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Original Article

Subcutaneous implantation of tooth germ stem cells over the masseter muscle in mice: An in vivo pilot study

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ABSTRACT

Objectives: This study aimed to explore the potential of tooth germ stem cells for regenerating tooth-like structures by subcutaneously implanting first molar tooth germ stem cells over the masseter muscle in mice.
Methods: Five pairs of house mice, *Mus musculus*, were selected for mating. At gestational day 14 (E14), the fetuses were extracted, and the first molar tooth germ at the cap stage was isolated. Tooth germ stem cells were prepared into a suspension and seeded onto scaffolds, which were then implanted subcutaneously over the masseter muscle in male mice. The control group (n = 5 male mice) received acellular scaffolds implanted at the same site. After 20 days, the regenerated tissues were resected and analyzed histologically using hematoxylin and eosin (H & E) staining, Masson's trichrome staining, and immunohistochemical (IHC) staining for cytokeratin (CK) and vimentin markers.
Results: H & E staining showed the formation of integrated oval structures at the implant site in all samples. Masson's trichrome staining identified dispersed accumulations of cellular mineralized matrix within the connective tissue. IHC staining was positive for vimentin, confirming the mesenchymal origin of the loose tissue at the center, indicating future dental pulp development. Positive CK staining indicated the ectodermal origin of dense peripheral tissues, suggesting the future formation of inner enamel epithelium. The combined immunohistochemical results for vimentin and CK confirmed the ecto-mesenchymal origin of the regenerated tissue, which resembled a late bell-stage tooth germ observed around gestational days 17.5–18 and showed early indications of dentin formation (D0).
Conclusion: The study indicates that tooth germ stem cells may have the potential to produce dense, tooth-like structures when implanted subcutaneously in mice. These findings provide preliminary insights into the possible applications of tooth germ stem cells in regenerative dental tissue engineering.

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1. Introduction

Regenerative medicine leverages host cells to repair or regenerate damaged tissues, offering a promising alternative to traditional organ transplantation [1]. The primary challenge of organ transplantation is the limited availability of donor organs, which significantly restricts the number of procedures that can be performed [2,3]. This has spurred growing interest in the regeneration of human organs ex vivo, an approach that has captured the attention of researchers in recent years.

Teeth, like other ectodermal organs, develop from complex interactions between ectodermal and mesenchymal tissues during fetal development [4]. Due to their accessibility in the oral cavity, teeth provide a unique opportunity for regeneration studies, as they can be re-implanted in the oral environment with minimal surgical complications [5,6]. While dental implants are currently the standard treatment for replacing missing teeth, they have limitations that can fail, such as peri-implantitis, bone loss, and mechanical complications [7]. Consequently, there is a strong preference among oral and maxillofacial surgeons for the use of a biological tooth instead of an artificial implant, should such an option become available [8].

Recent advances in biological tooth regeneration have demonstrated the potential for developing functional teeth using tissue engineering approaches [9]. Preliminary studies in animal models, including mice, rats, pigs, and dogs, have shown the feasibility of regenerating teeth using cells derived from engineered tooth germs [10,11]. Among these models, mice are particularly valuable for studying tooth development due to their genetic similarity to humans; only a single gene expression difference has been noted between murine and human tooth development processes, making them an excellent model for translational research [1,12].

The present study aimed to isolate tooth germ stem cells from early embryonic development using a straightforward technique, expand these cells ex vivo, and implant them in a mouse model. This approach seeks to pave the way for developing biological alternatives to conventional dental implants, potentially revolutionizing dental and regenerative medicine by providing a natural solution for tooth replacement.

2. Materials and methods

2.1. Animal husbandry

Fifteen house mice of the species *Mus musculus*, aged 6–7 weeks and weighing approximately 30 g, were obtained from the animal facility of the School of Pharmacy, Isfahan University of Medical Sciences. Ten mice, comprising five males and five females, were paired in separate cages and acclimated for one week with ad libitum access to rodent chow and water. Environmental conditions were maintained at 22 ± 2 °C, 55–60 % humidity, and a 12-h light/12-h dark cycle. After the quarantine period, breeding was carried out using a monogamous system, with each pair housed in individual cages. Day zero of pregnancy was confirmed by observing the formation of a vaginal plug. At embryonic day 14 (E14), when the first molar tooth germ was at the cap stage [10], fetuses were harvested (Fig. 1).

2.2. Tissue dissection

First molar tooth germs were isolated following the protocol described by Alfaqeeh et al. [13]. Under a magnifying loupe (BIOLOGICAL loupe XSZ-107 SERIES, VERYLION, Zhejiang, China), a transverse incision was made at the stomodeum, or primary mouth, of each fetus to resect the head and maxillary structures. A second incision was made at the neck to detach the mandible. Under a microscope, the mandibular first molar tooth germ was identified and extracted along with adjacent tissues. During dissection, the germ was maintained in Dulbecco's modified Eagle's medium (DMEM) on ice.

2.3. Scaffold fabrication

Nanofiber polyhydroxyalkanoate (PHA) scaffolds (1 × 1 mm) were used as cell carriers. The scaffolds were fabricated using a mixture of polyhydroxybutyrate, polyhydroxybutyrate co-valerate, and type I collagen (45:45:10 ratio) by electrospinning [14]. Scaffolds were sterilized by gamma radiation (25 kGy for 2 h), soaked in 70 % alcohol for 5 min, rinsed three times with phosphate-buffered saline (PBS), and incubated in DMEM at 37 °C prior to cell seeding. The acellular scaffolds used in the control group were subjected to identical preparation procedures, ensuring consistency in sterilization and handling across all samples.

2.4. Primary culture of tooth germ stem cells

To achieve a density of 5 × 10^5 cells/mL, tooth germs from each female mouse were pooled and immersed in an enzymatic solution containing 3 mg/mL collagenase (PluriSTEM™-Collagenase-Solution, MM_NF-SCM133) and 1 mg/mL dispase (PluriSTEM™-Dispase–II–Solution, MM_NF-SCM133) with 1 mL PBS. The mixture was incubated at 37 °C for 50 min. The cell suspension was filtered through a 70-μm filter to obtain single cells. PBS + DMEM with 10 % knockout serum, 1 % glutamine, and 1 % Non-Essential Amino Acids was added at twice the volume of the enzymatic mixture, and the cells were centrifuged at 1800 rpm for 5 min. The supernatant was discarded, and DMEM supplemented with 10 % knockout serum, 1 % glutamine, 1 % Non-Essential Amino Acids, and 1 % antibiotic solution containing 100 U/mL penicillin and 50 μg/mL streptomycin was added. The suspension was transferred into 15-mL centrifuge tubes and seeded onto a sterile scaffold in a 12-well plate at a density of 500,000 cells. Cells adhered within 16–18 h and primary colonies were formed by day 5. For implantation, one scaffold containing tooth germs derived from 10 to 12 fetuses of each female mouse was used per male mouse.

2.5. Subcutaneous implantation

General anesthesia was induced in five male mice through an intraperitoneal injection of acepromazine at a dose of 5 mg/kg and ketamine at a dose of 100 mg/kg. The implantation site was disinfected with betadine, and a 5-mm horizontal incision was made in the skin covering the right masseter muscle. The cell-seeded scaffold was implanted subcutaneously at the incision site, and the wound was sutured with 06 nylon sutures. Control mice (n = 5) received acellular unseeded scaffolds implanted at the same location. Following surgery, ampicillin was administered subcutaneously at a dose of 25 mg/kg. Mice were provided soft food and non-fat milk for 20 days. On day 20, the male mice were euthanized, and the experimental implants were resected for histological and immunohistochemical (IHC) assessment.

2.6. Post-implantation assessments

2.6.1. Histological analysis

Resected tissues were fixed in 10 % paraformaldehyde, sectioned at 6 μm thickness, and stained with hematoxylin and eosin (H & E) and Masson's trichrome.

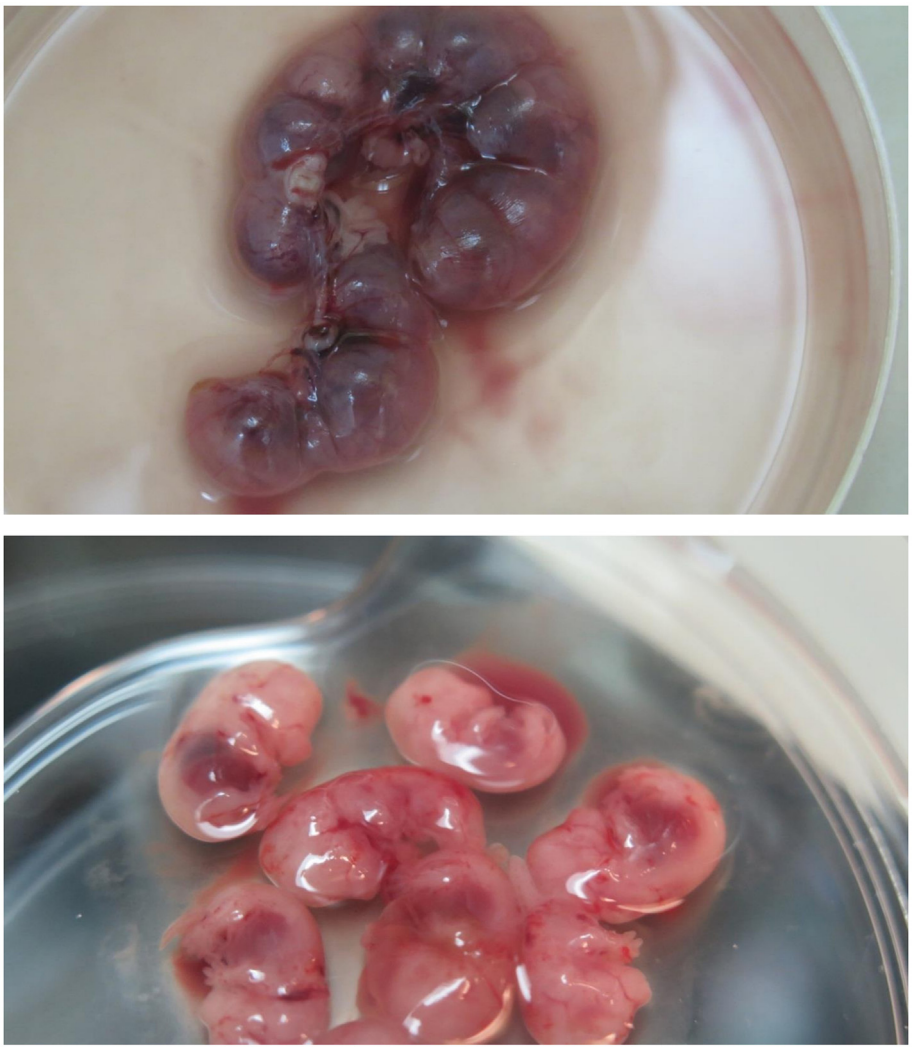

Fig. 1. Mouse fetuses at embryonic day 14 (E14).

2.6.2. Immunohistochemical (IHC) staining

Monoclonal mouse anti-human cytokeratin (CK) and monoclonal mouse anti-human vimentin antibodies (DAKO) were used for IHC staining according to the manufacturer's instructions. CK and vimentin markers were employed to confirm the ectodermal origin of epithelial cells and the mesenchymal origin of connective tissue cells, respectively.

2.7. Ethical considerations

All procedures were performed following the guidelines for the care and use of laboratory animals and the ethical standards set by Isfahan University of Medical Sciences.

3. Results

3.1. Gross observation

After 20 days, the gross examination of the mice in the experimental group revealed an increase in the size and volume of the implanted tissue at the implant site, indicating successful tissue growth (Fig. 2).

3.2. H & E staining results

Histological assessment of H & E-stained sections from the experimental group showed the formation of integrated, oval-shaped, non-mineralized structures at the implant site in all samples. These structures exhibited a uniform pattern of cell orientation, suggesting organized tissue development (Fig. 3A and B). In contrast, the control group did not show any evidence of dental tissue or newly formed structures (Fig. 3C).

Microscopic examination of the structures in the experimental group revealed a high infiltration of basophils at the periphery, with a central area containing fewer cells, primarily eosinophils. The formed structures comprised two distinct tissue types: a dense area with a high basophil concentration and a loose matrix with scattered cells. Higher magnification revealed various cell types, including neutrophils and giant cells. Further analysis showed organized internal epithelial cells with columnar morphology, external epithelial cells, a stellate reticulum-like tissue, odontoblast-like cells, a thin membrane, and a cell-free zone within the connective tissue (Fig. 4A). Blood vessels were also observed within the connective tissue, indicating active vascularization (Fig. 4B).

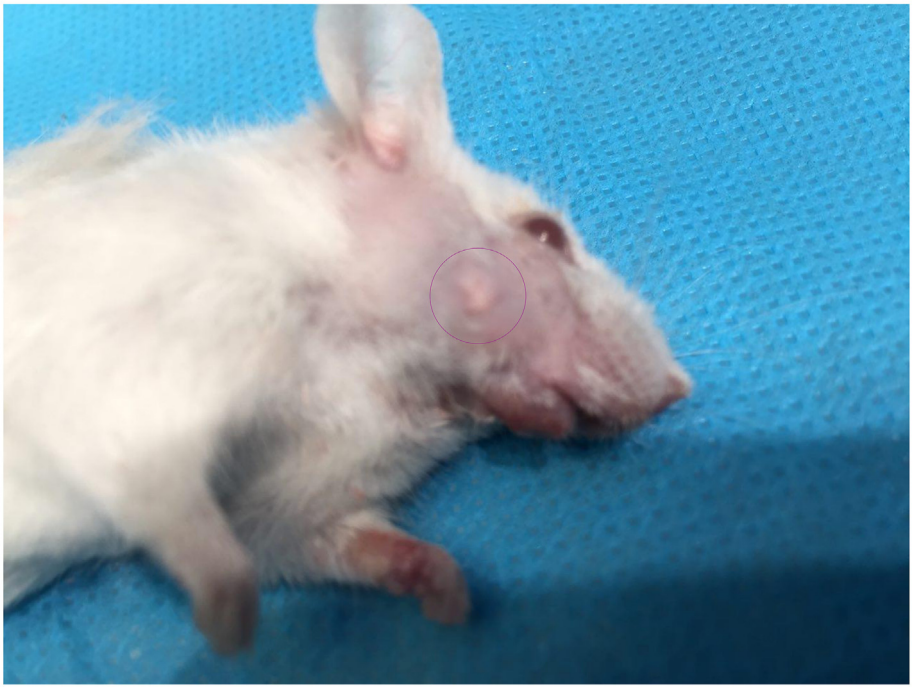

Fig. 2. Macroscopic view of the implanted tissue 20 days post-implantation.

3.3. Masson's trichrome staining results

Masson's trichrome staining was utilized to enhance the visualization of connective tissue, collagen fibers, and cell-free zones. The staining identified components such as dentin matrix, loose connective tissue, blood vessels, and inner enamel epithelium (IEE), demonstrating the complexity and differentiation of the implanted structures (Fig. 5A–F).

3.4. IHC staining results

IHC staining with epithelial (CK) and mesenchymal (vimentin) cell markers was performed to characterize the origins of the cell types observed in H & E staining. Vimentin staining was positive in the experimental tissues, indicating their mesenchymal origin, with dermal connective tissue serving as a positive internal control and epithelial tissue of the hair as a negative control (Fig. 6A). CK staining was positive in the superficial epithelium and central loose tissue, confirming their ectodermal origin, with the epithelial lining of the skin and hair sheath as a positive control and dermal connective tissue as a negative control (Fig. 6B). The positive staining for both vimentin and CK indicates the ecto-mesenchymal origin of the tissue, supporting the potential of tooth germ stem cells to regenerate ecto-mesenchymal structures resembling tooth germ components.

4. Discussion

Several researchers have proposed techniques for developing biological teeth using fetal tooth germs implanted in the kidney capsule, a favorable site for tissue growth [15,16]. In the present study, the authors utilized the slice culture method, which allows excellent access to tooth germs [13,17]. The earliest study involving fetal tooth germ implantation was conducted by Glasstone et al., in 1952, who split tooth primordia in half at early fetal stages, confirming that each half could independently develop into a normal-sized tooth [18].

In 2002, Young et al. [19] created tooth-shaped scaffolds and seeded them with cells isolated from early-stage third molar tooth germs of pigs and rats. These seeded scaffolds were then implanted

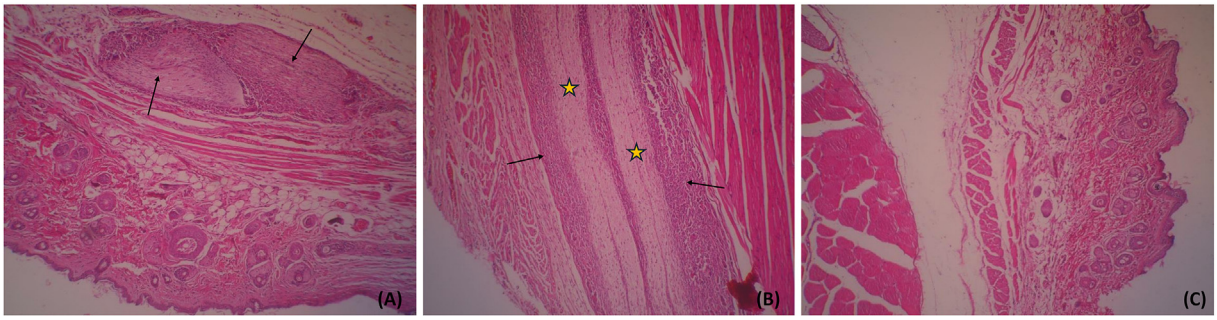

Fig. 3. (A) H & E staining of the implanted tissue in the experimental group at ×100 magnification, showing the formed structure (arrow). (B) Formation of dental structures displaying a highly cellular dense tissue component (arrowhead) and a loose tissue component (star) at ×100 magnification. (C) No tissue formation in the control group at ×100 magnification.

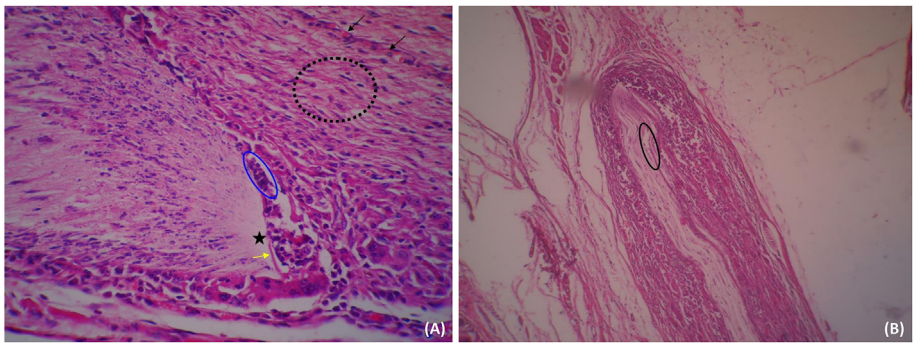

Fig. 4. (A) The Blue circle highlights a row of internal epithelial cells along the basement membrane; the black circle marks stellar cells; black arrows point to a row of external epithelial cells; the black star denotes a cell-free zone within the connective tissue, and the yellow arrow indicates the basement membrane. (B) Blood vessels observed within the connective tissue.

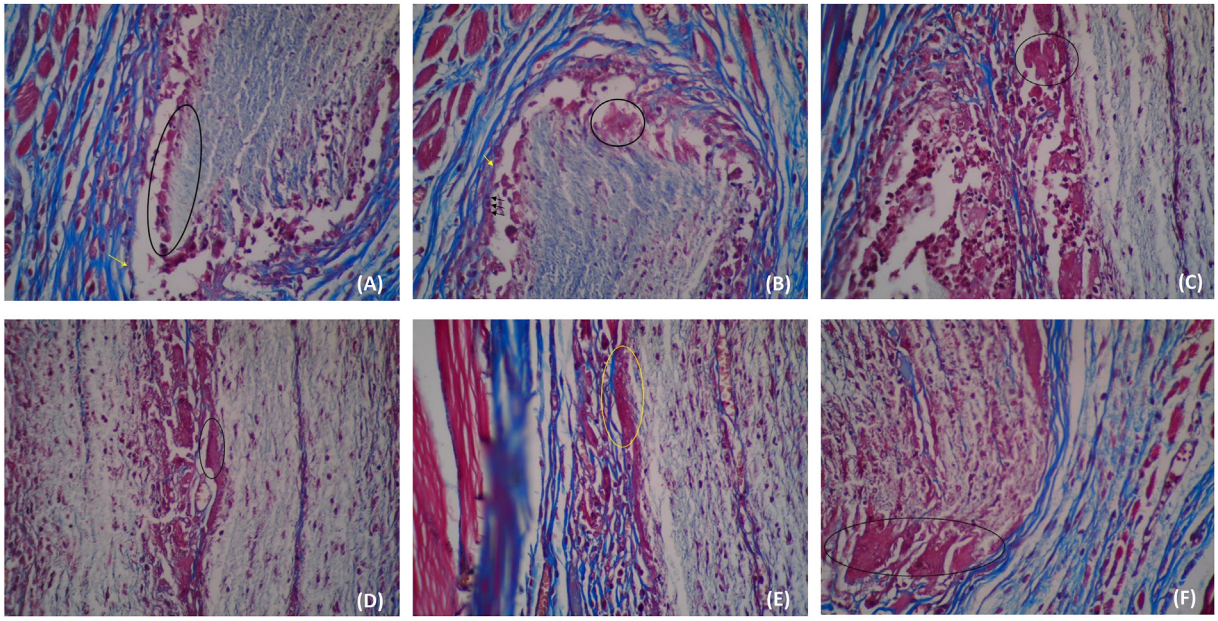

Fig. 5. (A) Masson's trichrome staining at ×400 magnification showing a closed-ended structure with loose connective tissue (dental papilla) on the right side, externally lined by a row of regularly arranged cells (black oval). (B) Formation of a homogenous acellular, pink-colored matrix (circle) at ×100 magnification. (C) Pink matrix formation adjacent to the pulp, with odontoblast cells encircled by a black circle. (D) Pink matrix formation adjacent to loose connective tissue. (E) Masson's trichrome staining showing loose connective tissue containing blood vessels next to the dentin matrix secreted by odontoblast cells (yellow circle) at ×400 magnification. (F) Formation of a homogenous pink matrix containing several nuclei, likely dentin secreted by odontoblasts.

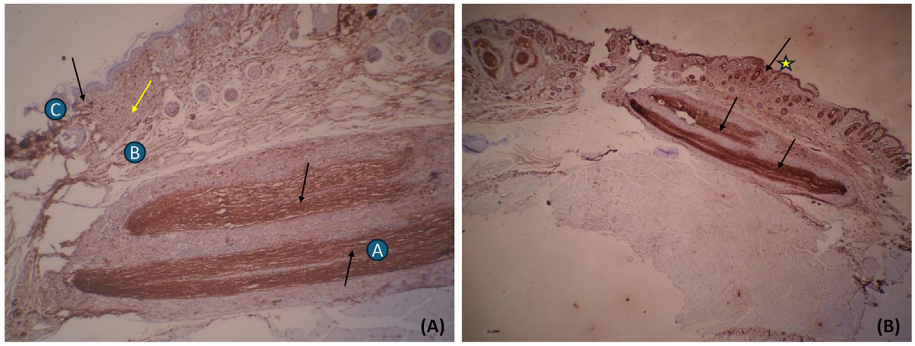

Fig. 6. (A) IHC staining of the tissue in the experimental group showing positive vimentin staining in the dermal connective tissue and central loose tissue. (B) IHC staining of the experimental group showing positive CK staining in the superficial epithelium and central loose tissue.

into the omentum of immunocompromised rats. Histological analysis showed the formation of a fine crown with a thickness of 1–2 mm after 20–30 weeks in a porcine model [19]. Yamamoto et al. used pellets of intact epithelium combined with mesenchymal cells from late bud-stage tooth germs [20], while Ohazama et al. demonstrated that adult bone marrow-derived stem cells could form regularly shaped teeth [21].

Hu et al. combined epithelial and mesenchymal pellets at gestational day 14 in culture plates, showing they could develop into teeth resembling naturally developing teeth at the early stages of crown formation. Notably, the cuspal pattern formed in vitro was similar to that of naturally developing teeth, although no root formation was observed [22]. Iwatsuki et al. reported similar results, isolating cells from gestational day 14 cap stage tooth germs, which were then seeded on 3D scaffolds and successfully regenerated regular tooth structures in the kidney capsule [15]. Differences between their study and the present one included the scaffold type, timing of tooth germ isolation, cell density on each scaffold, and implantation site. Iwatsuki et al. resected implants at various time points (5, 7, 10, 14, and 21 days), observing accelerated tooth development compared to natural teeth. They reported cap stage development by day 7 and late bell stage morphology by day 10, with adequate enamel and dentin formed by day 14. Two distinct crown-cusp morphologies—smooth and prominent—were identified histologically, though root formation was absent even after 21 days. The faster development in their study was likely due to the implantation site and high initial cell density, with 500,000 cells seeded per scaffold in their preliminary experiments [23].

Overall, multiple methods exist for regenerating complete biological teeth, often categorized by the cell source: prenatal or postnatal tooth germs [8,24]. Results vary significantly depending on the source; prenatal tooth germs generally exhibit a higher potential for forming the crown shape compared to postnatal germs. Studies utilizing fetal cells typically show a pattern of central pulp and dentin surrounded by enamel, consistent with findings from the present study. Conversely, studies using postnatal tooth germs often lack such structured organization [24]. Honda et al. (2006) resected third molar tooth germs bilaterally in dogs after birth and placed them in first molar extraction sockets. They successfully regenerated dentin but not enamel, suggesting that dental epithelium may lose its odontogenic induction potential after birth [25].

The IHC staining results in this study confirmed the ectodermal origin of peripheral cells and the ectomesenchymal origin of central tissues, aligning with the normal histological development of teeth. The epithelial lining of the oral cavity proliferates at future tooth sites and signals the underlying ectomesenchyme, inducing odontogenic gene expression. This leads to the differentiation of ectodermal cells into ameloblasts and ectomesenchymal cells into odontoblasts and dental pulp. Blood vessel formation is crucial for the development of mineralized tissues, and vascular structures were observed in the engineered tooth germs in this study, highlighting the potential of this approach for future applications in dental tissue engineering [26]. IHC staining was performed using CK and vimentin markers to characterize the origins of the regenerated tissue and to assess its potential for dental tissue development. CK, an epithelial marker [26], was positive in the superficial epithelium and central loose tissue, confirming the ectodermal origin of these components. This ectodermal contribution is critical in early tooth development, as epithelial cells differentiate into the inner enamel epithelium, which later contributes to enamel matrix production [27]. Vimentin, a mesenchymal marker [20], was positive in the loose connective tissue, indicating its mesenchymal origin. Mesenchymal cells play a pivotal role in the development of the dental papilla and subsequently differentiate into odontoblasts, which are responsible for dentin formation. The dual positivity for CK and vimentin highlights the ecto-mesenchymal nature of the regenerated tissue, reflecting the complex interplay between epithelial and mesenchymal cells during odontogenesis. This interaction is essential for the formation of tooth structures, as epithelial cells induce mesenchymal differentiation, and mesenchymal signals regulate epithelial cell behavior [28]. While the presence of CK and vimentin confirms the origins of the regenerated tissue, the absence of other key markers, such as amelogenin for enamel matrix formation, suggests that the developmental stage of the tissue had not yet progressed to the mineralization phase. Amelogenin is a key protein expressed by ameloblasts during enamel formation [29], and its absence aligns with the timing of sample collection, which occurred before hard tissue mineralization could be observed. These findings demonstrate that the tissue is in an early stage of development, characterized by cellular organization indicative of tooth germ regeneration.

To the best of the authors' knowledge, the only available study on the exact timing of tooth development was conducted by Gaete et al., in 2004 on ICR/JCI mice [30]. They found that the first evidence of tooth formation is the proliferation of the dental plate, occurring on gestational day 12.5. The bud stage follows on day 13.5, and the cap stage occurs on day 14.5, characterized by the initial development of the cervical loop. By day 15, the fetal mesenchyme differentiates into dental papilla and dental follicle cells. The bell stage is reached around day 17 when the tooth germ contains the enamel organ, dental papilla, and dental follicle; the dental papilla further differentiates into odontogenic cells, including odontoblasts and dental pulp stem cells. On day 18.5, predentin forms, and dentin secretion begins one or two days after birth (D0). Enamel secretion starts approximately three days after birth, coinciding with the late bell stage. Notably, dental development in this species is faster compared to other known species [30]. In this study, the engineered tooth germs were at gestational days 17.5–18, consistent with the late bell stage, suggesting that extending the implantation time could result in the mineralization of dentin and enamel.

During the early bell stage, several key events occur, including the formation of cubic external enamel epithelium and columnar internal enamel cells with high glycogen content, the separation of the dental papilla from the enamel organ by a basement membrane, and the entry of vascular bundles into the dental papilla, which peak later in this stage [31]. These parameters were observed in the histological assessment of the present study. The transition from cap to bell stage is marked by the development of enamel organ tissues. At birth (day 0), the enamel organ and stellate reticulum disappear, and by three days post-birth, dentin and enamel develop, with ameloblasts secreting enamel and odontoblasts secreting dentin [23]. In the present study, however, the stellate reticulum did not disappear, likely due to differences in strain development; the NMRI mice used in this study developed approximately 12 h sooner than other strains, such as C3H [32]. The authors concluded that a longer implant removal time would be more favorable, as other studies have shown more differentiated structures at 20 days post-implantation. The exact reasons for the differences in timing remain unknown. Still, they may be related to variations in methodology, such as seeded cell density or the degree of direct contact between epithelial and mesenchymal cells in the tooth germ. In this study, seeded scaffolds were implanted subcutaneously over the masseter muscle, a site chosen for its excellent surgical access, rich blood supply, ample space for cell growth, and lower risk of infection compared to intraoral sites. Subcutaneous implantation of dental pulp stem cells and stem cells from extracted deciduous teeth has previously shown promising results in forming dentin-pulp complexes [12].

For several reasons, the masseter muscle was chosen as the implantation site. The pilot study involving the implantation of scaffolds and cells in the sockets of central teeth resulted in cell death, likely due to the narrow dental space and insufficient blood supply. The kidney capsule, a common site for tissue regeneration, was not considered due to limited organ availability and the need for complex surgical techniques [1]. Instead, the masseter muscle provided an accessible, well-vascularized environment with sufficient space for scaffold placement and minimal mechanical pressure on the cells [12]. This approach also eliminated the need for major abdominal or chest surgery, reducing animal stress. Moreover, this innovative choice allowed us to explore a novel surgical site for tooth bud cell transplantation, as no similar reports were available at the time of the study [20]. The present study utilized polyhydroxyalkanoate scaffolds, selected for their well-documented biocompatibility, biodegradability, and favorable mechanical properties, which make them highly suitable for applications in tissue repair and regeneration [33]. To further enhance their surface properties and bio-functionality, the scaffolds were treated with type I collagen and peptide.

Recent advancements in tissue engineering introduced polyalkane-based scaffolds, which have demonstrated successful outcomes in regenerating bone [34], skin, nerve, and cartilage tissues [35–38]. The present study utilized a polyhydroxyalkanoate scaffold treated with type I collagen and peptide to enhance its surface properties and bio-functionality.

Despite progress, several challenges remain in reproducing biological teeth. Key obstacles include identifying ideal scaffolds, ensuring adequate vascularization, determining the precise shape and size of regenerated teeth, and ensuring the availability and functionality of dental epithelium. Controlling directional growth and minimizing implant rejection in the jaws are also unresolved issues [9]. Evidence suggests that using a 3D culture of bioengineered organ germs with cell compartmentalization and collagen gel drops can produce teeth with correct structures, mimicking natural tooth development [25]. However, tooth germ bioengineering using a patient's cells is not feasible for older individuals, as they lack growing tooth germs [11].

Pluripotent cells, including fetal stem cells and induced pluripotent stem cells (iPSCs), are promising candidates because they can differentiate into ectoderm, mesoderm, and endoderm. iPSCs can be derived from various tissues, such as dental pulp, periodontal ligament, gingiva, and oral mucosa [39]. Recently, Cai et al. used iPSCs from human urine, combined with murine tooth mesenchymal stem cells, to successfully regenerate teeth in mice after three weeks of implantation in the subrenal capsule [40].

In 2006, a team of 25 experts from various fields developed a comprehensive blueprint for human tooth regeneration, focusing on steps to reproduce teeth along with supporting tissues. The proposed steps for achieving the cap stage include: (I) isolating mature stem cells (from bone marrow or teeth) or using human fetal stem cells approved by the National Institute of Health; (II) culturing cells in a lab with banking for future organ reconstruction; (III) seeding cells onto a smart peptide scaffold with an amphiphilic base; (IV) inducing cells with molecular signals or porcine tissue induction; (V) confirming gene expression profiles; and (VI) repeating these steps until cap stage-related gene expression is achieved [41]. This ongoing work highlights the complexity of dental tissue engineering and underscores the need for further research to refine these approaches for clinical application.

This study has several limitations that should be considered when interpreting the findings. As a pilot study, the small sample size precluded statistical analyses, and results were presented descriptively to provide preliminary data for future research. Considering that in this study, the samples were removed on the twentieth day, it appears that this time was insufficient for the developmental formation of hard tissues like enamel and dentin. As a result, it was not possible to analyze the surface of the hard tissue using software like ImageJ. Future studies with longer observation periods, larger sample sizes, and the incorporation of additional markers such as amelogenin for enamel formation and dentin sialophosphoprotein (DSPP) for dentinogenesis will enable more comprehensive analyses and help elucidate the potential of the regenerated tissue to form mineralized structures.

In conclusion, the present study demonstrated that tooth germ stem cells are capable of forming integrated tooth-like structures, with epithelial and mesenchymal cells reorganizing in a manner similar to that seen in natural teeth. However, the enamel secretion stage was not observed. These findings highlight the potential of engineered tooth germs in replicating key aspects of natural tooth development, though further optimization is needed to achieve complete tooth formation, including enamel secretion.

Ethical approval and consent to participate

The study received approval from the Ethics Committee of Isfahan University of Medical Sciences.

Consent for publication

Not Applicable.

Data availability statement

The data presented in this study are available on request from the corresponding author.

Author contributions

Conceptualization: N.N and G.B; Methodology: A.T, N.N, M.H.N.E, and G.B; Software: S.T and S.A.M; Validation: A.T, and N.N; Formal analysis: G.B, N.N, and N.N; Investigation: G.B, B.M, M.M, F.E, and E.M; Resources: A.T, N.N, G.B, M.H.N.E, and B.M; Data Curation: N.N, S.T, and M.M; Writing - Original Draft: G.B, S.T, F.E, and E.M; Writing - Review & Editing: N.N, A.T, M.H.N.E, and S.A.M; Visualization: S.T, F.E, and E.M; Supervision: N.N; Project administration: A.T. All authors have read and approved the published version of the manuscript.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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