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Characteristics of large animal models for current cell-based oral tissue regeneration.

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ABSTRACT

The recent advances in the field of cell-based therapeutics open promising perspectives for oral tissue regeneration. The development of large animal models, which overcome the limits of the rodent models and allow to emulate clinical situations, is crucial for the validation of regenerative strategies to move towards clinical application.

Currently, porcine, canine and ovine models are the mainly developed for oral regeneration and their specific characteristics have an impact on the outcomes of the studies.

Thus, this systematic review investigates the application of porcine, canine and ovine models in present cell-based oral regeneration, according to the species characteristics and the targeted tissue to regenerate.

A customized search of PubMed, EMBASE, Scopus, and Web of Science databases, from January 2015 to March 2020 was conducted. Relevant articles about cell-based oral tissues engineering in porcine, canine and ovine models were evaluated. Among the evaluated articles, fifty-eight relevant studies about cell-based oral regeneration in porcine, canine and ovine models matched the eligibility criteria and were selected for full analysis.

Porcine models, the most similar specie with humans, were mostly used for bone and periodontium regeneration; tooth regeneration was reported only in pig except for one study in dog.

Canine models were the most transversal models, successfully involved for all oral tissues regeneration and notably in implantology. However, differences with humans and ethical concerns affect the use of these models.

Ovine models, alternative to porcine and canine ones were mainly used for bone and, scarcely, for periodontium regeneration. The anatomy and physiology of these animals restrain their involvement.

If consistency was found in defects specificities and cells trends among different species animal models of bone, dentin-pulp complex or tooth regeneration, variability appeared in periodontium.

Regeneration assessment methods were more elaborate in porcines and canines than in ovines.

Risk of bias was low for selection, attrition and reporting but unclear for performance and detection.

Overall, if none of the large animal models can be considered as an ideal one, they are of deemed importance for oral cell-based tissue engineering and researchers should consider their relevance to establish favorable conditions for a given preclinical cell-based therapeutics.

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IMPACT STATEMENT

This systematic review investigates porcine, canine and ovine models for current oral cell-based regeneration procedures, and researchers could refer to it for the choice of the most pertinent pre-clinical model for a given cell-based therapeutics.

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INTRODUCTION

Injuries and pathologies affecting the oral region as well as lesions resulting from invasive and destructing therapeutic approaches can determine extensive loss of tissues and function. Moreover, due to the heterogeneity of the tissues of this area, their reconstruction is particularly complex[1].

In recent years, thanks to the exponential growth of tissue engineering, new perspectives have been opened in cell-based oral regeneration[2]. In fact, the use of new sources of stem cells[2,3], the development of high-performance biomaterials and promising biotechnologies, such as 3D bioprinting[3,4], allowed a considerable progress towards human application of cell-based oral tissue regeneration procedures. Prior to clinical trials, validation in animal models is required.

It has been established that rodents' substantial anatomic and physiologic dissimilarities with humans affect extrapolation of results from murine studies to patients. Thus, the development of large animal models, overcoming the limits of the rodent ones and allowing the reproduction of near-to-real clinical situations, plays a crucial role in the translation of cell-based regenerative procedures from bench to bedside[5,6].

The identification of the most relevant animal model is a crucial step of the study conception. However, this selection is far from being a simple process, since multiple factors are at stake. Literature on oral regeneration reports large animal models that significantly contributed to the current knowledge on the field. In particular, non-human primates, porcines and canines have been involved, over decades, to investigate surgical procedures, pathogenesis of periodontal and endodontic diseases, guided-tissue regeneration and implantology[7-9].

The identification and characterization of dental stem cells, in 2000, allowed a significant development of cell-based approaches of oral tissue regeneration[10-12]. Therefore, the above-mentioned animal species have been used to validate key models of bone[3,9,13], periodontium[14,15], dentin-pulp complex[16-18] and tooth organ regeneration[19], which opened the way to current research and still represent the benchmarks in the field.

More recently, animal research faced considerable changes. Indeed, the introduction of the ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines supported transparency and systematization in reporting on animal studies, addressing the problem of poor reproducibility of scientific findings[20]. Moreover, an emerging debate on the protection of animals used for scientific purposes led to substantial revisions of the existing regulation, notably introducing the 3Rs principle (Replacement, Reduction and Refinement) and restricting the use of specific species, with an actual full application by different countries only in the last few years.

This results in the exclusion of non-human primates' models for cell based oral tissue regeneration, leaving the choice of porcines, canines and ovines[21-23].

Porcines are widely used due to their similitudes with humans in terms of anatomy (tab 1, fig 1), physiology and pathology, as well as for ethical reasons. Nevertheless, their temperament can be difficult to manage[5].

Canines are one of the most common large animal models for oral and dental regeneration, notably due to their familiar behavior and the comparable growth, physiology and pathology with humans. However, dogs are considered companion animals and their use in medical research is negatively perceived by society[7] (tab 1, fig 2).

As an alternative, the use of ovines increased over the last decade, but the ruminant nature leads to substantial anatomical differences in comparison with humans[7] (tab 1, fig 3).

Hence, this review aimed to investigate porcine, canine and ovine models for current cell-based oral tissue regeneration procedures, in order to i) provide an exhaustive analysis of the present potential application of each model, and ii) support researchers in the choice of the most pertinent one for a given study, according to the animal characteristics and the tissue to regenerate.

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METHODS

Search Strategy

The review process followed the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines[24] and the protocol was registered in PROSPERO (International Prospective Register of Systematic Reviews) under the number CRD42020201550.

The peer-reviewed literature reporting about large animal models on most recent cell-based oral regeneration procedures was systematically searched in PubMed (National Library of Medicine, NCBI), Embase, Web of Science and Scopus databases, from January 2015 to March 2020. The following combination of key words was used: Oral AND (regeneration OR tissue engineering) AND stem cells AND (pig OR dog OR sheep). A manual review of articles' references was also performed.

Eligibility criteria

Inclusion criteria were: 1) cell-based oral tissues regeneration studies, 2) large animal studies, 3) English language available full text, 4) publication between 2015 and 2020.

Exclusion criteria were: 1) murine studies, 2) *in vitro* studies, 3) non-cell-based studies, 4) ectopic and semi-orthotopic regeneration models, 5) literature reviews.

Two independent reviewers (FM and SV) screened all relevant titles and abstracts against eligibility criteria. If the abstract did not provide sufficient information, the full text article was analyzed. A third reviewer (BS) was involved to resolve disagreements.

Data Extraction and Analysis

The selected articles were assigned, depending on the regenerated tissue, as follow: a) bone regeneration, b) periodontium regeneration, c) dentin-pulp complex regeneration, d) tooth/tooth-root regeneration studies.

For each tissue, articles were subsequently classified according to the involved animal; reproduced clinical context/pathology, source of stem cells, scaffolds, follow-up and assessment techniques were considered.

Assessment of quality of the studies

Risk of bias for the included studies was evaluated by SYRCLE (SYstematic Review Centre for Laboratory animal Experimentation) risk of bias tool. The following criteria were used: 1) selection bias, 2) performance bias, 3) detection bias, 4) attrition and 5) reporting bias. Studies were scored with a ''yes'' for low risk of bias, ''no'' for high risk of bias, and ''?'' for unclear risk of bias by the two reviewers independently. Disagreements were resolved by a third reviewer (BS).

RESULTS

As presented in the flowchart based on PRISMA (fig 4), the initial search resulted in a total of 148 articles. Eight relevant publications were manually added from reference lists of the articles identified. After duplicate removal, a total of 123 articles was identified. The review and selection procedure resulted in the exclusion of 43 articles at title screening-stage and 11 articles based on the content of the abstract. Of the remaining 69 articles, 11 were excluded at the full-text reading stage for the following reasons: 1) 3 articles were not *in vivo* studies, 2) 1 article reported ectopic cell-based regeneration, 3) 3 articles reported studies not developed on large animal models, 4) 4 studies presented non cell-based regeneration. The entire selection process therefore resulted in a total of 58 articles, included in the present systematic review.

Characteristics of the included studies

Results are summarized in tables 2 to 5 (tab 2-5). Considering the regenerated tissues, 24 articles focused on bone[25-48], 17 on periodontium[49-65], 10 on dentin pulp-complex[66-75] and 7 on tooth or tooth root[76-82]. Regarding bone regeneration, 8 studies developed a porcine model[25-32], 14 a canine[33-46] and 2 an ovine one[47,48]. Eight studies reported periodontium regeneration in pig[49-56], 8 in dog[57-64] and 1 in sheep[65]. Among the articles about dentin

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pulp-complex regeneration, 2 studies were developed in pig[66,67] and 8 in dog[68-75]. Except for 1 canine model[82], in all the studies, tooth or tooth root regeneration was reported in porcines[76-81].

a) Bone regeneration (tab 2)

Porcine models

The clinical situations were exclusively acute defects such as mandible non critical[26] and critical size bone defects[27,29,31], mandible extraction socket[32], alveolar cleft[25,30] as well as ramus and condyle defects[28].

Employed cells were mostly porcine cells such as pMSCs[25,30,32], pBMSCs[26,29,31] and pADSCs[28], except in one study in which human DPSCs were used[27]. The used scaffolds were PLGA[25,29,30], b-TCP[26,27] and decellularized bone scaffolds[28,31]. In one article, cell sheets were involved[32].

Follow-up was performed during 4 weeks (or 30 days)[25,31], 6 weeks[32], 8 weeks[26,27], 12 weeks (or 90 days)[29,30] and 6 months[28].

Regenerated tissues were assessed by histology in all studies[25-32], in association with CT and/or µCT evaluation[25,28-31], immunohistochemistry[25,26,30], histomorphometry[26,27,32], mechanical tests[30] and fluorescence microscopy[32].

Canine models

The studies focused on acute models of mandible peri-implant bone defects[33,39,43,45,46], mandible non critical[34] and critical size bone defects[37,42,44], alveolar cleft[35], bilateral sinus lift[38], mandible segmental defect[41]. Two studies developed chronic mandible peri-implant bone defects[36,40].

The employed cells were cBMSCs[34,35,38,41,45], rhPDGF- cBMSCs[39], cADSCs[37,43], cEPCs[42], BMP2-cADSCs[40], BMP2-cPDLSCs[36], cPDLSCs[46]. Only 2 studies used hADCs[33,44]. Cell sheets were used in 2 articles[41,46].

Cells were seeded into various scaffolds such as b-TCP[35,39,45], TCP[40], HA-TCP[33,44], TCP-fibronectin[37,43], PLGA[44], b-TCP coated with PLGA releasing VEGF[42], FDB[41] and HA-collagen[36].

The follow-up was performed during 4 weeks[33], 8 weeks[41,42,44], 11 weeks[46], 12 weeks or 3 months[35-37,39,43,45], 16 weeks[34], 6 months[38] and 10 months[40].

In 13 out of 14 studies, assessment was made by histology and histomorphometry[33-40,42-46]; these techniques were also associated with 2D and/or 3D (µCT) radiographic analysis[38,40,41,44,45], BIC evaluation[33,36,39,40,43,45,46], hardness mechanical tests[34,38] and fluorescence microscopy[39]. Only one study evaluated regenerated tissues by combining histology, immunohistochemistry and radiographies[41].

Ovine models

One study focused on sinus lift[47], the other one on acute mandible segmental bone defect[48]. oMSCs[47] and oBMSCs[48] were used respectively associated with autologous serum and BBM scaffold. Follow-up was made for 16[47] and 32 weeks[48].

Histology and histomorphometry were performed in both studies[47,48], in one case also combined with CT and µCT evaluations[48].

b) Periodontium regeneration (tab 3)

Porcine models

The experimental models of periodontitis reproduced acute or acute-chronic mandibular class II furcation defects[53,56], acute-chronic maxillary and/or mandibular alveolar 3 walls bone defects[49-52,54,55].

Porcine cells such as pMSCs[50], pADCs[56] and pPDLSCs[53] were used in 3 studies. Human cells like hPDLSCs transfected with HGF[49], hDPSCs[52], IGFBP5-hMSCs[51] and human SCAPS[54] transfected or not with SFRP2[55] were employed in the other articles.

Cells were seeded into fibrin gel complex[56], IL1-HyA-sECM[50] hydrogel and collagen[53] scaffolds. In the other studies cells were injected[49,51,52,54,55] and in one case also associated with cells sheets[52].

The follow-up was performed during 4 weeks[56], 12 weeks or 3 months[49,51-55] and 16 weeks[50].

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Assessment was made by clinical, radiographic and histological evaluations in 5 studies[49,50,52,54,55], 3 out of which also performed histomorphometry[49,54,55].

One article presented clinical, photographic and histological but no radiographic analysis[51]. Two articles reported histology and histomorphometry respectively associated with µCT and immunohistochemistry[56] as well as fluorescence microscopy[53] but no clinical evaluation.

Canine models

The experimental models of periodontitis reproduced acute mandible alveolar bone dehiscence[59] and maxillary-mandibular class II furcation defects[63], acute-chronic mandible class III or II furcation defects[58,60], mandibular alveolar bone dehiscence[62,64], as well as chronic maxilla and mandible alveolar bone defects[61]. One model of tooth reimplantation was reported[57].

The employed cells were cBMSCs[58,59,63] also transfected with GFP[63] or TRL2[64], cESEHT with PAB[60], cPDLSCs[57], b-defensin-3-cPDLCs[61]. Human cells (hPDLCs) were used once[62].

One study used cells sheets[61]. Each article reported a different scaffold material such as atelocollagen with b-TCP[58], HA-collagen[59], collagen[64], BCP[62], PRP and fibrin glue[63], decellularized dental root with calcium phosphate (CaP)-fibronectin coating[57]. In 1 study grafting materials were not specified[60].

The follow-up was performed during 2 weeks[64], 8 weeks[57,58,60,61,63], 12 weeks[62] and 6 moths[59].

The assessment was performed in half of the studies by histology and histomorphometry[58-60,63] also associated with µCT analysis[59], immunohistochemistry and TRAP[58]. Histology and µCT analysis[57,62,64], in 1 case also associated with fluorescence microscopy[62], were reported. In 1 study assessment consisted of histology and immunohistochemistry[61].

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Ovine model

The periodontal defect was an acute dehiscence in the mandibular premolar-molar area. Ovine PDLSCs and BMSCs sheets were used associated with polycaprolactone biphasic scaffold.

The follow-up was 10 weeks. The assessment of regenerated tissues was made by histology, histomorphometry and µCT[65].

c) Dentin-pulp complex regeneration (tab 4)

Porcine models

One study focused on partial pulp regeneration[66], the other one investigated total pulp regeneration[67]. In both cases, upper and lower mature multirooted teeth were involved and the pulp defects were acutely induced.

Porcine DPCs and pDPSCs were used. The cells were seeded in a nanopeptide[66] and in HyA or Collagen hydrogels[67] respectively.

The follow-up was performed during 21 days[66] and 4 months[67].

In one study assessment was made by histology, immunohistochemistry, µCT and histomorphometry[66], in the other one histological, immunohistochemical and 2D radiographic analysis were performed[67].

Canine models

Models of partial pulp regeneration involving upper and lower multirooted teeth were reported in 2 studies[71,75]. Authors used immature[71] and mature[75] teeth respectively. Five studies focused on total pulp regeneration[68-70,73,74]. The involved teeth were upper and lower mature incisors[69,70,74], upper immature incisors[73] as well as upper and lower mature multirooted teeth[68]. Only 1 study evaluated a model of pulp chamber floor perforation in upper and lower mature premolars[72]. Expect for 1 article[73], dentin-pulp complex defects were acutely generated.

Regarding the source of cells, cDPSCs[68,71-73], (G-CSF)MDPSCs[69,70,74] as well cBMSCs[75] were involved.

Cells were implanted with atelocollagen scaffold[69,70,74], PRF[68], TDM or TCP[72], gelatin sponge associated with simvastatin[71] and chitosan hydrogel releasing VEGF-2, PDGF, NGF and BMP7[73]. In 1 study cells were injected[75].

Follow-up was performed during 2 weeks[69], 8 weeks[68], 9 weeks[75], 10 weeks[71], 3 months or 12 weeks[72,74], 4 months[73] and 6 months[70].

Histological assessment was reported in all studies[68-75], associated with histomorphometric analysis[68,69,72], 2D radiographies[71,73] MRI[70], *in situ* hybridization[69], immunohistochemistry[69] as well as blood and urine tests[75].

d) Tooth or tooth root regeneration (tab 5)

Porcine models

Three studies focused on tooth root regeneration in the mandibular incisor[78] and premolar area[76,77]. Three articles described whole tooth regeneration[79-81] in the upper incisor and premolar region[81], in lower canine and premolar region[80] as well as in lower premolar and premolar region[79].

Porcine DFCs transfected or not with GFP were used in 2 studies[76,77]. One article described re-associated tooth germs implantation associated with BMSCs systemically infused[81]. In 2 cases, pDPSCs were combined with pPDLSCs sheets[78] or epithelial cells from gingiva[79]. In 1 study pECs were associated with hDPCs and hUVEC1[80].

The scaffolds in which cells were seeded were TDM[76,77], HA-TCP[78], gelatin-chondroitin-hyaluronan scaffold[79] and dTB[80].

The follow-up was performed during 12 weeks[76], 150 days[81], 6 months[77,78,80] and 13,5 months[79].

Histology, radiography (2D, µCT, CT, CBCT) and immunohistochemistry analysis were reported in 5 studies[76,77,79-81], in 1 of which clinical oral assessment was performed too[77].

In only 1 study, clinical, radiographic (CT, µCT), biomechanical and elemental analysis were combined[78].

Canine model

Whole tooth regeneration was performed in lower premolar area. The regenerated teeth underwent orthodontic traction to test periodontal ligament remodeling.

Canine tooth buds cells were used. The follow-up consisted of 6 months plus 1 month for orthodontic treatment evaluation. The assessment was made by histology, 3D radiography (CBCT and µCT), scanning electron microscopy and energy dispersive X-ray spectroscopy[82].

Assessment of quality of studies

In 98% of the studies a low risk of selection bias (baseline characteristics) was found[25-42,44-64,66-82]. Performance bias was considered unclear in 98% of cases[25-47,49-82], because no information about random housing was given. Random outcome assessment was scored as low risk for 24% of the studies[25-29,33,34,36-40,42,43,46-48,50,52,54,57-60,62-74,76-82] and unclear for the rest of them. In none of studies blinding was described and the risk was rated as unclear[25-82]. A low risk of attrition and reporting bias was estimated for all studies[25-82] (fig 5, supplemental fig 1-4).

DISCUSSION

Large animal models according to regenerated tissues

Bone regeneration

Our research highlights that porcine, canine and ovine models were developed for cell-based regeneration of acute critical maxillary and mandible jawbone defects, mimicking congenital lack of tissues as well as traumatic or postsurgical sequels, such as clefts or segmental osteotomies[25-48]. Moreover, procedures requiring long healing process were possible, since reported follow-up went from 1 month even up to 10 months[25-48].

As supported by broad literature on cell-based bone regeneration in large animals[83], these models make up for multiple limitations of widespread murine calvarial defects, such as the impossibility to perform long term studies, the lack of biomechanical loading and faster tissue healing than in humans[5,84,85].

In accordance with previous studies[86-88], it appears that porcine models are preferred to the other models for higher challenge bone regeneration procedures, because of their similarities with humans, in terms of anatomy, morphology, healing, remodeling and mechanical properties[25,28,30].

For instance, Bhumiratana *et al*. demonstrated regeneration of ramus-condyle unit by using an autologous, anatomically shaped, living graft, made by decellularized bovine trabecular bone and pADSCs[28].

Furthermore, since congenital clefts occurring in pig resemble those in humans, Caballero *et al.* reported porcine alveolar cleft regeneration using porcine umbilical cord mesenchymal stem cells sheets associated with nano-microfiber PLGA scaffold[25,30].

In implantology, canines play a key role[14,89]. In this review, numerous peri-implant bone defects and re-osteointegration models were identified[33,36,39,40,43,45,46]; in fact, dogs' bone turnover, composition and mechanical properties are the most similar to humans among large animal models, even if jaws show a denser and more resistant bone[7,89].

Even if ovine models of cell-based bone regeneration are reported in literature[90,91], in our research, only two studies developed maxillary sinus lift and mandible segmental osteotomy. Indeed, ovine bone dissimilarities with humans such as higher density and mechanical resistance, as well as age related changes in structure and remodeling can limit the relevance of ovine models for follow-up studies[7,14,25,89,92].

Periodontium regeneration

Since murine periodontium and bacterial resistance sensibly differ from humans, porcine, canine and ovine models of periodontitis are developed[14,89].

In this review, similar types of periodontal lesions were reported in pig and dog. However, variability in defects' standardization and follow-up was encountered between the two species.

Only one study developed an acute ovine model of mandible dehiscence[65]. Sheep periodontium displays constant cement apposition as a compensation response to teeth ware which is typical in ruminants[14]. Hence, this periodontal physiology is likely to have an

impact on regeneration mechanisms, which represents a non-neglecting bias for the potential extrapolation to patients[14,92,93].

Dentin-pulp complex regeneration

Regenerative endodontics opens up the perspective of an alternative to millions of endodontic treatments each year[94-96].

Validated in murine ectopic models, dentin-pulp regeneration is hardly performed orthotopically due to frequent dental fractures and differences with human pulp reparation process. Thus, large animals are required to address these limitations[97,98].

Interestingly, despite a more important similarity of pig dental anatomy and physiology with humans in comparison with dogs, mostly canine and only two porcine models were developed[66-75.]

Besides, in both animals, partial and total dentin-pulp complex regeneration procedures, involving upper and lower mature or immature single-rooted or multi-rooted teeth, were evaluated, meaning that several clinical situations can be reproduced in these models. In addition, in dog also a pulp chamber floor perforation model was reported[72].

However, regarding partial pulp regeneration, contrasting findings were reported. In 2017, our team demonstrated, after 3 weeks follow-up, no pulp regeneration but reparative osteodentinogenesis in minipig mature multi-rooted permanent teeth by implanting pDPCs into a self-assembling injectable hydrogel scaffold in a pulpotomy model[66]. In dog, after 9 to 10 weeks, normally organized pulp tissue with a complete dentin bridge was found in single-rooted immature teeth as well as in multirooted mature teeth, using cDPSCs seeded in a gelatin sponge scaffold releasing simvastatine and injected cBMSCs respectively[71,75].

In line with previous studies[99-102], canine models as well as the only porcine model of total pulp regeneration used autologous DPSCs[67-70,73,74] mostly combined with collagen-based scaffolds[67,69,73,74] in mature single-rooted upper and lower teeth[69,70,73,74]. However, the role of neoangiogenesis was solely investigated in dog, since cells were constantly conditioned or associated with angiogenetic factors. One could assume that such a difference in

regenerative environments between animal models influenced the duration of the regenerative process[84] since functional dentin-pulp complex was obtained after 4 months in pig[67] and in 2 weeks to 6 months in dog[68-70,73,74].

Tooth regeneration

The challenging regeneration of tooth organ, which depends on the recombination of dental mesenchymal and epithelial stem cells, has been demonstrated in several animal models[19,103,104]. However, it has been shown that the dental functionality can only be assessed in large animals[19,103].

In this review, tooth regeneration was reported in numerous porcine models[76-81] and in only one canine model[82]. In particular, in pig, consistently with literature[87,105,106], two procedures were studied: tooth-root and whole tooth regeneration. Functional bio-root formation was reported after implantation of HA/TCP/DPSC/PDLSC sheets[78]. Positives outcomes were obtained also combining TDM with minipig DFCs[76,77]. Whole single-rooted tooth regeneration was achieved by allo-transplanted re-associated tooth germs into minipigs jaws associated with systemic infusion of pBMMSCs as well as recellularized dTBs seeded with porcine dental epithelial cells, human dental pulp cells, and human umbilical vein endothelial cells, with an average follow-up of 6 months[80,81].

In line with porcine models, premolar regeneration was achieved, in dog, 6 months after transplantation of bioengineered tooth germs made with autologous germs cells, in the lower jaw. Periodontal functionality was eventually confirmed by 4 weeks long orthodontic traction[82].

Defects characteristics

The reported oral tissues defects were mostly acute[25-35,37-39,41-48,53,56,57,59,62,63,65-72,74,75]. Indeed, surgically made lesions imply standardized configuration, clear understanding of the regenerative process and reduced experimental time. However, these models reproduce simplified regenerative environments[89].

The bacterial component of oral pathologies was considered in few chronic[36,40, 61,64,73] or acute-chronic[49-52,54,55,58,60] models of periimplantitis, periodontitis and pulp necrosis.

Certainly, these models, requiring time-consuming procedures, result in a variable degree of standardization of the defect, complicating the comparisons between studies[89].

Stem cells trends

The pertinence of an animal model for oral tissue-engineering also relies on the potentiality to study different stem cell populations/sources. Thus, the accessibility to autologous stem cells as well as the feasibility of allogenic grafts are crucial criteria of choice.

Similar trends of cell-based bone regeneration were reported in all large animal models with comparable results. In fact, most of the studies demonstrated increased bone formation in critical size defects using autologous BMSCs, ADCs or MSCs principally seeded into b-TCP, PLGA or demineralized bone matrix scaffolds[25,26,28-32,34,35,37-45,47,48].

Unlike the ovine models, in pig and dog large jaws reconstruction and peri-implant defects were also successfully treated by implanting autologous or human ADSCs and/or MSCs co-seeded with endothelial progenitor cells associated with b-TCP and/or PLGA[25,28-30,32,33,40,42-44] as well as autologous or human stem cells from dental tissues alone or combined with b-TCP or HA/collagen[27,36,46].

A substantial discrepancy between trends of periodontal defects regeneration was found; in fact, in pig hMSCs, hPDLSCs and hSCAPS[49,51,52,54,55] were mostly involved, while in dog and sheep mostly autologous mesenchymal stem cells or periodontal ligament stem cells and no SCAPs were used[57-61,63-65]. Moreover, porcine stem cells were mostly injected or used as sheets[49,51,52,54,55], whereas in dog and sheep they were implanted combined with a large variety of grafting materials[57-61, 63-65] which even more complicate comparisons within studies[28,89].

Regarding pulp and root or whole tooth regeneration, in both porcine and canine models mostly autologous dental stem cells[66-71,73-79,82] were used which is coherent with the manageable accessibility to this source of cells in animals showing similar loco-regional anatomy and dental eruption physiology with humans[6,7,25].

Regeneration assessment

Regeneration assessment is essential for the validation of tissue-engineering procedures. A pertinent large animal model should allow an appropriate follow-up duration for a given procedure as well as the quali-quantification of the newly formed tissues and their relation with the surrounding structures[7,25].

In the porcine, canine and ovine studies included in this systematic review, mineralized as well as non-mineralized oral tissues regeneration was assessed within periods even up to 13,5 months and the analysis was performed by similar approaches in the three models.

Overall, most of the studies reported histological, histomorphometric and/or 2D/3D imaging analysis[25-40,42-60,62-73]. Regardless for the µCT, which is an *ex-vivo* technique, imaging assessment was made by technologies currently used in patients such as intraoral 2D radiography, CT, CBCT and MRI. Furthermore, reiterative blood and urine tests, impossible in murine, were reported[74], which highlights the importance of large animals for mimicking clinical conditions[5,25].

However, specific animals' characteristics, data/means unavailability and the necessity to contain the number of samples, according to the 3Rs principle, give raise to some technical boundaries restraining tissues assessment[5,25,66,107].

For example, immunohistochemistry was not constantly performed in pig or dog and not reported at all in sheep models; indeed, some tissue-specific markers cannot be revealed, because of the lack of suitable antibodies. Moreover, due to their size, specimens require even up to several months for the demineralization prior to histological analysis. Thus, aggressive acids or techniques used to accelerate the process can impair antigenic sites and limit antibodies bond[66,107].

Risk of bias

Overall, the included studies presented a low risk of bias in terms of animals' selection (notably ARRIVE guidelines were respected), attrition and reporting. However, poor reporting in terms of performance and detection affected evaluations and synthesis of results. Thus, SYRCLE guidelines should be followed especially for randomization protocols,

animal housing facilities and blinding, which could improve homogeneity of large animal models' trials in oral cell-based regeneration.

CONCLUSION

The development of large animal models for oral tissue engineering is crucial for human application. Pig, dog and sheep are the most relevant species allowed by current regulation, but they can have significant drawbacks, including functional dissimilarities when compared to the human craniofacial and dental anatomy.

Porcine models, the most similar with humans, were successfully developed for bone and periodontium regeneration, but very little was demonstrated about dentin-pulp complex. Interestingly, tooth/tooth root regeneration was reported only in pig, except for one canine study.

Canines are indeed the most transversal models as they showed positive outcomes for the regeneration of bone, in particular in implantology, as well as periodontium and dentin-pulp complex; however, canines substantially differ from humans and ethical concerns arise from their involvement.

Ovines are the least developed models, as they emerged as an alternative to dog and pig. Besides the economic and ethical advantages, these animals display essential dissimilarities with humans. Hence, ovine were mainly used for bone and very little for periodontium regeneration.

If a consistency was found in defects specificities and cells trends among different species animal models of bone, dentin-pulp complex or tooth regeneration, a variability appeared in periodontium.

Indeed, methods of regeneration assessment were more elaborate in porcines and canines than in ovines.

Overall, preclinical models display specific properties to take into account for oral tissues engineering. Thus, studies of different regenerative procedures should be related to the choice of the most pertinent large animal model for a given cell-based therapeutics.

AUTHORS' CONTRIBUTION

Conceptualization, F.M. and S.V.; writing/original draft preparation, F.M.; writing/review and editing, F.M., S.V., M.E., B.S., C.C. and R.J.

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Figures legends

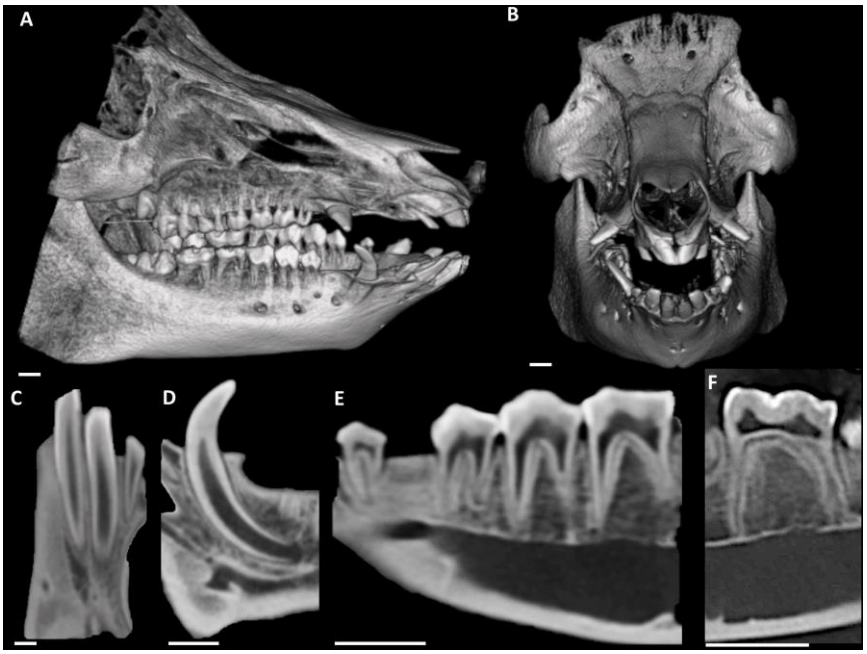

Figure 1. Pig anatomy- Cone Beam CT. **A)** Skull: lateral view. **B)** Skull: frontal view. **C)** Lower incisor region. **D)** Lower canine. **E)** Lower premolars region. **F)** Lower molar anatomy.

Scale bar: 1 cm.

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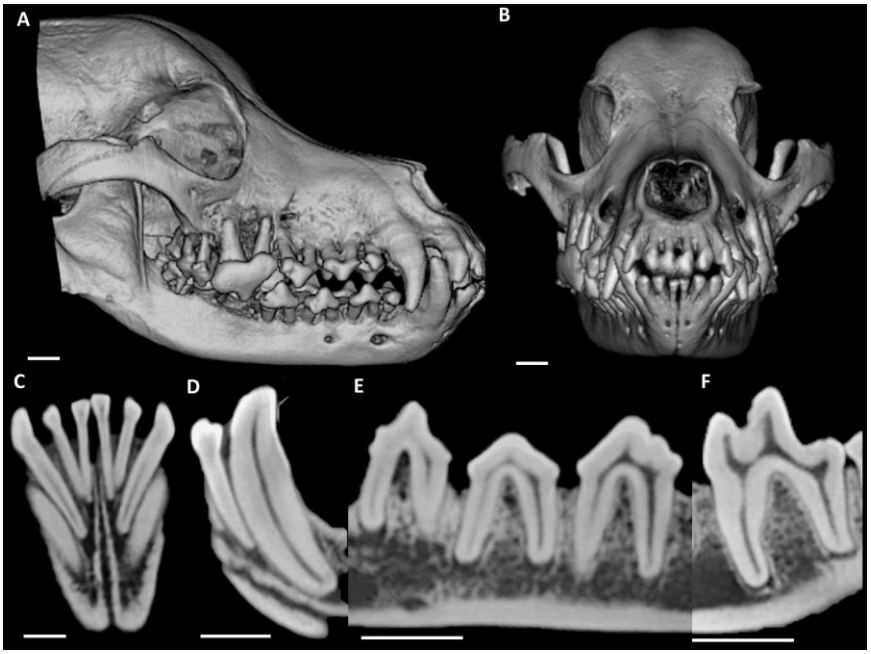

Figure 2. Dog anatomy- Cone Beam CT. **A)** Skull: lateral view. **B)** Skull: frontal view. **C)** Lower incisor region. **D)** Lower canine. **E)** Lower premolars region. **F)** Lower molar anatomy.

Scale bar: 1 cm.

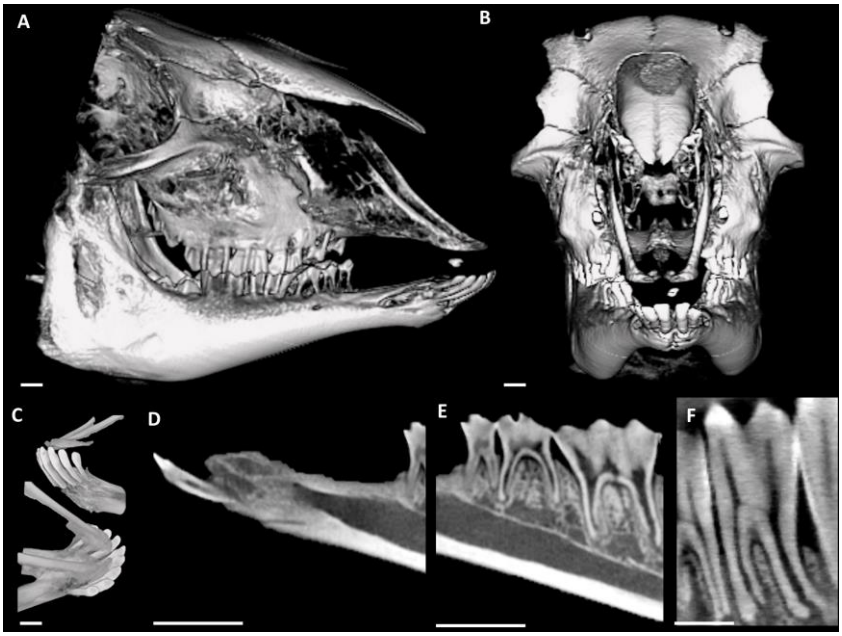

Figure 3. Sheep anatomy- Cone Beam CT. **A)** Skull: lateral view. **B)** Skull: frontal view. **C)** Upper jaw dental pad and lower incisor regions. **D)** Diastema (absence of the canine between incisors and premolars). **E)** Lower premolars region. **F)** Lower molar anatomy.

Scale bar: 1 cm.

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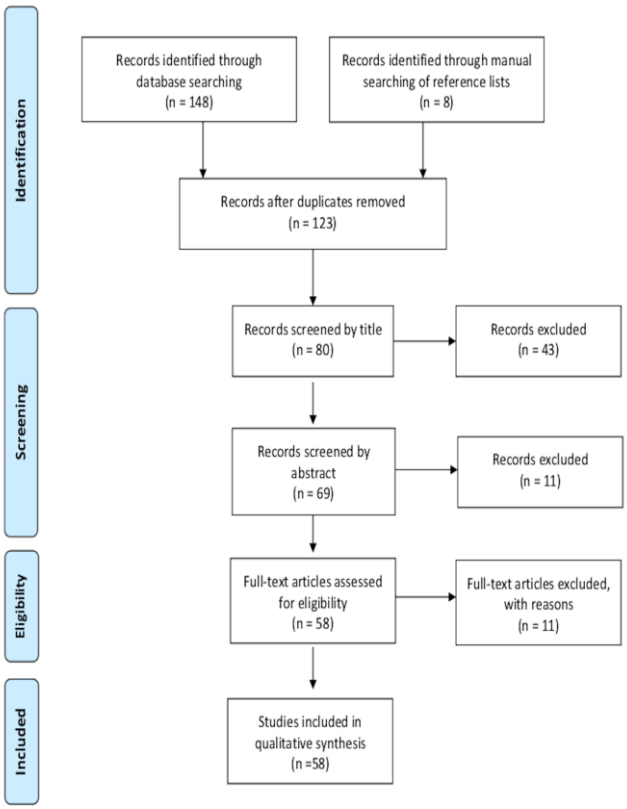

Figure 4. Flowchart of the manuscript selection process.

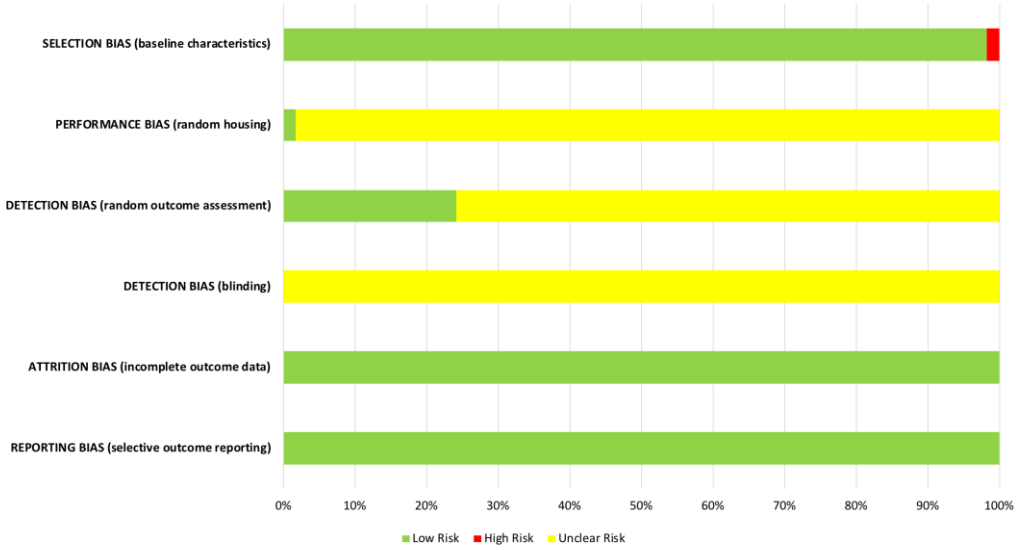

Figure 5. Risk of bias assessment evaluated according to the SYstematic Review Centre for Laboratory animal Experimentation (SYRCLE). Selection bias: baseline characteristics. Performance bias: random housing. Detection bias: random outcome assessment; blinding. Attrition bias: incomplete outcome data. Reporting bias: selective outcome reporting.

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Table 1. Characteristics of the large animal models.

ANIMAL MODEL	SIMILARITIES WITH HUMANS	DISSIMILARITIES WITH HUMANS	ADVANTAGES	DISADVANTAGES
PORCINES	- Size of the body - Jaws bone anatomy, morphology, healing, remodeling and mechanical properties - Mineral density and concentration of lamellar bone - Jaws blood supply - Diphyodonty, dental formula, eruption sequence	- Denser jawbone trabecular system - Continuously growing canine	- Reduced ethical issues	- Voluminous size - Uncooperative behavior
CANINES	- Growth, physiology and pathology - Bone turnover, composition and mechanical properties - Diphyodonty,	- Size and shape of the oral cavity - Denser and more resistant jaws bone	- Extensive knowledge of the model for orofacial tissues engineering - Easy handling	- Ethical issues

	dental anatomy, dental formula			
OVINES	- Size of the body - Diphyodonty, dental formula and shape of permanent teeth	- Ruminant digestion - pH of saliva - Absence of upper incisors, upper and lower canines - Jawbone density and biomechanical properties - Age related bone structure and remodeling	- Reduced ethical issues - Extensive availability - Easy handling - Less expensive housing	- Lack of literature - Limits of comparisons between studies using different ages animals

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Table 2. Bone regeneration.

AUTHOR/YEAR	CLINICAL CONTEXT	FOLLOW-UP	CELLS/SCAFFOLD	ASSESSMENT
Porcine models				
Caballero *et al.*, 2015 [25]	Alveolar cleft*	30 days	pUC-MSCS /nano-microfiber PLGA	Histology Immunohistochemistry CT
Konopnicki *et al.*, 2015 [26]	Mandible inferior border bone defect*	8 weeks	pBMPCs/ 3D printed b-TCP-polycaprolactone	Histology Immunohistochemistry Histomorphometry
Kuo *et al.*, 2015 [27]	Mandibular corner critical size bone defect*	8 weeks	hDPSCs/b-TCP	Histology Histomorphometry
Bhumiratana *et al.*, 2016 [28]	Ramus and condyle critical size bone defect*	6 months	pASCs/ decellularized bovine trabecular bone	Histology µCT
Tee *et al.,* 2016 [29]	Mandible lateral aspect critical size bone defect*	12 weeks	pMSCs /PGLA	Histology CT / µCT

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Caballero *et al.*, 2017 [30]	Alveolar cleft*	90 Days	pUC-MSCS /nano-microfiber PLGA	Histology Immunohistochemistry CT Mechanical tests
Cui *et al.,* 2018 [31]	Mandible inferior border critical size bone defect*	4 weeks	pBMSCs/DBM	Histology µCT
Mu *et al.,* 2018 [32]	Mandible extraction socket	6 weeks	pMCSs sheets	Histology Florescence microscopy Histomorphometry
Canine models				
Bressan *et al.*, 2015 [33]	Mandible premolar-molar region peri-implant bone defect*	4 weeks	hADCs / HA-TCP	Histology Histomorphometry
Du *et al.,* 2015 [34]	Mandible body bone defect*	16 weeks	cBMSCs/ PLGA	Histology Histomorphometry Mechanical analysis
Huang *et al.*, 2015 [35]	Alveolar cleft*	12 weeks	cBMSCs/ b-TCP	Histology Histomorphometry

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Park *et al.*, 2015 [36]	Mandible premolar-molar region peri-implant bone defect**	3 months	BMP2-dPDLSCs / HA-collagen	Histology Histomorphometry
Alvira-Gonzalez *et al.*, 2016 [37]	Mandible premolar-molar region critical size alveolar ridge bone defect*	3 months	cADSCs/ fibronectin-TCP	Histology Histomorphometry
Wang *et al.*, 2016 [38]	Bilateral sinus lift	6 months	cBMSCs/ HA	Histology Histomorphometry µCT Microhardness test
Xu *et al.*, 2016 [39]	Mandible premolar region peri-implant bone defect*	12 weeks	rhPDGF-cBMSCs/ b-TCP	Histology Histomorphometry CLSM Fluorescence microscopy
Xu *et al.*, 2016 [40]	Mandible premolar region peri-implant bone defect**	10 months	BMP2or GFP-cADSCs/TCP	Histology Histomorphometry 2D radiographies µCT

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Zeng *et al*., 2016 [41]	Mandible segmental defect*	8 weeks	cBMSC sheets/FDB	Histology Immunohistochemistry 2D radiographies
Khojasteh *et al*., 2017 [42]	Mandible bone defect*	8 weeks	cEPCs/ b-TCP coated with PLGA microspheres releasing VEGF	Histology Histomorphometry
Sánchez-Garcés *et al.*, 2017 [43]	Mandible peri-implant bone defect*	3 months	cADSCs/ TCP-fibronectin	Histology Histomorphometry
Shafeian *et al.*, 2017 [44]	Mandible critical size bone defect*	8 weeks	PRP-assisted hADSCs/ HA-TCP	Histology, Histomorphometry µCT 2D radiographies
Wang *et al.*, 2018 [45]	Mandible peri-implant bone defect*	12 weeks	Alveolar cBMSCs/ b-TCP	Histology Histomorphometry BIC
Washio *et al.*, 2018 [46]	Mandible peri-implant bone defect*	11 weeks	cPDLSCs sheets	Histology, Histomorphometry
Ovine models				
Ardjomandi *et al*., 2015 [47]	Sinus lift	16 weeks	oMSCs/ BBM	Histology, Histomorphometry

Gallego *et al*., 2015 [48]	Mandible segmental bone defect*	32 weeks	oBMSCs/ autologous serum	Histological Histomorphometry CT/ µCT

:** acute defect model; *:** chronic defect model; *****:** acute-chronic defect model.

pUC-MSCS: porcine umbilical cord-mesenchymal stem cells**; PLGA:** poly-co-glycolic acid; **CT:** computed tomography; **3D:** tridimensional; **pBMPCs:** porcine bone marrow progenitor cells; **b-TCP:** beta tricalcium phosphate; **hDPSCs:** human dental pulp stem cells; **pASCs:** porcine adipose-derived stromal/stem cells; **µCT**: micro-CT; **pMSCs:** porcine marrow stem cell; **pBMSCs:** porcine bone marrow stem cells; **DBM:** demineralized bone matrix; **hADCs:** human adipose-derived cells; **HA:** hydroxyapatite; **TCP:** tricalcium phosphate; **cBMSCs:** canine bone marrow stem cells; **BMP2**: bone morphogenetic protein 2; **dPDLSCs:** dog periodontal ligament stem cells; **cADCs: canine** adipose-derived cells ; **rhPDGF:** recombinant human platelet derived growth factor; **CLSM:** confocal laser scanning microscopy; **GFP**: green fluorescence protein; **FDB:** freeze-dried bone; **2D:** bidimensional; **EPCs:** endothelial progenitor cells; **VEGF:** vascular endothelial growth factor; **PRP:** platelet rich plasma; **hADSCs:** human adipose derived stem cells; **BIC:** bone to implant contact; **cPDLSCs:** canine periodontal ligament stem cells; **oMSCs:** ovine mesenchymal stem cells; **BMM:** bovine bone mineral; oBMSCs: ovine bone marrow stem cells.

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Table 3. Periodontium regeneration.

AUTHOR/YEAR	CLINICAL CONTEXT	FOLLOW-UP	CELLS/SCAFFOLD	ASSESSMENT
Porcine models				
Cao *et al.*, 2015 [49]	Mesial mandible and maxillary first molar 3 walls bone defect***	12 weeks	hPDLSCs transfected with HGF	Clinical examination Histology Histomorphometry CT
Fawzy El-Sayed *et al*., 2015 [50]	Mesial mandible and maxillary premolar-molar 3 walls bone defect***	16 weeks	pMSCs/ (HyA-sECM)	Clinical examination Histology CT
Liu *et al*., 2015 [51]	Mesial mandible first molar 3 walls bone defect***	12 weeks	Injected IGFBP5-hMSCs	Clinical examination photography histology
Hu *et al.*, 2016 [52]	Mesial mandible and maxillary first molar 3 walls bone defect***	12 weeks	hDPSCs sheets or injection	Clinical examination Histology CT

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Basan *et al.*, 2017 [53]	Mandible third premolar and first molar class II furcation defect*	120 days	pPDLSCs/ collagen	Histology Fluorescence microscopy Histomorphometry
Li *et al.*, 2018 [54]	Mesial mandible first molar 3 walls bone defect***	12 weeks	hSCAPs	Clinical examination Histology Histomorphometry CT
Li *et al.,* 2019 [55]	Mesial mandible first molar 3 walls bone defect***	12 weeks	SFRP2-hSCAPs	Clinical examination Histology Histomorphometry CT
Venkataiah *et al.*, 2019 [56]	Mandible premolars class II furcation defect*	4 weeks	pADMPC/ fibrin gel complex	Histology Histomorphometry µCT
Canine models				
Lee *et al.*, 2015 [57]	Tooth reimplantation onto maxillary extraction socket	8 weeks	cPDLSCs/ decellularized dental root + (CaP)-fibronectin coating	Histology µCT
Nagahara *et al.*,	Mandible first,	8 weeks	cBMMSC/	Histology

2015 [58]	second and third premolars class III furcation defect***		atelocollagen-b-TCP	Immunohistochemistry TRAP Histomorphometry
Liu *et al*., 2016 [59]	Mandible alveolar buccal plate defect*	6 months	cBMSCs/HA-collagen	Histology Histomorphometry µCT
Luo *et al.*, 2016 [60]	Mandible class II furcation defect***	8 weeks	ESEHT- PAB / not specified two graft materials	Histology Histomorphometry
Zhu *et al*., 2017 [61]	Mandible and maxillary third premolar, fourth premolar and first molar defect**	8 weeks	Beta-defensin-3-cPDLCs sheets	Histology, Immunohistochemistry
Shi *et al.*, 2018 [62]	Mandible second premolars buccal plate dehiscence*	12 weeks	hPDLCs/ BCP	Hstology flurescence microscopy µCT
Rezaei *et al.*,	Mandible and	8 weeks	GFP transfected	Histology

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2019 [63]	maxillary premolars class II furcation defect*		cBMMSCs/ fibrin glue and PRP	Histomorphometry
Zhou *et al.* 2019 [64]	Mandible premolar defect**	2 weeks	TRL2-transfected-cBMSCs/collagen	Histology µCT
Ovine model				
Vaquette *et al.* 2019 [65]	Mandible premolar-molar buccal plate dehiscence*	10 weeks	oPDLSCs and oBMSCs sheets/ polycaprolactone biphasic scaffold	Histology Histomorphometry µCT

:** acute defect model; *:** chronic defect model; *****:** acute-chronic defect model.

hPDLSCs: human periodontal ligament stem cells; **HGF:** hepatocyte growth factor; **CT:** computed tomography; **pMSCs**: porcine mesenchymal stem cells; **HyA-sECM:** hyaluronic acid-synthetic extracellular matrix; **IGFBP5:** insulin like growth factor binding protein 5; **hMSCs:** human mesenchymal stem cells; **pPDLSCs:** porcine periodontal ligament stem cells; **hSCAPs:** human stem cells from apical papilla; **SFRP2:** secreted frizzled-related protein 2; **pADMPC:** adipose-derived multi-lineage progenitor cells; **µCT**: micro computed tomography; **cPDLSCs**: canine periodontal ligament stem cells; **CaP**: calcium phosphate; **cBMMSC:** canine bone marrow mesenchymal stem cells; **b-TCP:** beta tricalcium phosphate; **TRAP:** tartrate-resistant acid phosphatase; **cBMSCs:** canine bone marrow stem cells**; HA:** hydroxyapatite; **ESEHT:** extraction socket early healing tissue stem cells; **PAB:** proper alveolar bone; **cPDLCs**: canine periodontal ligament cells; **BCP:** biphasic calcium phosphate; **GFP:** green fluorescent protein; **PRP: platelet rich plasma; TRL2:** toll-like receptor 2; **oPDLSCs:** ovine periodontal ligament stem cells; **oBMSCs:** ovine bone marrow

stem cells

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Table 4. Dentin-pulp complex regeneration.

AUTHOR/YEAR	CLINICAL CONTEXT	FOLLOW-UP	CELLS/SCAFFOLD	ASSESSMENT
Porcine models				
Mangione *et al*., 2017 [66]	Partial pulp regeneration on mature multi-rooted posterior mandible and maxillary teeth*	3 weeks	pDPCs/ nanopeptide hydrogel	Histology Immunohistochemistry Histomorphometry µCT
Zhu *et al.*, 2018 [67]	Total pulp regeneration on mature multi-rooted posterior mandible and maxillary teeth*	4 moths	pDPSCs/ HyA or Collagen hydrogel	Histology Immunohistochemistry 2D radiographies
Canine models				
Chen *et al.*, 2015 [68]	Total pulp regeneration on mature upper premolars and lower double-rooted premolars*	8 weeks	cDPSCs/ PRF granules	Histology Histomorphometry
Murakami *et*	Total pulp	2 weeks	(G-CSF)cMDPSCs,	Histology

al., 2015 [69]	regeneration on mature upper and lower incisors*		(G-CSF)cMBMSCs, (G-CSF)cMADSCs / atelocollagen	Histomorphometry In situ hybridization Immunohistochemistry
Iohara *et al.*, 2016 [70]	Total pulp regeneration on mature lower second and third incisors*	180 days	(G-CSF)cMDPSCs/ atelocollagen	Histology MRI
Jia *et al.*, 2016 [71]	Partial pulp regeneration on immature upper and lower premolars*	10 weeks	SIM + cDPSCs +absorbable gelatin sponge	Histology 2D radiographies
Bakhtiar *et al.*, 2017 [72]	Pulp chamber floor perforation on mature upper and lower premolars*	3 months	cDPSCs/TDM or TCP	Histology Histomorphometry
El Ashiry *et al.*, 2018 [73]	Total pulp regeneration on immature necrotic upper incisors with periapical periodontitis**	4 months	cDPSCs / chitosan hydrogel + VEGF-2, PDGF, NGF, BMP7	Histology 2D radiographies

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Iohara *et al.*, 2018 [74]	Total pulp regeneration on mature upper and lower incisors*	12 weeks	cMDPSCs with DLA/atelocollagen	Histology Blood tests Urine tests
El-Zekrid *et al.*, 2019 [75]	Partial pulp regeneration on mature upper and lower multi-rooted teeth*	9 weeks	Injected cBMSCs	Histology

:** acute defect model; *:** chronic defect model; *****:** acute-chronic defect model.

pDPC: porcine dental pulp cells; **µCT:** micro computed tomography; **pDPSCs:** porcine dental pulp stem cells; **HyA:** Hyaluronic acid; **2D:** bidimensional; **PRF:** platelet rich fibrin**; (G-CSF)cMDPSCs:** granulocyte-colony stimulating factor mobilized dental pulp stem cells; **(G-CSF)cMBMSCs:** granulocyte-colony stimulating factor canine mobilized bone marrow stem cells; **(G-CSF)cMADSCs:** granulocyte-colony stimulating factor canine mobilized adipose derived stem cells**; MRI:** magnetic resonance imaging; **SIM:** simvastatin**; TDM:** treated dentin matrix**; TCP:** tricalcium phosphate**; VEGF-2:** vascular endothelial growth factor-2; **PDGF:** Platelet-derived growth factor; **NGF:** nerve growth factor; **BMP7:** bone morphogenetic protein-7; **DLA:** dog leukocyte antigen; **cBMSCs:** canine bone marrow stem cells.

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Table 5. Tooth/ tooth root regeneration.

AUTHOR/YEAR	CLINICAL CONTEXT	FOLLOW-UP	CELLS/SCAFFOLD	ASSESSMENT
Porcine models				
Chen *et al*., 2015 [76]	Tooth root regeneration on lower premolar region	12 weeks	GFP transfected pDFCs/TDM	Histology Immunohistochemistry µCT
Luo *et al*., 2015 [77]	Tooth root regeneration on lower premolar region	6 moths	pDFCs/TDM	Clinical examination 2D radiographies Histology Immunohistochemistry µCT
Gao *et al*., 2016 [78]	Tooth root regeneration on lower incisors region	6 months	hDPSC + hPDLSC sheets/HA-TCP	Clinical examination CT/ µCT Biomechanical tests Elemental analysis
Yang *et al*., 2016 [79]	Whole tooth regeneration on lower premolar and molar	13,5 months	pDPSCs + epithelial cells from gingiva/ gelatin-chondroitin-hyaluronan	Histology Immunohistochemistry 2D radiographies
Zhang *et al*.,	Whole tooth	6 months	pECs+hDPCs +	Histology

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2017 [80]	regeneration on lower canine and premolar region		hUVEC/ dTBs	Immunohistochemistry µCT
Wu *et al*., 2019 [81]	Whole tooth regeneration on upper incisor, canine and premolar region	150 days	Re-associated human tooth germs cells and systemically infused hBMMSCs	Histology Immunohistochemistry CT CBCT
Canine model				
Ono *et al*., 2017 [82]	Whole tooth regeneration on lower premolar region	180 days (+ 30 days of orthodontic treatment)	Canine tooth buds cells	Histology CBCT/µCT SEM Energy-dispersive X-ray spectroscopy

GFP: green fluorescent protein; **pDFCs:** porcine dental follicles cells; **TDM:** treated dentin matrix; **µCT:** micro computed tomography; **2D:** bidimensional; **hDPSC:** human dental pulp stem cells; **hPDLSC:** human periodontal ligament stem cells; **HA-TCP:** hydroxyapatite-tricalcium phosphate; **CT:** computed tomography; **pDPSCs:** porcine dental pulp stem cells; **pECs:** porcine epithelial cells; **hDPCs:** human dental pulp cells; **hUVECs:** human umbilical vein endothelial cells; **dTBs:** decellularized tooth buds; **hBMMSCs:** human bone marrow mesenchymal stem cells; **CBCT:** cone beam computed tomography; **SEM:** scanning electron microscopy.