From the
Policlinic for Conservative Dentistry and Periodontology
at Rostock University
Director: Prof. Dr. med. dent. Hermann Lang

PERIODONTAL REGENERATION USING COLLAGEN MATERIALS, DENTAL STEM CELLS AND GROWTH FACTORS

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A COMPARATIVE STUDY IN MINIPIGS

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Tanja Basan
born on 11.08.1986 in Rostock, Germany

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Dean: Prof. Dr. med. habil. Emil Christian Reisinger

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Gutachter:

1. Gutachter:
   Prof. Dr. Hermann Lang,
   Poliklinik für Zahnerhaltung und Parodontologie,
   Universität Rostock
2. Gutachter:
   PD Dr. rer. nat. Christian Andressen,
   Institut für Anatomie,
   Universität Rostock
3. Gutachter:
   Prof. Dr. James Deschner
   Zentrum für Zahn-, Mund- und Kieferheilkunde
   Universität Bonn

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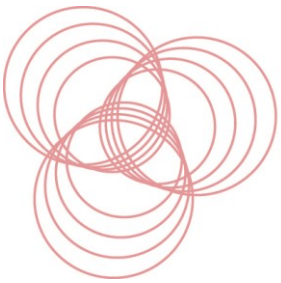

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All things are difficult
before they are easy.

Thomas Fuller (1608-1661)

Contents

List of abbreviations

ANOVA	analysis of variance
BMPs	bone morphogenetic proteins
BSE	bovine spongiform encephalopathy
CD	cluster of differentiation
CO_2	carbon dioxide
CTA	connective tissue attachment
DMEM- F_{12}	Dulbecco's Modified Eagle's Medium
DMS V	fifth german oral health study
DMSO	dimethyl sulfoxide
EA	epithelial attachment
ECM	extracellular matrix
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
FBS	fetal bovine serum
FGF	fibroblast growth factor
GF	growth factor
GTR	guided tissue regeneration
HA/TCP	hydroxyapatite/tricalcium phosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLA-DR	Human Leukocyte Antigen - antigen D Related
IGF	insulin-like growth factor
Kg	kilogram
LPS	lipopolysaccharide
M1	first molar
mg	milligram
ml	milliliter
MSCs	mesenchymal stem cells
NA	new attachment
NaCl	sodium chloride
$NaHCO_3$	sodium hydrogen carbonate
NC	new cementum
NHS	N-hydroxysuccinimide
P3	third premolar
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PDL	periodontal ligament

PDLSCs	periodontal ligament stem cells
Pen/Strep	Penicillin Streptomycin
pH	potentia hydrogenii
PMNs	polymorphnuclear neutrophils
rh	recombinant human
rpm	rounds per minute
SD	standard deviations
TGF	transforming growth factor
TSE	transmissible spongiform encephalopathy
VEGF	vascular endothelial growth factor
µl	microliter

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1 INTRODUCTION

1.1 Periodontitis

1.1.1 Periodontal apparatus

The healthy periodontal apparatus is composed of two hard and two soft tissues: the cementum and the alveolar bone as well as the gingiva and the periodontal ligament (Bartold et al. 2006). Figure 1 shows the components of the periodontal apparatus. It represents the supporting apparatus of teeth in function and occlusal relationships (Palumbo et al. 2011).

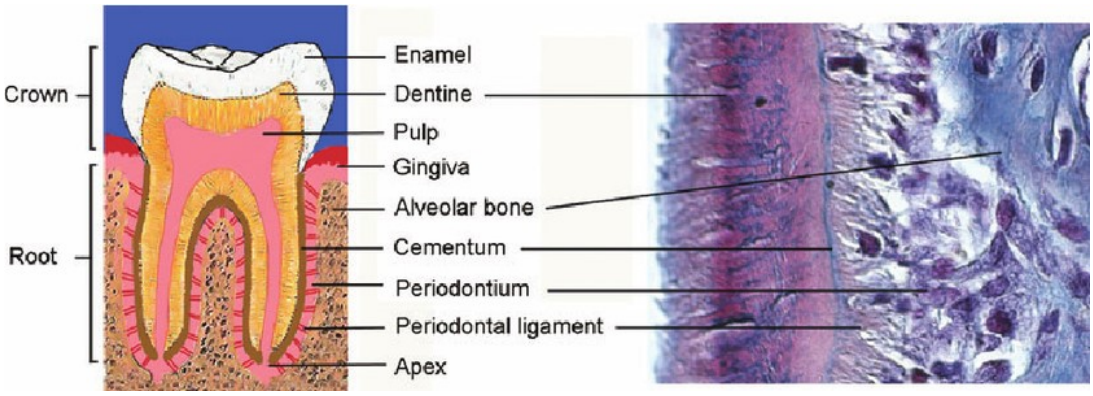

Figure 1: Components of the periodontal apparatus. left: illustration of a tooth and its surrounding tissues, right: histological picture of periodontal apparatus (Catón 2011).

The first soft tissue is the gingiva, which surrounds the tooth and overlays the jaw bone, whilst being tightly bound to the underlying bone. This presents an effective barrier against invasive pathogens as long as the periodontium is healthy. A healthy gingiva shows no reaction, such as bleeding, to periodontal probing or tooth brushing and has a coral pink color (Rateitschak 1989). The second soft tissue is a specialized connective tissue that represents the connection between cementum and alveolar bone of the tooth. Its complex fiber network is an elastic structure and is firmly anchored by Sharpey´s fibers (Beertsen et al. 1997). The periodontal ligament attaches the tooth to the alveolar bone socket and is also important for propriception. It is mainly composed of collagen fibers, is highly vascularized and contains many cells, including fibroblasts, cementoblasts or osteoblasts (Lekic et al. 1996). The cementum, a hard tissue and the substance that covers the root of a tooth, is a specialized calcified substance. It is an avascular mesenchymal tissue that attaches the teeth to the alveolar bone by anchoring the periodontal ligament and has the highest fluoride content of all mineralized tissues. The tissue is formed continuously throughout lifetime to guarantee an intact attachment (Rateitschak 1989). There are different types of cementum, including cellular and acellular.

Findings from previous studies showed that the cementum formed after periodontal treatments is mostly cellular (Sculean et al. 2005). The fourth component of the periodontium is the alveolar bone, which surrounds the tooth. It is also known as *dental alveoli* or *alveolar process.* The alveolar bone is attached to the cementum and also to the root of the tooth by the periodontal ligament. It is also modified throughout life, animated by various external factors (Rateitschak 1989).

1.1.2 Pathogenesis

One of the most common causes for tooth loss in adults is periodontitis, which is defined as a bacteria-induced inflammatory disease of the periodontal tissues (Park 2011). The disease progresses distinctly and is marked by clinical attachment loss, alveolar bone resorption, periodontal pocketing and gingival inflammation (Pihlstrom et al. 2005). The destruction of the periodontal apparatus is caused by the inflammation of the host as reaction to the microbial challenge (Darveau et al. 2010). The host response is modulated through a multitude of factors, such as genetics, general health, smoking habit, diet or other social determinants (Bartold et al. 2013). The latest epidemiological data in the USA has shown a very high prevalence of periodontitis of over 47% of adults (Eke et al. 2012). The fifth German oral health study (DMS V) from 2014 showed that there is still the need for periodontal treatments, although young adults show less periodontal diseases, due to an impending demographic change in Germany. In 2030, the majority of the population will be senior citizens and therefore the regeneration of lost periodontal tissues will still be a challenge in the German health system (Jordan et al. 2014). Periodontitis is not only a risk for early tooth loss, it can also lead to other systemic diseases like atherosclerosis, diabetes mellitus, rheumatoid arthritis and adverse pregnancy outcomes (Kebschull et al. 2010), (Madianos et al. 2013). Bacteria associated with periodontitis are a group of Gram-negative predominantly anaerobic species, the more prominent being the “red complex”, which are *Porphyromonas gingivalis* (*P. gingivalis*), *Treponema denticola* (*T. denticola*) and *Tannerella forsythia* (*T. forsythia*) (Holt 2005). Another bacterium responsible for periodontitis is *Aggregatibacter actinomycetemcomitans,* mainly associated with aggressive periodontitis (Schacher et al. 2007). Substances released from bacterial biofilm, consisting of the populations of above-named bacteria, are e.g. endotoxins, lipopolysaccharides (LPS), antigens or other virulence factors. They gain access to the gingival tissue and initiate an inflammatory immune response. This response is composed of a cellular activation of the host defence cells, which are inflammatory

mediators including cytokines, arachidonic acid metabolites and proteolotic enzymes. The activation of these cells leads to tissue destruction and bone resorption (figure 2) (Yucel-Lindberg and Båge 2013).

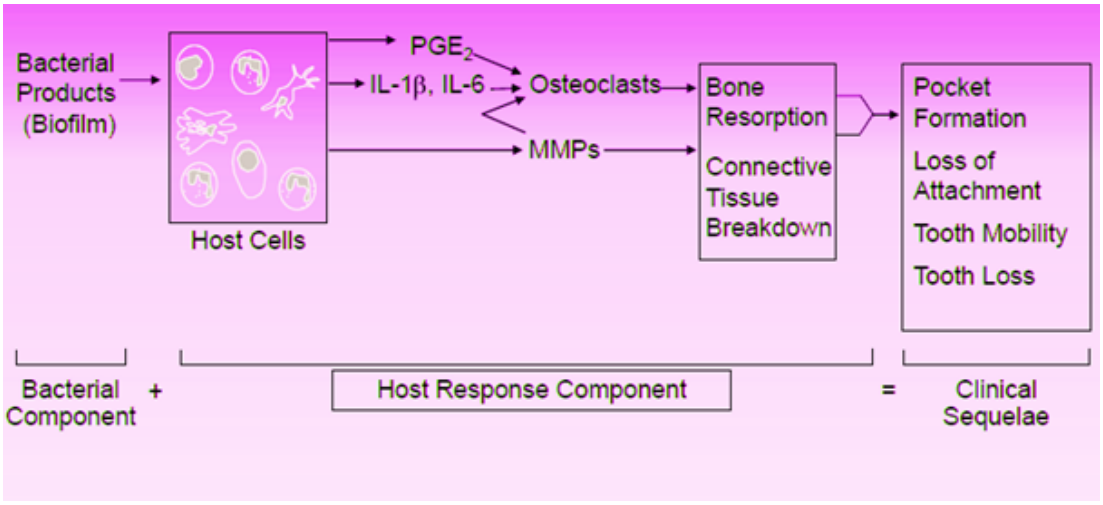

Figure 2: Pathogenesis. Bacterial products lead to activation of the host cells, via several intermediate steps to the activation of osteoclasts and MMPs which ends in the clinical picture of periodontitis (Golub et al. 1998).

The immunoinflammatory response is described as a “double-edged sword”, because on the one hand it causes destruction and on the other it provides specific antibodies and polymorphnuclear neutrophils (PMNs), which represents the dominant natural factors responsible for control of the bacterial challenge (Kornman 1999).

Once periodontitis is established, the collagen tissues are lost and the periodontal ligament is reduced in height and volume. These tissues are replaced by the downgrowth of gingival epithelium. By then, there is a defect marked clinically by bone and periodontal ligament loss, gingival pockets and recession of the gingival margin (figure 3) (Amar 1996).

In a US study from 2012 (with data from the 2009 and 2010 National Health and Nutrition Examination Survey (NHANES) cycle) over 47% of the adults aged 30 years and older, representing 64.7 million adults, suffer from periodontitis (Eke et al. 2012). This huge number clarifies the importance of researching and developing new and effective treatment options for periodontitis. A common and reliable procedure for regenerating the lost periodontal tissues has to be established. The conventional treatment of periodontitis is described in the following section.

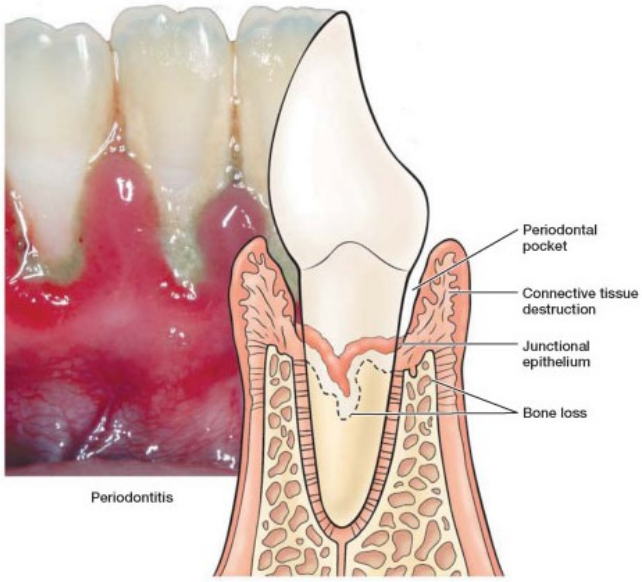

Figure 3: Clinical signs of periodontitis. Inflammation of the supporting structures of the tooth, progressive destruction of the periodontal ligament and loss of the alveolar bone (http://www.dent-wiki.com/foundations_of_periodontics/characteristics-of-chronic-periodontitis).

1.1.3 Treatment of periodontitis

One of the most difficult challenges in periodontics is the regeneration of the tooth supporting structures, which reduce as a result of periodontal disease progression (Lee et al. 2005). A periodontal therapy may cause either a repair or a regeneration of the surrounding tissues. Repair means healing of the tissues without maintaining its original morphology and function, like a non-functional scarring, whereas regeneration is defined as a complete recovery of the periodontal tissues in both structure and function. Therefore, regeneration is equivalent to the formation of alveolar bone and the building of collagen fibers that insert in newly formed cementum along the root (Illueca et al. 2006) A complete new periodontal apparatus is the entirety of bone, cementum and periodontal ligament (Sharpey´s fibers) (LeBlanc and Reisz 2013). The natural healing of wounds normally results in scarring or repair, therefore it is necessary to manipulate the natural healing process.

Conventional therapies for periodontitis are scaling and root planning as well as various surgical procedures. Their goal is to eliminate inflamed tissues and bacterial contamination with the result of these treatment options is more a repair than regeneration. There is a limitation for conventional treatments. On these grounds research focuses on root surface alteration, progenitor cell manipulation, cell exclusion, wound stabilization and growth factor enrichment (Amar 1996). The most

approved techniques so far are guided tissue regeneration (GTR) (Siciliano et al. 2011) and osseous grafting (Chen et al. 2013).

Frequently, the loss of teeth is caused by the progressive bone resorption (Duan et al. 2011). Current periodontal surgical treatment options, like scaling and rootplaning or open - flap debridement, have been established to eliminate inflammation and thus a disease progression, but are limited in periodontal regeneration (Crea et al. 2014). The regeneration of the lost alveolar bone is promoted by autografts (bone marrow), allografts (demineralized freeze-dried bone) and alloplastic (ceramics, hydroxyapatite, polymers and bioglass) materials, but the regeneration of alveolar bone is not the major challenge (Reynolds et al. 2003). Further researches should be focused on the regeneration of the periodontal ligament and newly formed cementum (Bartold 2006).

The goal is a complete regeneration, which can only be achieved with a network of stem cells, growth factors and environmental conditions (Lee et al. 2005). Periodontal treatments are limited by the formation of a long junctional epithelium and an insufficient formation of new cementum (Mardinger et al. 2012) (Dan et al. 2014). The tooth only disposes of a stable periodontal apparatus if the connection between root surface and alveolar bone is composed of newly generated periodontal ligament fibers inserting into new cementum (Duan et al. 2011).

The complex process of periodontal regeneration depends on locally derived progenitor cells, which are able to differentiate into periodontal ligament-forming cells, mineral-forming cementoblasts and bone-forming osteboblasts (Bartold and Narayanan 1998). One of the most difficult parts in regenerating the periodontal apparatus is the cementogenesis. The precise mechanism of cementum formation is still unclear. The theory is that a special matrix is generated on the newly formed dentin surface, although the precise function of this matrix is still unclear (Lindskog 1982; Hirooka 1998).

The most reliable cells for regenerating cementum, alveolar bone and PDL (periodontal ligament) are cementoblasts, osteoblasts and fibroblasts (Catón et al. 2011). The precursors of these cells are mesenchymal stem cells. Stem cells have the potential to differentiate into several cell types and can be cultured under defined tissue culture conditions *in vitro* (Estrela et al. 2011). There are many different kinds of dental stem cells that have been isolated and characterized: periodontal ligament stem cells (PDLSCs), dental pulp stem cells (DPSC´s), stem cells from human exfoliated deciduous teeth, stem cells from apical papilla and dental follicle precursor cells (Nakahara 2011). PDLSCs seem to have a great potential in

regenerative medicine (Tsumanuma et al. 2011). Due to the fact that stem cells always need a suitable carrier material, materials science is another important field, because it brings regenerative dentistry from the laboratory to the clinic (Mitsiadis et al. 2012).

New alternatives to conventional treatment options are methods based on the principles of guided tissue-engineering (GTR), which is based on the principles of cell biology and biomaterials and describes a technique to build new tissues to replace damaged or diseased tissues (Bartold and Narayanan 1998). There are three essential factors for a successful regeneration: a conductive scaffold/extracellular matrix, signaling molecules and stem or progenitor cells (Lynch et al. 1999). GTR may include the gene therapy (stem cells) and the use of biocompatible scaffolds with or without growth factors (Giannobile et al. 2001). There are a number of possibilities to treat periodontitis nowadays, but none of them appear to regenerate the original tissues totally and as a complex whole (Aljateeli et al. 2014), (Soo et al. 2012). In guided tissue regeneration, a barrier membrane is placed between the implanted materials and the surrounding soft tissue. This anticipates the invasion of gingival epithelial cells and guides the healing process (Buser et al. 1994). A barrier membrane should be degradable, but must not degrade uncontrolled. Otherwise it loses its barrier function. Therefore bi-layered membranes were developed to improve their stability and avoid cellular invasion. Osteoblasts and other periodontal structure building cells, like fibroblasts and cementoblasts, should be able to develop along with cells for the oral soft tissues (Behring et al. 2008). But only with therapeutic intervention it is possible to stimulate regeneration once periodontitis becomes established (Bartold et al. 2000).

1.1.4 Application of materials and scaffolds

A lot of biomaterials can serve as carrier materials, such as natural or synthetic polymers, extracellular matrix, self-assembling systems, hydrogels or bioceramics. Scaffolds qualified for transporting stem cells are for example different types of collagen (Galler et al. 2011). Growth factors in combination with biocompatible matrices play another important role in the reformation of the PDL (Kaigler et al. 2011). There are a variety of materials that were used to support periodontal regeneration so far (table 1).

Table 1: Materials used as scaffolds (Palwankar et al. 2014).

Natural	Synthetic
Ceramic	Polymers - Polyglycolic, Polyactic
Hydroxyapatite	Polycaprolactone
Tricalcium Phosphate	Co-polymers
Polymers - Hyaluronic Acid,	Polyphosphagonos
Alginate, Agarose, Chitosan,	Nano Calcium Sulphate
Collagen and Albumin	

Materials have been examined in a range of studies, including bone replacement grafts, such as autografts, allografts, xenografts and alloplasts. Guided tissue regeneration materials such as barrier materials, including both non-resorbable and bioabsorbable membranes have also been investigated in addition to growth factors such as bone morphogenetic proteins (BMPs) (Chen et al. 1995). Furthermore, bone replacement grafts have been well reviewed. There are many studies that investigate the benefit of those materials, like hydroxyapatite or tricalcium phosphate. They are easy to place and promise good clinical results (Reynolds et al. 2003).

Stem cells (see 1.1.6) need a carrier material for implantation. Carrier materials, also called scaffolds, should provide a 3D substratum in which cells can proliferate and migrate. The introduced cells produce a matrix and form a functional tissue once they are in the scaffold (Pandit et al. 2011). Therefore, the scaffold must have a microstructure and chemical composition, which is necessary for normal cell growth and function (Oh et al. 2006). Other functions of the matrices include structural reinforcement, a barrier to in-growth of surrounding tissues and regulation of cell function due to its interaction with certain integrins (Pandit et al. 2011). Biocompability and biodegradability are required qualities of a scaffold (Yang et al. 2001).

Collagen can also be used as a scaffold/carrier material. A scaffold should provide “excellent biocompatibility, controllable biodegradability, appropriate mechanical strength, flexibility as well as the ability to absorb body fluids for delivery of nutrients” (Khan and Khan 2013). Collagen fulfils these requirements, making it a popular biomaterial. Collagen is biocompatible and degradable, making a second surgical intervention unnecessary. It has been shown to support angiogenesis (Rothamel et

al. 2004), making it an ideal candidate for scaffolds. It is a versatile material and has many capabilities including keeping the wound pliable and flexible, promoting the development of granulation tissue, minimizing pain and providing mechanical protection against physical and bacterial damage (Mitchell and Lamb 1983). The collagen protein has a complex hierarchical conformation divided into four structures: primary (amino acid triplet), secondary (the α-helix), tertiary (triple helix) and quaternary (fibrils) (Gelse et al. 2003). Collagen can be easily modified into different physical forms such as powder/particles, gel/solution, membranes, sponges and composites (with ceramics) making it easy to apply in dental medicine (Ferreira et al. 2012).

1.1.5 Growth factors

At cellular level, wound healing and regeneration involves a complex sequence of events, including cell proliferation and differentiation. These processes are known to be modulated by growth factors (Graziani et al. 2006). With better understanding of periodontal regeneration process polypeptide growth factors play an increasingly important role in research. Growth factors are major growth-regulatory molecules and polypeptides, which stimulate cell proliferation *in vitro* and probably also *in vivo* (Goustin et al.1986). Various types of growth factors, for example epidermal growth factors, fibroblast growth factors, insulin-like growth factors, platelet-derived growth factors, tumor-derived growth factors and bone morphogenetic proteins were described (table 2) (Giannobile et al. 2001). Combinations of these growth factors do also exist, such as platelet-rich plasma preparations (Carlson and Roach 2002).

Growth factors are naturally occurring substances capable of stimulating cellular growth, proliferation, healing and cellular differentiation. Usually they are proteins or steroid hormones. They play a crucial role in information transfer between cells by binding to specific receptors on the surface of target cells. The chemical identity, concentration, duration and context of these growth factors contain information that dictates cell fate (Tayalia and Mooney 2009).

Within the complex “wound healing cascade”, GFs are known to play a central role in information transfer between a wide range of cells and their ECM. They stimulate endogenous repair mechanisms by providing the right signals to cells. This leads to a functional restoration of damaged or defective tissues (Chen et al. 2010).

Table 2: Functions of the main GFs in relation to different healing processes (Ramos-Torrecillas et al. 2014, Sigurdsson et al. 1997).

Growth factor	Most representative function
Platelet-derived growth factor	Chemotaxis, inducing cells to migrate to the wound bed.
Transforming growth factor-ß	Cell proliferation inhibition, increase in synthesis of extracellular matrix and inhibition of degradation; it favors neutrophil and monocyte chemotaxis, although its specific action also depends on the cell environment.
Bone morphogenetic proteins	Repair of epidermis in more superficial layers of skin and inhibition of keratinocyte proliferation in deeper layers, ability to induce bone formation.
Fibroblast growth factor	Mitogenic for endothelial cells, fibroblasts, chondroblasts, and osteoblasts; it favors angiogenesis.
Epidermal growth factor	Proliferation and mobility of fibroblasts and keratinocytes.
Vascular endothelial growth factor	Angiogenesis and increase in capillary permeability.
Insulin-like growth factor	Favoring reepithelization and production of granulation tissues.
Interleukins	General proinflammatory function, regulation of immunological cell growth and/or differentiation.

1.1.6 Dental stem cells

Stem cells are immature progenitor cells with regenerative potential. Based on a process of asymmetric mitosis, in which progenitor stem cells and daughter cells occur, they are capable for cell renewal and multi-lineage differentiation (Estrela et al. 2011). Stem cells can self-renew and produce different cell types. Therefore, they can offer new possibilities to regenerate missing tissues and treat diseases. In the field of dentistry, adult mesenchymal stem cells (MSCs) represent the most promising stem cells. They have been identified in several oral and maxillofacial tissues (Egusa et al. 2012). MSCs are the most versatile stem cells among all adult stem cells, because they can change their phenotype easily during differentiation.

They are also available in a large number and can be isolated and cultured relatively easy (Vemuri et al. 2011).

Recent studies showed that oral tissues are a rich source of stem cells. This might bring new opportunities, because those tissues are easily accessible for dentists. Once extracted, the removed tissue samples can be used for *in vitro* cell cultivation and play an important part in tissue engineering (Langer and Joseph 1993). Autologous stem cells can be embryonic or adult stem cells. Embryonic stem cells originate from embryonic tissues, like placenta or umbilical cord (Nadig 2009), which are pluripotent because they can develop into all types of cells from all three germinal layers. The adult stem cells, which are mostly multipotent, can only differentiate into a limited number of cell types (Egusa et al. 2012).

Only 30 years ago Melcher published that the three cell populations of the periodontium (cementoblasts: building cementum; fibroblasts: building periodontal ligament and osteoblasts: building alveolar bone) are all derived from one population of stem cells (Melcher 1985). Melcher also determined that only cells from the periodontal ligament can synthesis and secrete cementum and at the same time attach newly-formed collagen fibers.

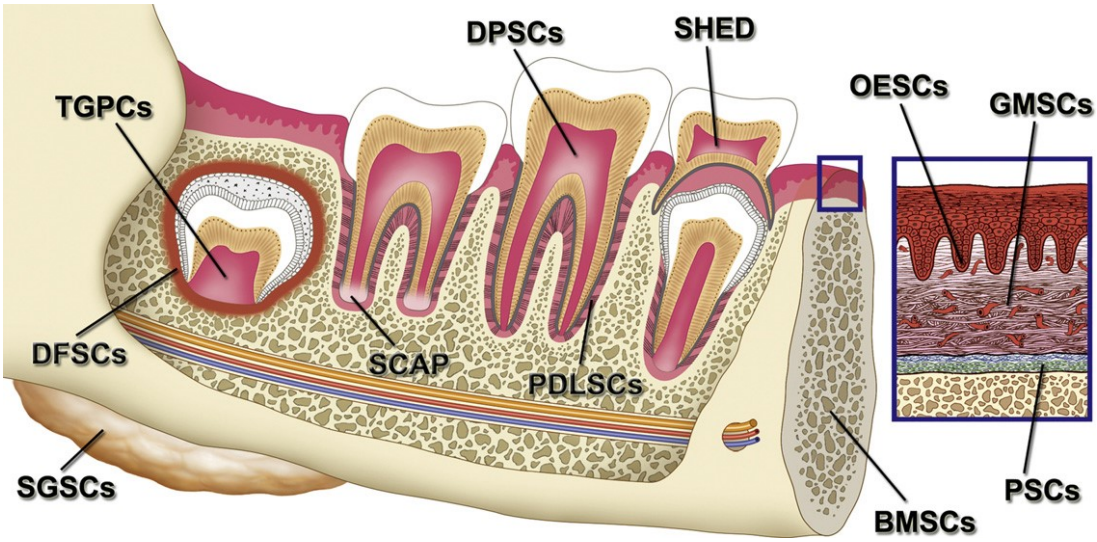

Figure 4: Sources of adult stem cells in the oral and maxillofacial region. BMSCs: bone marrow-derived MSCs from orofacial bone; DPSCs: dental pulp stem cells; SHED: stem cells from human exfoliated deciduous teeth; PDLSCs: periodontal ligament stem cells; DFSCs: dental follicle stem cells; TGPCs: tooth germ progenitor cells; SCAP: stem cells from the apical papilla; OESCs: oral epithelial progenitor/stem cells; GMSCs: gingiva-derived MSCs, PSCs: periosteum-derived stem cells; SGSCs: salivary gland-derived stem cells (Egusa et al 2012).

Adult stem cells can self-renew and differentiate to maintain healthy tissues and repair injured tissues. They can be found in many oral tissues and many sources have been found in the maxillofacial region (figure 4). It is believed that the adult stem cells reside in a specific area of each tissue, called “stem cell niche”. A lot of these stem cells appear in mesenchymal tissues, why they are called mesenchymal stem cells (MSCs) (Egusa et al. 2012). MSCs are adherent to tissue-cultured-treated plastic, which is a well-established method to identify those (Horwitz et al. 2005). Another criterion for identifying stem cells is the expression of certain cell surface markers like CD105, CD73 and CD90 and a lack of the expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR surface molecules. Further, they must be able to differentiate to osteoblasts, adipocytes and chondroblasts *in vitro* (Dominici et al. 2006).

MSCs are the most promising stem cells for clinical applications. They were initially found in bone marrow first and can be found in several oral and dental tissues yet, like orofacial bone, dental pulp, periodontal ligament etc. (figure 5) (Ding et al. 2011). The regenerative potential of some dental tissues can be observed. The periodontal tissues are able to regenerate by a natural process after an orthodontic treatment. Another example is the building of reparative dentin after a root canal treatment or a deep carious lesion (Cox et al. 1992).

Periodontal ligament stem cells (PDLSCs) are part of the MSCs and can be isolated from extracted teeth. In animal models it has been established that PDLSCs can regenerate any kind of periodontal tissues (cementum, periodontal ligament and alveolar bone) (Seo et al. 2005).

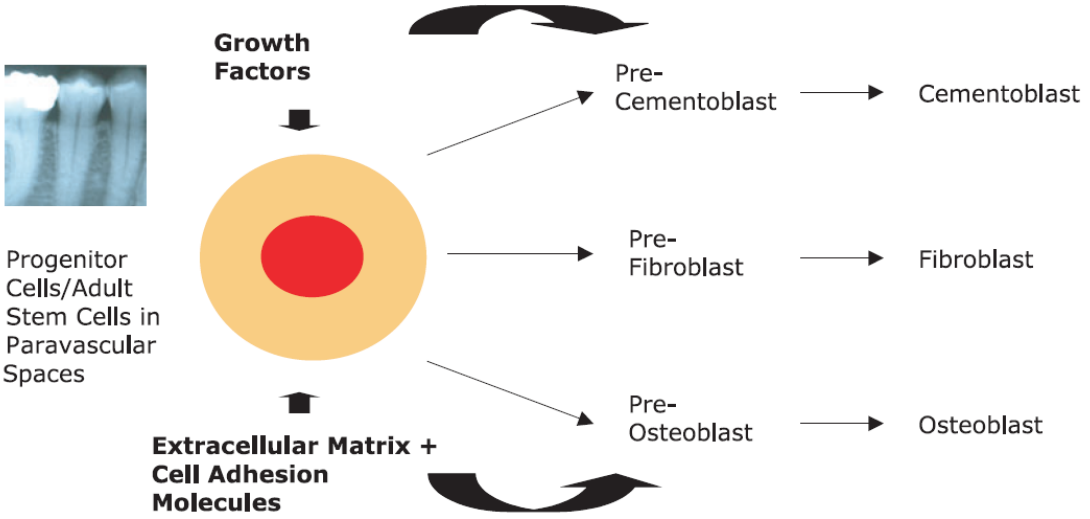

Figure 5: Stem cell differentiation/role of periodontal stem cells in periodontal regeneration. Progenitor stem cells can differentiate into cementoblasts, fibroblasts or osteoblasts (Bartold et al. 2006).

PDLSCs need a suitable scaffold being implanted, such as hydroxyapatite/tricalcium phosphate (HA/TCP), to induce the formation of cementum, periodontal ligament and bone *in vivo* (Gronthos et al. 2000).

Studies showed that it is also possible to regenerate other tissues such as skin, cartilage, bone, pancreas, etc. with the help of stem cells (Persidis 1999). Therefore, it seems achievable that autologous periodontal ligament stem cells, cultured within a suitable scaffold in combination with growth factors presented in autologous blood, will lead to new periodontal tissue attachment (Bartold et al. 2006).

An example for testing the potential of PDLSCs *in vivo* is a study with rats. Cultured human periodontal ligament stem cells have been implanted into surgically created periodontal defects in immunosuppressed rats. The result was a “periodontium-like-structure” consisting of cementum, inserting periodontal ligament and alveolar bone (Seo et al. 2004). Many studies have been performed with minipigs or beagles (Lang et al. 1997, Pieri et al. 2009, Kawaguchi et al. 2004).

1.2 Purpose of this study

In this study we used a minipig model to analyze the potential of different collagen carrier materials with or without periodontal ligament stem cells or a growth factor cocktail for periodontal regeneration in class II furcation defects. The aim of the present study was to examine the regenerative potential of a) different collagen support versus control, b) different collagen support +/- a growth factor cocktail (GF) and c) a collagen powder versus collagen powder + periodontal ligament stem cells (PDLSCs) comparatively in a large animal model.

2 MATERIALS AND METHODS

2.1 Materials

A collagen membrane, a collagen matrix and a collagen powder were implanted solely or combined with either dental stem cells or a growth factor cocktail into class II furcation defects. All defects were covered with a semipermeable membrane (bredent medical, angiopore selective permeable membrane, Senden, Germany) with a thickness of 0,3 mm – 0,5 mm and a size of 35 mm × 45 mm (REF: AP053545). The membrane consisted of compact collagen fibre structures and offered a combination of safe barrier function and enhanced angiogenesis. The compact fibre structure hindered the invasion of gingival epithelial cells to avoid an epithelial downgrowth, although the micro-fibrillary angiopores served as guidance for the infiltration of blood vessels, which were important for the regeneration process.

2.1.1 Collagens

Three different collagen materials were used in this study. The collagen membrane (DOT, Rostock, Germany) consisted of 1% collagen mixed with hydroxylapatit/tricalcium phosphate (BONITmatrix) in a mass relation of 4:1. Hydroxylapatit and tricalcium phosphate have been investigated thoroughly in intrabony and furcation defects (Saffar et al. 1990), (Bowen et al. 1989). BONITmatrix is a synthetic resorbable bone graft material. It consists of hydroxyapatit (HA) and ß-tricalciumphosphat (ß-TCP) in the proven ratio of 60/40. A specific particularity is the incorporation of biological active silicon in form of a silicon dioxide xerogel. The material is characterized by an interconnecting pore system in the nano- and micrometer range. Biological fluids can diffuse due to the high capillarity and the adsorptive capacity of the surface and the binding of growth factors leads to osteogenesis.

Furthermore, a collagen matrix (Bioserv, Rostock, Germany) was applied. Therefore, collagen from jellyfish *Rhopilema spec.,* a biopolymer with natural ECM (extracellular matrix) characteristics and low risks for BSE was used in this study. Collagen is a highly conserved protein across multiple species and can be obtained from different sources, like bovine or porcine skin. However, there is still the problem of potential bovine spongiform encephalopathy (BSE) or transmissible spongiform encephalopathy (TSE). To avoid this risk jellyfish from marine sources, precisely from jellyfish *Rhopilema spec.* was chosen to design a 3D scaffold for tissue engineering applications. In earlier tests these scaffolds promoted excellent cell

growth, biocompatibility and suitable degradation characteristics (Hoyer et al. 2014). A jellyfish contains more than 95 % water. When they are dried, more than 40 % of their dry weight is collagen, making them an excellent source of collagen (Miura and Kimura 1985).

Also, a commercially available collagen powder was used as filling material and carrier for the dental stem cells. The collagen powder was provided by MedSkin Solutions Dr. Suwelack AG, Billerbeck, Germany. The material was obtained from bovine skin and either implanted solely or used as a carrier material for the periodontal ligament stem cells. The collagen powder in spherical shape was easy to apply and consisted of the collagen types I, III and V.

2.1.2 Periodontal ligament stem cells

The stem cells used in this study were periodontal ligament stem cells – PDLSCs. Periodontal ligament stem cells are part of the mesenchymal stem cells (Acharya et al. 2010). PDLSCs can produce cementum- and periodontal-like structures *in vivo*. Therefore, they can play an important role in regenerative dentistry (Mrozik et al. 2010). Under standard growth conditions, i.e. 10 % fetal bovine serum (FBS) - containing medium, PDLSCs can be isolated from the periodontal ligament and cultured in a culture dish. Periodontal ligament stem cells are heterogeneous, adherent to plastic and do have a fibroblast-like morphology (Vemuri et al. 2011). PDLSCs do have the ability to generate clonogenic adherent cells (Seo et al. 2004).

2.1.2.1 Isolation of PDLSCs

The PDLSCs were isolated and cultured according to Haddouti´s protocol with slight modification (Haddouti et al. 2009). In the first surgery, the teeth were extracted and surrounding tissues were taken from a total of 15 minipigs. The extracted teeth plus the surrounding tissues were used to isolate and culture mesenchymal stem cells. All surgical procedures were carried out under aseptic conditions. All extracted teeth and viable materials were treated repeatedly with a rinsing solution, consisting of cooled phosphate buffered saline (PBS, 20 ml PBS 1x) and antibiotics (200 µl penicillin/streptomycin 1x, PenStrep, gibco, Grand Island, NY, USA) to avoid bacterial contamination. The samples were transported in DMEM-F_{12} (gibco, Grand Island, NY, USA) including 2 % antibiotics (PenStrep, gibco, Grand Island, NY, USA). They had to be kept on a constant temperature of + 4 °C.

In the laboratory, PDL tissues were separated from the root using a scalpel and were minced to the smallest size possible under aseptic conditions. The tissue

samples were incubated in DMEM-F_{12} (gibco, Carlsbad, USA) with 2.5 mg/ml of dispase (Sigma-Aldrich, St. Louis, USA) for 1.5 hours at 37 °C and 5 % CO_2. After incubation, the tubes were centrifuged at 400 g for 4 minutes at 4 °C. The supernatant was discarded, the remaining tissues transferred into a cell culture flask (greiner-bio one GmbH, Kremsmünster, Austria) and 3 ml cell culture medium of DMEM-F_{12} including 10 % fetal bovine serum (BIOCHROM, Berlin, Germany) and 1 % PenStrep (gibco, Carlsbad, USA) was added. The cell culture flasks were incubated at 37 °C and 5 % CO_2.

The following day, floating cells were removed and the medium was replaced with fresh medium. All samples were checked for bacterial contamination under a light microscope. Flasks without bacterial contamination were incubated for 1 – 2 weeks and attached cells were fed with fresh medium every 3 days. The cells were cultured in DMEM-F_{12} and passaged with trypsin (gibco, Grand Island, NY, USA). Daily control of the samples was necessary. Cell culture stocks were frozen. Therefore, the cells were trypsinated with 2 ml trypsin (gibco, Grand Island, NY, USA) and centrifuged at 400 g for 1 minute at 4 °C. Cryotubes were used to freeze cell passages with FBS (fetal bovine serum, BIOCHROM, Berlin, Germany) and 10% DMSO (dimethyl sulfoxide, Carl Roth GmbH, Karlsruhe, Germany) at -70 °C. For reimplantation 10^6 cells of passage (p) 3 – 4 were applied.

2.1.2.2 Characterization of PDLSCs

Periodontal ligament stem cells (figure 6) are a unique population of the mesenchymal stem cells and can be characterized by the expression of different surface markers like CD 29, CD 44, CD 90 and many more (table 3).

Table 3: Characteristics of human dental tissue and gingiva-derived MSCs. PDL: periodontal ligament; differentiation lineages: dent (dentinogenic lineage), mes (mesodermal lineage), (Egusa et al. 2012).

Stem cells	CD antigen expression		Other representative markers	*In vivo* tissue formation capacity
	positive	negative		
PDLSCs	CD9, CD10, CD13, CD29, CD44, CD49d, CD59, CD73, CD90, CD105 CD106, CD146, CD166	CD31, CD34, CD45	STRO-1, Scleraxis	dent (cementum, PDL), mes (alveolar bone)

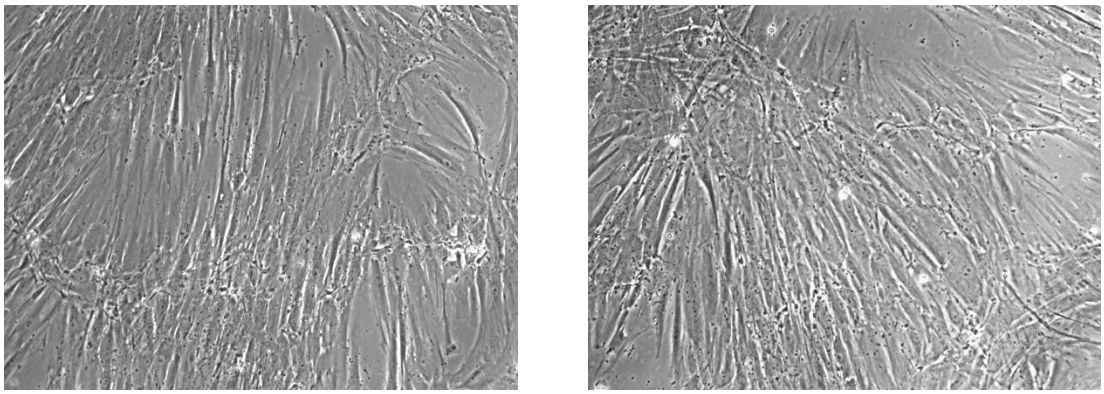

Figure 6: Light microscopically pictures of periodontal ligament stem cells.

The periodontal ligament stem cells were characterized by the presenting specific surface molecules. For characterization, the stem cells (p4) were trypsinated with 2 ml trypsin (gibco, Grand Island, NY, USA) and 10 ml of DMEM-F_{12} (gibco, Carlsbad, USA). Following, 10 % FBS (BIOCHROM, Berlin, Germany) was added to deactivate the trypsin. Then, the cells were centrifuged at 300 g for 10 minutes at 21°C. The dissolved cells had to be washed twice to eliminate any remaining FBS.
The cell pellet was then dissolved in a 99 µl FACS buffer consisting of 1 % PBS with a pH of 7, 2 and 0, 2 % sterile bovine serum albumin (BSA). Afterwards, 1 µl of the respective antibodies was added. The solution had to be cooled at 4°C for 30 minutes.
Afterwards, 1 ml of the FACS buffer was added and all was centrifuged at 300 g for 10 minutes at 4°C. The supernatant was removed and a further 100 µl FACS – buffer added. Next, the flowcytometrical measurement started.
The flow cytometer (BD Accuri C6; Becton-Dickinson, Mansfield, MA, USA) validated the presence of the earlier named antibodies.

2.1.3 Growth factors

The growth factor cocktail used in this study was provided by DOT (DOT, Rostock, Germany). The growth factors were dissolved in 0, 9 % sodium chloride, resulting in a concentration of 2 mg/ml, handed in 1 ml syringes. One syringe contained a mixture of VEGF, b-FGF, IGF-1 and TGF-ß1 (table 4). Per defect 0, 15 ml were used with an amount of 0, 3 mg GF.

Table 4: Content of growth factors in 10 mg lyophilisat.

Sample	Total weight of lyophilisat [g]	TGF-ß3 [pg]	VEGF [pg]	b-FGF [pg]	IGF-1 [pg]	TGF-ß1 [pg]
648	0.1520	0	799.953	159.448	62.297	87683.92
881	0.2243	0	893.536	323.861	45.206	260118.88
268	0.2933	0	491.804	440.465	15.217	103345.92

There are different kinds of growth factors that stimulate different cell differentiations. In this study a mix of diverse growth factors was used (table 5).
VEGF means vascular endothelial growth factor. VEGFs stimulate vasculo- and angiogenesis (Koch et al. 2011).
FGF stands for fibroblast growth factors. More precisely, b-FGF means basic fibroblast growth factor. They are also mediating the formation of new blood vessels (Itoh and Ornitz 2011).
IGF-1 is the insuline like growth factor. It binds to its specific receptor which is present on many cell types in many tissues. It is a stimulator of cell growth and proliferation and a potent inhibitor of programmed cell death (Pollak 2012).
The fourth component of this mixture is TGF, equivalent to transforming growth factor. TGFs are proteins that control proliferation, cellular differentiation and other functions in most cells (Dobaczewski et al. 2011). More precisely, TGF-β1 plays an important role in cell proliferation, matrix formation and odontogenesis. Furthermore, it also affects growth, collagen turnover and the differentiation of apical papilla cells (Chang et al. 2015).

Table 5: Content of GF per syringe.

Charge	VEGF [pg]	b-FGF [pg]	IGF-1 [pg]	TGF-ß1 [pg]
881	178.7	64.8	90.2	52023.8

2.1.4 Material testing prior to implantation

Prior to the implantations, several testings with the stem cells were necessary. Stem cells need a suitable carrier material for implantation. Also, a scaffold is important to guarantee that the cells remain in place. Those scaffolds have to provide a 3D substratum on which cells can proliferate and migrate (Ponticiello et al. 2000). Therefore, the PDLSCs were paired with the collagen matrix, a collagen hydrogel and the collagen membrane. The stem cells were transferred into these materials overnight, then after 24 hours a life-death-stain was performed to evaluate the survival rate of the stem cells.
Another carrier was also tested. The collagen powder (MedSkin Solutions Dr. Suwelack AG, Billerbeck, Germany) was paired with the stem cells. Therefore, the acid pH value (pH ~ 4-5) of the collagen powder was buffered to a neutral pH value, because stem cells would not survive in an acid pH value (Monfoulet et al. 2014).

A test series with different buffers (HEPES and sodium hydrogen carbonate ($NaHCO_3$) followed (table 6). For a 24 hour testing, the stem cells were put into the above named buffers overnight:

Table 6: Test series with different buffers, the collagen powder and medium.

Test	mixture
A	0.1 g collagen powder + 400 µl medium + 400 µl 2 % $NaHCO_3$
B	2 x 0.1 g collagen powder + 300 µl medium + 500 µl 2 % $NaHCO_3$
C	0.1 g collagen powder + 800 µl HEPES

The samples were incubated at 4°C for 30 minutes and subsequently for another 24 hours at 37°C. Safranin was added. Afterwards, the cells were trypsinated and centrifuged at 400 g for 4 minutes at 4°C and 1900 rpm. The supernatant was retarded and the cell pellet dissolved in 160 µl HEPES. Cells were counted and evaluated under a fluorescence microscope.

Finally, a potassium buffer was tested. It turned out to be the most suitable and was used in the following implantations. Therefore, per defect 0, 05 g collagen powder, 100 µl potassium buffer and 300 µl medium were applied.

Prior to the implantations, the stem cells were trypsinated and counted in a Neubauer counting chamber (BRAND, Wertheim, Germany) for a precise cell concentration. After centrifugation the cell pellet was dissolved in medium without FBS. In the laboratory and under aseptic conditions, a 24 wells microplate was prepared. Each well of one row contained the following materials: 1. row: 0, 05 g collagen powder, 2. row: 100 µl potassium buffer, 3. row: 300 µl medium without FBS and stem cells.

For the transport to the operating room the microplates had to be constantly kept on ice. Chairside, the prepared materials were mixed. The buffer and the collagen powder had to be mixed first to eliminate the acid pH value. Finally, the stem cells were added and the mixture was applied into the furcation defect with the help of a sterile spatula.

2.2 Methods

2.2.1 Experimental design of animal research

Fifteen “Göttinger” miniature pigs (22 ± 3 months old), weighing 35 ± 10, 8 kg, each exhibiting a fully erupted permanent dentition, were used for this study. The minipigs were housed under conventional conditions with free access to water. Diet had to be modified to support wound healing. The study protocol was approved by the appropriate local authority (German Decree on the Reporting of Laboratory Animals 7221.3-1.1-075/11, Regional Authority for Agriculture, Food Safety and Fisheries, State of Mecklenburg-Western Pomerania, Germany). All surgical procedures were performed under anaesthesia. In addition to general anaesthesia the animals received a further local anaesthesia. All efforts were made to minimize animal suffering. The perioral tissues and the gingival were disinfected with povidone iodine and the perioral hair was cut. The 1st and 2nd premolar of each animal were extracted in both quadrants of the lower jaw. The extracted teeth were used for isolating and culturing stem cells. 90 days after tooth extraction class II furcation defects were created at the 3rd premolar and 1st molar in the mandible on both sites. The defects were randomly filled with different treatment groups:

- Group I - control,
- Group II - collagen membrane,
- Group III - collagen membrane plus growth factors,
- Group IV - collagen matrix,
- Group V - collagen matrix plus growth factors,
- Group VI - collagen powder,
- Group VII - collagen powder plus stem cells.

Afterwards, all defects were covered with a semipermeable membrane and a polychrome sequential labeling was performed 14, 28 and 84 days after the replantation. Therefore, three different fluochromes were administered by subcutaneous injection: xylenol orange (6 %, 2-5 g/animal), calcein green (1 %, 0.8-1.5 g/animal) and alizarine complexone (3 %, 1-1.5 g/animal, all from Thermo Fisher Scientific, Waltham, USA). To ensure a good wound healing, a clinical examination of the animals occurred simultaneously. The animals were sacrificed after 120 days of healing followed by a histological assessment (figure 7).

This study was part of a third-party funded project supported by the state of Mecklenburg-Western Pomerania. As the author was still a student by then, her tasks were to assist in the surgeries (surgeons were Dr. Daniel Welly and Malte Scholz, Department of Operative Dentistry and Periodontology, University of

Rostock, Rostock, Germany), the isolation and culturing of the periodontal ligament stem cells, histological assessment of the specimens and statistical analysis.

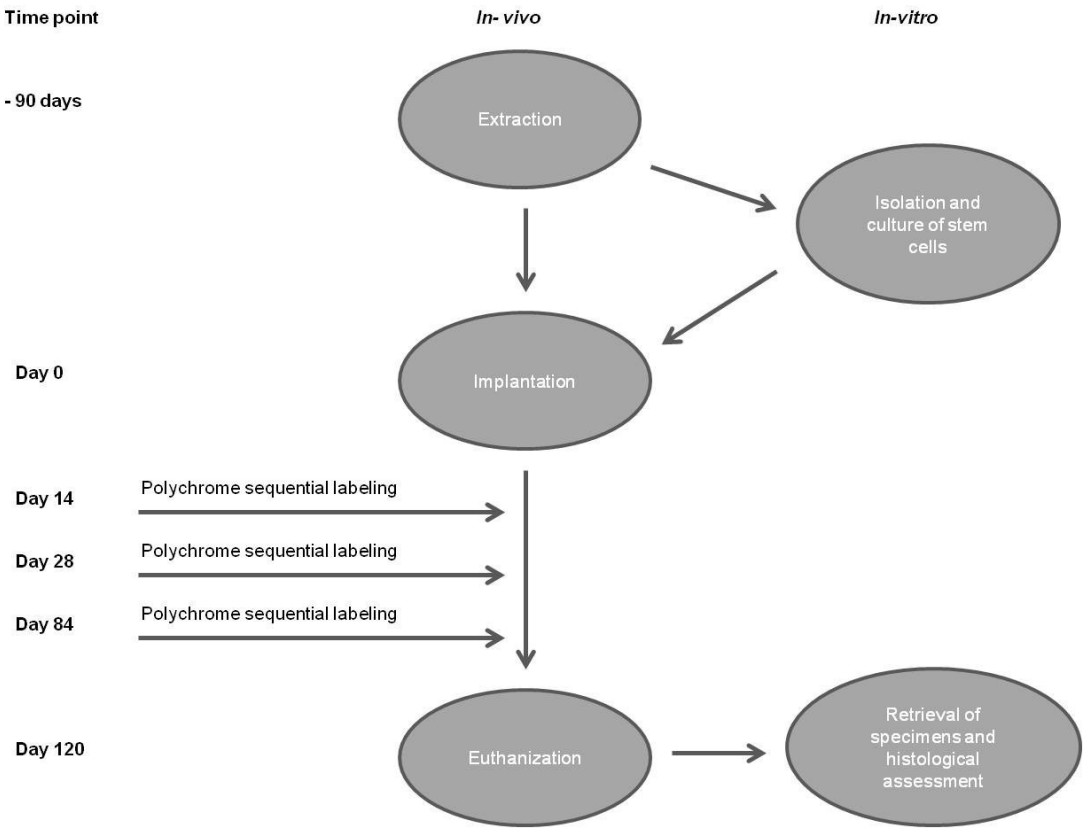

Figure 7: Experimental workflow. Within 210 days the total procedure occurred, from extracting teeth over implantation to histological assessment of the specimens.

2.2.2 Anaesthesia

All surgical procedures were carried out under aseptic conditions and general anaesthesia. Prior to each surgical intervention, the animals received a pre-medication with ketamine 10 %, (Pfizer AG, NY, USA) plus 1.5 ml midazolam (Sanochemia Pharmazeutika AG, Neufeld, Österreich) by intramuscular injection. Intravenous access was established through the ear vein. For muscle relaxation, an induction was continued by the injection of 0.25 ml – 0.4 ml pancuronium (Organon Teknika, Eppelheim, Germany). Following oral intubation anaesthesia was continued by inhalation of isoflurane (AbbVie AG, Baar, Switzerland) and injection of 0.5 ml - 0.8 ml/min fentanyl (Janssen - Cilag, Neuss, Germany). The administration of oxygen was about 1, 5 l/min.

After the beginning of the general anaesthesia, the perioral hair was cut and the perioral tissues and the gingiva were disinfected with povidone iodine (Betaisodona ®, Mundipharma GmbH, Limburg an der Lahn, Germany). Additionally, the animals received a further local anaesthesia (Ultracain D-S forte, 1:100 000, 2 ml, Sanofi-Aventis Deutschland GmbH, Germany). Intraoperative, antibiotics were administered as ampicillin/sulbactam 1000 mg/500 mg i.v. (HEXAL AG, Holzkirchen, Germany). For postoperative analgesia treatment the animals received an oral Metacam ® - suspension (15 mg/ml) with a dose of 2.7 ml/100 kg body weight and synulox 250 mg (Pfizer AG, NY, USA).

2.2.3 Extractions

The 1st and 2nd premolars of the minipigs were extracted in both quadrants of the mandible. The teeth were loosened with a lever and pulled with extraction forceps. The periodontal ligament of the extracted teeth was obtained to isolate PDLSCs. All extracted teeth and viable materials were treated repeatedly with a rinsing solution (table 7), consisting of cooled phosphate buffered saline (PBS, 20 ml PBS 1x) and antibiotics (200 µl penicillin/streptomycin 1x, PenStrep, gibco, Grand Island, NY, USA). The rinsing was necessary to minimize bacterial contamination. The samples were transported in a transport medium (DMEM-F_{12}, gibco, Grand Island, NY, USA) including 2 % antibiotics (PenStrep, gibco, Grand Island, NY, USA). The samples had to be kept on a constant temperature of + 4 °C.

Table 7: Rinsing solutions for extractions. Six different rinsing solutions were used in order to delete excessive blood and bacteria. Finally the samples were transported in the transport medium to the laboratory.

Rinsing solution	**Antibiotics**
50 ml PBS 1x	
50 ml PBS 1x +	500 µl Penicillin/Streptomycin (1x)
20 ml PBS 1x +	200 µl Penicillin/Streptomycin (1x)
20 ml PBS 1x +	2 ml Penicillin/Streptomycin (10x)
20 ml PBS 1x +	200 µl Penicillin/Streptomycin (1x)
20 ml PBS 1x +	200 µl Penicillin/Streptomycin (1x)
Transport medium	
20 ml DMEM-F12 +	400 µl Penicillin/Streptomycin (1x)

2.2.4 Implantations

In the second surgical intervention, class II furcation defects were created at the 3rd premolar and 1st molar bilaterally of the mandible. Initially, a mucoperiosteal flap was raised with the help of a raspatory. The periodontal furcation defects were created with a bud burr (Henry Schein dental Deutschland GmbH, Langen, Germany). The bottom of the created furcation defects were marked with a notch for later histological evaluation. Subsequently, the defects were randomly filled with the following materials: Group I - control, Group II - collagen membrane, Group III - collagen membrane plus growth factors, Group IV - collagen matrix, Group V - collagen matrix plus growth factors, Group VI - collagen powder, Group VII - collagen powder plus stem cells (table 8). With a sterile Heyman type spatula (Henry Schein dental Deutschland GmbH, Langen, Germany) all materials were filled into

the furcation defects. All defects were covered with a 35 X 45 mm semipermeable membrane (bredent medical, angiopore selective permeable membrane, Senden, Germany) and closed with a mucoperiosteal flap using absorbable sutures (ETHICON, VICRYL, 3-0, Polylactin 910, SH-1 plus) to ensure a transmucosal healing for 120 days (figure 8).

Table 8: Overview of implanted materials. The furcation defects were created at the 3rd premolar (P3) and 1st molar (M1) and filled with below named materials. * defects treated with particles were not part of this study.

minipig	3. quadrant		4. quadrant	
	P3	M1	P3	M1
207627	collagen membrane	control	hydrogel	collagen matrix
208054	collagen membrane	control	collagen matrix	hydrogel
207574	collagen membrane + GF	control	hydrogel + GF	collagen matrix + GF
207882	collagen membrane + GF	control	collagen matrix + GF	hydrogel + GF
208178	collagen powder + 2,9 x 10^5 cells	collagen powder + 3,8 x 10^5 cells	collagen matrix	collagen membrane
208380	collagen powder	collagen powder	collagen powder + 2,2 x 10^5 cells	collagen powder + 2,9 x 10^5 cells
208248	collagen powder + 6,4 x 10^5 cells	collagen powder + 5,6 x 10^5 cells	collagen powder + 2,3 x 10^6 cells	collagen powder + 1 x 10^6 cells
209356	abscess	abscess	control	control
208196	collagen membrane + GF	collagen membrane	collagen matrix	collagen matrix
207733	collagen membrane + GF	collagen membrane	collagen membrane	collagen membrane + GF
211715	collagen powder	collagen powder	collagen powder + cells + particles *	collagen powder + cells + particles *
211343	collagen powder + cells+ particles*	collagen powder + cells+ particles*	abscess	abscess
211717	collagen powder	collagen powder	collagen powder + cells+ particles*	collagen powder + cells+ particles*
305148	collagen membrane + GF	collagen membrane + GF	collagen membrane	collagen membrane
305068	collagen matrix + GF	collagen matrix	collagen matrix + GF	collagen matrix + GF

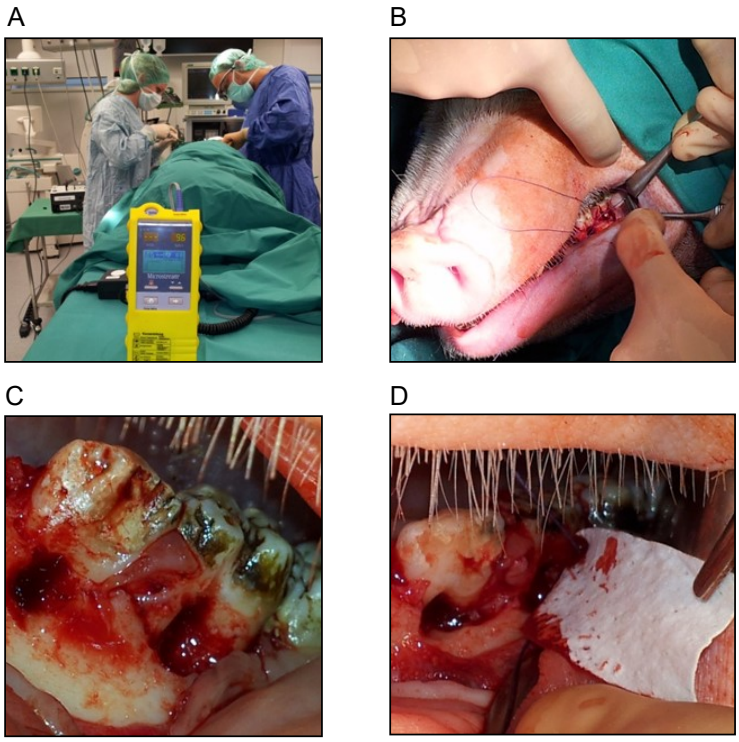

Figure 8: Implantations. A: at surgery: arterial oxygen saturation was measured with a method called pulse oximetry, B: the minipig was covered with sterile blankets and perioral tissues were disinfected with beta isodoma, C: 3rd quadrant, mucoperiostal flap mobilized, furcation defects visible, D: angiopore selective permeable membrane before placing.

2.2.5 Polychrome sequential labeling

The technique of polychrome sequential labeling was used in order to microscopically investigate the state of new bone formation and remodeling processes at different time intervals (figure 9). Therefore, three different fluochromes (Thermo Fisher Scientific, Waltham, USA) were administered by intravenous injection 14, 28 and 84 days after implantation: xylenol orange (6 %, 2-5 g/animal), calcein green (1 %, 0.8-1.5 g/animal) and alizarine complexone (3 %, 1-1.5 g/animal). Thus, the animals were sedated by ketamin 10 %. Simultaneously, a clinical examination of the animals was performed to ensure a good wound healing and to check the medical condition of the animals.

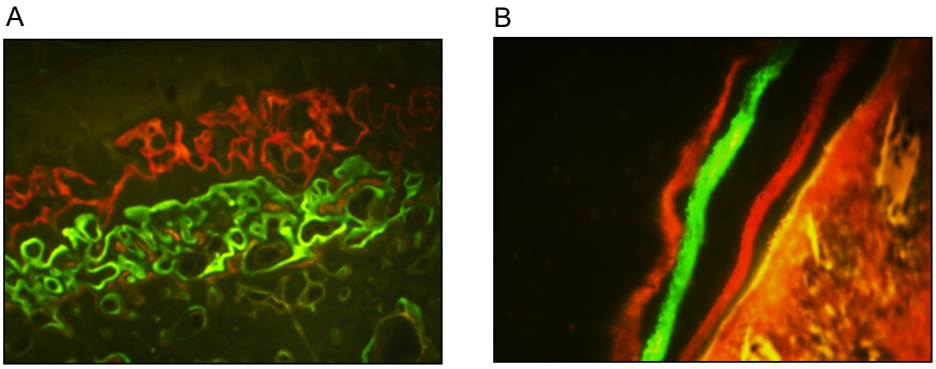

Figure 9: Fluorescence microscopy of furcation defect. A: different fluochromes mark bone formation at different time intervalls, B: newly formed cementum and the desmodont is visible.

2.2.6 Histological analysis

Histological samples were used to evaluate the healing processes. For the production of the samples, the animals were euthanized by an overdose of thiopental (Ospedalia AG, Hünenburg, Schweiz) 120 days after the last surgery. Therefore, a premedication with ketamine (Pfizer AG, NY, USA) was applied. The oral tissues were fixed by perfusion with 10 % buffered formalin (Helm Austria GmbH, Wien, Austria) administered through the carotid arteries. The mandible was exarticulated and cut into segments. It was fixed in formafix (Formafix Switzerland AG, Hittnau, Switzerland) 4 % for 7 days and kept in ethanol for a further 14 days. The segments were then block-embedded in technovit® (Heraeus, Hanau, Germany) 7200 VLC over 28 days. Next, the specimens were cut into 250 µm-thick sections in the sagittal direction using a saw microtome (EXAKT Advanced Technologies GmbH Norderstedt, Germany) under permanent cooling. Finally, the specimens were grinded down to 15 µm and stained with toluidine blue. In an observer blinded fashion, the specimens were evaluated with respect to morphologic and morphometric aspects. A light optical microscope (Carl Zeiss, Axio Imager M2, Jena, Germany) with scanning stage was used. With the help of the microscope all samples were scanned at a magnification of 20 using an Axiocam MRC5 digital microscope camera (Carl Zeiss Microscopy GmbH, Jena, Germany). The evaluation and measurement of the samples occurred with the help of AxioVision (microscope software, Carl Zeiss Microscopy GmbH, Jena, Germany). The bottom of the defects showed the notches. In case the notches could not be identified in the histological samples, the fluochrome labeling was used to determine the defect area. Due to the fact that fluorescent colors accumulate in calcified tissues with a higher formation rate (Cheng et al. 2013), newly formed tissues could be identified with the fluochrome labelling. The visualization of the newly formed

tissues was observed via a fluorescence microscope (Carl Zeiss, 4×, Axiovert 40 CFL, Axiocam MRC5, Jena, Germany) with a filter of 490 – 520 nm especially excitating the calcein green fluorescence. With the help of AxioVision each tissue was marked with its specific color in every specimen. The measured lengths were summed up and the percentage for every measured tissue of the total defect length was calculated (e.g. the new attachment formation for the control group is 13.0 ± 18.0 %, this means 13 % of the total defect length). Based on the results a statistical analysis followed. The total defect length was defined as the distance along the root, from one notch to the other (= 100 %). The total defect length was comprised by different tissues such as new attachment (NA), connective tissue (CT), epithelial attachment (EA) and new cementum (NC). AxioVision SE64 Rel. 4.8 (microscope software, Carl Zeiss Microscopy GmbH, Jena, Germany) was used for the histomorphometric measurement. Each tissue was marked by different colours (NA – green, CT – yellow, EA – red, NC – blue, see figure 11).

The histological assessment of the specimen occurred in several steps. First, the defect area was determined with the help of the fluorescent colors (figure 10).

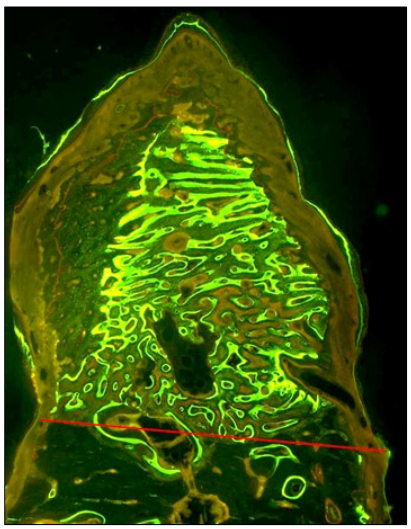

Figure 10: Fluorescence microscopy of furcation defects. Red line marks the margin between the older and newly formed bone; red line represents bottom of furcation defects. The distinction between older (low fluorescence) and newly formed bone (highly fluorescent) is visible; defect area is marked by fluorescent tissues.

A systematic drawing illustrates a furcation defect and the different tissue types and its specific colors used in this study (figure 11). Figure 12 shows the measured tissues in a histological picture of a class II furcation defect. The three main tissue types that were measured in the histological specimens are shown in figures 13-15.

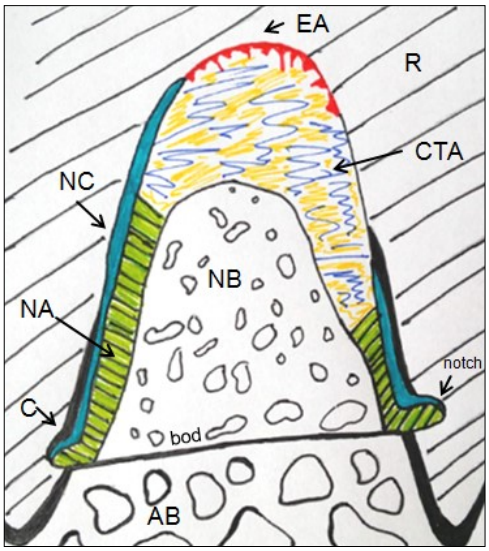

Figure 11: Drawing of furcation defect with measured sections for histometric assessment. AB: alveolar bone, bod: bottom of defect, C: cementum, NC: newly formed cementum, NA: new attachment, EA: epithelial attachment, R: root (dentin), CTA: connective tissue attachment, NB: newly formed bone, B) measured sections in histological sample: green: NA, yellow: CTA, red: EA, not in this picture: turquoise: CTA+NC, white: concrement, purple: old attachment.

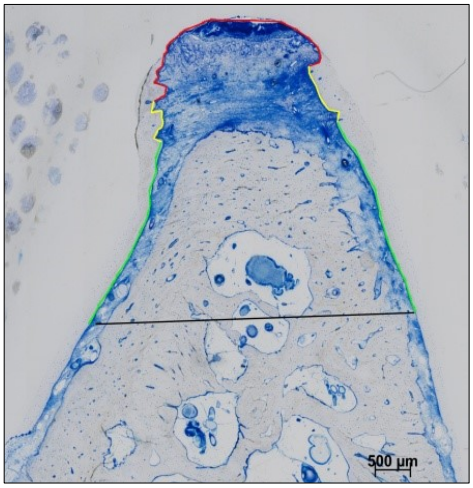

Figure 12: Representation of the different tissues marked in specific colour and measured sections. green: NA, yellow: CTA, red: EA, not in this picture: turquoise: CTA+NC, white: concrement, purple: old attachment.

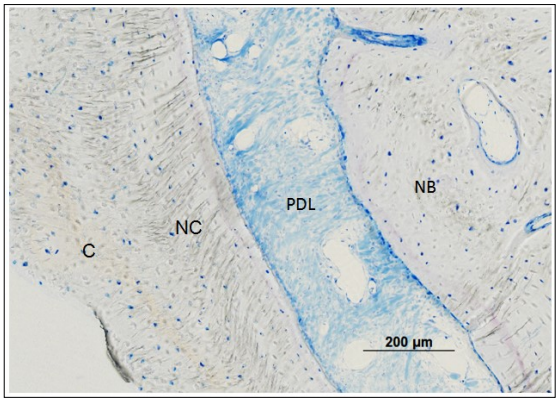

Figure 13: New attachment (NA). The NA is marked by newly formed collagen fibers inserting into newly formed cementum along the root surface. New cementum is rich in fibers (see grey lines). The PDL is marked by directional collagen fibers inserting into newly formed cementum. C: cementum, NC: newly formed cementum, PDL: newly formed periodontal ligament (collagen fibers), NB: newly formed alveolar bone; scale on micrograph indicates original dimensions in µm.

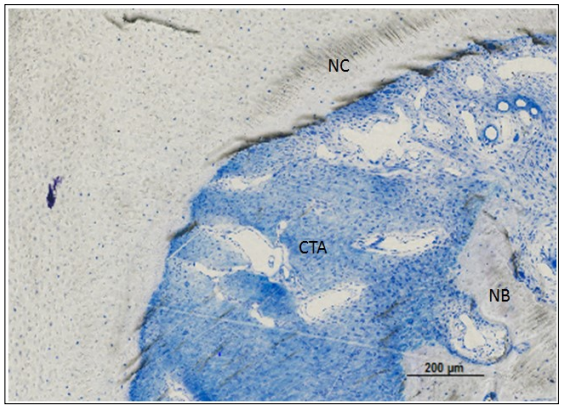

Figure 14: Connective tissue attachment (CTA). The CTA is marked by undirected collagen fibers, blood vessels and fibroblasts. The new cementum (NC) is cellular and marked by filamentary darker structures. NC: newly formed cementum, NB: newly formed bone, scale on micrograph indicates original dimensions in µm.

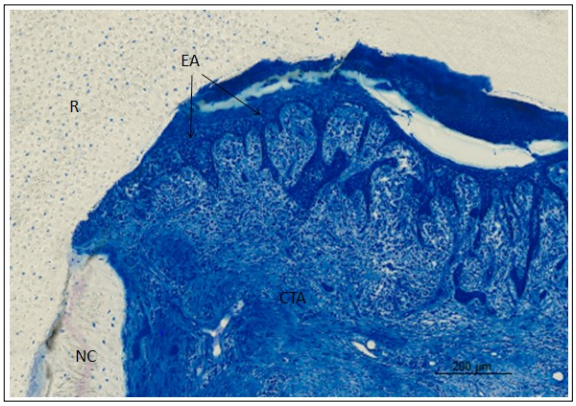

Figure 15: Epithelial attachment (EA). The EA, also called long junctional epithelium, is the primary pattern of healing that occurs after periodontal debridement, there is no periodontal ligament or bone formation. It is marked by spherical cells building long septa fencing in the connective tissue. NC: new cementum, R: root (dentin), CTA: connective tissue attachment, scale on micrograph indicates original dimensions in µm.

2.2.7 Statistics

The Kolmogorov-Smirnov test was used to prove the normal distribution of the data. The ANOVA test followed to determine the significant differences between the data of the measured defects' lengths. The level of statistical significance was set at $p \leq 0.05$. The data was evaluated with Excel (Microsoft Excel 98, Microsoft Co.) and presented as mean ± SD. The graphs were also crafted with Excel.

3 RESULTS

The aim of this study was to examine the regenerative potential of various collagen materials with or without dental stem cells or growth factors in periodontal class II furcation defects in minipigs. Therefore, the materials were implanted randomly in a total of 60 defects. Prior to the implantations, several pre-tests were done.

3.1 Material testings prior to implantations

To find an appropriate carrier material for the periodontal ligament stem cells, several preliminary studies were performed. The PDLSCs were paired with the collagen matrix, a collagen hydrogel and the collagen membrane. 24 hours later a Live/Dead staining was performed. With the help of Live/Dead stainings a differentiation between living and dead cells (Moreau et al. 2009) was possible. Dead cells were stained red (figure 16). It turned out that most of the cells did not survive in combination with the initially tested scaffolds.

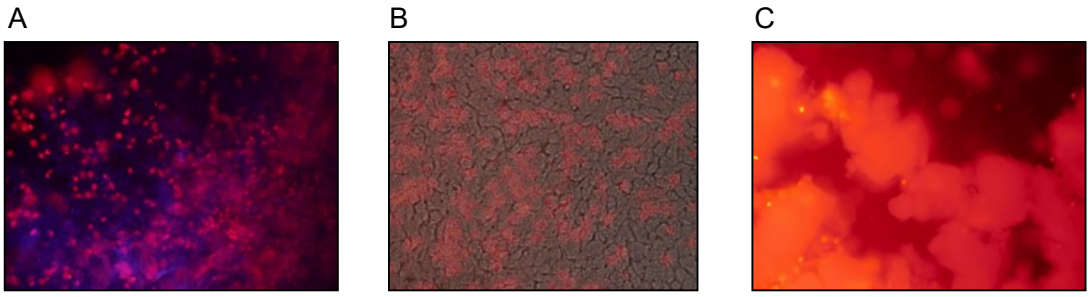

Figure 16: Material testing prior to implantations. The above named materials were not appropriate to be a carrier for the PDLSCs, A: collagen matrix: stem cells died after 24 hours, Live/Dead staining, B: overlay of Live/Dead staining and light microscope image of collagen hydrogel and stem cells, most of the cells died after 24 hours, C: collagen membrane: stem cells died after 24 hours.

Further materials were tested in combination with the PDLSCs. The collagen powder (see 2.1.1) appeared to be a potential carrier material for dental stem cells. The acid pH value of the collagen powder was buffered to a neutral (~ 7) pH value with different buffers. First, HEPES was used (table 9). Subsequently, sodium hydrogen carbonate (table 10) was also tested and a Live/Dead staining with both buffers was performed (figure 17).

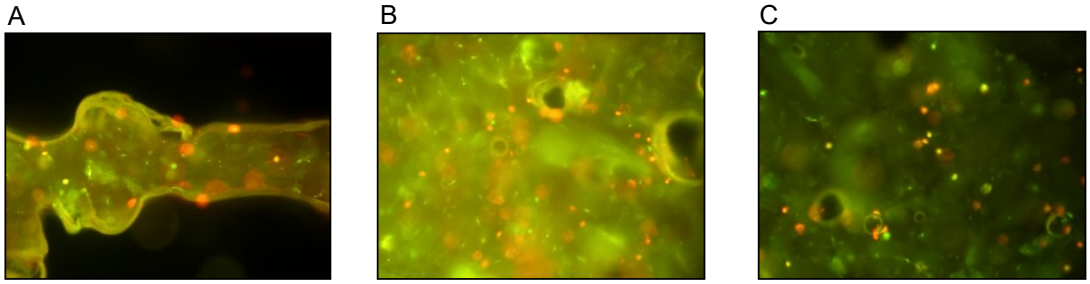

Figure 17: Live/Dead staining of PDLSCs. PDLSCs were put into the following mixtures: A: 0,1 g collagen powder + 400 µl medium + 400 µl 2% NaHCO3, B: 2 x 0,1 g collagen powder + 300 µl medium + 500 µl 2% NaHCO3, C: 0,1 g collagen powder + 800 µl HEPES.

Table 9: Testings with HEPES.

Collagen powder	HEPES	pH	conclusion
0.1 g	1 ml	4.9 – sunk down to 4.8 after 20 minutes	no stable values
0.1 g	800 µl	4.3 – sunk down to 4.2 after 20 minutes	no stable values

Table 10: Testings with sodium hydrogen carbonate.

Collagen powder	$NaHCO_3$	DMEM-F_{12}	pH
0.1 g	0.1 g	200 µl	9.1
0.1 g	0,1 g	500 µl	8.1
0.1 g	0.1 g	800 µl	8.1
0.1 g	0.07 g	800 µl	7.9
0.1 g	0.01 g	800 µl	7.6
0.2 g	0.01 g	1.6 ml	7.8
0.1 g	800 µl 2 % $NaHCO_3$	0 µl	7.6
0.1 g	400 µl 2 % $NaHCO_3$	400 µl	7.1
0.1 g	600 µl 2 % $NaHCO_3$	200 µl	7.5

Finally, a potassium buffer (table 11) was tried. The use of the potassium buffer resulted in stable pH values. Therefore, a Live/Dead staining occurred (figure 18) and the potassium buffer was used for later implantations (see 2.1.4). Per furcation defect 0.05 g collagen powder, 100 µl potassium buffer and 300 µl medium, combined with the dental stem cells, were applied.

Table 11: Testings with potassium buffer.

Collagen powder	potassium buffer	DMEM-F_{12}	pH
0.1 g	300 µl	500 µl	8.0
0.1 g	200 µl	600 µl	7.5 = optimal

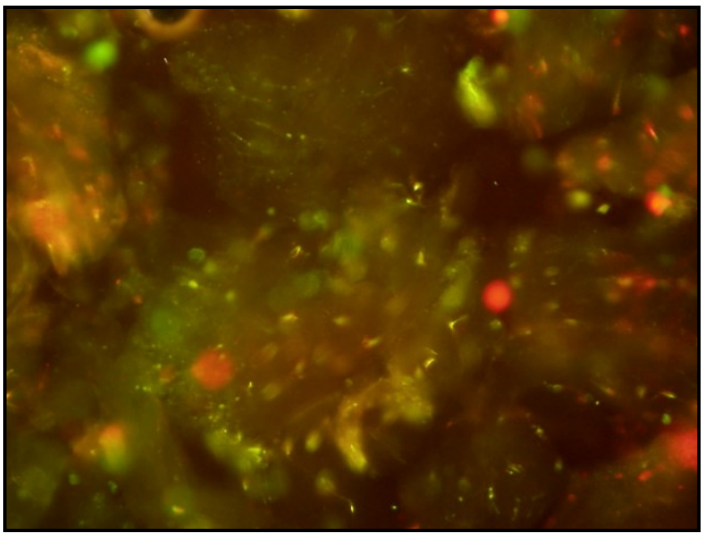

Figure 18: Potassium buffer with PDLSCs. Live/Dead staining of stem cells located in potassium buffer and collagen powder, 24 hours after inserting; most cells are alive (colored in green), collagen powder presents high self-fluorescence.

3.2 Phenotyping of periodontal ligament stem cells

The dental stem cells used in this study could be successfully isolated and characterized. Compared with the isotypes, a positive shift along the x-axis was obvious for the surface markers CD 29 and CD 44. For CD 45 a negative antigen expression was shown. The following surface markers were proven (figure 19-21):

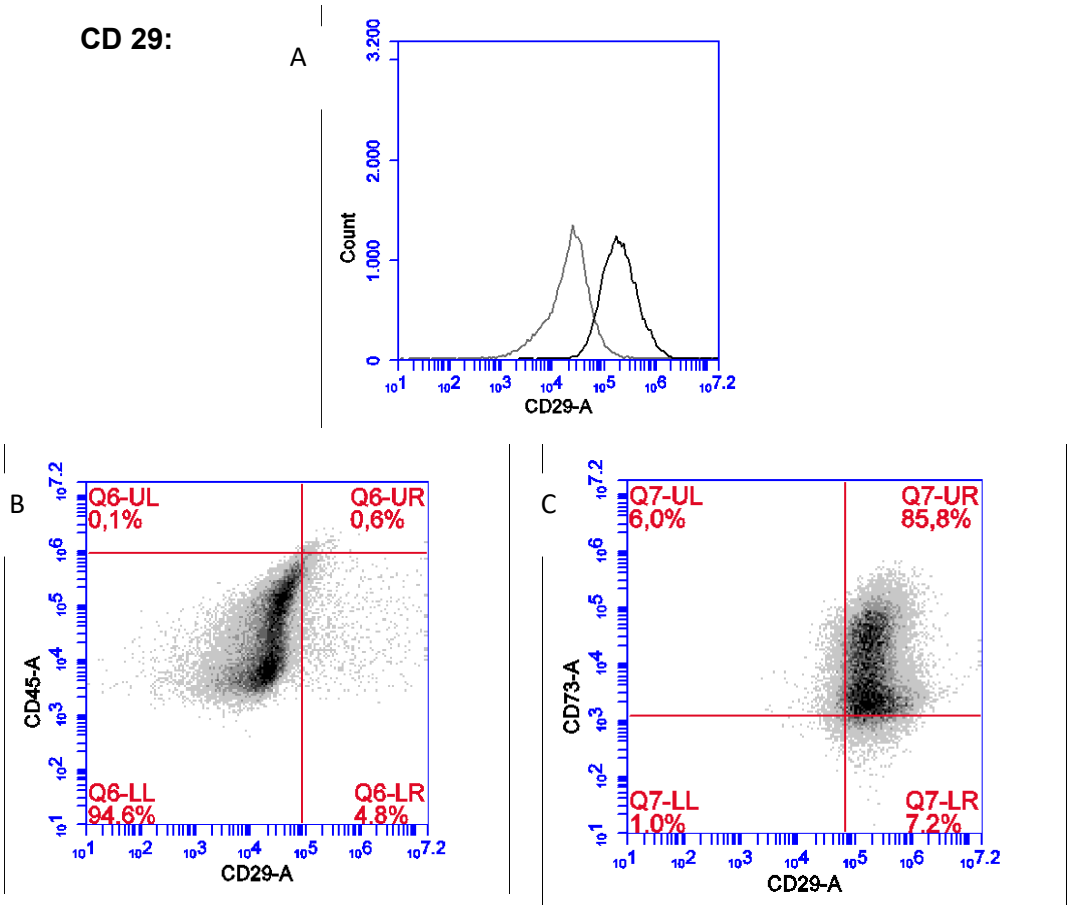

Figure 19: Detection of surface molecule CD29. A: deferment of the graphs proves existence of CD 29. First (grey) graph demonstrates isotype = negative control, second graph (black) demonstrates surface marker CD 29, B: iso type of CD 29; C: CD 29 – positive shift visible.

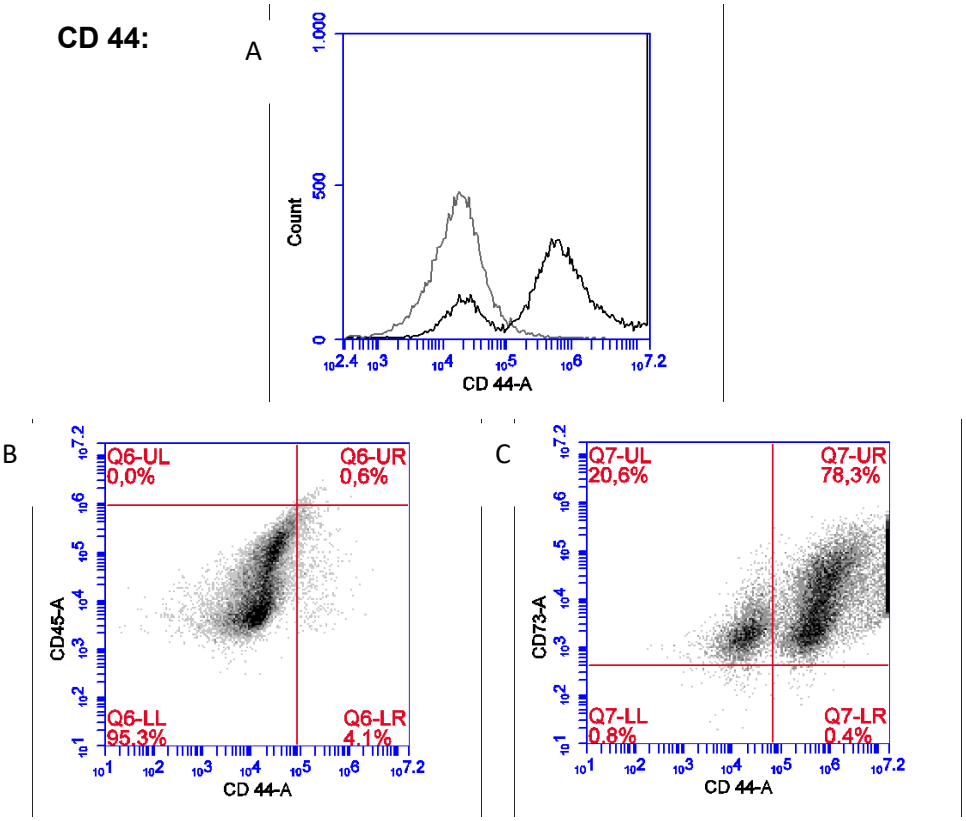

Figure 20: Detection of surface molecule CD44. A: deferment of the graphs proves existence of CD 44. First (grey) graph demonstrates isotype = negative control, second graph (black) demonstrates surface marker CD 44, B: iso type of CD 44; C: CD 44 – positive shift visible.

CD 45:

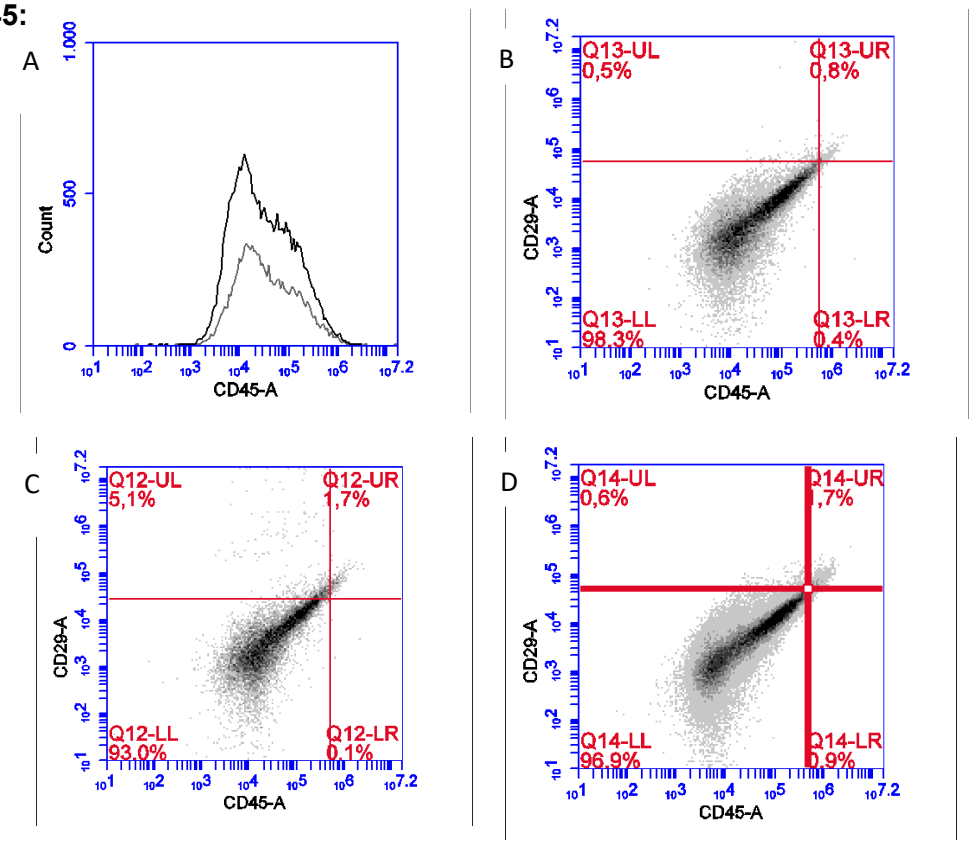

Figure 21: Detection of surface molecule CD45. A: no deferment of the graphs shows any existence of CD 45. The hemopoietic stem cell marker, first (grey) graph demonstrates isotype = negative control, second graph (black) demonstrates surface marker CD 45 B: iso type of CD 45; C: CD 45; D: uncoloured sample.

3.3 All implanted materials improved regeneration

The aim of this study was to examine the regenerative potential of different collagen materials with or without PDLSCs or GF. Therefore, 60 furcation defects were filled randomly with the mentioned materials and the healing was compared to control. The different filling materials and their newly formed new attachments are presented in figure 22. It is apparent that all experimental groups show an increased new attachment formation. In contrast to control, all results varied significantly with the exception of the collagen matrix plus GF.

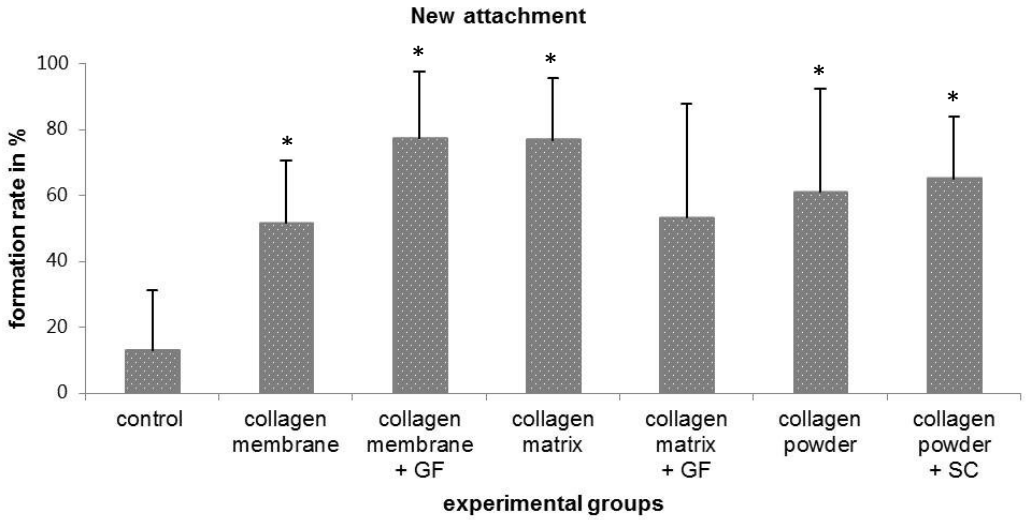

Figure 22: Overview of NA of all implanted materials. Y-axis shows percentages of measured tissue in relation to total defect length, x-axis shows different implanted materials and their significances in relation to the control group. P was set at $p \leq 0.05$ and all data are presented as mean ± SD, * = significant to control.

The collagen membrane plus GF achieved the highest new attachment level of approximately 77.3 ± 20.3 % (figure 23 A). The lowest new attachment level was found in the control group (13.0 ± 18.0 %, figure 23 B). In comparison to the control group, all tested implanted materials achieved higher new attachment levels. The second-highest result was achieved with the collagen matrix (76.9 ± 18.6 %, figure 23 C), followed by the collagen powder (61.1 ± 31.4 %, figure 23 D), the collagen powder plus PDLSCs (65.1 ± 18.9 %, figure 23 E) and the collagen membrane (51.7 ± 18.8 %, figure 23 F). All results varied statistically significantly compared to the control, with the exception of the collagen matrix plus GF (53.3 ± 34.6 %, figure 23 G).

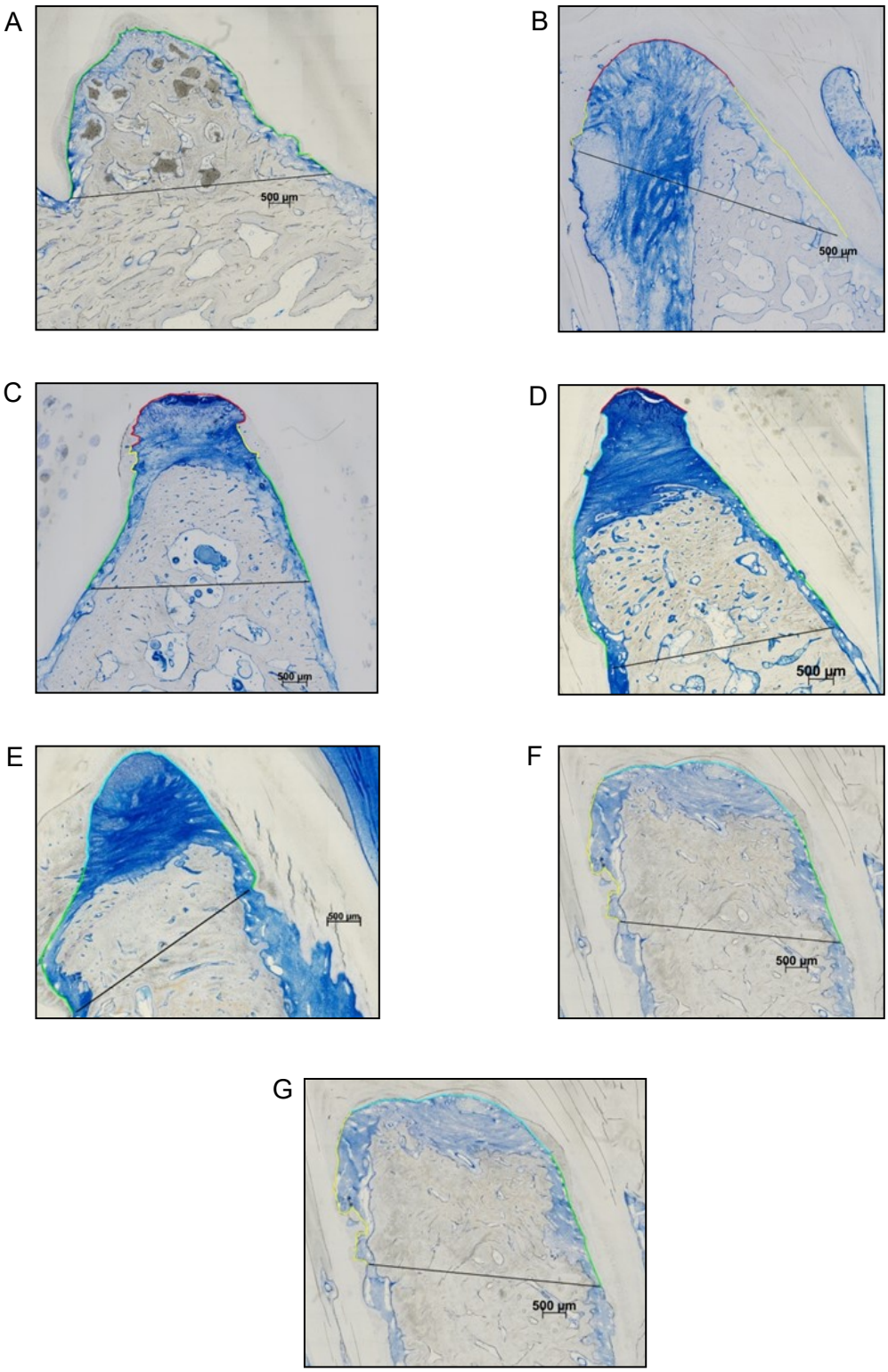

Figure 23: Histological pictures of new attachment formation. A) Collagen membrane plus GF: NA is 77.3 ± 20.3 %, B) control group: NA is 13.0 ± 18.0 %, C) collagen matrix: NA is 76.9 ± 18.6 %, D) collagen powder: NA is 61.1 ± 31.4 %; E) collagen powder plus PDLSCs: NA is 65.1 ± 18.9 %, F) collagen membrane: NA is 51.7 ± 18.8 %; G) collagen matrix plus GF: NA is 53.3 ± 34.6 %.

Moreover, the epithelial attachment could be decreased when compared to control in all experimental groups (figure 24). In comparison to control (epithelial attachment formation rate 39.5 ± 28.6 %, figure 25 A) four of the six experimental groups had a statistically significant decrease in epithelial attachment formation (collagen membrane: 4.7 ± 9.3 %; figure 25 B, collagen membrane plus GF: 0.0 ± 0 %; figure 25 C, collagen powder: 7.0 ± 15.4 %; figure 25 D, collagen powder plus SC: 2.2 ± 4.9 %; figure 25 E). In two groups the epithelial attachment was decreased, but not statistically significant (collagen matrix: 6.9 ± 15.4 %; figure 25 F, collagen matrix plus GF: 20.4 ± 23.2 %; figure 25 G).

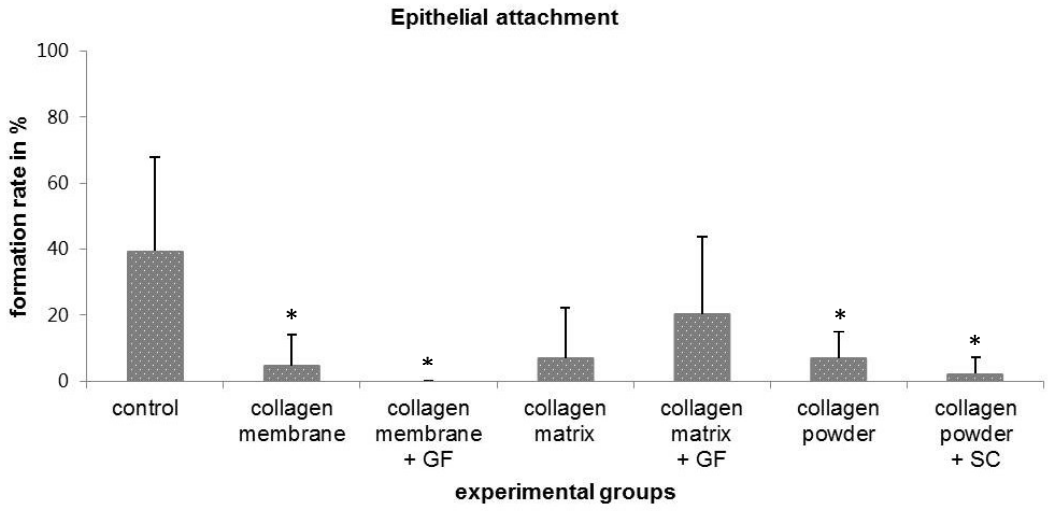

Figure 24: Overview of EA of all implanted materials. Y-axis shows percentages of measured tissue in relation to total defect length, x-axis shows different implanted materials and their significances in relation to the control group. P was set at $p \leq 0.05$ and all data are presented as mean ± SD, * = significant to control.

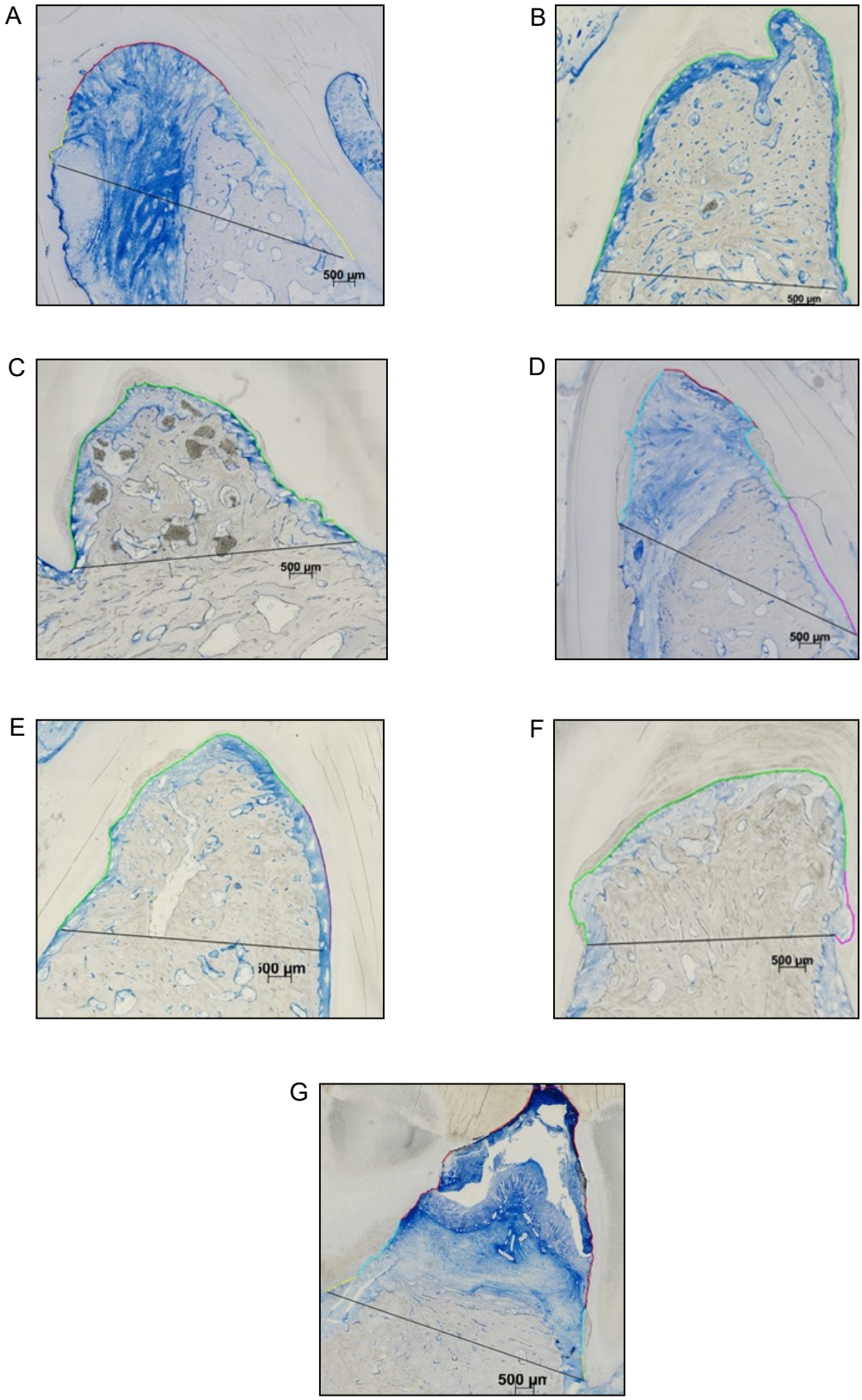

Figure 25: Histological pictures of epithelial attachment formation. A) Control group: EA is 39.5 ± 28.6 %, B) collagen membrane: EA is 4.7 ± 9.3 %, C) collagen membrane + GF: EA is 0.0 ± 0 %, D) collagen powder: EA is 7.0 ± 15.4 %, E) collagen powder + SC: EA is 2.2 ± 4.9 %, F) collagen matrix: EA is 6.9 ± 15.4 %, G) collagen matrix + GF: EA is 20.4 ± 23.2 %.

3.4 The use of additional stem cells improved periodontal regeneration, but not significantly

Considering the carrier materials solely by contrast with the carrier materials plus stem cells, it was shown that the stem cells lead to an increased new attachment formation. Comparing the collagen powder alone or combined with the periodontal ligament stem cells, the new attachment level went from 61.1 ± 31.4 % to 65.1 ± 18.9 %. This shows, that there is a tendency to improve regeneration, but not in a significant way. However, the enhancement of periodontal regeneration using periodontal ligament stem cells was not statistically significant in this examination.

3.5 Periodontal regeneration was not improved significantly by the addition of growth factors

There was no significant difference found between the use of the carrier materials solely compared to the carrier materials and the additional application of growth factors. The effect of the growth factors was controversial, because they showed opposing effects. On the one hand, they improved regeneration (collagen membrane alone: 51.7 ± 18.8 % versus collagen membrane plus GF: 77.3 ± 20.3 %). On the other hand, with the additional application of GF the new attachment formation decreased (collagen matrix alone: 76.9 ± 18.6 % versus collagen matrix plus GF: 53.3 ± 34.6 %).

3.6 There was no significant difference between stem cells and growth factors

Comparing the carrier materials by themselves to the carrier materials plus stem cells or growth factors, it could be shown, that the new attachment formation with the use of the additional application of PDLSCs or GF did not differ statistically. No significant difference in tissue formation was determined. All implanted materials improved regeneration, but the additional application of the stem cells or growth factors did not lead to a further significant enhancement in new attachment formation. Summarized, the addition of GF to the carrier materials resulted in an increased variability of the results with high standard deviations. The additional application of stem cells improved the outcome insignificantly. Both materials tended to improve regeneration, but not in a statistically significant way.

4 DISCUSSION

The aim of this study was to analyze the potential of different collagen carrier materials with or without periodontal ligament stem cells or a growth factor cocktail in a minipig model, to find new treatment options for periodontal regeneration. 120 days after surgery the animals were euthanized. With the help of histological specimens periodontal regeneration was evaluated to morphologic and morphometric aspects. Summarized, all implanted materials showed an increased new attachment formation and a reduction of the epithelial attachment. Partially, the additional application of periodontal ligament stem cells or growth factors slightly improved the healing processes, although this improvement was not statistically significant.

4.1 Carrier materials

In this study, different collagen materials were used as filling materials and carrier for the dental stem cells or the growth factor cocktail. The class II furcation defects were filled with the above named materials and additionally covered with a semipermeable membrane. The semipermeable membrane was used to avoid the formation of a long junctional epithelium. Gingival epithelial cells compete with other cells for the population of the root surface. Therefore, guided tissue regeneration was applied to support periodontal regeneration. With the help of a semipermeable membrane epithelial cells were excluded. Thereby, the sufficient supply of oxygen inside the tissues was ensured (Rakhmatia et al. 2013).

Previously, collagen has been proven to be a suitable carrier material in regenerative periodontology (Ferreira et al. 2012, Kosen et al. 2012, Zhang et al. 2006). Therefore, this study investigated if the regenerative potential could be further improved by the addition of periodontal ligament stem cells or growth factors. Various collagen materials were used in this study: a collagen membrane, a collagen matrix and a collagen powder (see 2.1). All implanted materials could achieve at least 50 % higher new attachment formation compared to control.

Collagen is a widely used material in regenerative therapies. It is mostly used as a membrane in guided tissue regeneration, but it can also be used as a filling material itself. The positive effect and regenerative potential of collagen materials has been shown in several studies. It was shown that the formation of functionally oriented periodontal ligament, cellular mixed fibre cementum and alveolar bone was stimulated in beagle dogs when filling periodontal defects with an absorbable collagen sponge in 2013 (Kim et al. 2013). In the same way, Kosen et al. filled class

II furcation defects with collagen hydrogel/sponge scaffolds in beagle dogs. After two and four weeks of healing the histometric parameters were compared to a control and the volume of reconstructed alveolar bone, cementum and new periodontal ligament was significantly greater. The new bone volume was 20 % greater than in the control group. After 4 weeks of healing, the new cementum level was 12 % higher and the new periodontal ligament showed an increase of 13% compared to control. The outcomes of the research from Kosen et al. are comparable to the results of our study. In our investigation, all treatment groups showed an increase of the new attachment formation of at least 28 %. Despite these regeneration processes, the collagen also showed a high biocompability and degradability (Kosen et al. 2012).

Another study delivered similar results using a bovine hydroxyapatite/collagen block in one-wall intrabony periodontal defects in dogs (Jung et al. 2011). In two out of five groups a regeneration of the periodontal tissues could be achieved. Without using a barrier membrane, the filling materials did not remain in place and 3 of 5 groups did not regenerate periodontal tissues. Again, it was shown, that collagen is a promising material for periodontal regeneration in combination with GTR.

Our study also confirmed that collagen alone, implanted solely, also increased the new attachment formation. When compared to control, all collagen materials showed a significantly increased periodontal regeneration. There are several studies that document that the additional application of dental stem cells or growth factors may lead to a further improvement of the periodontal regeneration (Zhang et al. 2006, Iwata et al. 2009). Zhang et al. used a porous chitosan/collagen scaffold in combination with human transforming growth factor-β1 (TGF-β1) in athymic rats. Human periodontal ligament cells were seeded into these scaffolds and it was shown that the cells did not only proliferate, but also recruited surrounding cells to grow in the scaffold. Another study from Sculean et al. tested the effectiveness of a composite bovine-derived xenograft and a bioresorbable collagen membrane in deep intrabony defects of thirty-two periodontitis patients. The control group with an open flap surgery achieved significantly less clinical attachment gain than the experimental group (Sculean et al. 2005). Moreover, the epithelial attachment could be decreased with all filling materials in this study. So, the implantation of the filling materials did not only improve the new attachment formation, it also prevented the formation of epithelial attachment. Once more, these results are comparable to the results of this research project. In our study, the epithelial attachment was decreased with all collagen materials. The results confirm the outcome from Kosen

et al. from 2012, where not only were periodontal tissues formed, but also the epithelial down-growth was suppressed by the application of collagen hydrogel. After four weeks of healing the long junctional epithelium decreased from about 35% in the control group to 13% in the experimental group. Also, Jung et al. could evince the effectiveness of using a collagen block in beagle dogs. The epithelial attachment could be decreased from 0, 93 mm in the control group to 0, 80 mm in the experimental group (Jung et al. 2011).

Taken together, collagen was frequently proven to be a suitable filling material in periodontal defects.

4.2 Stem cells

Stem cells play an increasingly important role in periodontal research. The stem cells used in this study were periodontal ligament stem cells. They were isolated from the periodontal ligament of extracted teeth. Periodontal ligament stem cells are part of the mesenchymal stem cells (Egusa et al. 2012; Horwitz et al. 2005). There are different kinds of dental stem cells, for example dental pulp stem cells, stem cells from human exfoliated deciduous teeth or dental follicle stem cells (Egusa et al. 2012).

The periodontal ligament stem cells were implanted with the collagen powder. Thereby, an increase of the new attachment formation and a reduction of the epithelial attachment could be achieved. Our outcomes confirm the results of other studies. In 2008, Liu et al. showed showed that periodontal regeneration could be significantly improved with the use of periodontal ligament stem cells in a porcine model (Liu et al. 2008). Those findings were confirmed by the research of Ding et al. from 2010. In this study, a significantly better periodontal tissue regeneration compared to control could be achieved with allogeneic and autologous periodontal ligament stem cells (Ding et al. 2010). Also, a systematic review from Bright et al. recapitulated that in 43 studies, using periodontal ligament stem cells in four different species of animals (dog, rat, pig and sheep) and various sizes of surgical defects, 70,5 % of the results showed an statistically significant improved periodontal regeneration (Bright et al. 2015).

Two studies from Dogan et al. and Akizuki et al. showed that periodontal ligament stem cells can prevent epithelial down growth and root resorption. Therefore, periodontal ligament stem cells were cultured *in vitro* and periodontal defects treated *in vivo*. A formation of connective tissue attachment, characterized by parallel bundles resting on the root dentin, and a reduction of the epithelial attachment could

be observed (Dogan et al. 2002; Akizuki et al. 2005). The efficiency of adipose-derived stem cells was examined by Tobita et al. in 2008. Their study demonstrated that eight weeks after the implantation of adipose-derived stem cells a periodontal-like structure could be seen in rats (Tobita et al. 2008).

As with most of the studies mentioned above, our study showed a significant gain of new attachment by using PDLSCs when compared to the control. However, there was no statistically significant difference when compared to the groups using GF or the carrier material alone. The lack of significance may be justified by the small number of samples used in this study, resulting in large standard deviations. More possible reasons for this phenomenon are discussed in methodological considerations (4.4).

4.3 Growth factors

The growth factors used in this study were implanted with two different collagen materials, the collagen matrix and the collagen membrane, and showed controversial results. It is supposed that growth factors increase new attachment formation and improve periodontal regeneration. Several studies showed that growth factors may create an environment which is adjuvant to support a de novo tissue formation. They have the ability to regulate various functions of cells originating in the periodontal tissues and thereby can improve periodontal regeneration (Stavropoulos et al. 2012). In a split-mouth designed study it was also shown that growth factors increased the clinical attachment level (Howell et al. 1997). There were test sites with one of two concentrations of rhPDGF (recombinant human platelet-derived growth factor) plus rhIGF-1 (recombinant human insulin-like growth factor-1) and a control site containing the carrier only. Clinical attachment level gain at test sites was significantly higher than compared to control group.

Nevins et al. and Jayakumar et al. showed that the clinical attachment level improved three months after surgery with the use of growth factors. Also, an increased rate of bone growth was observed (Nevins et al. 2013; Jayakumar et al. 2011). Both resulted in indicating clinical attachment level gain when using platelet-derived growth factor PDGF-BB (subgroup of the PDGF family) combined with β-tricalcium phosphate compared to control consisting of β-tricalcium phosphate (β-TCP) alone.

A recent study from *Kitamura* et al. from 2015 investigated if periodontal regeneration in intrabony defects could be increased by the use of trafermin, a recombinant human fibroblast growth factor. Therefore, a total of 328 periodontitis

patients with 4-mm and 3-mm or deeper probing pocket depths and intrabony defects were either treated with the fibroblast growth factor or no filling material during flap surgery. The study showed that the percentage of bone fill at 36 weeks was significantly greater in the growth factor group, but the gain of the clinical attachment level was not significantly different between the groups (Kitamura et al. 2015).

There is another current systematic review dealing with the clinical efficacy of growth factors to enhance tissue repair and tissue regeneration in the oral and maxillofacial region from 2015 (Schliephake 2015). This review concludes, that autogenous growth factors in platelet concentrates could improve clinical attachment level and bone fill significantly, but without any clinical benefit. Therefore, the study summarizes that the evidence of clinical efficacy of growth factors in regenerative processes in the oral and maxillofacial area is limited.

Our study confirms the statement from Schliephake. On the one hand, the new attachment formation increased when combining the collagen membrane with the growth factor cocktail. On the other hand, the new attachment formation decreased when the growth factors were combined with the collagen matrix. Thus, the effect of the growth factors was controversial in our research. They had different impacts, depending on the carrier material used.

The collagen matrix, made of collagen from the jellyfish *Rhopilema spec*., may contain endotoxins. Kawaguchi et al. described in a research from 1995, that the presence of endotoxins induce the production of tumor necrosis factor – alpha (TNF-alpha). TNF-alpha inhibits the effect of growth factors, for example fibroblast growth factor, in the site of the wound. Therefore, the wound healing and the collagen production may be decreased by the presence of endotoxins (Kawaguchi et al. 1995). This might be a possible reason for the lack of significance when using the collagen matrix as a filling material in this study. Other studies assert that collagen from a jellyfish activates the immune response *in vivo* (Morishige et al. 2011).

Growth factors, applied with the correct concentrations, are supposed to improve periodontal regeneration. The contentious impact of the growth factors used in this study could also be associated with different concentrations. In a study from Graziani et al. (Graziani et al. 2006) different concentrations of growth factors and their effect on osteoblasts and fibroblasts in the early phase of wound healing were examined. In their study, platelet-rich plasma was tested, consisting of seven different growth factors. The findings of this study showed that the proliferation of these cells is dose dependent, but it is not the highest dose resulting in best

proliferation. Another reason for the controversial impacts of the growth factor cocktail and also for different growth factor concentrations inside the furcation defect may be the different adhesion of the growth factors to the carrier materials. Perhaps, the growth factors could attach more easily to the collagen membrane than to the collagen matrix. Based on the fact that cell proliferation is a critical event during early wound healing, an increased growth factor concentration may not provide the optimal environment for wound healing. Therefore, the right concentration that would be ideal in periodontal class II furcation defect is yet to be determined.

4.4 Methodological considerations

In this study we used an animal model to find new treatment options for periodontitis. The periodontal ligament stem cells and the growth factor cocktail were implanted with different collagen materials in minipigs. The inflammatory processes in periodontal diseases in minipigs are similar to the ones in periodontal tissues in humans (Štembírek et al. 2012), making the minipigs ideal candidates for periodontal research. Minipigs are omnivores and the anatomical structure of their teeth and periodontal apparatus resembles humans and the wound healing processes are also comparable (Forster et al. 2010).

The materials were randomly assigned to surgically created class II furcation defects. The number of samples was limited. There was a minimum of 4 defects per test group due to the regulatory guidelines of animal testings in Germany. Due to the small number of samples, the standard deviations are high and an increased number of samples would have provided a more meaningful statistic.

The outcomes and the conclusion of this study are limited by several factors. It is difficult to compare the efficacy of the dental stem cells and the growth factors, because different carrier materials were used for implantation. The PDLSCs were implanted with the collagen powder, whereto the growth factors were implanted either with the collagen membrane or the collagen matrix. The use of the different carriers makes a direct comparison complex. The material testings prior to implantation (2.1.4) showed that the stem cells were not combinable with the collagen matrix. Maybe the pH-value of the collagen matrix was not appropriate for the survival of the stem cells. Another reason could be the presence of endotoxins (4.3). The periodontal ligament stem cells could also not be combined with the collagen membrane, wherefore they were only implanted with the collagen powder. Pre-trials showed that the stem cells survived at least 24 hours when combined with the collagen powder. Maybe further *in vitro* trials would have been necessary to

completely understand the interaction of the collagen powder and the stem cells. Furthermore, it is speculated that periodontal ligament cell differentiation is highly sensitive to differences in microenvironments. These facts may result in different tissue formations, as also seen in this study (Benatti et al. 2007). Considering the outcomes of this study, there was an improved periodontal regeneration using the PDLSCs.

The growth factors used in this study were mixed in a growth factor cocktail and may differ from growth factor concentrations used in other studies. Therefore, it does not provide any insight into the efficacy of the single growth factors used in this cocktail (Kaigler et al. 2011). Furthermore, the four different growth factors used in this cocktail, may potentially interact among themselves. As mentioned above, it is challenging to compare the effectiveness of the growth factor cocktail, because they were implanted with different carrier materials to the stem cells. Nevertheless, since all implanted collagen materials lead to an increased new attachment formation (from 51 % – 79 %), a tendency for the regenerative potential of the additional application of growth factors or dental stem cells can be drawn.

Progenitor cells and dental stem cells play an increasingly important role in periodontal research. They have the potential to differentiate into osteoblasts, fibroblasts or cementoblasts. Thus, new periodontal tissues can be formed, including periodontal ligament, cementum, alveolar bone and ginigiva (Yoshioka et al. 2015). Those cells need suitable scaffolds to guarantee a safe application and their interaction with the environment (Diomede et al. 2016). Growth factors are also supposed to improve periodontal regeneration, but more *in vitro* studies are necessary considering the existing controversies of their efficacy (Vahabi et al. 2015; Yan et al. 2015).

In clinical practice, there are several factors influencing periodontal regeneration - the health condition of the animal, metabolic processes, genetic factors or different immune systems can affect healing processes. Also, the healing process can be influenced by the surgeon’s abilities or his clinical experiences. Consequently, the regenerative processes *in vivo* cannot be easily compared with experimental studies with stem cells *in vitro.*

Environmental factors and individual variations affect wound healing *in vivo*, but have no influence on experimental investigations *in vitro.* Therefore, the assumed positive impact of stem cells or growth factors *in vitro* may be overlayed by a significant variation of individual factors *in vivo*. Those individual variations may be

presented by the large standard deviations in this study. It can thus be concluded that the complexity of a system also increases its variability.
Also, the results of this research showed that in particular the additional application of stem cells and in some cases also the addition of growth factors have a positive, but limited effect on regeneration *in vivo*. Due to the complexity of the clinical situation and the variety of factors that influence wound healing the improvement is not statistically significant.
In other fields of medicine similar effects were shown. Corneal transplants in combination with adipose tissue derived MSCs did not increase the survival of the transplants, but rather increased inflammation in a study with rabbits in 2015 (Fuentes-Julián et al. 2015). This led to a lower survival of the graft when compared to sham-treated corneal transplants. The authors also concluded that there are diverse parameters that must be established before MSCs can be useful in corneal transplants including cell source, time of injection, immune suppression or number of cells. Yeagy et al. used bone marrow stem cells for kidney repair (Yeagy et al. 2011). In their review they also question the impact of bone marrow stem cells, because some authors have shown that bone marrow stem cells can differentiate into renal cells and reverse renal dysfunction while others observed inconsistent results and doubt their efficacy.

There are several studies investigating the efficacy of stem cells alone in periodontal defects in rats. Periodontal fenestration defects were treated with allogeneic PDLSCs seeded onto an absorbable gelatin sponge. The results were compared to the material alone and a blank. A statistically significant difference was achieved within specimens retrieved on day 21 for analysis of regeneration of cementum/periodontal ligament (PDL)-like structures (Han et al. 2014). In addition, there is a study from Cai et al. from 2015, where mesenchymal stem cells were obtained from rats and then pre-cultured under different in vitro conditions. They were implanted and it could be shown that a prior *in vitro* chondrogenic differentiation lead to regeneration of alveolar bone and ligament tissues (Cai et al. 2015).
Also, there are studies that examine the effectiveness of growth factors alone in dogs and in humans. Intrabony 2-wall defects were created bilaterally on the second premolar and the first molar in nine dogs. The defects were filled with recombinant human platelet-derived growth factor or a ß-tricalcium phosphate matrix. The amount of new cementum was significantly higher with the platelet-derived growth

factors (Nevins et al. 2012). Another study tested the regenerative potential of GF in humans. 83 patients received a local application of β-tricalcium phosphate scaffold matrix with or without two different dose levels of recombinant human platelet-derived growth factor. After 36 months, there was a continuing increase in clinical attachment gain; linear bone growth and percentage bone fill over time, suggesting overall stability of the regenerative response (Nevins et al. 2013).

To the best of our knowledge there is no other study comparing the efficacy of periodontal ligament stem cells or growth factors in one study while using the same animal model. Our study showed that the carrier materials alone or in combination with stem cells or growth factors can induce a significantly improved periodontal regeneration when compared to the non-treatment control. Both, the addition of the dental stem cells or the growth factors to the carrier material did not enhance the extent of the periodontal regeneration in a statistically significant way. Also, there was no significant difference between stem cells and growth factors (Basan et al. 2017).

5 SUMMARY

In periodontology, a regeneration of the lost periodontal tissues is still challenging. Therefore, the aim of this study was to examine the regenerative potential of different collagen materials with and without stem cells or growth factors in a minipig animal model.

In a total of 60 surgically created class II furcation defects the following materials were applied: a) different collagen support versus control, b) different collagen support +/- a growth factor cocktail and c) a collagen powder versus collagen powder + periodontal ligament stem cells. The periodontal ligament stem cells were isolated from extracted teeth of 15 adult miniature pigs. The materials were applied and a polychrome sequential labeling followed. After 120 days of healing, a histological evaluation of the regenerative processes in the furcation defects followed.

In all treatment groups a statistically significant increase in new attachment formation was observed. The new attachment reached a maximum of 77 percent of the total defect length. On the contrary, in the control group a new attachment formation of 13 percent was measured. Equally, the formation of the epithelial attachment could be decreased with all materials. The results showed that the collagen carrier itself caused significant improvement of regeneration, which was already shown in many previous studies. An additional therapeutic effect of stem cells or growth factors could be observed, but did not change the outcome significantly. One common feature of the application of SC and/or GF was the presence of high standard deviations. There were no significant differences between all experimental groups. Due to the limitations of this project, it can be assumed that the lack of significant differences is caused by the complexity of the clinical setting (e.g. operator skills, individual wound healing and differences in defect morphology).

To sum up, stem cell research seems to be at a point where the basic approaches that could be achieved over the last couple of years need to be consolidated for promising use *in vivo*. Our results show that the possible therapeutic effect of stem cells or growth factors shown *in vitro* or in small animal testings cannot always be easily transferred into a clinical situation.

6 THESIS STATEMENTS

1. All implanted materials improved the formation of the new attachment when compared to the controls.

2. The additional application of stem cells did not significantly improve regeneration.

3. The additional application of growth factors did not significantly improve regeneration.

4. There was no difference in regeneration process between the growth factors and the stem cells.

5. Due to individual factors, the possible effects of stem cells and growth factors *in vitro* may not become evident when applied *in vivo*.

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9 APPENDIX

A Curriculum Vitae

Zahnärztin Tanja Basan

Geburtsdatum:	11.08.1986
Geburtsort:	Rostock
E-Mail:	tanja.basan@uni-rostock.de

Schulausbildung

07/2006	Abitur am Friderico Francisceum Gymnasium zu Bad Doberan

Hochschulausbildung

10/2007 – 09/2008	Studium der Zahnmedizin an der Universität Hamburg
10/2008 – 10/2013	Studium der Zahnmedizin an der Universität Rostock
10/2013	Abschluss des Staatsexamens, Note: „sehr gut“

Stipendien

01/2014 – 12/2014	Landesgraduiertenförderung Mecklenburg-Vorpommern

Berufliche Tätigkeit

seit 01/2015	Assistenzzahnärztin und wissenschaftliche Mitarbeiterin in der Poliklinik für Zahnerhaltung und Parodontologie der Universitätsmedizin Rostock

Publikationen

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B Selbstständigkeitserklärung

Ich versichere hiermit an Eides statt, dass ich die vorliegende Arbeit selbstständig angefertigt und ohne fremde Hilfe verfasst habe. Dazu habe ich keine außer den von mir angegebenen Hilfsmitteln und Quellen verwendet und die den benutzten Werken inhaltlich und wörtlich entnommene Stellen habe ich als solche kenntlich gemacht.

Tanja Basan Rostock, den 13.02.2017