.1M phosphate-buffered saline (PBS), and then resuspended in a wash of blocking buffer consisting of PBS containing 1% foetal bovine serum (FBS) (Lonza, South Africa). Samples were then assayed at a density of 1 x 10^5 cells/ml, in a 4 ml round bottom propylene tube. They were incubated at room temperature and in the dark, for approximately 20 minutes and stained with the fluorescence-conjugated monoclonal antibodies at the concentrations established during the titration process. The determined dilutions in 90 µl of wash buffer were the following: 2.5 µl of CD45 in (CD45 V450 mouse anti-human BD Horizon ™, Sigma-Aldrich, St Louis Missouri, USA), 1.25 µl of CD14 (CD14 APC-H7 mouse anti-human BD Pharmingen™, San Diego CA, USA), 2.5 µl of CD29 (CD29 PE anti-human BioLegend, San Diego CA, USA), 1.25 µl of CD44 (CD44 FITC anti-human BioLegend, San Diego CA, USA), 1.25 µl of CD90 (CD90 PE-Cy™ mouse anti-human BD Pharmingen™, San Diego CA, USA) and 2.5 µl of CD105 (CD105 APC anti-human BioLegend, San Diego CA, USA).

After the incubation period, the cell suspensions were then washed with 1 ml of wash buffer solution added to the tube and then centrifuged twice at 400×*g* for 5 minutes. The supernatants were aspirated and the cell pellets were fixed in 200 µl of 1% paraformaldehyde for 10 minutes at room temperature. 1 ml of PBS was then added to the cell suspensions that were then rinsed by centrifugation at 400×*g* for 5 minutes. Following

this, the decantation process was again performed by removing the supernatant and resuspending the resulting cell pellets in 1 ml of PBS for the flow cytometry analysis. The control cells included the unstained cells that were transferred in a 4 ml round-bottom propylene tube, washed in 1.ml of PBS and centrifuged at 200 × *g* for 10 minutes, then fixed in 200 µl 1% paraformaldehyde for 10 minutes at room temperature. Following the fixation, the cell suspensions were rinsed in 1 ml of PBS and centrifuged again at 200 × *g* for 10 minutes. The supernatant was removed and 1 ml of PBS was added to the suspension for the subsequent analysis by the flow cytometer.

As previously mentioned, approximately one hundred thousand cells were acquired and recorded by the flow cytometer for each analysis. The negative selection was first performed and fluorochrome conjugated monoclonal antibodies against haematopoietic cell markers CD45 and CD14 were used. This was then followed by the positive selection of mesenchymal stem cells using the established markers: fluorochrome conjugated monoclonal antibodies against CD29, CD44, and CD105. The data was obtained with BD FACDiva software and was then analysed using FlowJo software (v9.4.11 TreeStar Inc) following the specific developed gating strategy.

2.5. Neural induction

2.5.1. Neural markers in dental stem cells

It has been demonstrated that human dental pulp stem cells are able to express neural markers such as nestin, doublecortin (DCX) and neuN due to the migration of neural crest cells into the developing tooth germ (Arthur *et al.* 2008, Gervois *et al.* 2015). It has also been shown that human DPSCs secrete neurotrophic growth factors such as brain-derived neurotrophic factor (BDNF) (Martens *et al.* 2012), as well as angiogenic factors such as

vascular endothelial growth factor (VEGF) and other proangiogenic growth factors such as platelet-derived growth factors (PDGF) (Bronckaers *et al.* 2013) suggesting that hDPSCs can adequately sustain neuronal cells and this was demonstrated both *in vitro* but also in various *in vivo* following transplantation of hDPSCs in experimental animals (Sakai *et al.* 2012). In this experiment, only the secretion of doublecortin and nestin was investigated.

2.5.2. Neural differentiation

After the flow cytometry analysis, the now phenotyped DPSCs and SHEDs were seeded in 6-well plates at a concentration of 2×10^3 cells per ml of the neuroinductive medium: Neurobasal™ A Media (Gibco®, UK) supplemented with B27® Supplement XenoFree (Gibco®, UK), 50 U/ml penicillin, 50 µg/ml streptomycin (Lonza, Walkerville, USA) 20 ng/ml epidermal growth factor (EGF) (Sigma-Aldrich, St Louis Missouri, USA), 40 ng/ml basic fibroblast growth factor (FGF) (Sigma-Aldrich, St Louis Missouri, USA) (Appendix C). The cells were incubated at 37°C in a 5% CO_2 atmosphere in an air humidified incubator for 7 days. The cells were carefully washed and the medium changed every 3 days: the free-floating cells were collected, rinsed with 0.1M phosphate buffered saline (PBS) and resuspended in a new neurogenic medium. The cell colonies, referred to as neurospheres, were kept floating in the culture medium and their size closely monitored, so as not to interfere with the cell viability and the differentiation capacity (Gervois *et al.* 2014).

On day 7, the cells were again collected, washed and resuspended in a new medium consisting of Dulbecco's Modified Eagle's medium (DMEM) (Lonza, Verviers, Belgium), supplemented with Insulin-Transferrin-Selenium-X (Gibco®, UK), 50 U/ml penicillin, 50 µg/ml streptomycin (Lonza, Walkerville, USA) and 40 ng/ml basic fibroblast growth factor

(FGF) (Sigma-Aldrich, St Louis Missouri, USA) (Appendix C), and the cells were cultured for an additional 7 days. The experimental cells to be used for immunocytochemistry were seeded in 6-well plates at a concentration of 2×10^3 cells per ml of the neural progenitor basal medium. The control cells were seeded at the same concentration in the clonogenic culture medium.

On day 14, *all-trans* retinoic acid (ATRA) (Sigma-Aldrich, St Louis Missouri, USA) was added to the medium. Cells were kept under neurogenic maturation-promoting conditions until confluency was reached at approximately day 21. Again, the experimental cells to be used for immunocytochemistry were seeded in coated 6-well plates at a concentration of 2×10^3 cells per ml of the neuroinductive medium. The control cells were seeded at the same concentration in the clonogenic culture medium. After 7, 14 and 21 days of neural induction, corresponding to enzymatic passages 4 (P4), 5 (P5) and 6 (P6) respectively, the cells were processed for the immunocytochemistry analysis.

2.6. Immunocytochemistry analysis

2.6.1. Ki-67

Ki-67 is a non-histone nuclear antigen (395 kDa) with its gene located on chromosome 10. It is exclusively synthesized in the proliferative phases of the cell cycle (late G1, S, G2 and M phases) (Fisher *et al.* 2002; Potemski *et al.* 2006; Faratzis *et al.* 2009). Ki-67 is not exhibited during the resting phase of the cell, which renders it a reliable nuclear marker for the determination of the proliferative activity of a cell. Indeed, when it is conjugated with the specific mononuclear antibody to Ki-67, it is recognized and only the cells undergoing active division are stained (Fisher *et al.* 2002).

Before the culture of the neural induced dental stem cells, a 6-well culture plate was prepared by placing a sterilized glass cover slip (22mm X 22mm) in each of its wells. The cover slips were washed with sterile PBS (Appendix D) then the cells were seeded on to them at a concentration of 1×10^6 cells per ml. Afterwards, the cells were incubated at 37° C in a 5% CO_2 atmosphere in an air humidified incubator until confluency was reached.

At days 7, 14 and 21, the immunocytochemistry experiments were performed. The designated reagents were added to the cells in each of the wells, following which the fixative procedure with 4% paraformaldehyde was performed for 30 minutes at room temperature and under the laminar flow hood. After the fixing, the 4% paraformaldehyde was removed and each well was rinsed once with Tris-Buffered Saline (TBS) (Appendix D). The buffer was then thoroughly removed and replaced in each well, with 1 ml of the blocking buffer (TBS 5.5% goat serum) for 1 hour. The cells were then rinsed off with 1 ml of TBS and finally incubated with a mouse monoclonal anti Ki-67 (Sigma, South Africa) overnight and at 4°C.

In the negative controls specimens, TBS alone was added while the antibody was omitted. The following day, each well was thoroughly washed with 1 ml TBS/Triton twice for 5 minutes then once with TBS and finally incubated with a polyclonal goat anti-mouse biotinylated secondary antibody (Dako, South Africa) diluted in TBS 3% bovine serum albumin for 1 hour at room temperature.

Subsequent to this, the cells were incubated for 30 minutes in 0.6% hydrogen peroxide in TBS at room temperature. After the elapsed time, the solution was aspirated, and the cells were once again rinsed three times with 1ml TBS/Triton for 5 minutes before the

incubation with 800µl Vectastain ABC Kit (Dako, South Africa) for 1 hour at room temperature. The solution was completely aspirated, and the cells washed twice for 5 minutes with 1ml TBS. Then, 1 ml of diaminobenzidine tetrahydrochloride (DAB) reagent (Sigma, South Africa) (Appendix D) was added into each well. This reaction was carefully monitored using a microscope in order to reduce any background effect. The reaction was then terminated by adding 1 ml of sterile distilled water into each well. This solution was aspirated and the cells washed once again with 1 ml of sterile distilled water. The cover slips were dipped twice each in a graded series of alcohol and then in two changes of xylene before being mounted with Entellan (Merck, South Africa) on to glass slides (1mm X 76mm X 26mm). After reaction with Diamino benzidine reagent (DAB), the Ki-67 immunolabelled cells were counted in all sections on the Axiovision light microscope using X63 lens. The Ki-67 positive proliferative cells were counted at days 7, 14 and 22 after neural induction.

2.6.2. Doublecortin

Doublecortin (DCX) is a microtubule binding phosphoprotein (40kDa) which is expressed in actively proliferating and migrating immature neurons in embryonic and adult neurogenesis structures (Brown *et al.* 2003; Bohlen *et al.* 2007). It is associated with the migration of neuronal precursor' cells. DCX is encoded by a gene located on the X chromosome and was originally described in lissencephaly, one of the most severe malformations of the human cerebral cortex (des Portes *et al.* 1998; Gleeson *et al.* 1998). The DCX immunolabelling procedures were performed on free-floating sections of neural induced DPCSs and SHEDs.

Sections were firstly washed 3 times for 10 minutes in PBS, rinsed once with TBST for 5 minutes under gentle shaking and at room temperature. They were then transferred into an endogenous peroxidase inhibitor solution (49.2% 0.1M phosphate buffer, 49.2% methanol, 1.6% of 30% H_2O_2) for 30 minutes. After 3 rinses in 0.1M phosphate buffer for 10 minutes, the sections were treated with a blocking solution (3% normal rabbit serum, 2% bovine serum albumin, 0.25% Triton X-100 in 0.1M phosphate buffer) for 2 hours under gentle shaking at room temperature to avoid non-specific binding. The tissues were then transferred into the primary antibody solution (1:300, goat anti-DCX) (Biotechnology, Santa Cruz, USA) in the blocking buffer solution of TBST and incubated for 48 hours at 4°C under gentle shaking.

Following the incubation period, the tissues were removed from the refrigerator, placed on a shaker to equilibrate at room temperature and then washed 3 times in 0.1M phosphate buffer for 10 minutes and under gentle shaking before the incubation in the secondary antibody solution consisting of: 1:1000 dilution of biotinylated anti-goat IgG (BA-5000, Vector Laboratories, Burlingame, CA, USA) in 3% normal rabbit serum and 2% bovine serum albumin in 0.1M phosphate buffer for 2 hours at room temperature and under gentle shaking. Sections were then subjected to a 10 minutes rinse in 0.1M phosphate buffer 3 times and under gentle shaking. After the rinse, the sections were incubated in an Avidin-Biotin solution consisting of 1:125 A reactive and 1:125 B reactive (Vector Laboratories, Burlingame, CA, USA) in 0.1M phosphate buffer for 1 hour. The sections were once again washed into 0.1M phosphate buffer 3 times and for 10 minutes before being transferred for 5 minutes into a solution of 0.05% diaminobenzidine (DAB) in 0.1M phosphate. Each 1 ml vial of the solution was supplemented with 3.3 µl of 30 % H_2O_2, then mixed and allowed to develop under a low power stereo microscope until the

appearance of definite nuclear staining. The reaction was subsequently arrested by 3 final 10 minutes rinses of the sections in 0.1M phosphate buffer. The tissues were then mounted on 0.5% gelatinized slides, air-dried overnight, dehydrated in an ascending graded series of alcohols, cleared in xylene and then coverslipped with Depex. In the control sections, the same protocol was repeated but the primary antibody was omitted. After the reaction, the DCX immunolabelled cells were qualitatively analysed under the light microscope and quantitatively analysed using the click counter at days 7, 14 and 21 after neural induction.

2.6.3. Nestin

Nestin is a high molecular weight (240kDa) protein with its gene located on chromosome 1 (Guerette *et al.* 2007). Nestin belongs to the class VI of intermediate filaments of the cytoskeleton (Michalczyk *et al.* 2005; Guerette *et al.* 2007) and is exhibited in nervous, muscular, cardiac tissues and the neural crest cells and testis (About *et al.* 2000). In the central nervous system, nestin is mainly expressed at the early stages of development and is then replaced by neurofilaments and it is a neural-specific protein expressed in the neural stem cells (Bohlen *et al.* 2007).

The nestin immunolabelling procedures were performed on free-floating sections of neural induced DPCSs and SHEDs. Sections were firstly washed 3 times for 10 minutes in PBS, rinsed once with TBST for 5 minutes under gentle shaking and at room temperature. They were then transferred into an endogenous peroxidase inhibitor solution (49.2% 0.1M phosphate buffer, 49.2% methanol, 1.6% of 30% H_2O_2) for 30 minutes. After 3 rinses in 0.1M phosphate buffer for 10 minutes, the sections were treated with a blocking solution (3% normal rabbit serum, 2% bovine serum albumin, 0.25% Triton X-100 in 0.1M phosphate buffer) for 2 hours under gentle shaking at room temperature to avoid non-

specific binding. The tissues were then transferred into the primary antibody solution: 1:5000 nestin (Novocastra laboratory (NCL), Wetzlar, Germany) in the blocking buffer solution of TBST and incubated for 48 hours at 4°C under gentle shaking. Following the incubation period, the tissues were removed from the refrigerator, placed on a shaker to equilibrate at room temperature and then washed 3 times in 0.1M phosphate buffer for 10 minutes and under gentle shaking before the incubation in the secondary antibody solution consisting of: 1:1000 dilution of biotinylated anti-goat anti rabbit (Vector Laboratories, Burlingame, CA, USA) in 3% normal goat serum and 2% bovine serum albumin in TBST for 1 hour at room temperature and under gentle shaking. Sections were the subjected to a 10 minutes rinse in 0.1M phosphate buffer 3 times and under gentle shaking. After the rinse, the sections were incubated in an Avidin-Biotin solution consisting of 1:125 A reactive and 1:125 B reactive (Vector Laboratories, Burlingame, CA, USA) in 0.1M phosphate buffer for 1 hour. The sections were once again washed into 0.1M phosphate buffer 3 times and for 10 minutes before being transferred for 5 minutes into a solution of 0.05% diaminobenzidine (DAB) in 0.1M phosphate. Each 1 ml vial of the solution was supplemented with 3.3 µl of 30 % H_2O_2, then mixed and allowed to develop under a low power stereo microscope until the appearance of definite nuclear staining. The reaction was subsequently arrested by 3 final 10 minutes rinses of the sections in 0.1M phosphate buffer. The tissues were then mounted on 0.5% gelatinized slides, air-dried overnight, dehydrated in an ascending graded series of alcohols, cleared in xylene and then coverslipped with Depex. In the control sections, the same protocol was repeated but the primary antibody was omitted. After the reaction, the nestin immunolabelled cells were qualitatively analysed under the light microscope and quantitatively analysed in all sections on the Axiovision light microscope using X63 lens at day 7, 14 and 21 after neural induction.

CHAPTER 3

RESULTS

3.1. Cell culture

3.1.1. Descriptive analysis of culture-expanded cells

Cells from the pulp tissues of permanent and deciduous teeth were successfully isolated. The culture-expanded cells were examined under an Olympus CX 41 inverted phase contrast microscope at × 100, × 200 and × 400 magnifications. The results were always simultaneously noted in the two experimental groups. At day 1 of cell culture, the flasks were observed to ascertain that the cells were allowed to grow in a culture medium devoid of microbial contamination, and several floating, very small and round cells were seen in the two experimental groups (Fig. 10A). After 3 days of culture, the first signs of adherent clusters of cells were observed in each flask (Fig. 10B). The cells were exhibiting a less rounded and more elongated shape. Non-adherent floating cells and cell debris were also present in the culture medium and they were washed off using PBS.

After 7 days of culture, more colony forming units of cells (CFU) were noted in both deciduous (Fig. 11) and permanent culture flasks (Fig. 12); they were spindle-shaped fibroblast-like cells displaying the typical mesenchymal stem cell shape. Their adherence to the tissue culture flask was evident and they were expanding, although not showing cell to cell contact. In addition, very few isolated cobble-stone-like cell formations were observed in each population. After 14 days of culture, most of the cells extracted from the pulp of deciduous (Fig. 13) and permanent teeth (Fig. 14) became semi-confluent and exhibited limited cell to cell contact. Confluency was reached after 21 days of culture in

the permanent teeth (Fig. 16). However, some delay of 2 to 3 days in the culture-expanded cells extracted from the pulp of the deciduous teeth (Fig. 15) was observed.

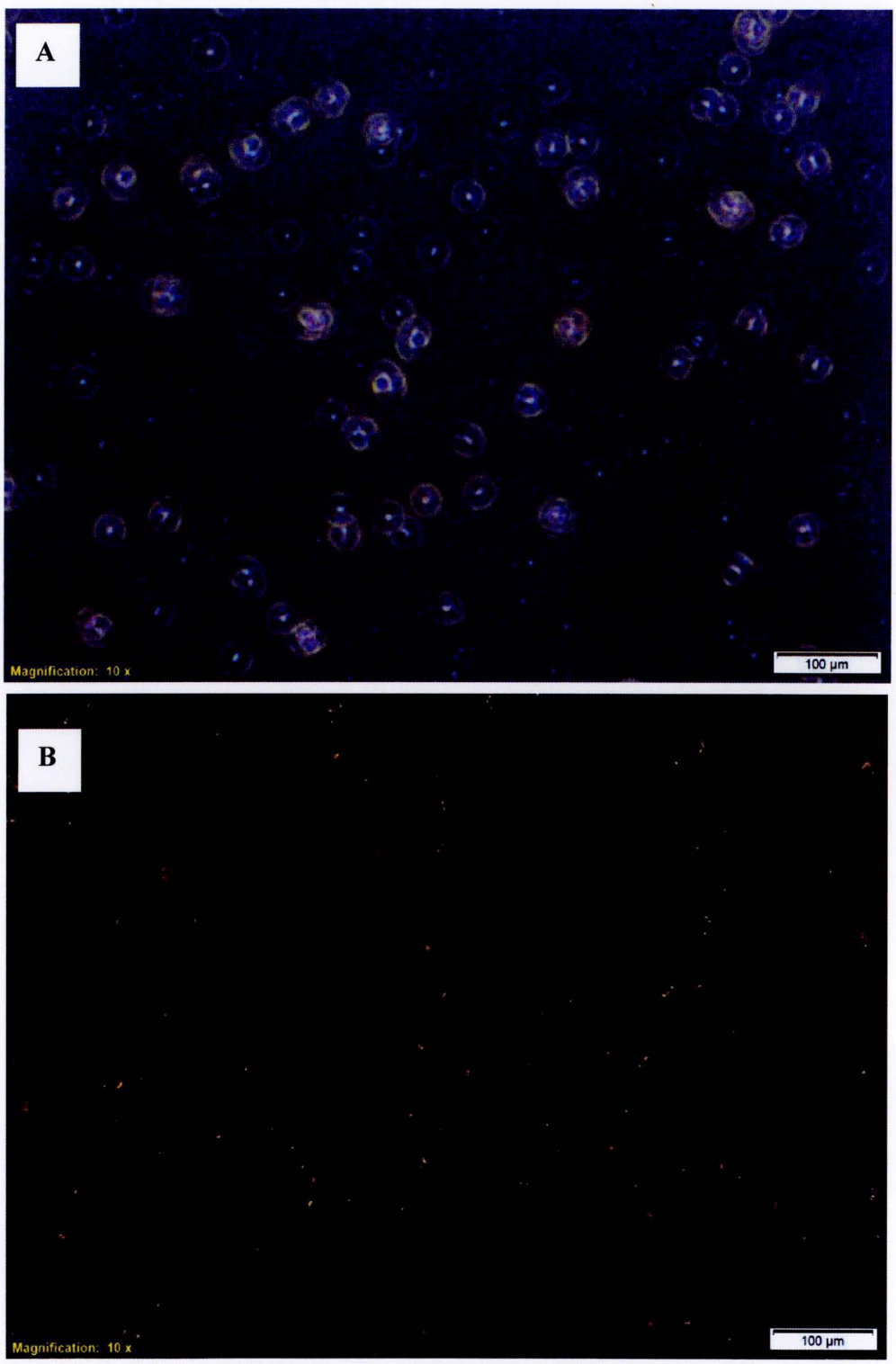


Figure 10: Representative photomicrographs of the cell culture observed in both **deciduous** and **permanent** teeth at day 1 (**A**) and day 3 (**B**) (×100).

DAY 7: DECIDUOUS TEETH

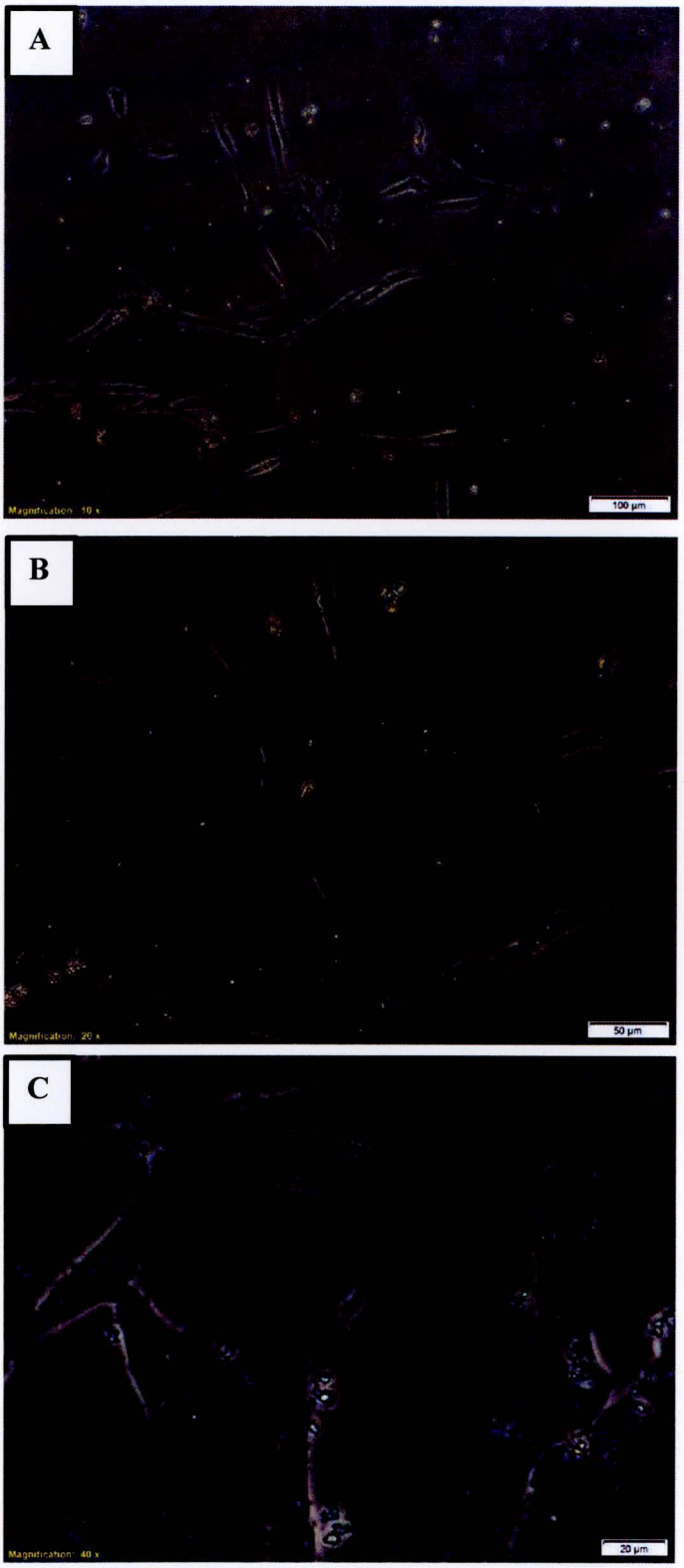


Figure 11: Representative photomicrographs of the cell culture in **deciduous** teeth at day 7, ×100 (**A**), ×200 (**B**) and ×400 (**C**).

DAY 7: PERMANENT TEETH

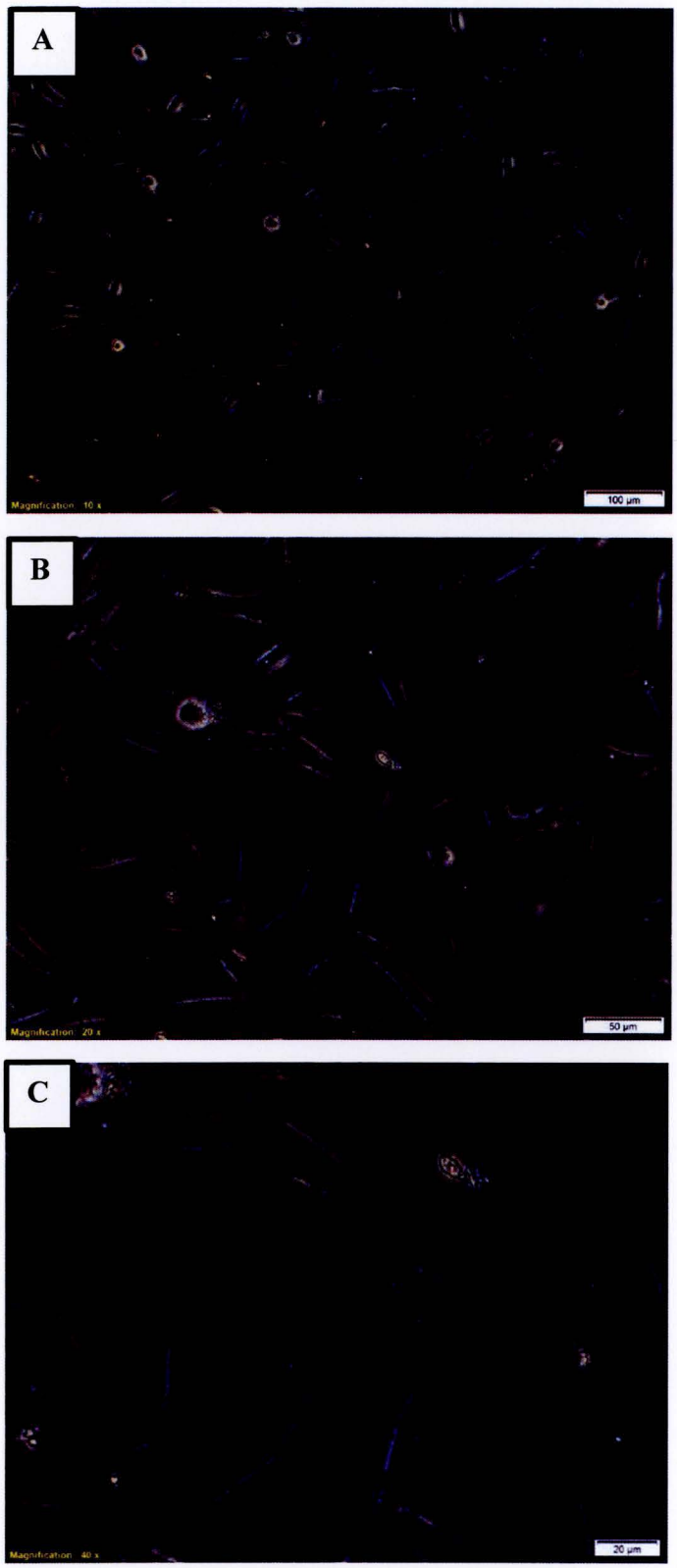


Figure 12: Representative photomicrographs of the cell culture in **permanent** teeth at day 7, ×100 (**A**), ×200 (**B**) and ×400 (**C**).

DAY 14: DECIDUOUS TEETH

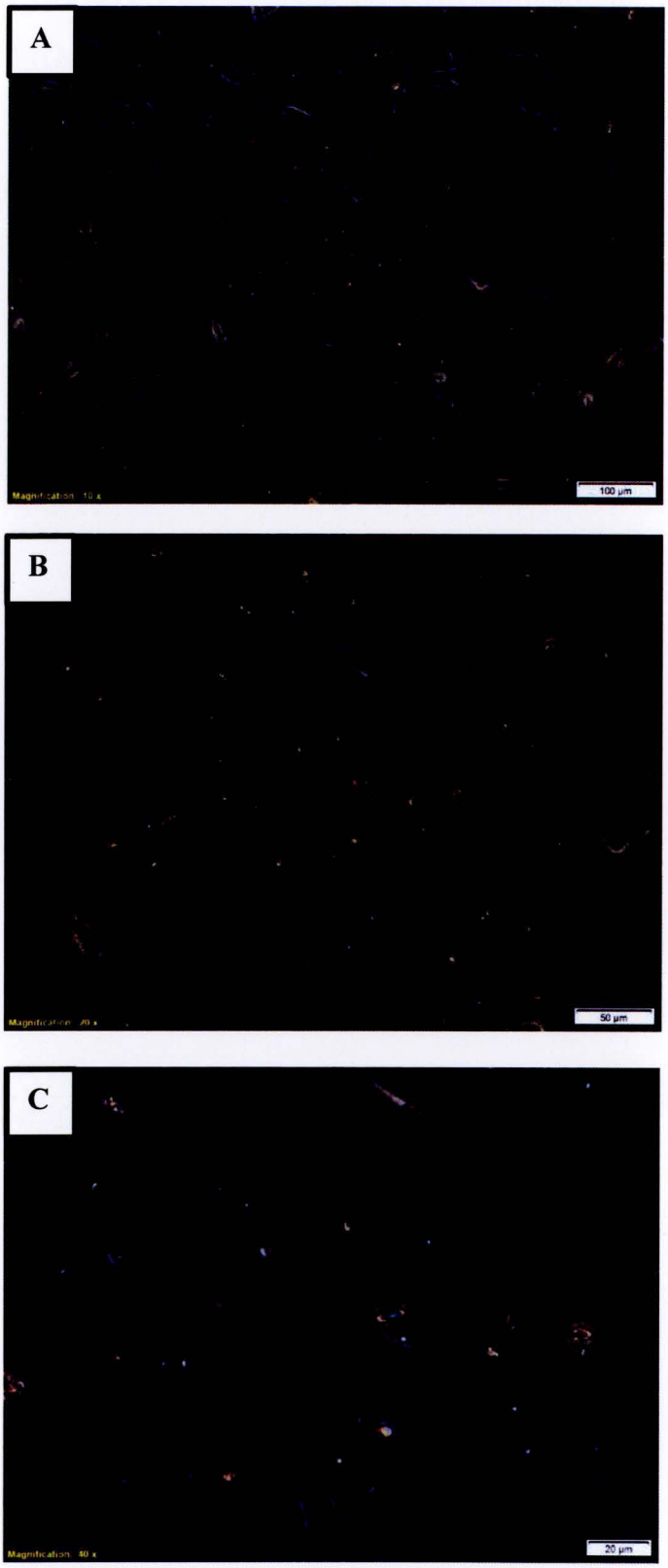


Figure 13: Representative photomicrographs of the cell culture in **deciduous** teeth at day 14, ×100 (**A**), ×200 (**B**) and ×400 (**C**).

DAY 14: PERMANENT TEETH

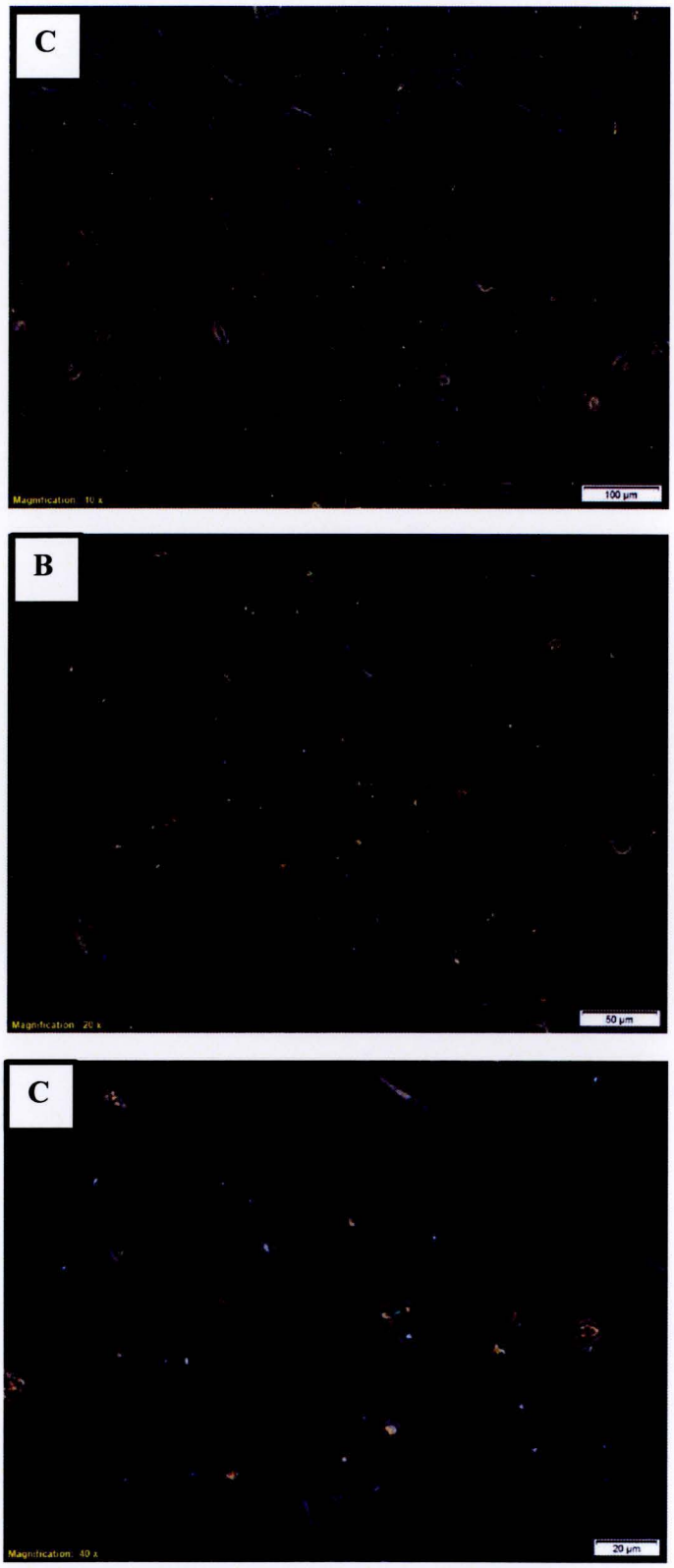


Figure 14: Representative photomicrographs of the cell culture in **permanent** teeth at day 14, ×100 (**A**), ×200 (**B**) and ×400 (**C**).

DAY 21: DECIDUOUS TEETH

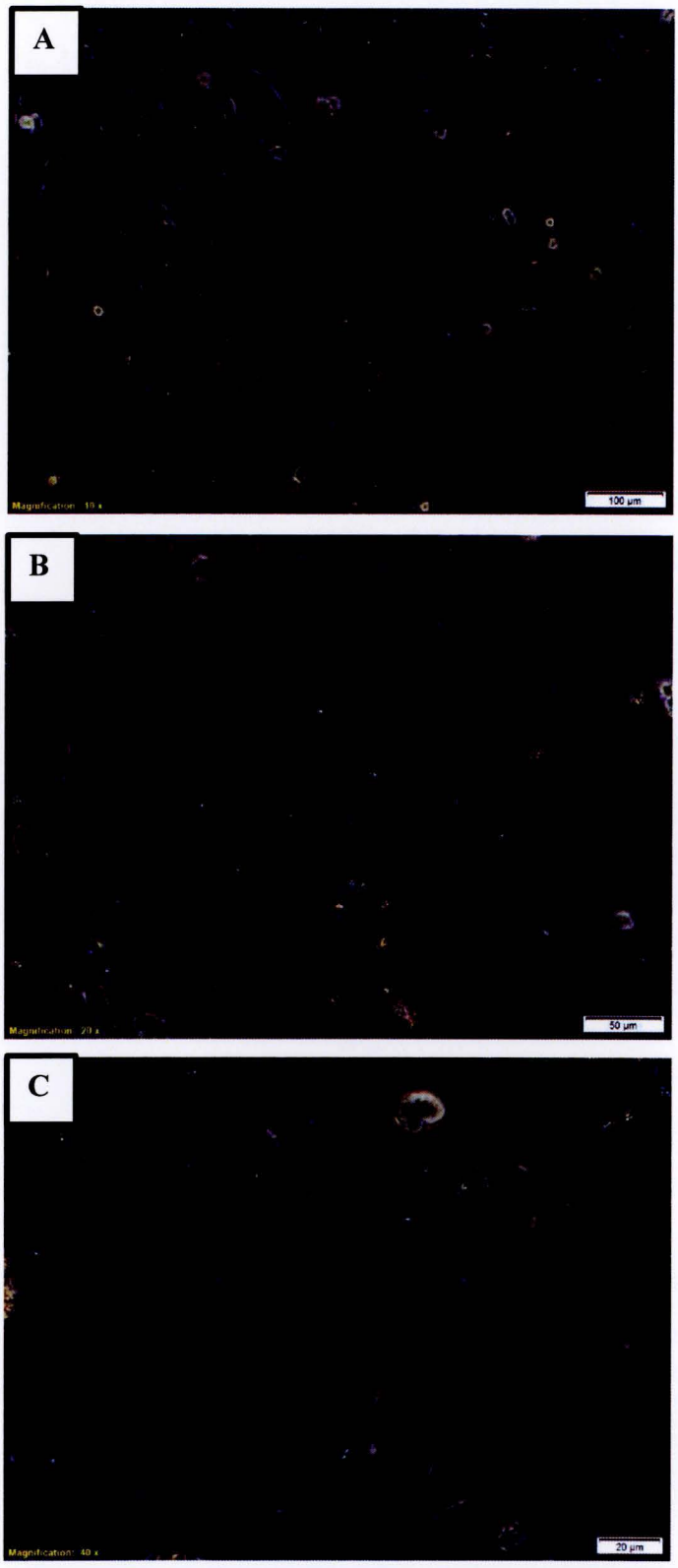


Figure 15: Representative photomicrographs of the cell culture in **deciduous** teeth at day 21, ×100 (**A**), ×200 (**B**) and ×400 (**C**).

DAY 21: PERMANENT TEETH

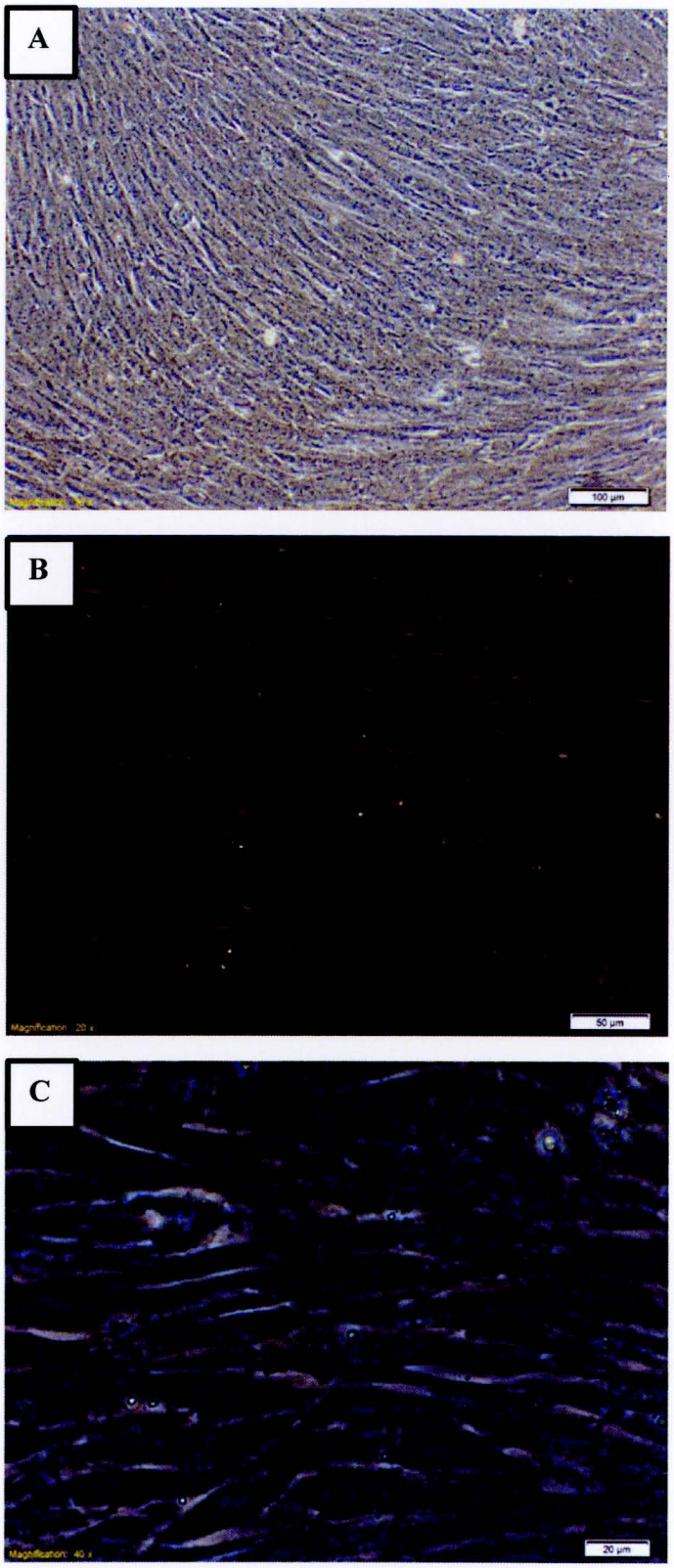


Figure 16: Representative photomicrographs of the cell culture in **permanent** teeth at day 21, ×100 (**A**), ×200 (**B**) and ×400 (**C**).

3.1.2. Quantitative analysis of culture-expanded cells

3.1.2.1. Percentage of viable culture-expanded cells

The descriptive analysis included the continuous variables of the percentage of viable culture-expanded cells, in both deciduous and permanent teeth, and was analysed and summarised by using the mean, standard deviation, median and interquartile range, and their distribution was illustrated with bar column graphs (Fig. 17). Furthermore, the relationship between the percentages of viable culture-expanded cells at days 7, 14 and 21 was determined by a one-way repeated analysis of variance (ANOVA).

The mean indices of the percentage of viable culture-expanded cells for both deciduous and permanent teeth were calculated at days 7, 14 and 21 and they are shown in Tables 1 and 2.

Table 1: Mean indices of the percentage of viable culture-expanded cells in **deciduous** teeth at days 7, 14 and 21.

Day	Number of teeth	Mean percentage of viable cells (SD)	Range
Day 7	30	73.4 (10.1)	48-89%
Day 14	30	63.3 (9.1)	42-81%
Day 21	30	54.4 (9.9)	35-75%

Table 2: Mean indices of the percentage of viable culture-expanded cells in **permanent** teeth at days 7, 14 and 21.

Day	Number of teeth	Mean percentage of viable cells (SD)	Range
Day 7	30	75.2 (9.9)	56-89%
Day 14	30	65.8 (10.5)	40-82%
Day 21	30	56.3 (11.0)	36-75%

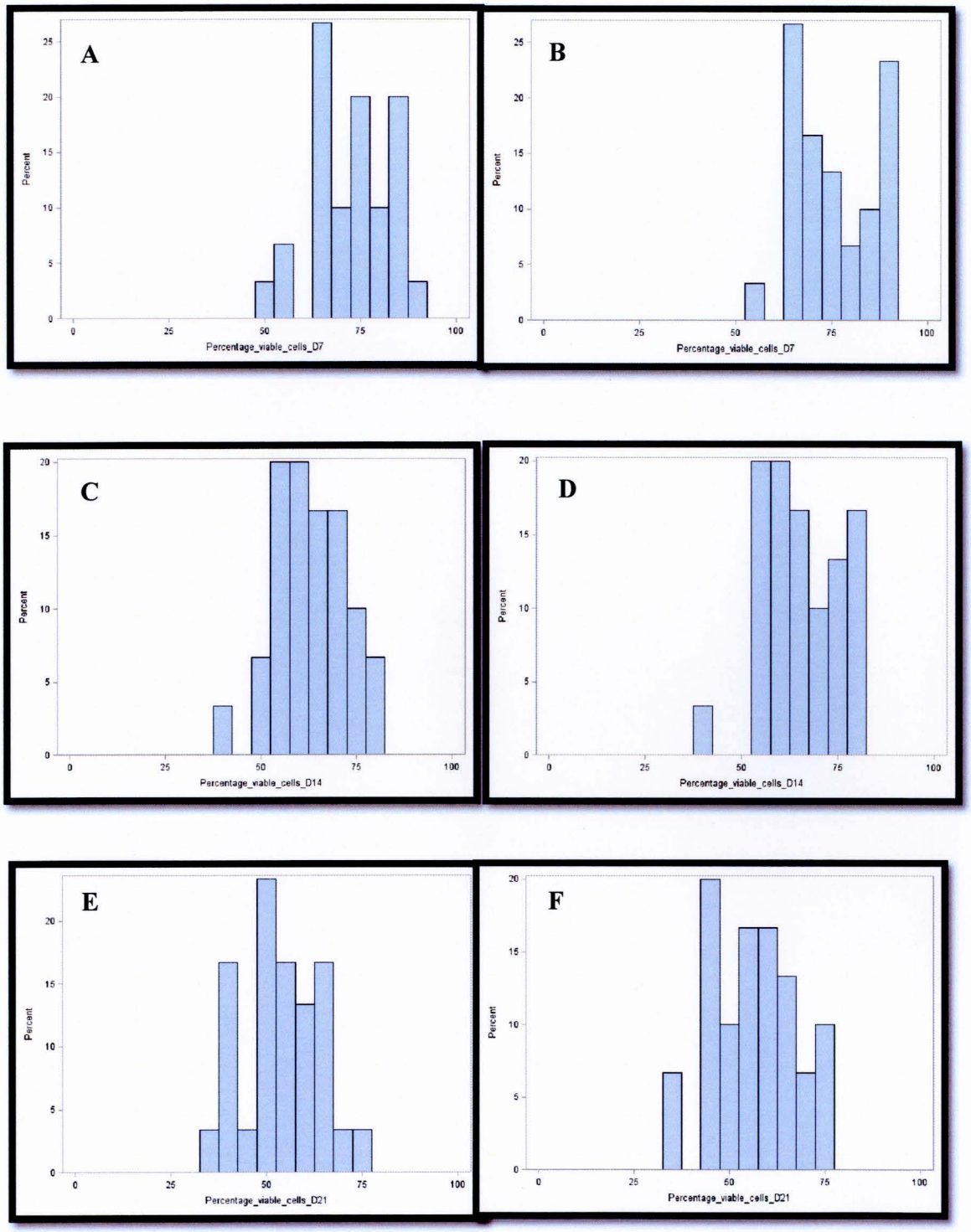


Figure 17: Representative histograms of the percentage of viable culture-expanded cells at day 7 for **deciduous** (**A**) and **permanent** (**B**) teeth, at day 14 for **deciduous** (**C**) and **permanent** (**D**) teeth and at day 21 for **deciduous** (**E**) and **permanent** (**F**) teeth. The percentage of cells within the total of 30 specimens, for each population, is represented in the y-axis.

3.1.2.2. Comparative analysis of viable culture-expanded cells

For each time point and in both populations, the mean percentage of viable culture-expanded cells were statistically compared using an analysis of variance (ANOVA) and they were all significantly different to each other ($p<0.0001$), as it is illustrated in Fig. 18.

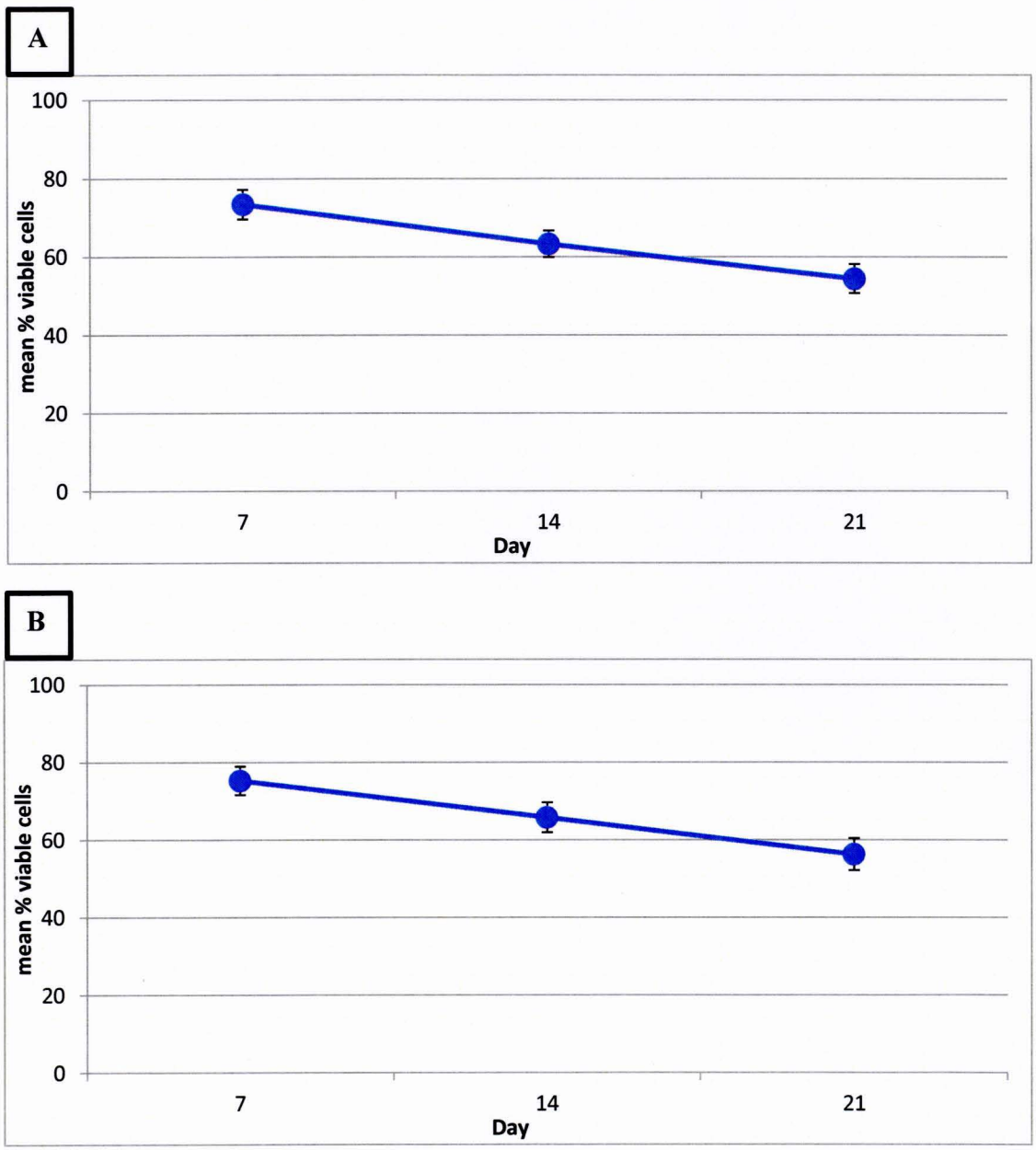


Figure 18: Representative graph of the comparison of the percentage of viable culture-expanded cells at days 7, 14 and 21, in **deciduous (A)** and **permanent (B)** teeth. The error bars denote the 95% confidence interval for the mean.

3.2. Flow cytometry

3.2.1. Descriptive flow cytometric analysis of culture-expanded populations

Flow cytometry analysis confirmed the $CD44^+$ $CD90^+$ CD105 and $CD29^+$ $CD90^+$ $CD105^+$ profile of the expanded cultured cells obtained from the dental pulp of permanent and deciduous teeth respectively. Furthermore, the $CD14^-$ $CD45^-$ profile was also established, thus confirming the absence or low percentage of haematopoietic stem cells. Cells exhibited slightly altered scattered properties possibly due to the persisting heterogeneity of the cells in the cultured pulp tissue, despite the enzymatic digestion in collagenase and dispase prior to the incubation. Thus, only those cells which exhibited the traditional side scatter (SSC) properties, associated with cell (DPSCs or SHEDs) granularity or internal complexity and forward scatter (FSC) properties, associated with cell size, were analysed further (Fig. 19A, Fig. 20A).

Following the flow cytometric interrogation, analysis of the co-expression of primarily CD29, CD90 and CD105 cell surface markers for the deciduous tooth and the co-expression of CD44, CD90 and CD105 cell surface markers for the permanent tooth was performed. In the both deciduous and permanent teeth, the first population obtained was designated $CD45^{bright+}$ $CD14^{bright+}$, and identified the cells of haematopoietic and monocytic origin (Fig. 19B, Fig. 20B). The percentage of these cells was 52.2% and 50.6% in both deciduous and permanent samples respectively (Fig. 21). The second flow cytometry event was gated on the remaining $CD45^-$ $CD14^-$ cells and demonstrated four distinct populations based on the total percentage of non-haematopoietic cells acquired (74.5% in both populations) (Fig. 19C, Fig. 20C). In the deciduous teeth, these populations were designated $CD44^{dim}$ $CD29^+$ $CD29^{bright+}$ (Fig. 19C), $CD105^+$ $CD29^{bright+}$ (Fig. 19D), $CD90^+$ $CD29^{bright}$ (Fig. 19E) and $CD105^+$ $CD90^+$ (Fig. 19F). In the permanent

teeth, the resulting populations were designated $CD44^{bright+}$ $CD29^{bright+}$ (Fig. 20C), $CD44^{bright+}$ $CD29^{dim}$ (Fig. 20C), $CD105^{intermediate+}$ $CD44^{bright+}$ (Fig. 20D), $CD90^{+}$ $CD44^{bright+}$ (Fig. 20E) and $CD90^{bright}$ $CD105^{bright}$ (Fig. 20F).

In the deciduous tooth sample, investigations into the $CD29^{bright+}$ population which comprised 77.2% (Fig. 19C) of the total population, revealed a negligible contaminating population of $CD29^{-}$ cells which nevertheless exhibited $CD90^{+}$ expression and $CD105^{intermediate+}$ expression (Fig. 19F). In the permanent tooth sample, investigations into the $CD44^{bright+}$ population which also dominated the total population (74.5%) (Fig. 20C) revealed a small contaminating population of $CD44^{-}$ cells which nevertheless also exhibited $CD90^{+}$ expression and $CD105^{intermediate+}$ expression (Fig. 20F). A relationship between CD44 and CD29 surface cell markers was noted as they were both exhibited, although in different percentages, in both types of tooth (Fig. 21). In the deciduous tooth, the $CD44^{dim}$ population (Fig. 21A), co-expressed $CD90^{dim}$ and $CD105^{dim}$ (Fig. 21C, Fig. 21D) and the dominating $CD29^{bright}$ population (Fig. 21A) expressed comparative levels of $CD90^{+}$ and $CD105^{intermediate}$ (Fig. 21C, Fig. 21D). In the permanent tooth, the $CD29^{dim}$ population (Fig. 21F), primarily co-expressed $CD90^{dim}$ (Fig. 21G) and the notably large $CD44^{bright}$ population (Fig. 21E) expressed comparative levels of $CD90^{+}$ $CD105^{intermediate}$ and $CD105^{+}$ (Fig. 21G, Fig. 21H). The flow cytometry analysis was repeated at day 7, 14 and 21 and each time, the results obtained analysed by the FlowJo software (v9.4.11 TreeStar Inc) were similar to the ones previously described.

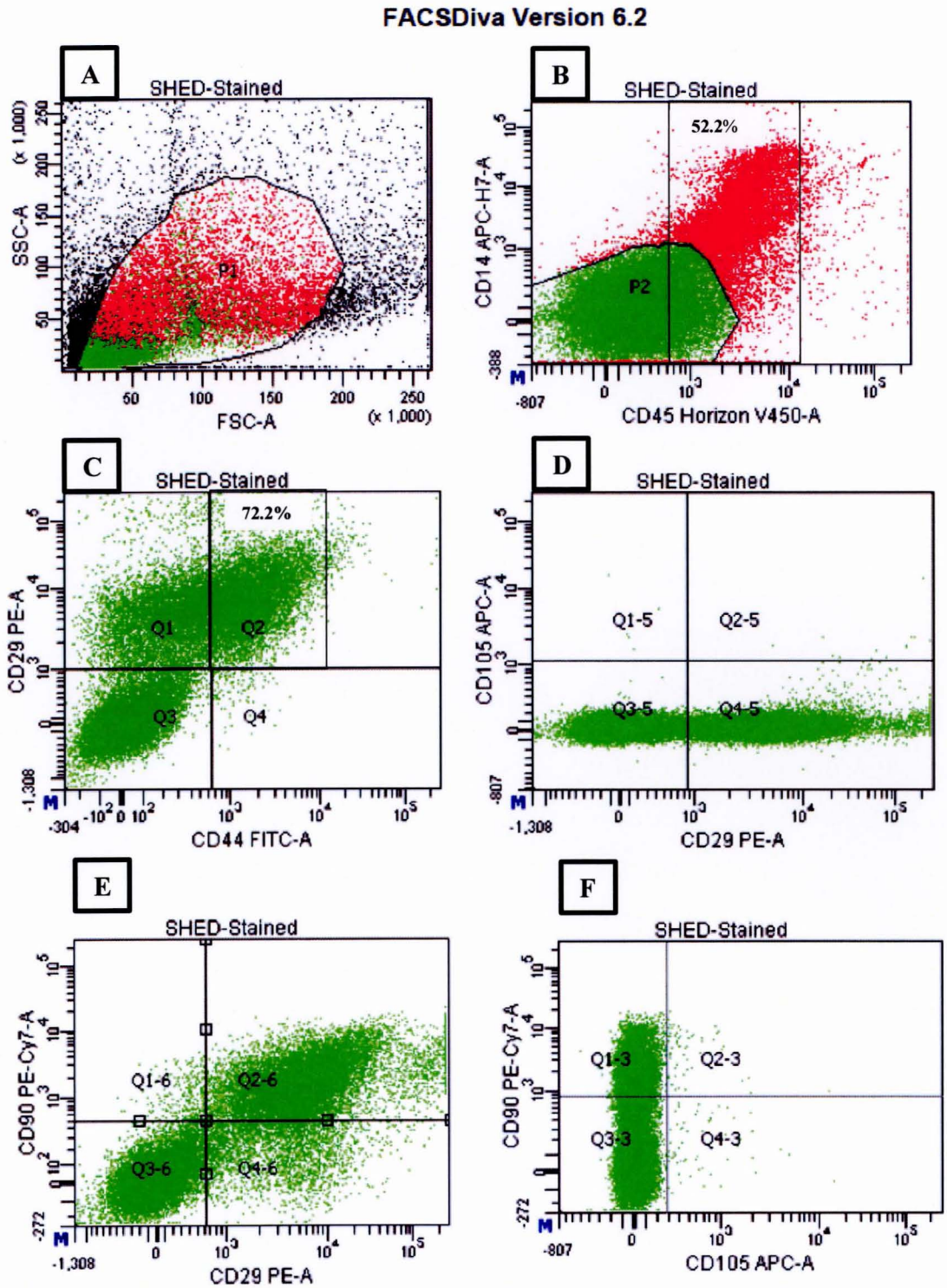


Figure 19: Representative Scatterplots illustrating flow cytometric analysis of the **CD29** cells population.

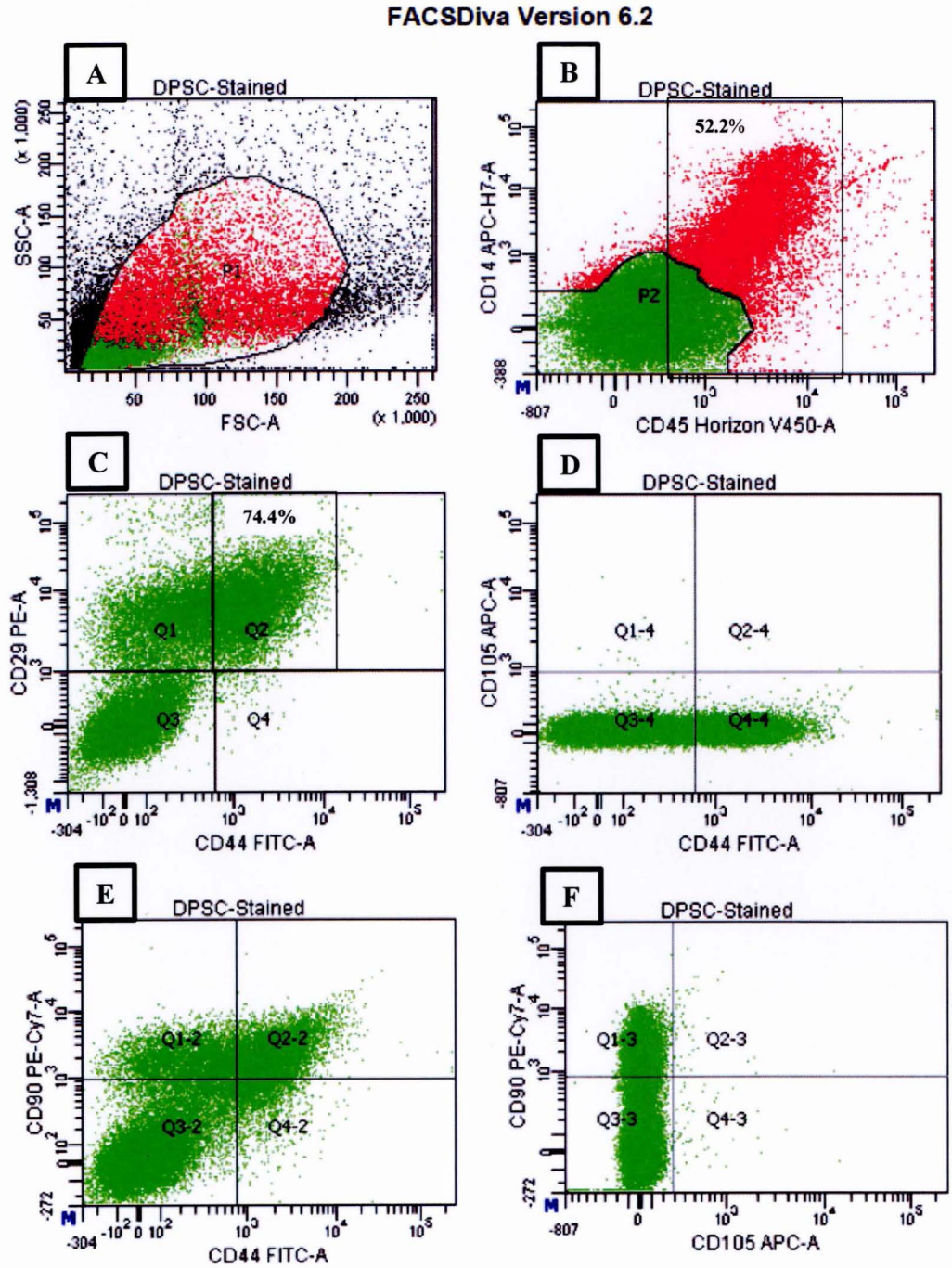


Figure 20: Representative Scatterplots illustrating flow cytometric analysis of the **CD44** cells population.

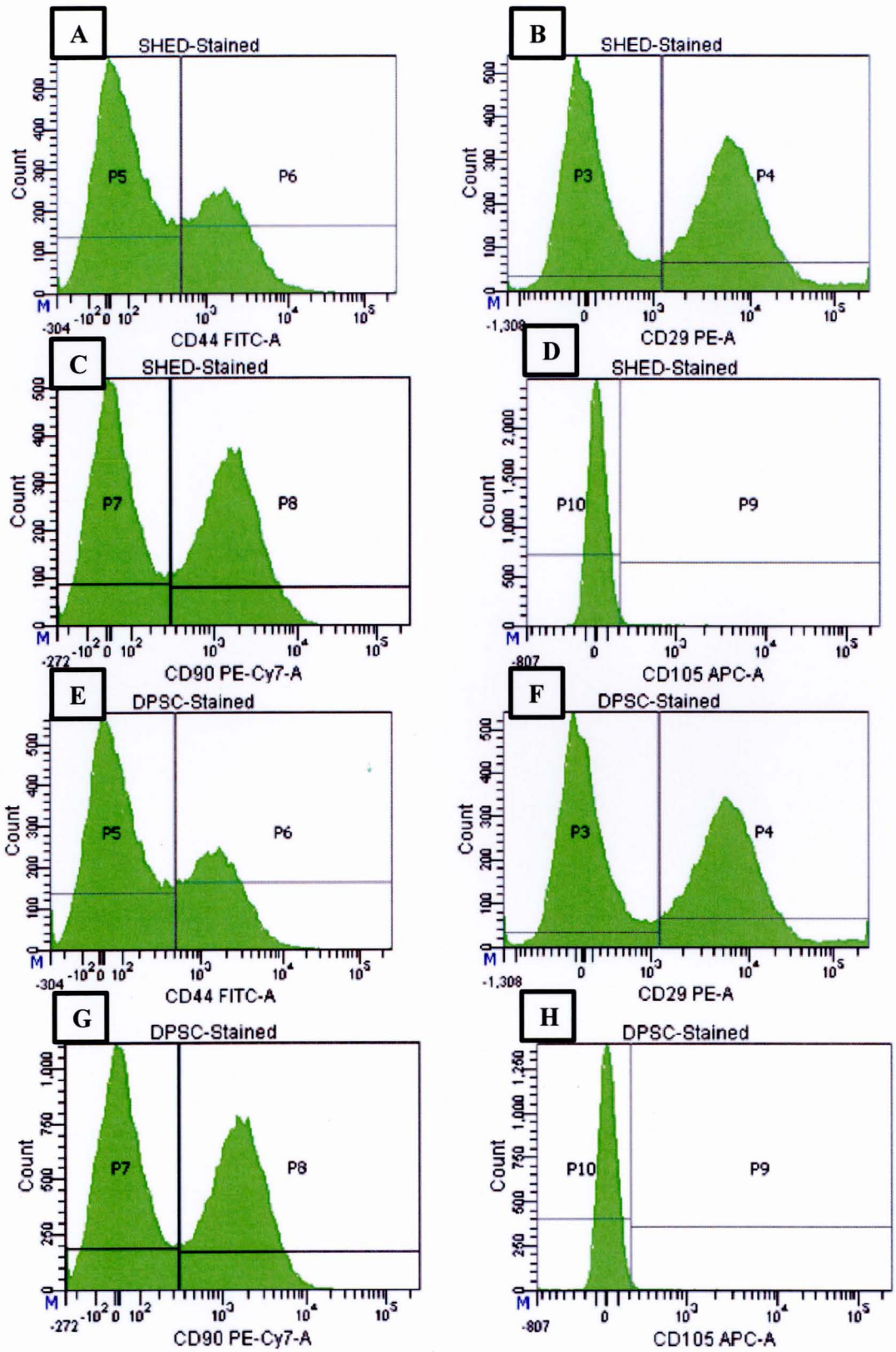


Figure 21: Representative histograms of the flow cytometric analysis of the **CD29** population (**A**, **B**, **C** and **D**) and the **CD44** population (**E**, **F**, **G** and **H**).

3.2.2. Quantitative flow cytometric analysis of culture-expanded populations

3.2.2.1. Percentage of $CD29^+$ and $CD44^+$ cells

The mean indices of the percentage of $CD29^+$ and $CD44^+$ cells was calculated by the FlowJo software (v9.4.11 TreeStar Inc) and the distribution of the data at days 7, 14 and 21 is shown in Tables 3 and 4.

Table 3: Mean indices of the percentage of **$CD29^+$** cells.

Day	Number of teeth	Mean percentage of $CD29^+$ cells (SD)	Range
Day 7	30	55 (7.0)	39-67%
Day 14	30	66.3 (9.1)	42-81%
Day 21	30	72.2 (10.0)	48-89%

Table 4: Mean indices of the percentage of **$CD44^+$** cells.

Day	Number of teeth	Mean percentage of $CD44^+$ cells (SD)	Range
Day 7	30	50.7 (7.9)	35-62%
Day 14	30	56.3 (7.1)	39-68%
Day 21	30	74.4 (2.1)	49-89%

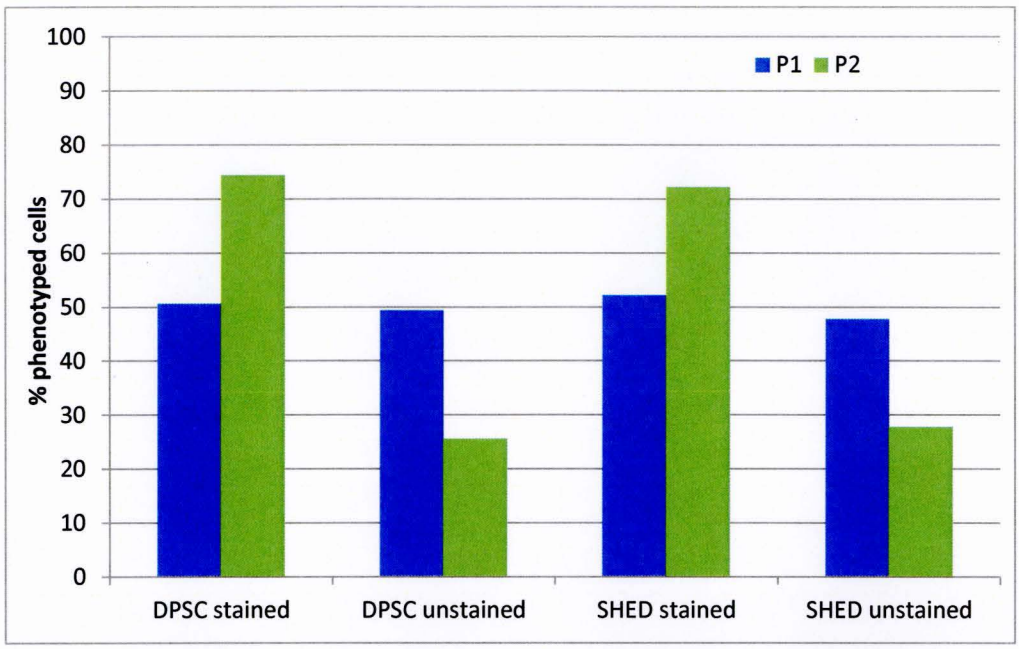


Figure 22: Representative bar column graphs of the percentage of $CD29^+$ (**A**) and $CD44^+$ (**B**) at day 21.

P1 corresponds to the percentage of $CD45^+$ $CD14^+$ haematopoietic cells that are excluded from the second flow cytometric event.

P2 represents the population of $CD29^+$ cells (SHEDs) and $CD44^+$ cells (DPCSs) acquired from the CD45-CD14- non haematopoietic cells.

3.3. Neural differentiation

3.3.1. Descriptive analysis of neuronal differentiated cells

The two populations of characterised dental stem cells, SHEDs and DPSCs transferred in the neural inductive medium exhibited distinct morphological changes during the differentiation process. Before the change of the neural basal medium to the DMEM, both types of dental stem cells were arranged in clusters of floating neurospheres. From day 7, their phenotype was similar to those of mesenchymal stem cells; however, they were more elongated and started exhibiting some dendritic process. After day 14 and until day 21, both SHEDs and DPSCs acquired the morphological characteristics of neuronal cells: they were showing a rather large and round cellular body as well as various cytoplasmic extensions that were arranged in an intercellular network (Fig. 23). It is worthy to note that these images were consistent in both stem cell populations and that the differentiation process followed the same steps as well.

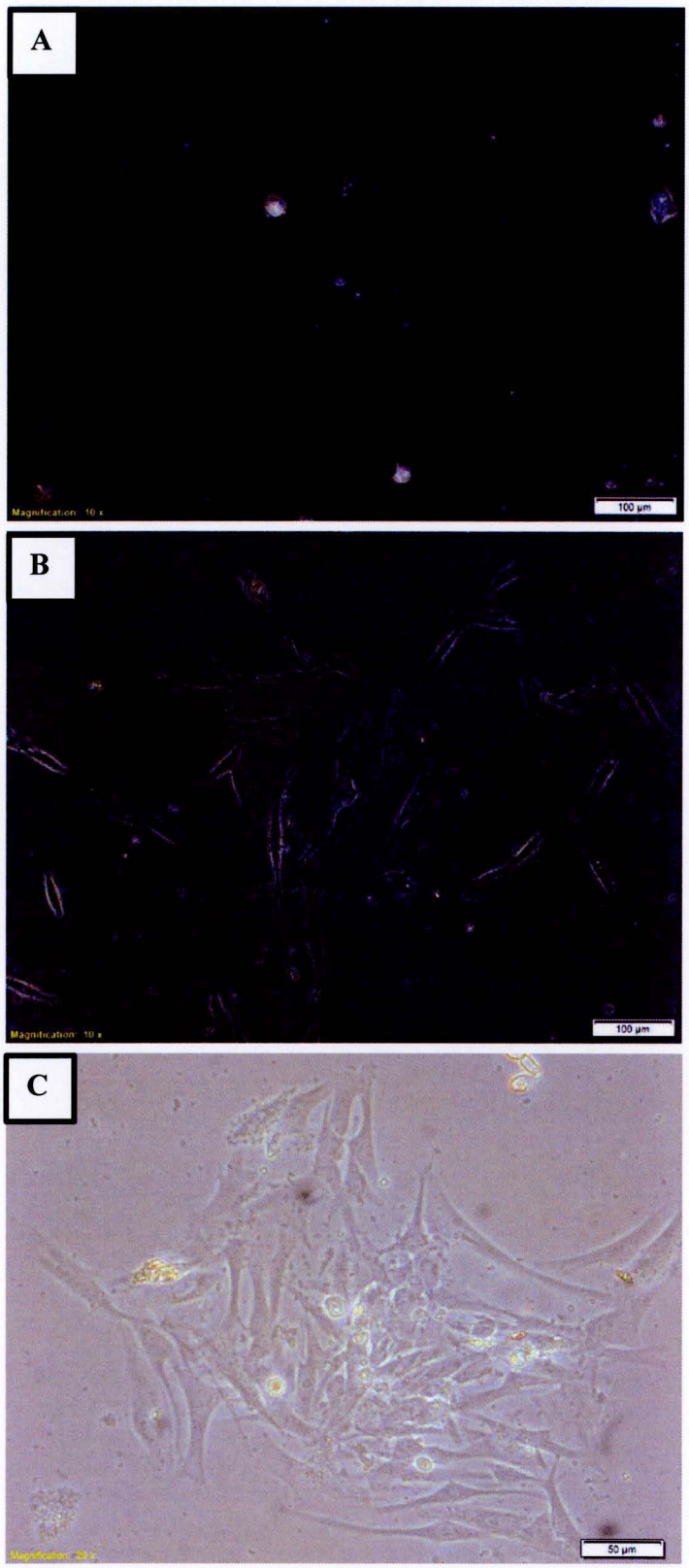


Figure 23: Representative photomicrographs of the neural differentiated cells in both **deciduous** and **permanent** teeth at days 7, ×100 (**A**), 14 ×100, (**B**) and 21 ×200 (**C**).

3.3.2. Quantitative analysis of neuronal differentiated cells

3.3.2.1. Percentage of viable neuronal differentiated cells

The descriptive analysis included the continuous variables of the percentage of viable neuronal differentiated cells, in both deciduous and permanent teeth, and was analysed and summarised by using the mean, standard deviation, median and interquartile range, and their distribution was illustrated with bar column graphs. Furthermore, the relationship between the percentages of viable differentiated neural cells at days 7, 14 and 21 was determined by a one-way repeated analysis of variance (ANOVA).

The mean indices of the percentage of viable differentiated neural cells for both deciduous and permanent teeth were calculated at days 7, 14 and 21 and they are shown in Tables 5 and 6.

Table 5: Mean indices of the percentage of viable neuronal differentiated cells in **deciduous** teeth, at days 7, 14 and 21.

Day	Number of teeth	Mean percentage of viable cells (SD)	Range
Day 7	30	51.2 (8.1)	36-66%
Day 14	30	53.3 (8.9)	39-67%
Day 21	30	68.1 (5.3)	56-78%

Table 6: Mean indices of the percentage of viable neuronal differentiated cells in the **permanent** teeth, at days 7, 14 and 21.

Day	Number of teeth	Mean percentage of viable cells (SD)	Range
Day 7	30	53.3 (8.2)	36-66%
Day 14	30	58.8 (8.8)	41-69%
Day 21	30	63.3 (9.1)	42-81%

3.3.2.2. Comparative analysis of viable differentiated neuronal cells

For each designated time point, the mean percentage of viable neuronal differentiated cells were statistically compared using an analysis of variance (ANOVA) and they were all significantly different to each other ($p<0.001$), as it is illustrated in Fig. 24.

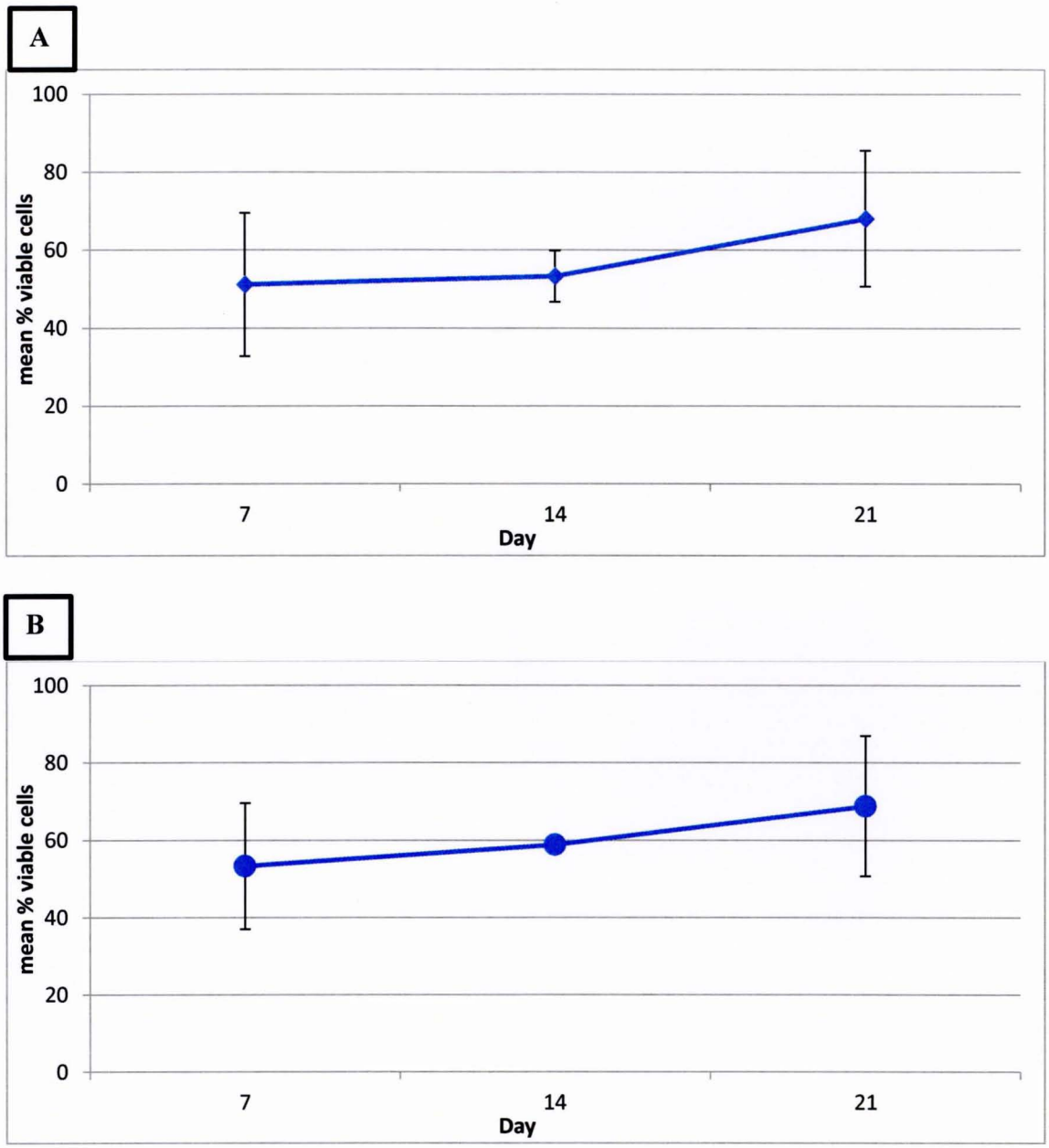


Figure 24: Representative graph of the comparison of the percentage of viable neural differentiated cells at days 7, 14 and 21, in **deciduous** (**A**) and **permanent** (**B**) teeth.

3.4. Immunocytochemistry analysis

3.4.1. Ki-67

3.4.1.1. Descriptive analysis of Ki-67 positive proliferative cells

Ki-67, the selected intrinsic marker for proliferative cells in this experiment, was positively labelled in neural differentiated cells, from both phenotyped DPSCs and SHEDs. The stained cells displayed crisp to dark brown nuclei and their distribution within the culture flasks appeared to vary according to the period of incubation. At day 7, the Ki-67 positive proliferative cells were observed in a greater number, in both populations and in accordance to the higher rate of self-renewal at that designated time. At day 21, the Ki-67 labelled cells were visualised as scattered throughout the cell cultures and this was due to the low replication rate of the more mature cells detected at that point of incubation (Fig. 25).

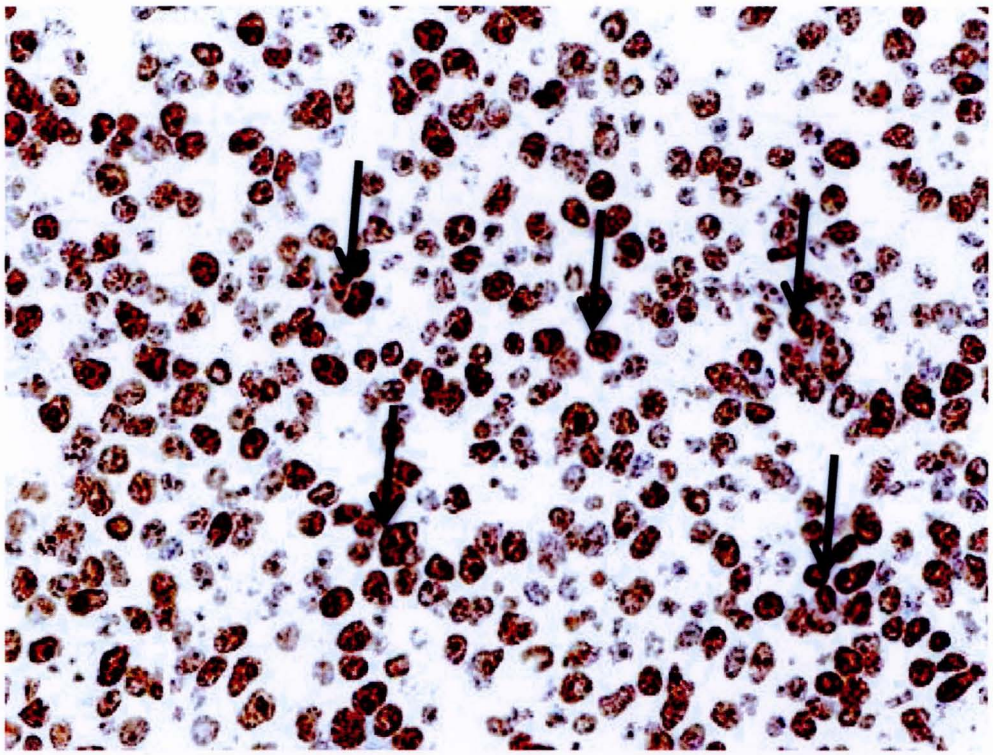


Figure 25: Representative photomicrograph of Ki-67 positive proliferative cells exhibited in both **deciduous** and **permanent** teeth at day 21.

3.4.1.2. Quantitative analysis of Ki-67 positive proliferative cells

3.4.1.2.1. Percentage of Ki-67 positive proliferative cells

The descriptive analysis included the continuous variables of the percentage of Ki-67 positive proliferative cells, in both deciduous and permanent teeth, and was analysed and summarised by using the mean, standard deviation, median and interquartile range, and their distribution was illustrated with bar column graphs. Furthermore, the relationship between the percentages of Ki-67 positive proliferative cells at days 7, 14 and 21 was determined by a one-way repeated analysis of variance (ANOVA).

The mean indices of the percentage of viable cells for both deciduous and permanent teeth were calculated at days 7, 14 and 21 and they are shown in Tables 7 and 8.

Table 7: Mean indices of the percentage of positive proliferative Ki-67 cells in **deciduous** teeth, at days 7, 14 and 21.

Day	Number of teeth	Mean percentage of Ki-67$^+$ (SD)	Range
Day 7	30	68.1 (5.3)	56-78%
Day 14	30	55.0 (7.0)	39-67%
Day21	30	43.5 (6.9)	28-64%

Table 8: Mean indices of the percentage of positive proliferative Ki-67 cells in **permanent** teeth, at days 7, 14 and 21.

Day	Number of teeth	Mean percentage of Ki-67$^+$ (SD)	Range
Day 7	30	69.3 (3.7)	59-78%
Day 14	30	55.4 (5.8)	44-66%
Day 21	30	44.5 (7.3)	31-62%

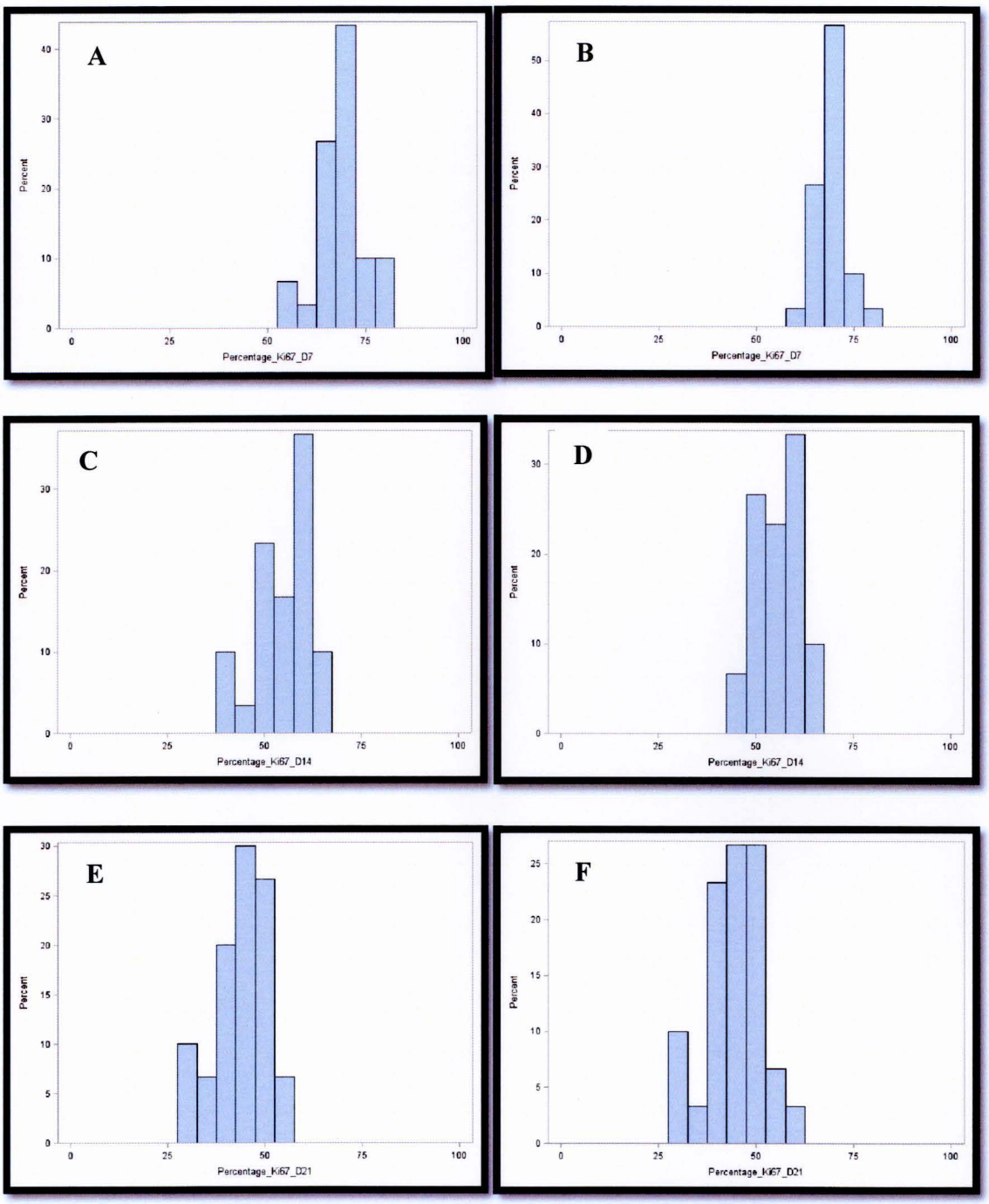


Figure 26: Representative histograms of the percentage of KI-67 positive proliferative cells for **deciduous (A)** and **permanent (B)** teeth at day 7, at day 14 for **deciduous (C)** and **permanent (D)** teeth and day 21 for **deciduous (E)** and **permanent (F)**.

3.4.1.2.2. Comparative analysis of positive proliferative Ki-67 cells

For each designated time point, the mean percentage of Ki-67 positive proliferative cells were statistically compared using an analysis of variance (ANOVA) and they were all significantly different to each other ($p<0.001$), as it is illustrated in Fig. 27.

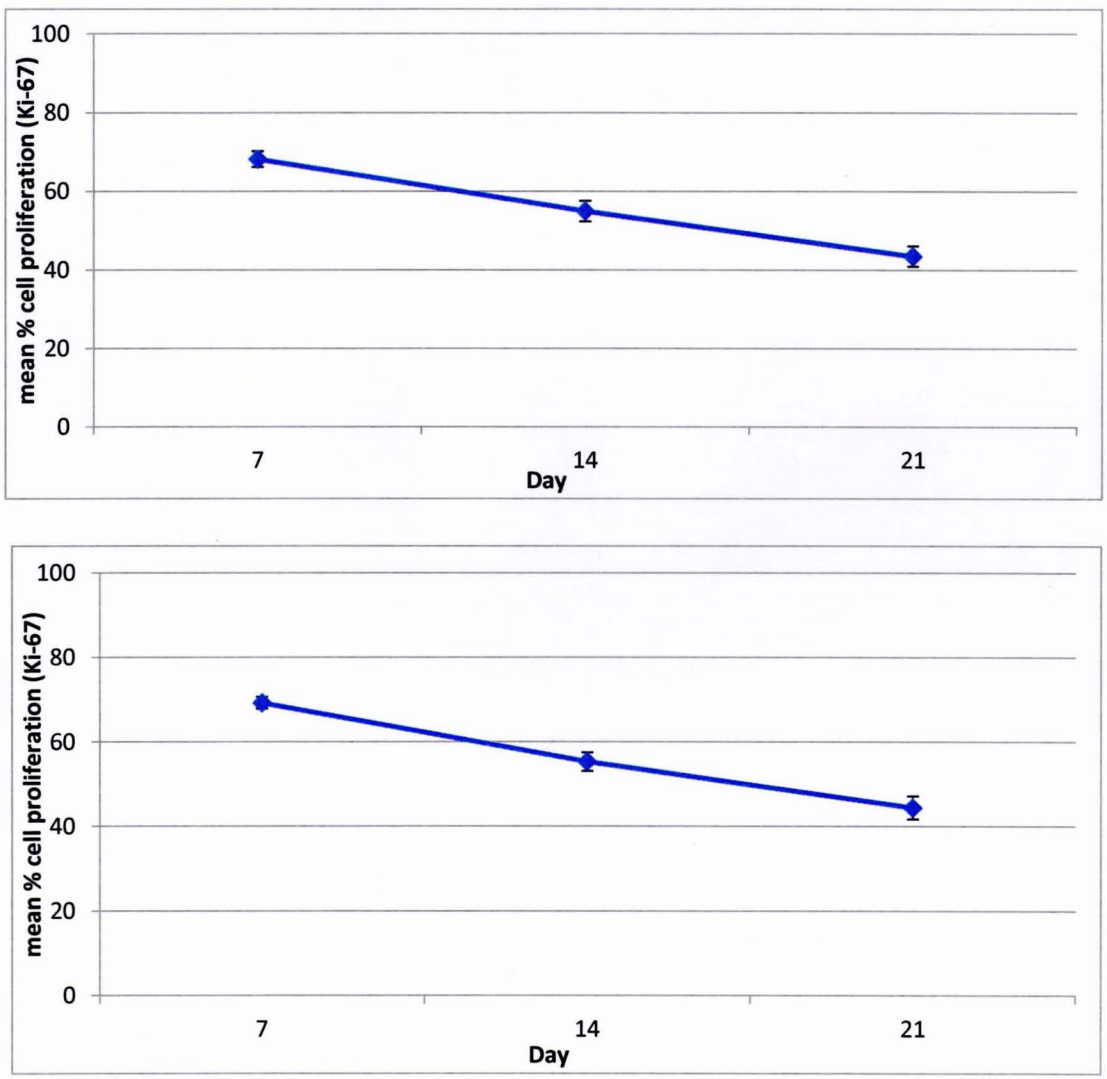


Figure 27: Representative graph of the comparison of the percentage of positive proliferative Ki-67 cells at days 7, 14 and 21, in **deciduous (A)** and **permanent (B)** teeth.

3.4.2. Doublecortin (DCX)

3.4.2.1. Descriptive analysis of positive DCX labelled cells

Characterised SHEDs and DPSCs cultured in the neuroinductive medium both exhibited cells that were positively labelled for doublecortin. Most of the differentiated neuronal cells exhibited stellate shaped morphology with growing cell processes. Although the expression was rather weak in all samples, it became clearly detectable from day 7 and progressively decreasing with the incubation time as the newly differentiated neurogenic cells were undergoing maturation (Fig. 28).

DOUBLECORTIN EXPRESSION

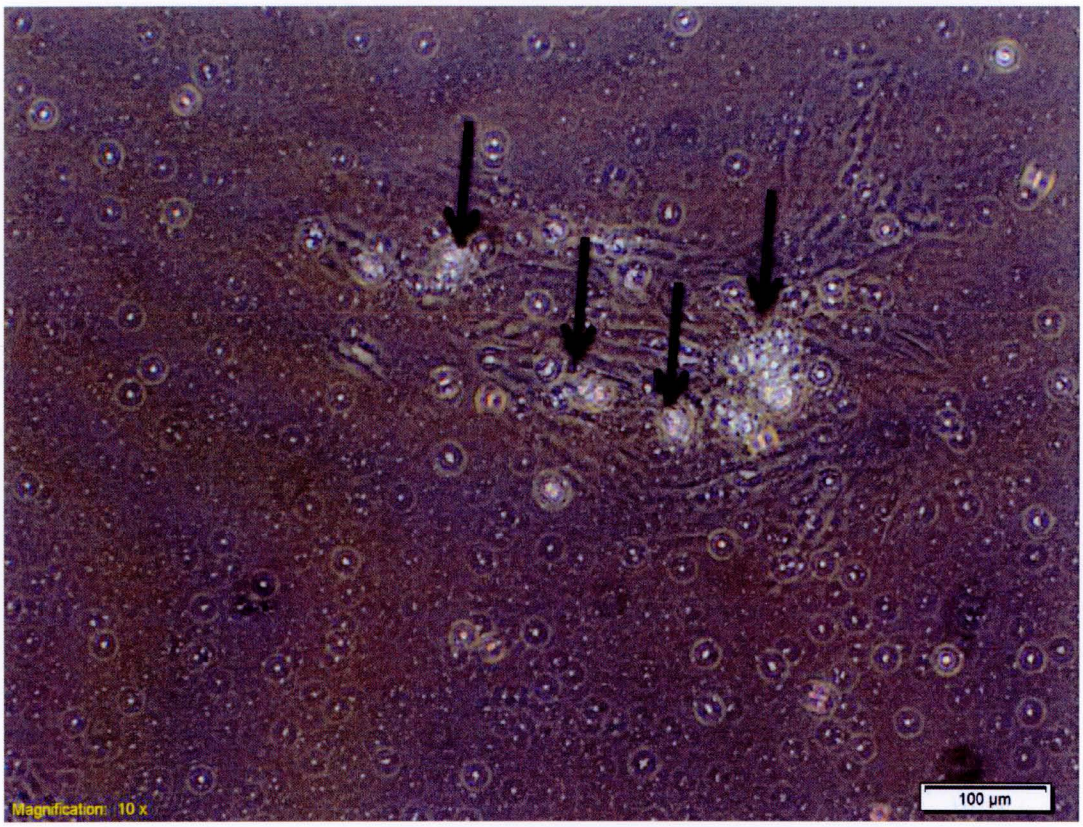


Figure 28: Representative photomicrograph of DCX positive proliferative cells exhibited in both **deciduous** and **permanent** teeth at day 7 (× 100).

3.4.2.2. Quantitative analysis of positive DCX labelled cells

The descriptive analysis included the continuous variables of the percentage of DCX labelled cells. positive proliferative cells, in both deciduous and permanent teeth, and was analysed and summarised by using the mean, standard deviation, median and interquartile range, and their distribution was illustrated with bar column graphs. Furthermore, the relationship between the percentages of DCX labelled cells at days 7, 14 and 21 was determined by a one-way repeated analysis of variance (ANOVA).

The mean indices of the percentage of viable cells for both deciduous and permanent teeth were calculated at days 7, 14 and 21 and they are shown in Tables 9 and 10.

Table 9: Mean indices of the percentage of DCX^+ labelled cells in **deciduous** teeth at days 7, 14 and 21.

Day	Number of teeth	Mean percentage of DCX^+ cells (SD)	Range
Day 7	30	51.2 (9.0)	42-61%
Day 14	30	50.3 (8.9)	42-87%
Day 21	30	24.7 (6.9)	17-52%

Table 10: Mean indices of the percentage of DCX^+ labelled cells in **permanent** teeth at days 7, 14 and 21.

Day	Number of teeth	Mean percentage of DCX^+ cells (SD)	Range
Day 7	30	53.3 (8.1)	42-76%
Day 14	30	49.8 (10.5)	42-78%
Day 21	30	25.6 (7.0)	17-54%

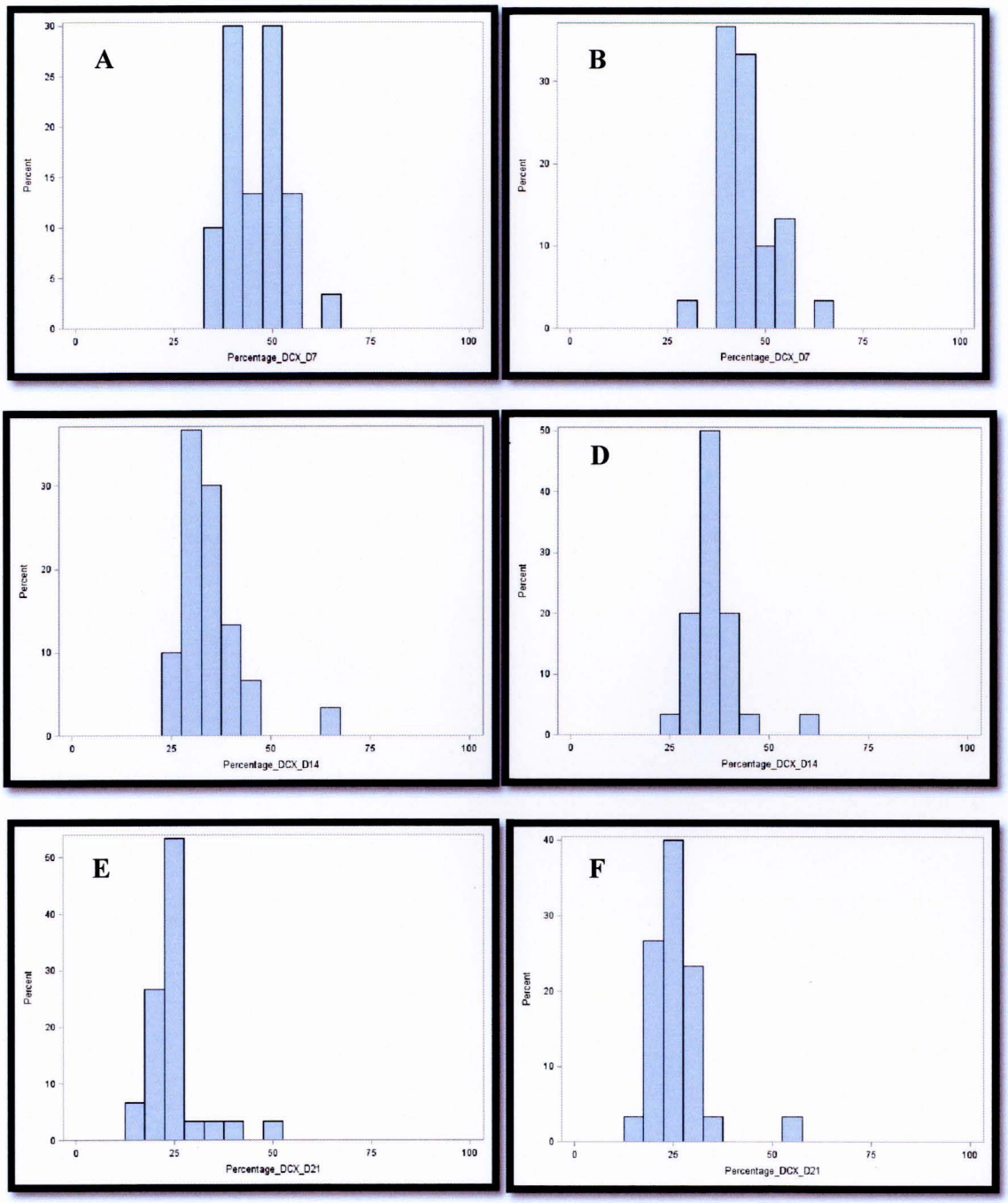


Figure 29: Representative histograms of the percentage of DCX^+ labelled cells at day 7 for **deciduous** (**A**) and permanent (**B**) teeth, day 14 for **deciduous** (**C**) and **permanent** (**D**) teeth and day 21 for **deciduous** (**E**) and **permanent** (**F**) teeth.

For each designated time point, the mean percentage of viable neuronal differentiated cells were statistically compared using an analysis of variance (ANOVA) and they were all significantly different to each other ($p<0.001$), as it is illustrated in Fig. 30.

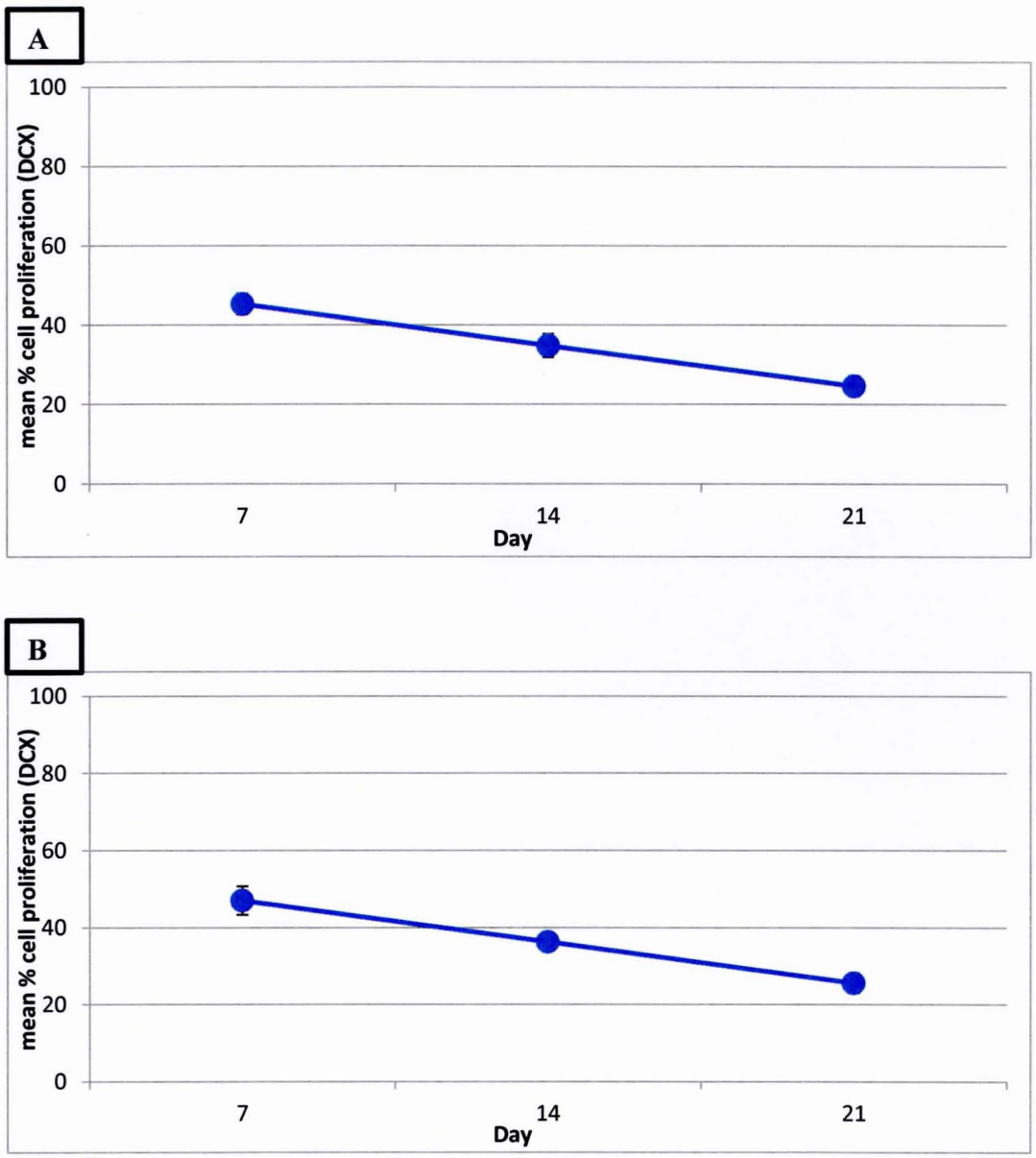


Figure 30: Representative graph of the comparison of the percentage of DCX^+ labelled cells at days 7, 14 and 21, in **deciduous (A)** and **permanent (B)** teeth.

3.4.3. Nestin

3.4.3.1. Descriptive analysis positive nestin labelled cells

Characterised SHEDs and DPSCs cultured in the neuroinductive medium both exhibited nestin positive labelled cells. Most of the differentiated neuronal cells exhibited stellate shaped morphology with growing dendritic processes. The expression of nestin, always exhibited in all samples, was opposite to that of doublecortin during the incubation period: it appeared as brown stains of varying size in the cells. It also became clearly detectable from day 7 and progressively increased as the newly differentiated neurogenic cells were undergoing maturation at day 21 (Fig. 31).

NESTIN EXPRESSION

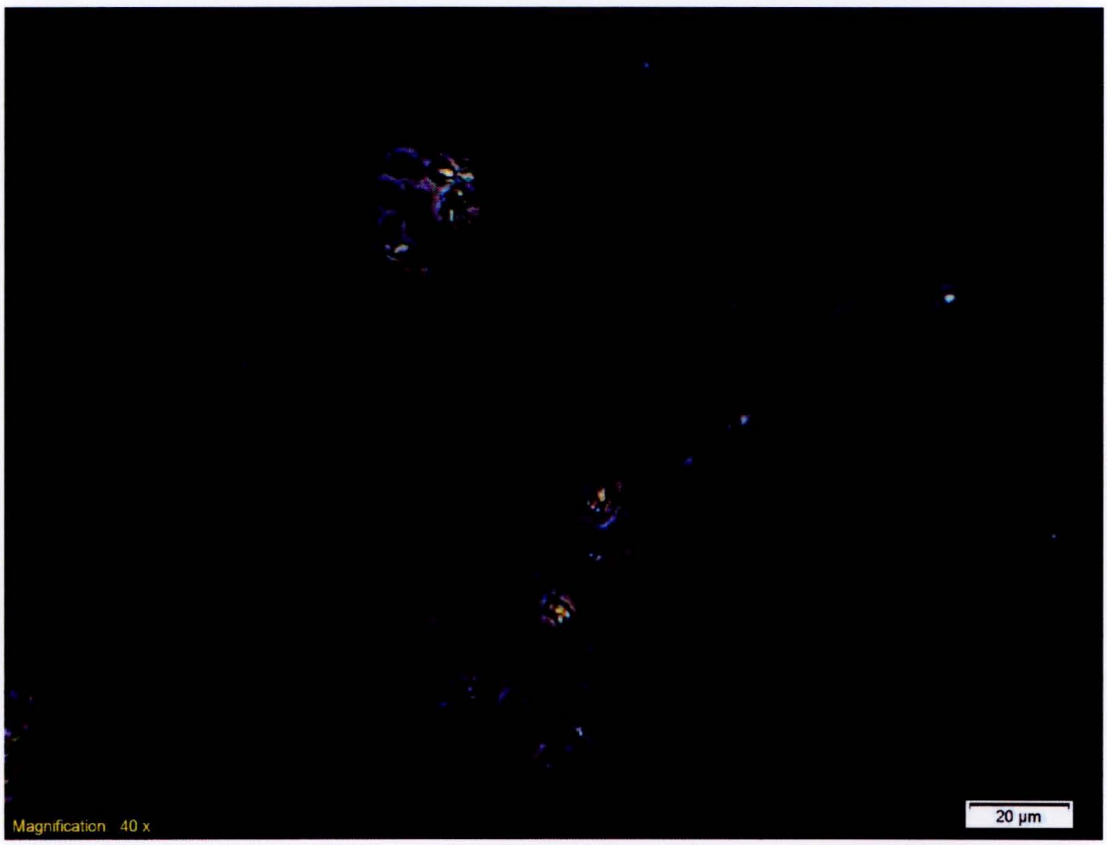


Figure 31: Representative photomicrograph of nestin positive cells exhibited in both **deciduous** and **permanent** teeth at day 21 (× 400). The brown and rounded stains are demonstrated as the cells are gradually reaching maturation.

3.4.3.2. Quantitative analysis of positive nestin labelled cells

3.4.3.2.1. Percentage of positive nestin labelled cells

The descriptive analysis included the continuous variables of the percentage of nestin. positive labelled cells, in both deciduous and permanent teeth, and was analysed and summarised by using the mean, standard deviation, median and interquartile range, and their distribution was illustrated with bar column graphs. Furthermore, the relationship between the percentages of nestin positive labelled cells at days 7, 14 and 21 was determined by a one-way repeated analysis of variance (ANOVA).

The mean indices of the percentage of viable cells for both deciduous and permanent teeth were calculated at days 7, 14 and 21 and they are shown in Tables 11 and 12.

Table 11: Mean indices of the percentage of positive nestin labelled cells in **deciduous** teeth at days 7, 14 and 21.

Day	Number of teeth	Mean percentage of nestin$^+$ cells (SD)	Range
Day 7	30	22.2 (4.8)	12-34%
Day 14	30	27.2 (6.4)	24-46%
Day 21	30	32.3 (7.1)	26-56%

Table 12: Mean indices of the percentage of positive nestin labelled cells in **permanent** teeth at days 7, 14 and 21.

Day	Number of teeth	Mean percentage of nestin$^+$ cells (SD)	Range
Day 7	30	22.4 (4.6)	16-35%
Day 14	30	27.5 (6.1)	21-45%
Day 21	30	32.8 (5.7)	28-52%

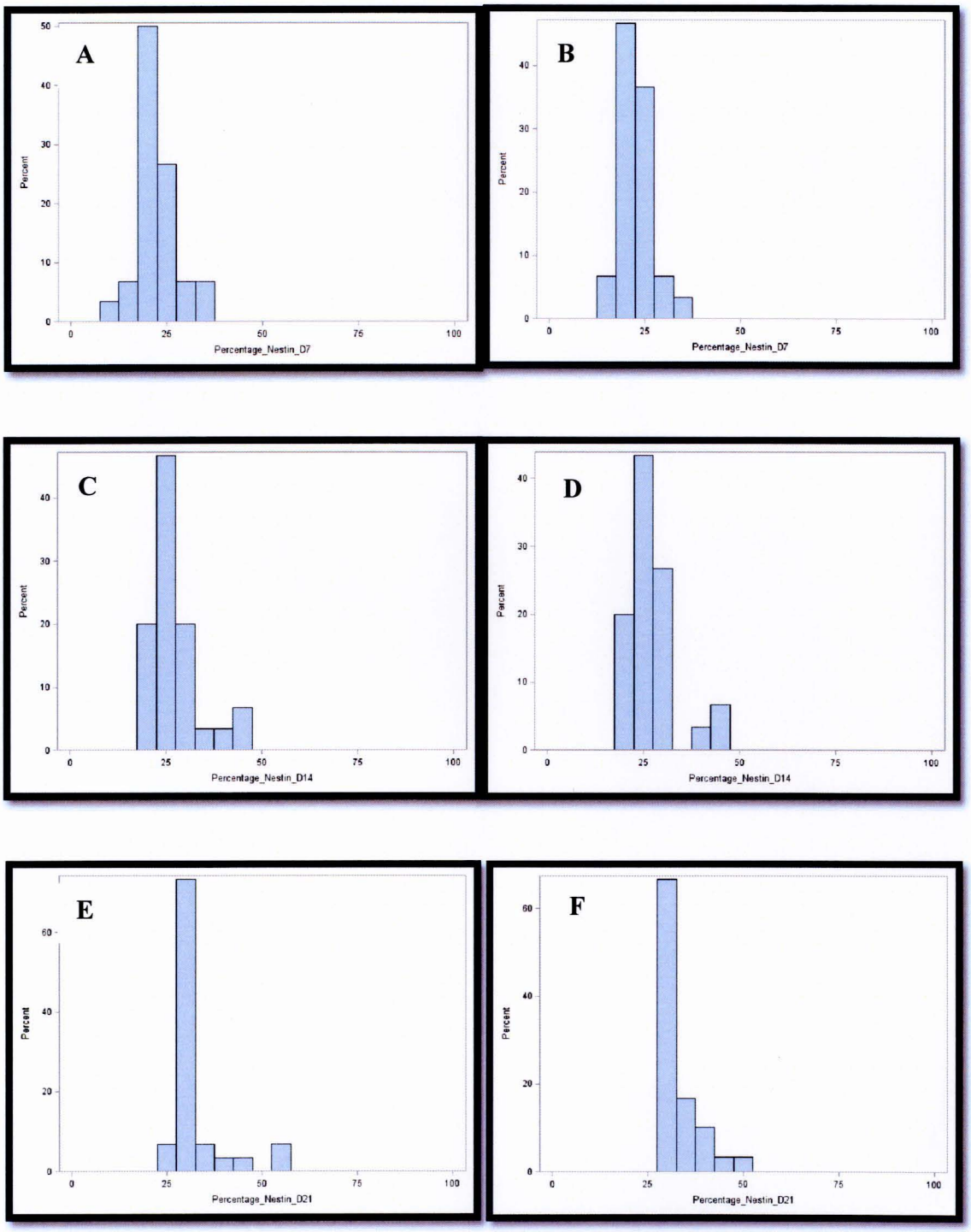


Figure 31: Representative histograms of the percentage of nestin positive labelled cells at day 7 for deciduous (**A**) and permanent (**B**) teeth, day 14 for deciduous (**C**) and permanent (**D**) and day 21 for deciduous (**E**) and permanent (**F**) teeth.

3.4.3.2. Comparative analysis of positive nestin labelled cells

For each designated time point and in the two populations, the mean percentage of nestin positive cells and DCX positive cells were statistically compared using an analysis of variance (ANOVA) and they were all significantly different to each other ($p<0.001$), as it is illustrated in Fig. 33.

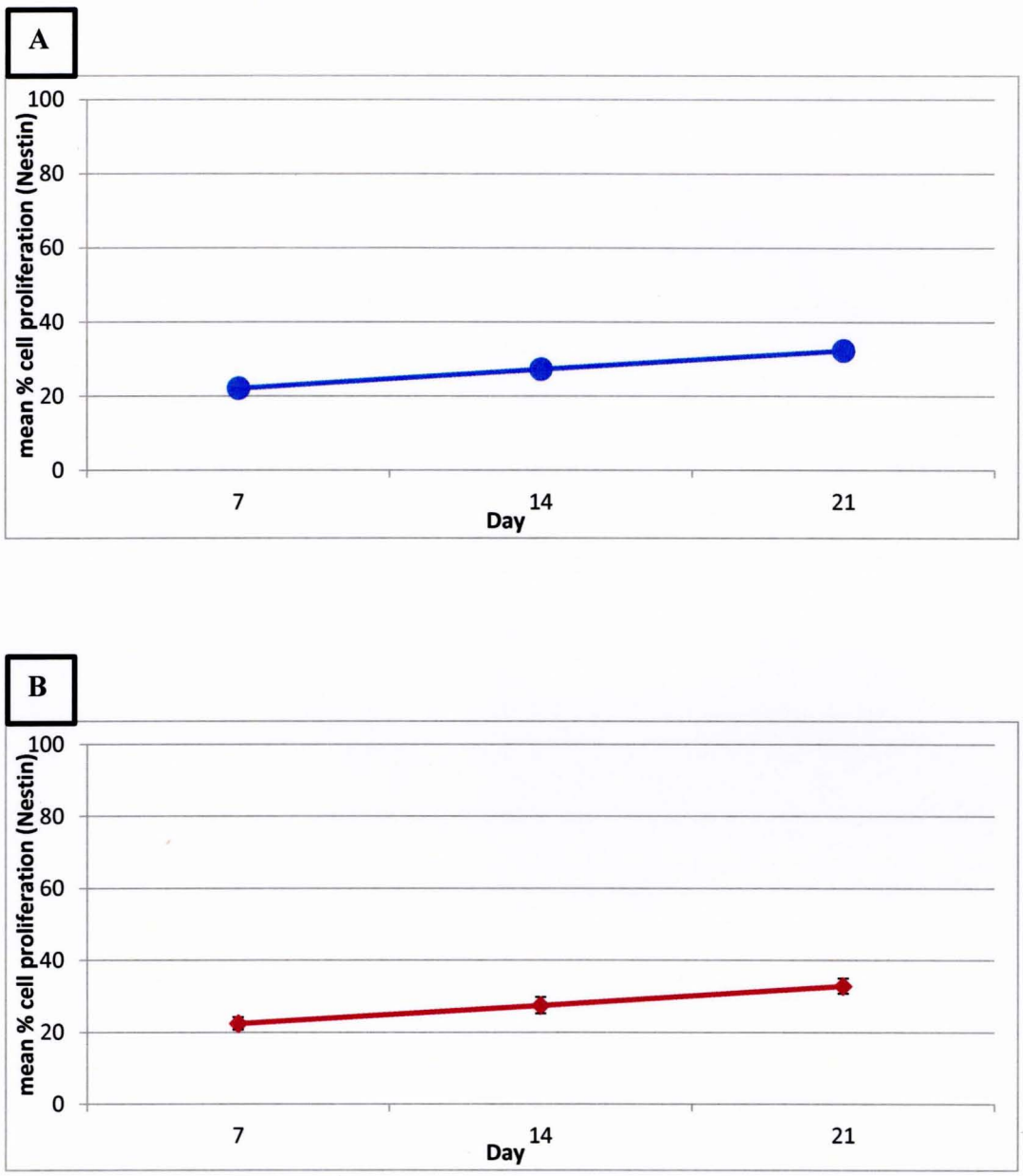


Figure 33: Representative graphs of the comparison of the percentage of nestin positive cells at days 7, 14 and 21 for **deciduous** (**A**) and **permanent** (**B**) teeth.

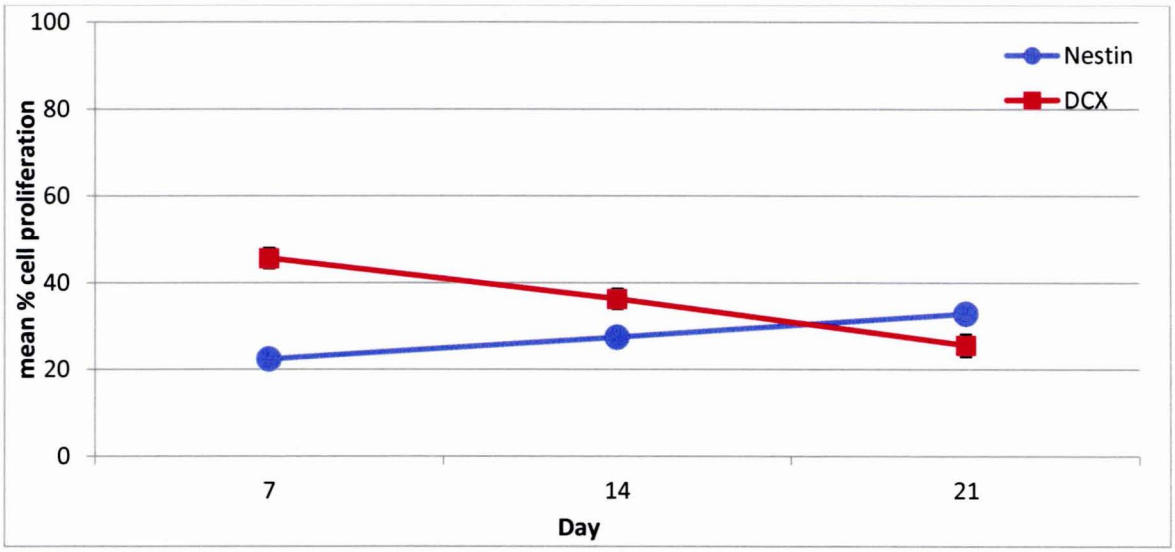


Figure 34: Representative graph of the comparison of the percentage of nestin$^+$ cells and DCX$^+$ cells at days 7, 14 and 21, in both **deciduous** and **permanent** teeth.

CHAPTER 4

DISCUSSION

Since the discovery of mesenchymal stem cells, various studies have provided evidence of their ability of continuous self-renewal and their differentiation into several specialised cell lineages. They have been successfully isolated from various adult tissues and used in regenerative medicine, thus paving the way for stem cell biology as a research field (Caplan *et al.* 1991). Human dental stem cells lately discovered in the teeth and their supportive tissues have singularised a unique pool of mesenchymal stem cells, easily isolated in a non-traumatic manner, thus abolishing some highly contentious ethical and clinical debate (Gronthos *et al.* 2002).

4.1. Statement of the principal findings

The present study has provided, in a South African cohort, descriptive and quantitative evidence of the neurogenic potential and differentiation of the dental stem cells identified in the pulp tissue of permanent and deciduous teeth namely, dental pulp stem cells (DPSCs) and stem cells of the human exfoliated deciduous teeth (SHEDs). These results, accomplished through a combination of cell culture, flow cytometry, neural induction and immunocytochemistry analysis, have granted conclusive evidence of the neurogenic potential of these two populations when cultured in the appropriate conditions.

4.2. Cell culture findings

The self-renewal potential of culture-expanded DPSCs and SHEDs, under the standard *in vitro* differentiating conditions, is well documented and has firstly been demonstrated by Gronthos *et al.* (2000) and Miura *et al.* (2003) respectively. One of the set criteria

proposed by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) to define human MSC for both laboratory-based scientific investigations as well as pre-clinical studies, and regardless of their tissue of origin, is their adherence to culture flasks (Dominici *et al.* 2006).

The findings in this study showed that cell proliferation was successful in both populations, with the first sign of cell renewal and cell adherence noted after one day and three days of culture respectively. In each population, the mean percentages of viable cells, at each designated time point, were all statistically significantly different to each other ($p<0.0001$). On the other hand, there was no statistically significant difference in comparative indices of viable cells between the deciduous and permanent groups. The sample size of 30 deciduous and permanent teeth was sufficient for descriptive analysis and paired comparison of the results obtained at different incubation times. The relationship between results at days 7, 14 and 21 for indices of viable cells, determined by a one-way repeated measures ANOVA test , was in accordance with previous studies reporting no significant difference ($p<0.0001$) in cellular proliferation between the two populations (Wang *et al.* 2012).

At day one, both SHEDs and DPSCs exhibited the same rounded and spherical morphology that has been well published, and they were still floating in the culture medium (Gronthos *et al.* 2000; Miura *et al.* 2003). From day 3, colony forming units of fibroblast-like cells (CFU-F) were discernible in the two populations, as reported by the latter researchers and Morszcek *et al.* in 2005. In addition, a significant increase in cell proliferation and percentage of viable cells in the culture medium, analysed at 7-days intervals was observed with prolonged incubation ($p<0.0001$). This was similar to the

early findings of Friedenstein *et al.* (1976) on mesenchymal stem cells cultured *in vitro*: he reported that the most tightly adherent cells in the flasks were spindled-shaped and aggregated in foci of two to four cells. These cells were initially inactive for 2 to 4 days, after which they began to rapidly multiply (Friedenstein *et al.* 1976). From day 7, the first enumeration of cells was possible with the first passage of the cells with a frequency of approximately 1:376. This value was in accordance with the frequency of cells (1:400) obtained by Gronthos *et al.* (2000) in their experiment on dental pulp stem cells.

Cell culture results in this present investigation correlate extremely favourably with most published studies. Proliferative cells in the two populations were morphologically similar; in addition, the expanded cells were almost indistinguishable when the phenotype displayed in the two populations was observed, as reported by Wang *et al.* (2012). Furthermore, they assumed the typical spindle-shaped appearance of mesenchymal stem cells showing cell processes. They all exhibited heterogeneity in size and they all showed definite signs of cell division as indicated by their round or oval nuclei with multiple nucleoli. These morphological characteristics were analogous to the ones observed in mesenchymal stem cells obtained from the bone marrow (Huang *et al.* 2009). Similarly, the detection of cell semi-confluency and confluency at day 14 and 21 respectively in the two populations cultures was also equivalent to the observations of Gronthos *et al.* (2000), Huang *et al.* (2009) and Miura *et al.*, (2003). Moreover, Gronthos *et al.* (2000) attributed the high rate of cell replication at day 21 to the several cell culture promoting supplements in the media.

Manas *et al.* (1998) examined the morphology, phenotype and *in vitro* functions of mesenchymal stem cells isolated from bone marrow samples. They noted that from

passage 1 corresponding to day 14 of incubation in their study, the primary cultures consisted of a heterogeneous population. They observed the co-existence of haematopoietic stem cells as well as the typical spindle-shaped mesenchymal stem cells (Manas *et al.* 1998). The former was indicated by several accumulations of cobblestone areas formation characteristic of haematopoietic stem cells. The authors concluded that a small percentage of the latter persisted in expanded culture of mesenchymal stem cells. This data then demonstrated that, in long-term culture assays, mesenchymal stem cells had the ability to support haematopoietic differentiation *in vitro* (Manas *et al.* 1998). In the present study, very few rounded and viable cells were also noted and remained scattered in the culture flasks after days 7 (passage 1) and 14 (passage 2). However, these cells, not included in the study objectives, were not investigated any further. It is then recommended that their supplementary analysis could perhaps be included in future projects.

4.3. Flow cytometry observations

In the present study, the findings of the flow cytometric experiment demonstrated that cells from both tooth type were expressing CD29, CD44, CD90 and CD105, with a predominance of CD29 in deciduous teeth and CD44 in the permanent teeth samples. There was a statistically significant difference in the $CD29^+$ and $CD44^+$ indices observed in permanent and deciduous teeth respectively ($p<0.0001$). Furthermore, CD90 and CD105 were also expressed with a higher display of CD90 in both populations (58.6% and 59.5% in deciduous and permanent teeth respectively) and a lower detection of CD105 (30.7% and 29.8% in deciduous and permanent teeth respectively). In addition, the CD14 and CD45 used for the negative selection in the gating strategy were not exhibited on the cell surface, except in the small percentage of contaminated $CD29^+$ and $CD44^+$ cells which showed a slight percentage of $CD14^-$ cells. As CD14 is a marker that primarily

discriminates monocytes and macrophages from mesenchymal stem cells, this expression was probably attributed to the large size of the former cells acquired and registered in the forward scatter.

The presence of $CD29^+$ and $CD44^+$ cells, supplemented by $CD90^+$, $CD105^+$, $CD14^-$ and $CD45^-$ cells was positively identified in this experiment, thus confirming the phenotype of the isolated pulp tissue cells from deciduous and permanent teeth as dental stem cells. These results were in accordance with the findings reported by Tsai *et al.* (2004) who isolated mesenchymal stem cells from amniotic fluid obtained by second-trimester amniocentesis. The cell cultures were characterised from passage 8 and were analysed for the following surface antigens: CD10, CD11, CD14, CD34, CD90, CD105, CD117 and HLA-A, B, C. This was the first identification of the positive expression of CD29 and CD44, the low positive expression of CD90 and CD105 and the lack of expression of CD14, in a pool of pluripotent mesenchymal stem cells. The early antigenic expression of CD73 demonstrated by Tsai *et al.* (2004) is an essential characteristic that defines mesenchymal stem cells. CD73 is an adhesion molecule that is thought be a signal transduction activator during the interaction of the mesenchymal stem cells with the rest of the stromal environment component (Barry *et al.* 2004). Although Tsai conducted his study in a foetal tissue, it later appeared to follow the general rules of positive and negative markers, as a minimal criterion of expression for mesenchymal stem cells therapy (Dominici *et al.*, 2006). This also corresponded to the results of Yu *et al.* (2010) and Orciani *et al.* (2010) who confirmed this phenotype in mesenchymal stem cells from various sources. Both authors stated the low expression of CD105 and the absence of CD14 and CD45 cell surface markers.

The surface antigen profile established in the culture-expanded cells was equivalent to the observations of Gronthos *et al.* (2006) who investigated the markers expressed in isolated adult stem cells of dental origin. In his analysis carried out in both deciduous and permanent teeth, the most expressed putative stem cell marker was STRO-1, a trypsin-resistant cell-surface antigen used to isolate and purify bone marrow stromal cells. This was followed by the expression of CD29 and CD44, as well as CD146, a perivascular stem cell marker. The expression of CD146 in a population of mesenchymal stem cells created much interest as it suggested that dental stem cells originated from the bone marrow perivascular niche (McCulloch *et al.* 1995; Bianco *et al.* 2001). It was also demonstrated that low expression of CD146 indicated the presence of unipotent mesenchymal stem cells with a low cloning efficiency (McCulloch *et al.* 1995; Bianco *et al.* 2001). Furthermore, as CD146 is a marker related to the regulation of haematopoiesis, Gronthos' conclusions corroborated the findings of Manas *et al.* (1998) describing the persistent detection of cobblestone-like haematopoietic stem cells in mesenchymal stem cells assays.

Evidence of the co-existence of a small percentage of haematopoietic stem cells with mesenchymal stem cells has also been provided by Okolicsanyi *et al.* (2015). Human mesenchymal stem cells isolated from the iliac crest of five different healthy donors and commercially available were culture-expanded, for a period of approximately 60 days. They established that, even after 20 enzymatic passages, the incubated cells retained their three mesenchymal lineage potentials (chondrogenic, adipogenic and osteogenic) as well as their neural potential. In addition, the expression of cell surface markers was examined and the generated cells consistently exhibited the positive low expression of the haematopoietic stem cell marker CD45. However, this low manifestation of CD45 did not

alter the expression of the other known mesenchymal stem cell markers (Okolicsanyi *et al.*, 2015).

4.4. Neural differentiation report

The ability of all five types of dental stem cells to differentiate into various specialised cell lineages, when cultured in the appropriate environment, is well documented (Gronthos *et al.* 2000; Miura *et al.* 2003; Seo *et al.* 2004; Morszeck *et al.* 2005; Sonoyama *et al.* 2008; Huang *et al.* 2009). However, the majority of the studies have particularly targeted their odontoblast-like and osteogenic differentiation potential for their further use in tooth mineralisation and repair (Gronthos *et al.* 2000; Laino et al. 2005; Huang et al. 2009). Recently, a few studies centred on the neurogenic phenotype of both SHEDs and DPSCs (Miura *et al.* 2003; Gronthos *et al.* 2009; Gervois *et al.* 2014), have successfully shown that they offer a reliable alternative source of mesenchymal stem cells as they can produce potential neurons and functional neurons *in vitro* (Arthur *et al.* 2008; Gervois *et al.* 2014;). Gronthos *et al.* (2000) heralded that the multipotent stem cells of the pulp of permanent teeth were able to express neural markers after their transplantation into mice. Three years later, Miura *et al.* (2003) described the differentiation of SHEDs into neural cells and established their persistent display of neuronal and glial cell markers after their *in vivo* transplantation into the dentate gyrus of immunocompromised mice.

Dental stem cells originate from the neural crest derived ectomesenchyme (Nanci 2008; Moore 2013). The dorsolateral migration of the neural crest cells into the developing tooth during odontogenesis suggest that dental stem cells are capacitated to differentiate into neurogenic cells (Arthur *et al.* 2008; Ernst *et al.*2009; Le Douarin *et al.*2012). This hypothesis has been demonstrated in several studies (Bianco *et al.* 2001) and dental stem

cells have now been progressively used for tissue repair and regeneration in neurodegenerative diseases (Sakai *et al.* 2012). Moreover, Nosrat *et al.* (1998) conducted neural differentiation experiments in the dental pulp of rats and demonstrated the production of neurotrophic factors by DPSCs *in vitro*. They further culture-expanded the neural differentiated DPSCs with embryonic neurons and found the viability of the latter were actually promoted by DPSCs (Nosrat *et al.* 2004).

In the present study, DPSCS and SHEDs were incubated in 6-well plates at a concentration of 2×10^3 cells per ml of the neuroinductive medium (Gronthos *et al.*2009; Gervois *et al.* 2014). Our results showed that cell differentiation was successful in both populations, with the first sign of floating neurospheres indicative of neural differentiation observed at early stages. As differentiation and maturation proceeded until day 21, the cells were acquiring a different elongated morphology with cytoplasmic extensions, typical of neural cells. The same period of differentiation was noted in the work of Pittenger *et al.* (1999), who investigated the multilineage potential of adult human mesenchymal stem cells. He demonstrated that after 3 weeks of incubation in a lineage-specific medium, culture-expanded cells were highly exhibiting the phenotype of the selected lineage-specific cell types. These results were also reported by Ernst and Morszeck (2009) who studied the neurogenic potential of murine dental follicle stem cells. They showed the morphological changes undergone by the cells cultured in neurogenic medium: after the appearance of neutrospheres at the beginning of the cultivation, differentiated cells exhibited a flattened shape with dendritic extensions from day 7 of expansion in a neuroinductive medium (Ernst *et al.* 2009). Furthermore, these findings were similar to those of Gervois *et al.* (2015) who also reported the two-step process in the acquisition of neuronal morphology during the differentiation process. At early stages the induced DPSCs cells were

transformed into free floating neurospheres while in the late stage of differentiation, cells that grew out of the neurospheres were characterised as elongated cells with multiple cytoplasmic extensions. Okolicsanyi *et al.* (2015) in their long-term culture of commercially available mesenchymal stem cells also reported the consistent formation of neurospheres in the initial phase of neural differentiation. This was then followed by the appearance of neural cells displaying a fibroblastic phenotype, even after an extended *in vitro* expansion corresponding to 20 enzymatic passages (Okolicsanyi *et al.* 2015).

4.5. Immunocytochemistry findings

The findings of this immunocytochemistry analysis ascertained the proliferation of newly differentiated neuronal cells through the Ki-67 positive proliferative cells and also established the persistence of the neural lineage throughout the incubation period with doublecortin and nestin immunoreactity.

4.5.1. Ki-67

One of the defining criteria of stem cells is their ability to self-regenerate and undergo multiple cycles of cell division (Kumar *et al.* 2013). Cell proliferation is characterised by DNA replication and mitosis occurring during the cell cycle (Kumar *et al.* 2013). A number of growth factors stimulate cells to the transition from cell quiescent phase into cell cycle division phase (Kumar *et al.* 2013). The intrinsic proliferative capacity of cells can be measured by indices of proliferation factors such as Ki-67 (Kumar *et al.* 2013). SHEDs and DPSCs have shown in previous studies, a significant proliferation rate and differentiation potential into many functional cell types (Gronthos *et al.* 2000; Miura *et al.* 2003; Sakai *et al.* 2012).

The results of this present experiment revealed, in both deciduous and permanent teeth, the presence of Ki-67 positive proliferative cells following their exposure to a neuroinductive medium. SHEDs and DPSCs proliferation rates were assessed at days 7, 14 and 21 and Ki-67 stained cells were predominant at the early stages of incubation. At day 7, the cultures of neural differentiated cells comprised small clusters of cells arranged into neurospheres, and rapidly proliferating (68.1%). However, from day 14, the cells exhibited an elongated and increased size, accompanied by a deceleration in their multiplication values. In each population, the mean percentages of Ki-67 positive proliferative cells, at each designated time point, were all statistically significantly different to each other ($p<0.0001$). On the other hand, there was no statistically significant difference in comparative indices of Ki-67 positive proliferative cells between the deciduous and permanent groups.

The findings in this study were in accordance with the reports of Gronthos *et al.* (2000) and Miura *et al.* (2003) who studied the proliferative activities of DPSCs and SHEDs respectively. In both populations, the presence of Ki-67 nuclear labelling in the early stages of cell differentiation was emphasised. Moreover, the researchers observed that the growth rate of both cells exhibited a mild decrease after a prolonged incubation period. Seo *et al.* (2004) then Coura *et al.* (2008) made the same conclusion in their experiments on another category of dental stem cells isolated from the periodontal ligament. In addition, they also noted that the areas of proliferative activity were in close proximity to the developing blood vessels in stem cell niches, again suggesting the perivascular origin of dental stem cells (Seo *et al.* 2004; Coura *et al.* 2008). It is worthwhile noting that the decreased values in time of Ki-67 indices were contradictory to the recent work of Okolicsanyi *et al.* (2015) who were able to demonstrate a high growth rate as well as a

maintained differentiation potential in long-term culture-expanded mesenchymal stem cells.

4.5.2. Doublecortin

The findings of the immunocytochemistry analysis of doublecortin (DCX) in this present study established the differentiation potential of SHEDs and DPSCs into immature neurons. To date, insufficient studies reporting the expression of DCX in neurogenic cells of dental stem cells origin have been undertaken. On the other hand, ßIII-tubulin, a protein almost exclusively active in immature neurons has been studied (Bohlen *et al.* 2007; Gunewardene *et al.* 2014).

Following the confirmation of the proliferative activity in the neuroinduced cultures, the expression of doublecortin (DCX) was investigated. In both SHEDs and DPSCs, a generally weak expression of DCX was observed. In addition, the estimated levels of DCX labelled cells were decreased with the incubation period. DCX levels of SHEDs and DPSCs were assessed at days 7, 14 and 21 and DCX stained cells were more discernible at the early stages of incubation. At day 7, the cultures of neural differentiated cells comprised DCX labelled cells scattered in the neurogenic medium and from day 14, there was a clear decrease in their number. In each population, the mean percentages of DCX positive labelled cells, at each designated time point, were all statistically significantly different to each other ($p<0.0001$). On the other hand, there was no statistically significant difference in comparative indices of DCX positive labelled cells between the deciduous and permanent groups.

Sakai *et al.* (2012) investigated the neuroregenerative properties of SHEDs and DPCSs by transplanting them into a transected rat's spinal cord. Following their neurogenic induction, both populations co-expressed amongst other neural lineage markers, DCX, ßIII-tubulin and nestin. The obtained data showed that secretions of DCX and ßIII-tubulin were predominant in the early stages of neural differentiation suggesting the higher percentage of immature neurons at that designated time (Sakai *et al.* 2012). The neuroregenerative properties of SHEDs and DPCSs were later evaluated by transplanting them into a transected rat's spinal cord. The findings revealed that during the acute phase of spinal cord injury, both SHEDs and DPSCs significantly improved the recovery of the locomotor function (Sakai *et al.* 2012).

The results in this present research were similar to that of Tamaki *et al.* (2013) who analysed the neurogenic induction of human dental stem cells and bone marrow mesenchymal stem cells *in vitro*. The researchers confirmed the endogenous production of ßIII-tubulin in the early stages of neural differentiation. From day 7, they noted that the cells exhibited a more neuron-like multipolar appearance (Tamaki *et al.*, 2013). Furthermore, in the dental stem cells group, the examination of the control cells cultured in the standard clonogenic growth medium revealed the continuous expression of ßIII-tubulin. They therefore concluded that, because of the contribution of neural crest cells during tooth development (Le Douarin *et al.*, 2012), dental stem cells had the intrinsic ability to differentiate into neuronal cells and neural maturation was then accomplished through the neuroinductive medium (Tamaki *et al.*, 2013).

4.5.3. Nestin

The findings of the immunocytochemistry analysis of nestin in this present experiment established the differentiation competency of SHEDs and DPSCs into mature neurons. The expression of nestin was evaluated in both populations of SHEDs and DPSCS and it exhibited varying patterns with the period of incubation. Nestin levels in SHEDs and DPSCs were assessed at days 7, 14 and 21 and nestin stained cells were predominant at the late stages of incubation. At day 7, the cultures of neural differentiated cells comprised nestin labelled cells scattered in the neurogenic medium and from day 14, there was an increase in their number. In each population, the mean percentages of nestin immunoreactive cells, at each designated time point, were all statistically significantly different to each other ($p<0.0001$). On the other hand, there was no statistically significant difference in comparative indices of nestin positive cells between the deciduous and permanent groups.

This result was in accordance with several reports in the literature showing the standard expression of nestin in dental stem cells, after the stage of neurospheres and at late stages of the incubation period (Gronthos *et al.* 2002 and 2006; Miura *et al.* 2003; Morszeck *et al.* 2005; Sakai *et al.* 2012). Gronthos *et al.* (2002, 2006) and Miura *et al.* (2003) detected the expression of nestin in DPSCs and SHEDS neurogenic cultures respectively, from day 14 of neural differentiation. Morszeck *et al.* (2005) reported the same observations in dental follicle stem cell neurogenic cultures and the high numbers of nestin positive cells was emphasised. This was attributed to the embryonic nature pertaining to the dental follicle and still maintained in the impacted third molar teeth.

However, these results were not similar to the findings of About *et al.* (2000) who explored the nestin expression in a sample of embryonic and adult human teeth. Active secretion of nestin was demonstrated in dental pulp tissue cultures of both embryonic and permanent teeth at all stages of development. In the embryonic samples. nestin labelled cells were shown in the developing odontoblasts from the bell stage of tooth development. In the developing third molar of young adult patients, nestin expression was also displayed in the odontoblasts and the authors attributed this immunoreactivity to the neural crest-derived nature of the odontogenic ectomesenchyme. They thus recommended the use of NeuN, a soluble nuclear protein commonly used for as a biomarker for most neuronal cells (Mullen *et al.* 1992), for the identification of mature neuronal cells in dental tissues (About *et al.*, 2000).

The latter results were also confirmed by Kerkis *et al.* (2007) who analysed cells isolated from deciduous pulp tissue samples. These cells were termed immature dental pulp stem cells (IDPSCs) as they expressed several embryonic stem cell markers. The spontaneous expression of neuronal markers (nestin and ßIII-tubulin) was noted in both the experimental cells incubated in the neurogenic medium and the control group cultured in the standard clonogenic medium. Furthermore, nestin positive cells were as numerous as ßIII-tubulin labelled cells during the entire differentiation process. The authors then suggested that IDPSCs could perhaps represent multipotent precursors of both SHEDs and DPSCs, however, recommended further studies in order to confirm their hypothesis.

CHAPTER 5

CONCLUSION

5.1. Cell culture

Cells isolated from the pulp of extracted deciduous and permanent teeth are highly proliferative, adherent in cell culture and they remain vital after a 3 weeks incubation period. The co-existence of haematopoietic stem cells with mesenchymal stem cells, although noted, has not been investigated further and this observation can be recommended for further studies.

5.2. Flow cytometry

The culture-expanded cells from the pulp tissue of deciduous and permanent teeth display the phenotypic cell surface markers described by the International Society of Cellular Therapy to characterise as mesenchymal stem cells: they were identified by various positive (CD29, CD44, CD90 and CD105) and negative mesenchymal stem cell markers (CD14 and CD45). However, in both populations, the low expression of $CD45^+$ cells observed in both populations again eludes to the expansion of haematopoietic stem cells in cultures of mesenchymal stem cells. This should be further examined in future experiments.

5.3. Neural differentiation

Characterised stem cells isolated from the human deciduous teeth and dental pulp stem cells exhibit the potential of self-renewal and differentiation in neuronal lineage. However, our study, restricted to an incubation period of three weeks, did not evaluate this

competency for a longer timeframe. A long term neural differentiation study could be envisaged in future work.

5.4. Immunocytochemistry

Ki-67 positive proliferative cells as well as doublecortin and nestin labelled cells in the neuroinduced SHEDs and DPSCs medium has been confirmed. In addition, an impaired proliferation rate has been noted after a prolonged culture period. Further investigation could perhaps intercept the exact time of cell incubation when this proliferation ceases. Furthermore, nestin expression throughout the life cycle of both the deciduous and permanent teeth, due to the neurocrestal origin of the odontogenic ectomesenchyme, has been reported. Future studies could perhaps focus on the expression of NeuN, a neuronal specific nuclear marker observed in most neuronal cell types in human. Finally, *in vivo* experiments in laboratory animals could also be undertaken in order to investigate the functional capacity of these neuronal differentiated cells

APPENDIX

APPENDIX A: Ethical Clearance Certificate

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Dr N Masumbuko Kahumba

CLEARANCE CERTIFICATE	**M120506**
PROJECT	Isolation, Culture and Neurogenic Differentiation of Human Dental Stem Cells
INVESTIGATORS	Dr N Masumbuko-Kahumba
DEPARTMENT	School of Anatomical Sciences
DATE CONSIDERED	25/05/2012
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 22/06/2012 **CHAIRPERSON** ..... (Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable

cc Supervisor: Dr Amaze Ihunwo

DECLARATION OF INVESTIGATOR(S)
To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES.

APPENDIX B: Cell count using a haemocytometer chamber

The culture-expanded cells from a confluent culture in a 25ml Nunc® culture flask were enzymatically treated.

- 1 ml of accutase-treated cells was suspended in 4ml culture of growth medium
- 20 µl of this cell suspension was mixed with an equal volume of 0.4% trypan blue (a dilution factor of 2) and then mixed thoroughly
- 20 µl of this suspension was then placed into the two compartments of a clean and dry haemocytometer chamber, using a pipette.
- Only the viable cells (clear cells) within the five squares were counted using the following formula below:
- Cells per ml = average cell count per square X dilution factor X 104

The desired cell concentration was obtained by diluting the cell suspension.

APPENDIX C1: Media

Phosphate buffered saline (PBS) (pH 7.4)

The following salts were added to 1 litre of distilled water

Na_2HPO_4	10.9 g
NaH_2PO_4	3.2 g
NaCl	9g

The pH of the solution was adjusted to 7.4 by adding 1M NaOH, then the solution was autoclaved and stored at 4°C.

Digestive solution:

The digestive solution consisted of the following supensions:

Collagenase:	6 mg / ml (0.06 g / 10 ml) in PBS.
Dispase:	8 mg / ml (0.08 g / 10ml) in PBS.

The abovementioned suspensions were added to a 50 ml tube, at a ratio of 1:1 to obtain the digestive solution which was stored at -20°C and warmed to 37°C before use.

Clonogenic medium:

Add the following reagents to 80 ml of Alpha MEM:

FBS:	20 ml of FBS
L-glutamine:	1 ml
Stock of ß-mecaptoethanol:	250 µl
Stock of L-ascorbic acid 2-phosphate:	100 µl of (1000 × stock) in 100 ml
Penicillin/Streptomycin:	50 µl

The solution was mixed and then stored at 4°C.

APPENDIX C2: Neurogenic Media

Neuroinductive medium A

The following reagents were added to 100 ml of Neurobasal A media:

B27 Supplement:	2 ml
Basic fibroblast growth factor:	160 µl
Penicillin/Streptomycin:	50 µl
Epidermal growth factor:	2 µl

Neuroinductive medium B

The following reagents were added to 100 ml of a solution made of 50 ml of DMEM and 50 ml of F12 media:

Insulin transferrin-sodium selenite supplement:	1 ml
Basic fibroblast growth factor:	160 µl
Penicillin/Streptomycin:	50 µl

APPENDIX D: Immunocytochemistry solutions

Phosphate buffered saline (PBS):

The following salts were added into 1 litre de-ionized water:

NaCl	8.00g
Na_2HPO_4	1.15g
KCl	0.20g
KH_2PO_4	0.20g

The pH was adjusted to 7.4 by adding 1M NaOH and the solution was then stored at 4°C.

Tris-Buffered Saline (TBS):

The following salts were added into 1 litre de-ionized water:

TRIS-BASE	6.055g
NaCl	8.766g

The pH was adjusted to 7.4 by adding 1M NaOH and the solution was then stored at 4°C.

Diaminobenzidine tetrahydrochloride (DAB) reagent:

DAB reagent consisted of the following suspensions:

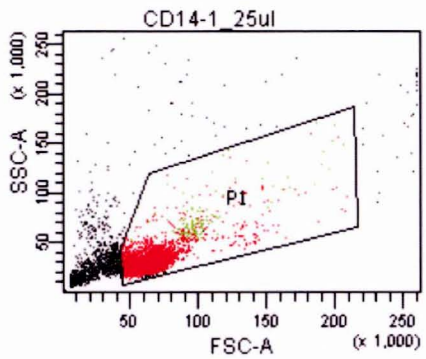
7 µl of 30% H_2O_2 added to 10 ml of distilled water

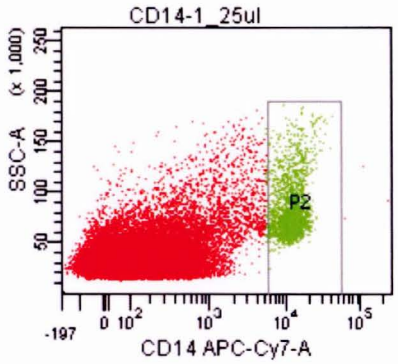
1 mg/ ml DAB was added into 10 ml PBS

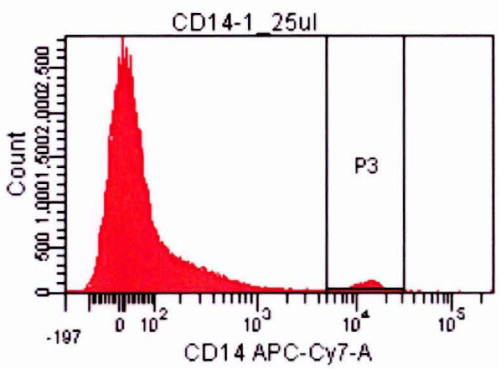
The two solutions were then mixed and filtered through a filter paper.

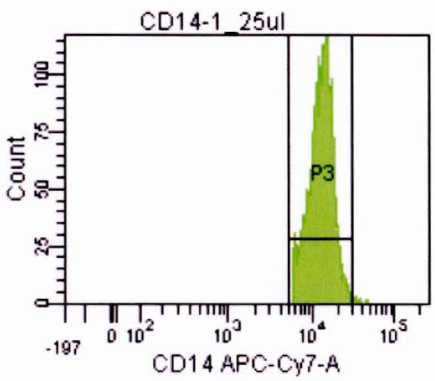
APPENDIX E1: Antibodies titration (CD14)

FACSDiva Version 6.2









Tube: 1_25ul

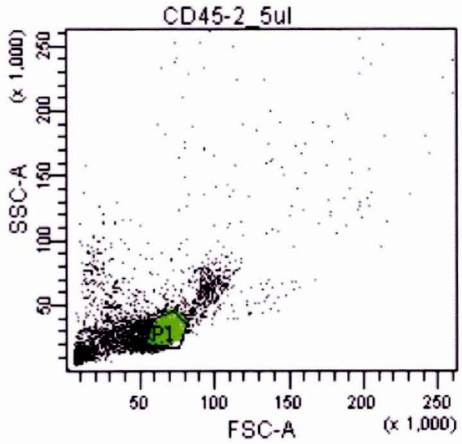
Population	#Events	%Parent	%Total
All Events	100,000	####	100.0
P1	76,688	76.7	76.7
P2	2,217	2.9	2.2
P3	2,256	2.9	2.3

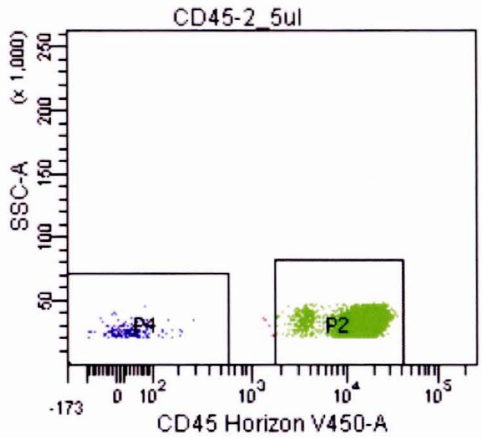
Experiment Name: CD14 APC H7
Specimen Name: CD14
Tube Name: 1_25ul

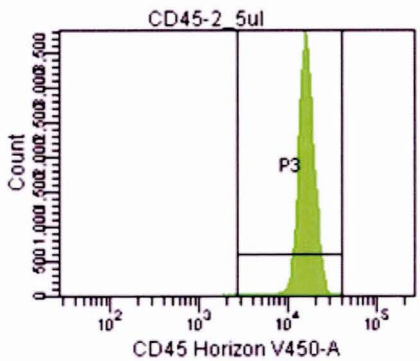
Population	%Parent	CD14 APC-Cy7-A Mean	CD14 APC-Cy7-A Median
P2	2.9	12,918	12,563
P3	2.9	12,651	12,416

APPENDIX E2: Antibodies titration (CD45)

FACSDiva Version 6.2







Tube: 2_5ul

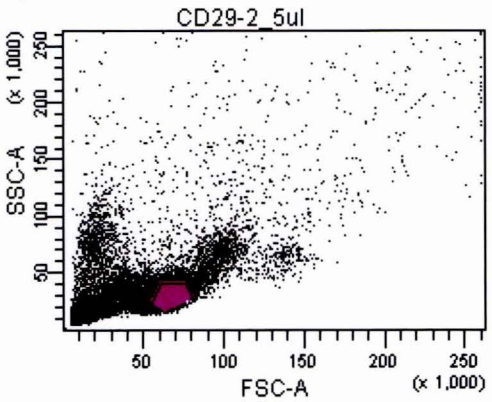
Population	#Events	%Parent	%Total
All Events	100,000	####	100.0
P1	48,410	48.4	48.4
P2	48,251	99.7	48.3
P3	48,229	99.6	48.2
P4	154	0.3	0.2

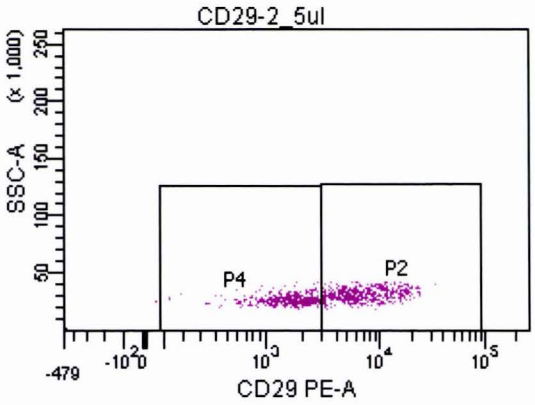
Experiment Name: CD45 V450
Specimen Name: CD45
Tube Name: 2_5ul
Record Date: Oct 3, 2013 1:31:00 PM

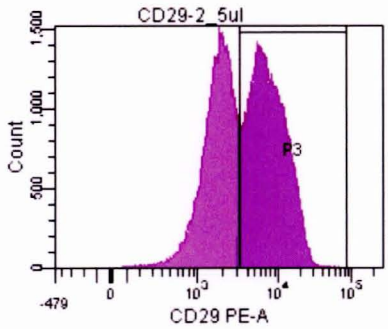
Population	%Parent	CD45 Horizon V450-A Mean	CD45 Horizon V450-A Median
P2	99.7	16,516	16,185
P3	99.6	16,523	16,187
P4	0.3	29	23

APPENDIX E3: Antibodies titration (CD29)

FACSDiva Version 6.2







Tube: 2_5ul

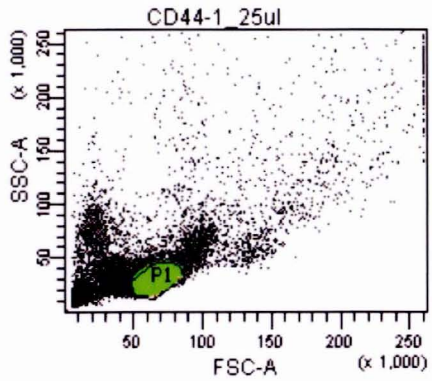
Population	#Events	%Parent	%Total
All Events	200,000	####	100.0
P1	92,340	46.2	46.2
P2	52,087	56.4	26.0
P3	50,646	54.8	25.3
P4	40,079	43.4	20.0
P2 OR P4	92,166	99.8	46.1

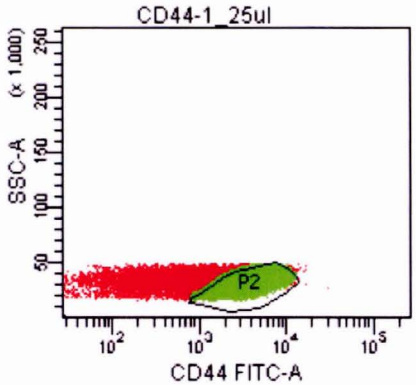
Experiment Name: CD29 PE
Specimen Name: CD29
Tube Name: 2_5ul

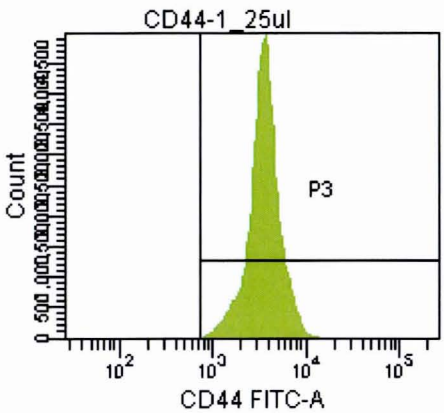
Population	%Parent	CD29 PE-A Mean	CD29 PE-A Median
P2	56.4	8,479	6,999
P3	54.8	8,634	7,140
P4	43.4	1,712	1,695
P2 OR P4	99.8	5,536	3,800

APPENDIX E4: Antibodies titration (CD44)

FACSDiva Version 6.2







Tube: 1_25ul

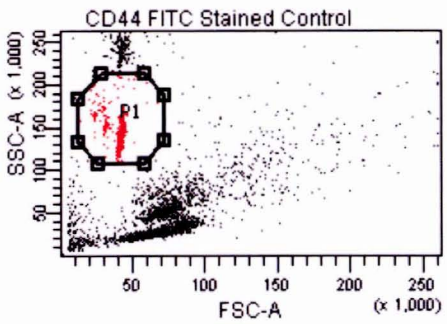
Population	#Events	%Parent	%Total
All Events	200,000	####	100.0
P1	120,042	60.0	60.0
P2	104,533	87.1	52.3
P3	110,663	92.2	55.3

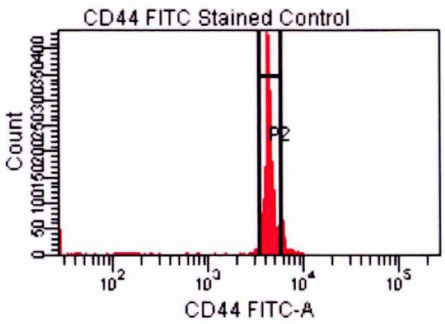
Experiment Name: CD44 FITC
Specimen Name: CD44
Tube Name: 1_25ul
$OP: Nyota
GUID: 5af3fcf0-f84d-43af-8f60-80aa6d5181b5

Population	#Events	%Parent	SSC-A Mean	CD44 FITC-A Mean	CD44 FITC-A Median
P1	120,042	60.0	26,430	3,415	3,396
P2	104,533	87.1	25,858	3,815	3,596
P3	110,663	92.2	26,161	3,670	3,516

APPENDIX F: Compensation beads (experimental group)

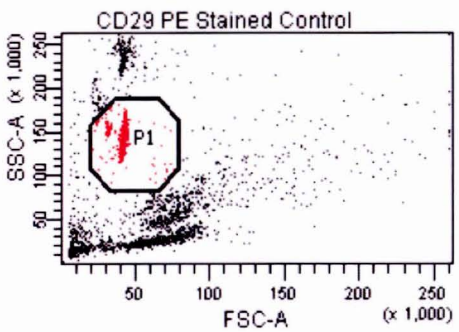
FACSDiva Version 6.2

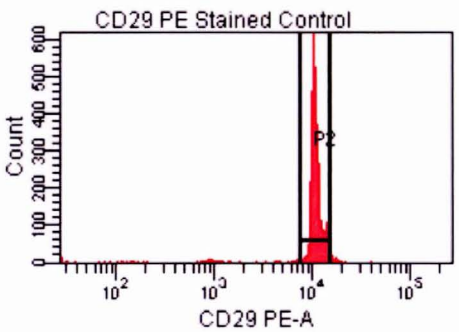




Specimen Name:	Compensation Controls
Tube Name:	CD44 FITC Stained Control
$OP:	Nyota
GUID:	e04f816b-f62e-4228-938f-8b45abc2f8db

Population	#Events	%Parent	CD44 FITC-A Mean	CD44 FITC-A Median
P1	2,491	49.8	4,342	4,303
P2	2,090	83.9	4,357	4,295



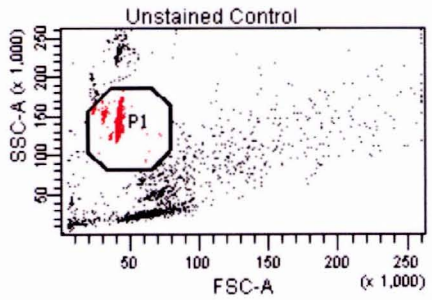


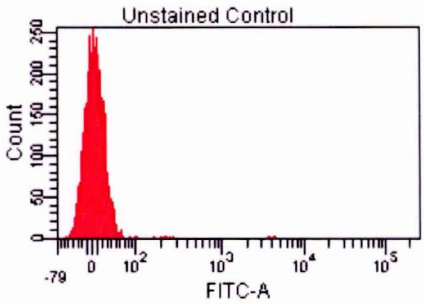
Specimen Name:	Compensation Controls
Tube Name:	CD29 PE Stained Control
$OP:	Nyota
GUID:	c8259635-b39e-47cc-b324-6b87204575e2

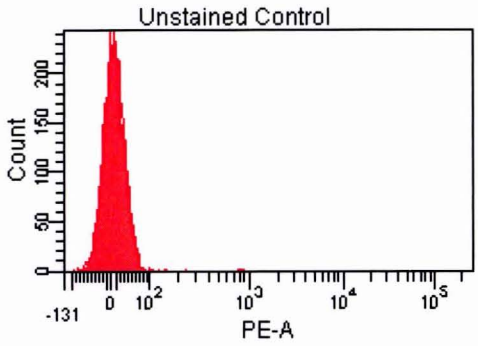
Population	#Events	%Parent	CD29 PE-A Mean	CD29 PE-A Median
P1	2,961	59.2	10,900	10,677
P2	2,766	93.4	11,050	10,682

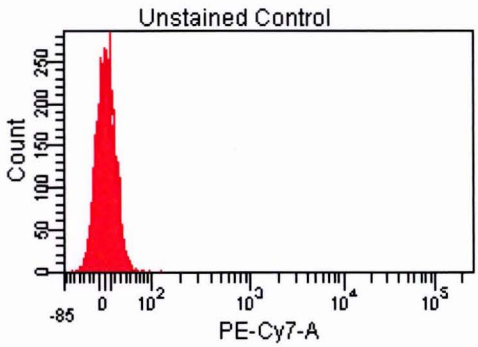
APPENDIX F: Compensation beads (control group)

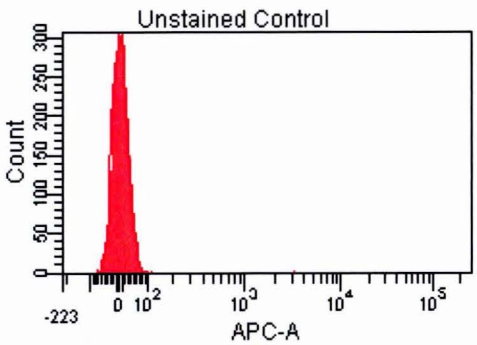
FACSDiva Version 6.2

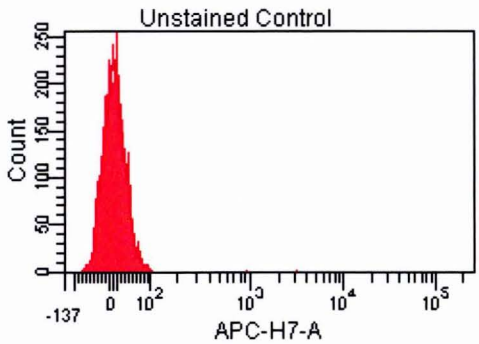


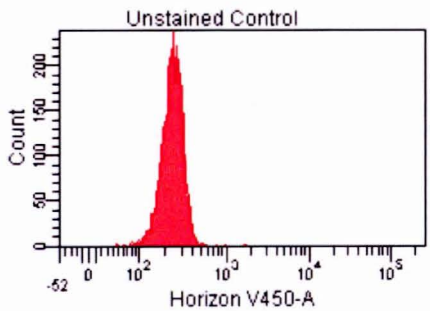












Specimen Name: Compensation Controls
Tube Name: Unstained Control

Population	FITC-A Mean	FITC-A Median	PE-A Mean	PE-A Median	PE-Cy... Mean	PE-C... Median	APC-A Mean	APC-A Median	APC-... Mean	APC-... Median	Horiz... Mean	Horiz... Median
P1	8	3	4	3	0	1	1	0	2	0	240	236

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