The combined application of rat bone marrow mesenchymal stem cells and bioceramic materials in the regeneration of dental pulp-like tissues

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Research article

Keywords: Pulp regeneration, iRoot BP, Bone marrow mesenchymal stem cells, Revascularization

Posted Date: January 23rd, 2020

DOI: https://doi.org/10.21203/rs.2.21651/v1

Abstract

Background: To observe the effects of combined application of rat bone marrow mesenchymal stem cells (rBMSCs) and a bioceramic material on pulp-like tissue formation. Methods: Rat incisor root fragments without pulp tissues were prepared and filled with a collagen scaffold seeded with rBMSCs, while one side of the root segment was covered by a bioceramic material (iRoot BP). After culture for 12 h, the root fragments were implanted subcutaneously for 3 months. Hematoxylin and eosin (HE) staining was applied to observe biocompatibility and the formation of pulp-like tissues. Incisor root fragments were divided into three parts (BP1/3 , M1/3, and D1/3) to analyze the area and number of new vessels. Immunohistochemical staining of neuroendocrine marker PGP9.5, dentin sialophosphoprotein (DSPP), and vascular endothelial growth factor (VEGF) was applied to observe the formation of pulp-like tissues. Root fragments filled with the collagen scaffold only were used as a control. Results: Three months after implantation, root fragments were collected, which were surrounded by a transparent tissue membrane with a good blood supply. The root fragment cavity was filled with pink vascularized pulp-like tissue. According to HE results, iRoot BP had good biocompatibility with new pulp-like tissues and few infiltrating inflammatory cells. Increases in the number and area of new blood vessels were observed in BP1/3 compared with the other two parts. Expression of PGP9.5 and DSPP showed that the newly formed tissues were similar to normal pulp tissues. Conclusion: iRoot BP has good biocompatibility and increases the number and area of new blood vessels. The combined application of stem cells and bioceramic materials may be a better method for pulp revascularization .

Background

In recent years, pulp regeneration has become a research focus and new trend in the field of pulp disease treatment with the progress of cell biology and bioengineering. The main purpose of pulp regeneration is to regenerate the necrotic or infected pulp, and to restore its normal activity and physiological functions including immune functions, nutritional functions of its blood vessels, and sensory functions of nerves. Evidence has shown that the bioceramic material iRoot BP enhances adherence, migration, attachment, proliferation, and mineralization of dental pulp stem cells [1] and induces mineralization of human dental pulp cells and expression of genes related to odontoblast differentiation in vitro [2]. However, the effects of combined application of rat bone marrow mesenchymal stem cells (rBMSCs) and a bioceramic material on pulp-like tissue formation in vivo are not fully understood

In this study, rat incisor root fragments filled with a collagen scaffold seeded with rBMSCs with one side covered by iRoot BP were implanted subcutaneously to observe the combined effect of rBMSCs and a bioceramic material on pulp-like tissue formation.

Methods

Disinfection of root segments

Mandibular incisor teeth of Wistar rats(weight 220–260 g, Laboratory Animal Center, Shandong University) were removed completely, the pulp was removed with a pulp extraction needle first, and 5.25% sodium chloride were used to remove the remaining pulp. The incisors were ground into 3-mm long root segments. Root fragments were sterilized in 75% alcohol at room temperature. Before use, the fragments were rinsed three times with PBS containing penicillin (100 U/ml) and streptomycin (100 μg/ml). After drying, one side of the root segment was covered by iRoot BP in a thin layer for cell seeding under aseptic conditions.

BMSC culture and cell seeding

The protocol for rBMSC culture has been described previously [3]. Each collagen membrane was cut into small pieces of several triangular segments with lengths of 0.5 and 3 mm to allow maximal cell attachment under the aseptic conditions. The collagen membrane was packed into the pulp cavity compactly and then placed into a 96-well plate. Cells were suspended at a density of 1×10^6/ml and seeded on the collagen scaffold. The cells were cultured in a CO_2 constant temperature incubator for 12 h. Scanning electron microscopy (SEM) was used to observe the attachment and morphology of the scaffolds (Fig. 1).

Application of iroot BP at one end of the root segment

One side of the root segment was covered by iRoot BP ((Innovative Bioceramix, Vancouver, BC, Canada) under aseptic conditions and placed in a 37℃ incubator for 2 h to harden the iRoot BP. An rBMSC suspension was placed on the root canal with the collagen membrane and incubated in the CO_2 incubator for 12 h.

Establishment of a rat subcutaneous model

After inducing intraperitoneal anesthesia with 10% chloral hydrate at 4 ml/kg, Wistar rats were routinely disinfected. A longitudinal incision of approximately 3 cm was made on the central skin of the back of the rats by surgical scissors. Blunt separation was applied on both sides with hemostatic forceps to find the location of abundant blood supply, and the root segments were placed in the subcutaneous mucosa (Fig. 1A–1C). Experimental groups were as follows: rBMSC + iRoot BP group (collagen scaffold composite of rBMSCs covered by iRoot BP placed in the root canal), rBMSC group (collagen scaffold composite of rBMSCs placed in the root canal), collagen group (only collagen scaffolds placed in the root canal); collagen group(only collagen membrane placed in the canal); blank group (only the root canal). Three months after the implantation, all rats were euthanized with a lethal dose (150 mg/kg) of sodium thiopental, specimens were obtained for histological and immunohistochemical analyses. Protocols of the study met the approval of the Ethics in the Care and Use of Laboratory Animals Committee of the Jinan Stomatological Hospital.

Tissue preparation and histological assessment

The specimens were fixed in 4% polyformaldehyde/phosphate buffer for 24 hours. The samples were then demineralized in 10% EDTA for 2 months and embedded in paraffin. Sections with a thickness of 5 μm were prepared for HE staining, vascular area analysis, and immunohistochemistry (IHC).

Statistical analysis

All data are expressed as mean ± standard deviation. One-way analysis of variance and the Newman-Keuls test were conducted using GraphPad Prism 5 software.

Results

rBMSC seeding

rBMSCs (passage 3) were seeded onto a collagen scaffold for 12 hours and then observed by SEM. SEM showed that a large number of rBMSCs had attached to the collagen scaffold, and the cells and collagen membrane fiber scaffold had a certain adhesion and relatively uniform distribution (Fig. 1).

General observations of root fragments after implantation

Root fragments were placed in well blood-supplied subcutaneous areas of rats. After 3 months, the specimen was removed from the rat for general observations. The surface was surrounded by a transparent tissue membrane with a good blood supply (Fig. 2A, 2B). In the rBMSC + iRoot BP group, the pulp cavity was filled with pink or red tissues with obvious blood vessels on the side without iRoot BP, and some red blood vessels covered the surface of the side where the iRoot BP was placed (Fig. 2C). In the control group, both sides of the root segment had pink or red tissues with obvious blood vessels filling the medullary cavity (Fig. 2D).

Histological observations of iRoot BP biocompatibility

The samples were observed by optical microscopy after H&E staining, which showed that some collagen was not completely absorbed near the iRoot BP, and cells in the pulp-like tissue were scattered in the iRoot BP space, contacted with BP directly, and grew well. Few infiltrating inflammatory cells were found without any adverse reactions (Fig. 3A–3D). In addition to root segments, cells in the tooth body were perfectly fused with the iRoot BP (Fig. 3E–3F).

Analysis of neovascularization in new pulp-like tissues

In the rBMSC + iRoot BP groupa large number of new blood vessels were seen near the BP site, which was significantly more than in the middle and opening of the root (Fig. 4A–4C). The number and area of new blood vessels were larger in BP1/3 than in M1/3 and D1/3, which further indicated that iRoot BP significantly promoted the formation of neovascularization in pulp-like tissue (Fig. 4D, 4E). In addition, closure by BP affected the absorption rate of the collagen scaffold to some extent. Collagen scaffolds in the middle of the root were not absorbed as thoroughly as those at the opening (Fig. 4).

Expression of neuroendocrine marker PGP9.5 and DSPP in the newly formed tissues

DSPP (the complex of DSP and DPP) is a kind of non-collagen, which plays an important role in dentin mineralization. After DSPP immunohistochemical staining, the immunoreactive cells were mainly distributed in the newly formed pulp-like tissues, especially cells arranged with the newly formed dentin-like tissues. The results revealed that immunoreactivity for DSPP in the rBMSC group was obvious throughout the regenerated soft tissues as a light brown unevenly stained area (Fig. 5A). Overall moderate PGP9.5 staining was also seen in the regenerated pulp-like tissues as a non-uniform area of cable-like tan (shown by the red arrow), which was particularly intense in nerve-like cells (Fig. 5B). This observation suggested the formation of nerve endings in pulp-like tissues. Immunoreactivity for VEGF in the rBMSC + iRoot BP group was obvious throughout the regenerated soft tissues as a light brown unevenly stained area (Fig. 5C). This observation suggested angiogenesis in new pulp tissues of the teeth.

Histological observation of control groups

In the rBMSC group, the root was filled with new pulp-like tissues, and regeneration of blood vessels was clearly seen (Fig. 6A, 6B). In the collagen group, only the collagen membrane was placed on the lumen. After staining, most of the collagen on the lumen was not degraded completely, and fibrous tissue grew on both sides of the root canal, which was continuous with the lateral tissue (Fig. 6C). In the blank group, only the root was implanted subcutaneously in rats. There was fibrous tissue growing in the opening of both sides of the root, and no new tissue formation was found in the middle part (Fig. 6D).

Discussion

There are two strategies for pulp regeneration at present: cell transplantation and homing. The former method is based on cells by transplanting exogenous stem cells onto scaffolds [4]. Root fragment models for pulp regeneration have been discussed in many studies, which show that pulp-like tissues can be produced in vivo by transplantation of dental pulp or other stem cells into tooth slices or fragments [5, 6, 7, 8]. Previous studies have selected human single premolar fragments as carriers for implantation into animals. In our study, we chose the rat incisor root as a carrier to avoid immune rejection of the experimental animals to the greatest extent. Rat bone marrow mesenchymal stem cells (rBMSCs) combined with collagen membrane scaffolds were implanted into rat incisor teeth fragments, and new tissue filling the pulp cavity was observed in the root segment of rat incisor teeth. H&E and immunohistochemical staining showed the appearance of odontoblast-like cells and neovascularization.

iRoot BP as a new nano-bioceramic material is a recently developed bioceramic-based endodontic cement with improved performance compared with MTA [9]. Its main components include calcium trisilicate, calcium disilicate, calcium phosphate, tantalum oxide, and zirconi [10]. Compared with MTA,

iRoot BP plus has the same cytotoxicity, apical closure, and antimicrobial activity [11]. In addition, it overcomes the shortcomings of MTA. Therefore, iRoot BP plus has recently been considered as an alternative to MTA.

Several studies have shown that iRoot BP can be used for direct pulp capping without pulp inflammation [12, 10]. However, there is no report on its application to pulp regeneration. In our study, one side of the root segment was closed by iRoot BP, which showed that iRoot BP had good biocompatibility with new dental pulp tissues, and odontoblast-like cells and neovascularization were seen in the new tissues.

Angiogenesis is a crucial cellular morphogenesis process through which new blood vessels grow from existing blood vessels, penetrate the extracellular matrix, and generate new blood vessels to meet local metabolic needs [13, 14]. Promoting angiogenesis has been emphasized as a key strategy in regenerative medicine, which stimulates the repair of damaged tissues, such as bone, cartilage, muscles, and nerves, by providing effective nutrition and oxygen [15, 16]. Angiogenesis is thought to be an important factor in pulp regeneration, because only blood vessels are generated in the canal space, leading to long-term stability of the newly formed tissues [5]. In the iRoot BP + rBMSC group, iRoot BP slowed absorption of the collagen, but neovascularization near BP1/3 was significantly more than that at other sites, indicating that iRoot bp significantly promotes the regeneration of blood vessels.

Markers such as DMP1, DSPP, ALP, OCN, and RUNX2 are commonly used to evaluate odontogenic differentiation of stem cells from different sources [17, 18]. In our study, we chose DSPP as a marker of odontogenic differentiation. VEGF plays an important role in angiogenesis by promoting endothelial cell proliferation, increasing vascular permeability, and changing the biological effects of the extracellular matrix [19]. In this study, PGP9.5 protein was selected to reflect the nervous system, because it is a specific marker of neurons and nerve fibers, which reflects the degree of damage and repair of neurons and nerve fibers [20]. IHC of DSPP, VEGF, and PGP9.5 showed that iRoot BP promoted the differentiation of mesenchymal stem cells into dentin and generated pulp-like tissue with blood vessels and nerves.

Conclusions

In summary, this study shows that iRoot BP has a significant role in promoting pulp regeneration and the formation of new blood vessels, which has good biocompatibility with pulp-like tissues in the body.

Declarations

Ethics approval and consent to participate

Protocols of the animal study met approval from Ethics in the Care and Use of Jinan Stomatological Hospital and complied with the guidelines for the use of animals in research.

Consent for publication

Not applicable.

Acknowledgements

Not applicable.

Funding

Jinan Health Science Development Fund (2017-1-14) and Dean’s Research Fund of Jinan Stomatological Hospital (2017-02) awarded to Xijiao Yu. The funder in the design of the study, the collection, analysis, and interpretation of data and in writing the manuscript gave financial support.

Competing interests

The authors declare no conflict of interest.

Availability of data and materials

All data generated or analyzed during this study are included in this article.

Authors' contributions

All the authors involved in the work of data collection, manuscript modification. YD and YXJ conceived this work, and all supported the design. WRX,WX,and LYC participation in animal experiments ,data collection and analysis .WRX and WX wrote the manuscript, YD and YXJ proofread the manuscript .All authors approved the final manuscript.

Abbreviations

BMSCs: Bone marrow mesenchymal stem cells

HE: Hematoxylin-eosin staining

DSPP: Dentin sialophosphoprotein

VEGF: Vascular endothelial growth factor

EDTA: Ethylenediaminetetraacetic acid

PBS: Phosphate buffered saline

SEM: Scanning electron microscopy

IHC: Immunohistochemistry

DMP1: Dentin matrix protein-1

ALP: Alkaline phosphatase

OCN: Osteocalcin

Runx2: Runt-related transcription factor 2

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Figures

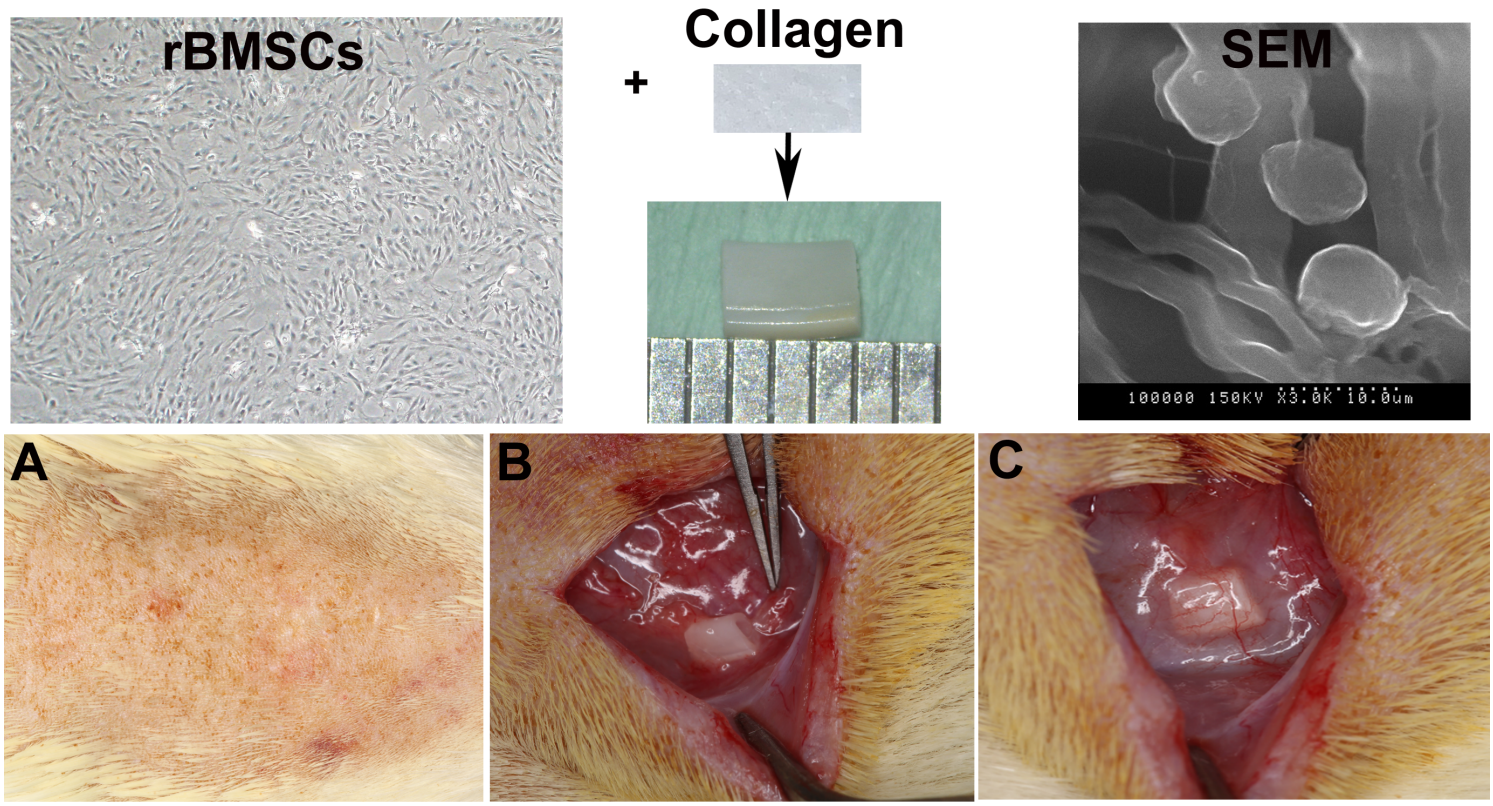

Figure 1

Protocol for rBMSC seeding and subcutaneous implantation. After 12 hours of culture, a large number of rBMSCs had attached to the collagen scaffold. A: Skin preparation for surgery. B: Finding the location of abundant blood supply. C: Placing the root segments in the subcutaneous mucosa.

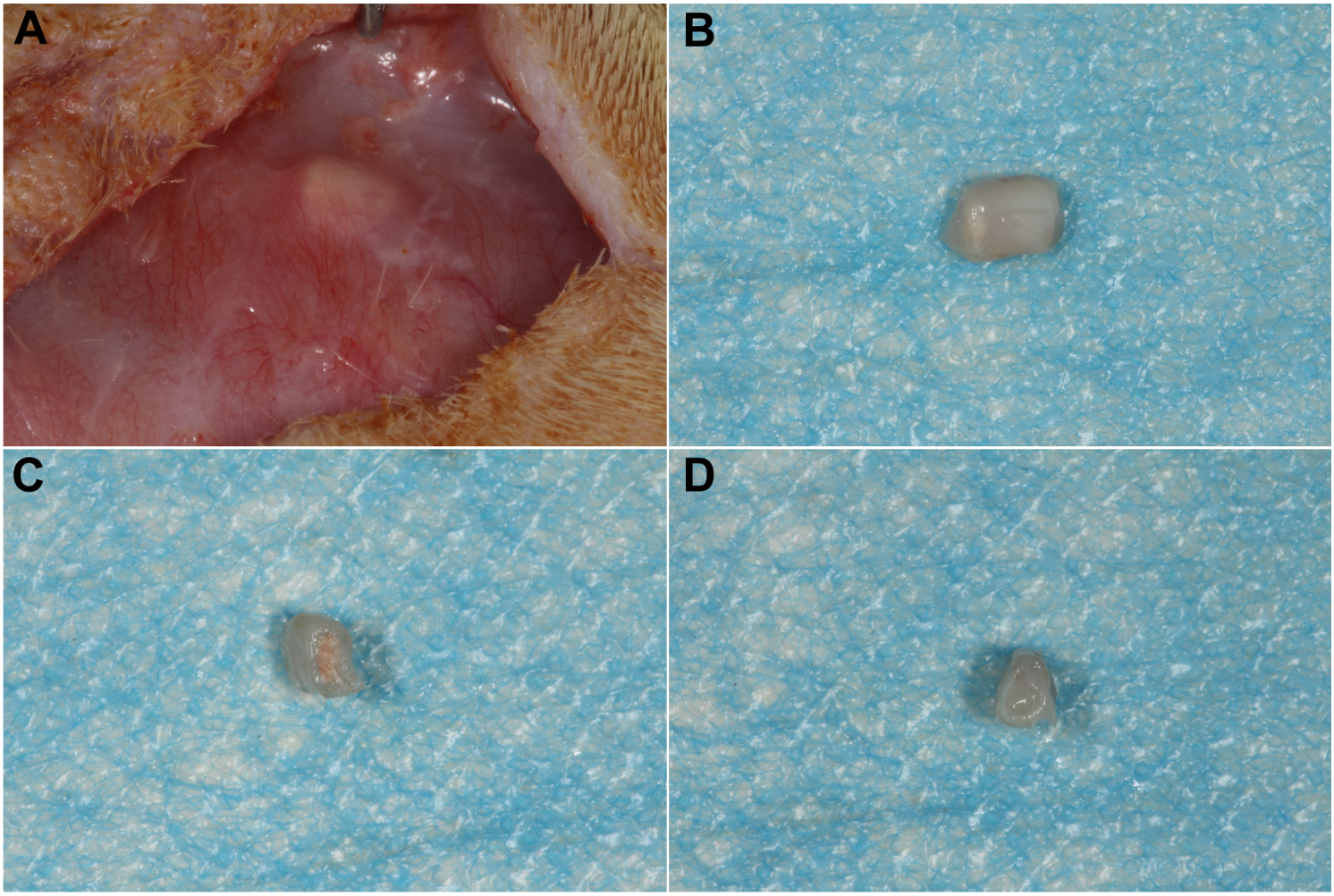

Figure 2

General observations of root fragments after implantation. A: The blood supply radiated around the tooth root. B: When the specimen was removed, the surface of the specimen was surrounded by a transparent tissue membrane. C: In the rBMSC+iRoot BP group, new tissue filled the root segment, and some red blood vessels covered the side of BP. D: In the control group, the pulp cavity was filled with pink or red tissues with obvious blood vessels on both sides of the root segment.

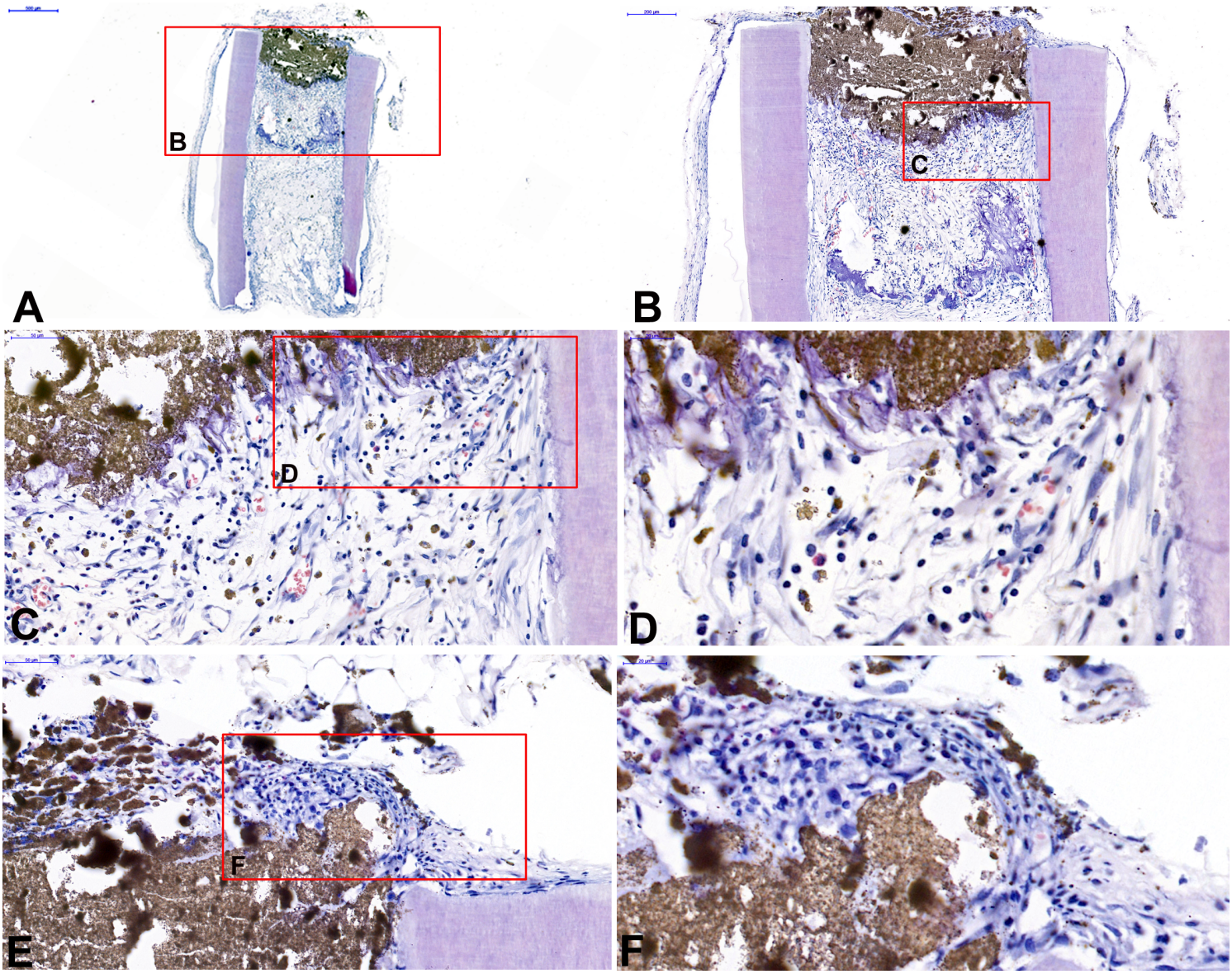

Figure 3

Histological analysis of iRoot BP biocompatibility. A–D: iRoot BP was in direct contact with new dental pulp-like tissue without any adverse reactions. E–F: iRoot BP had a good compatibility with the tissue of the tooth root.

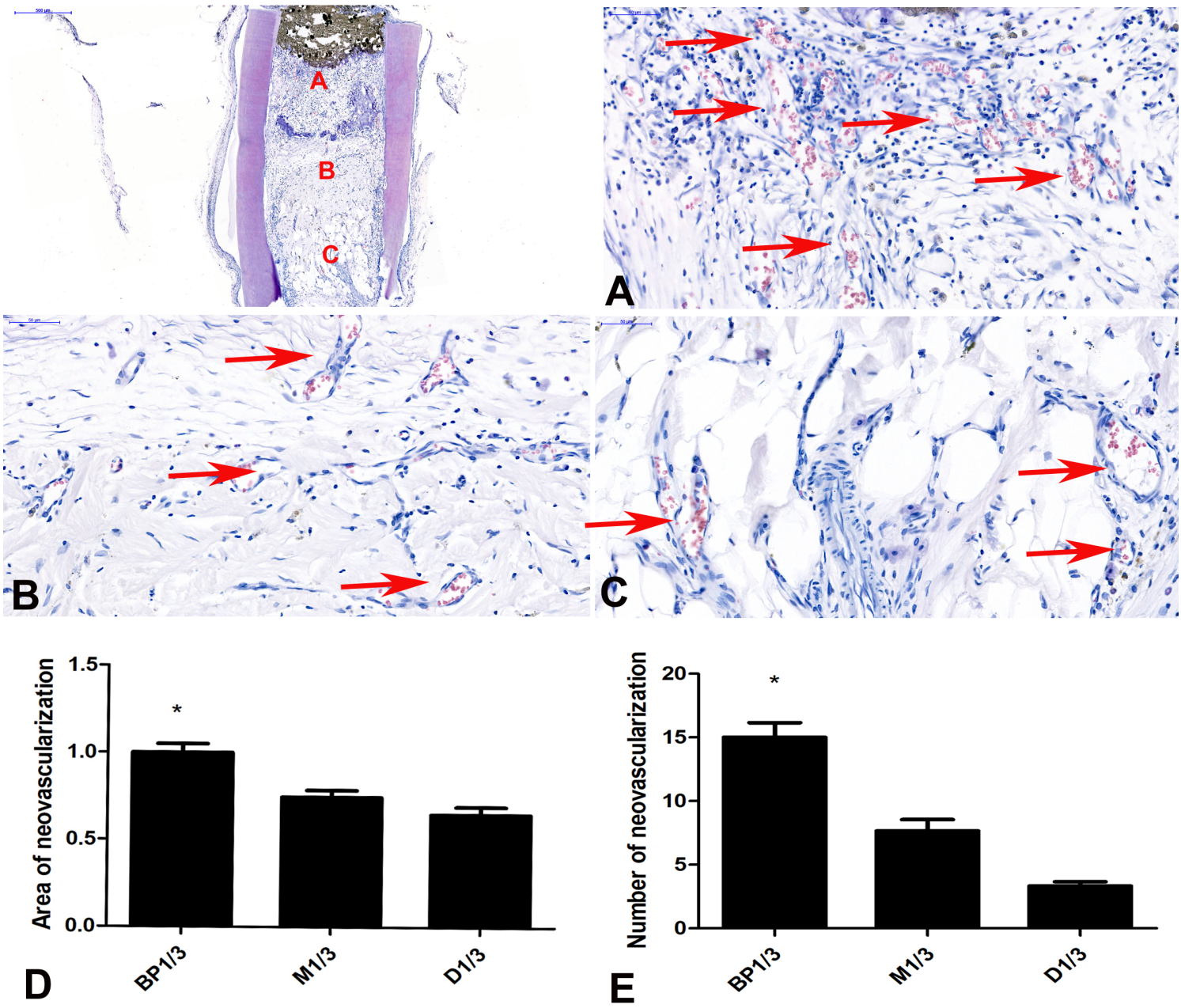

Figure 4

Analysis of neovascularization in new pulp-like tissues. A: The number of new blood vessels in BP1 / 3. B: The number of new blood vessels in M 1 / 3 .C: Number of new blood vessels in the opening. D: Comparison of the area of neovascularization of BP1/3,M1/3 and D1/3 . *P < 0.05.E: Comparison of the number of neovascularization of BP1/3, M1/3 and D1/3 . *P < 0.05.

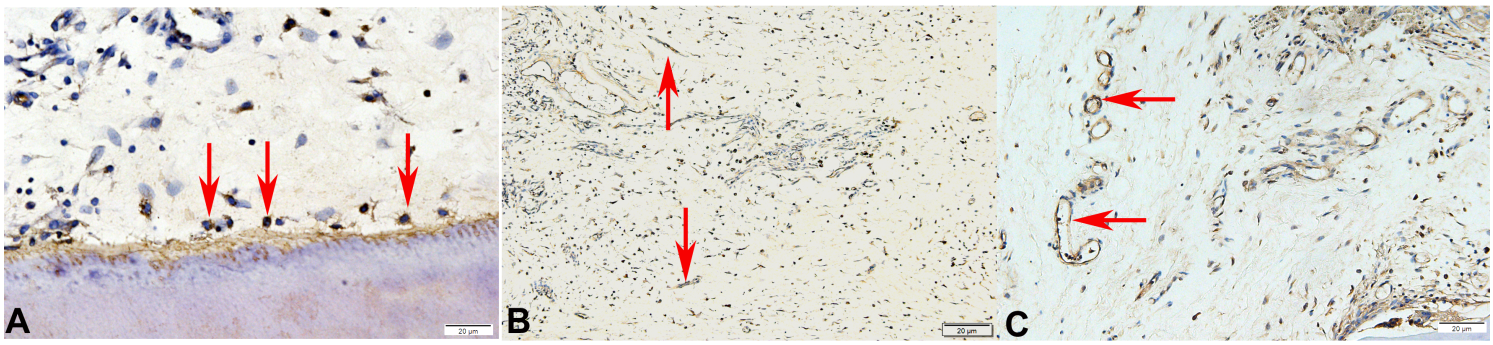

Figure 5

Immunohistochemical staining PGP9.5 and DSPP in the newly formed tissues. A: The arrow points to DSPP positive staining. B: The PGP9.5 immunohistochemical staining (The arrow points to a positive stain). C: The VEGF immunohistochemical staining (The arrow points to a positive stain).

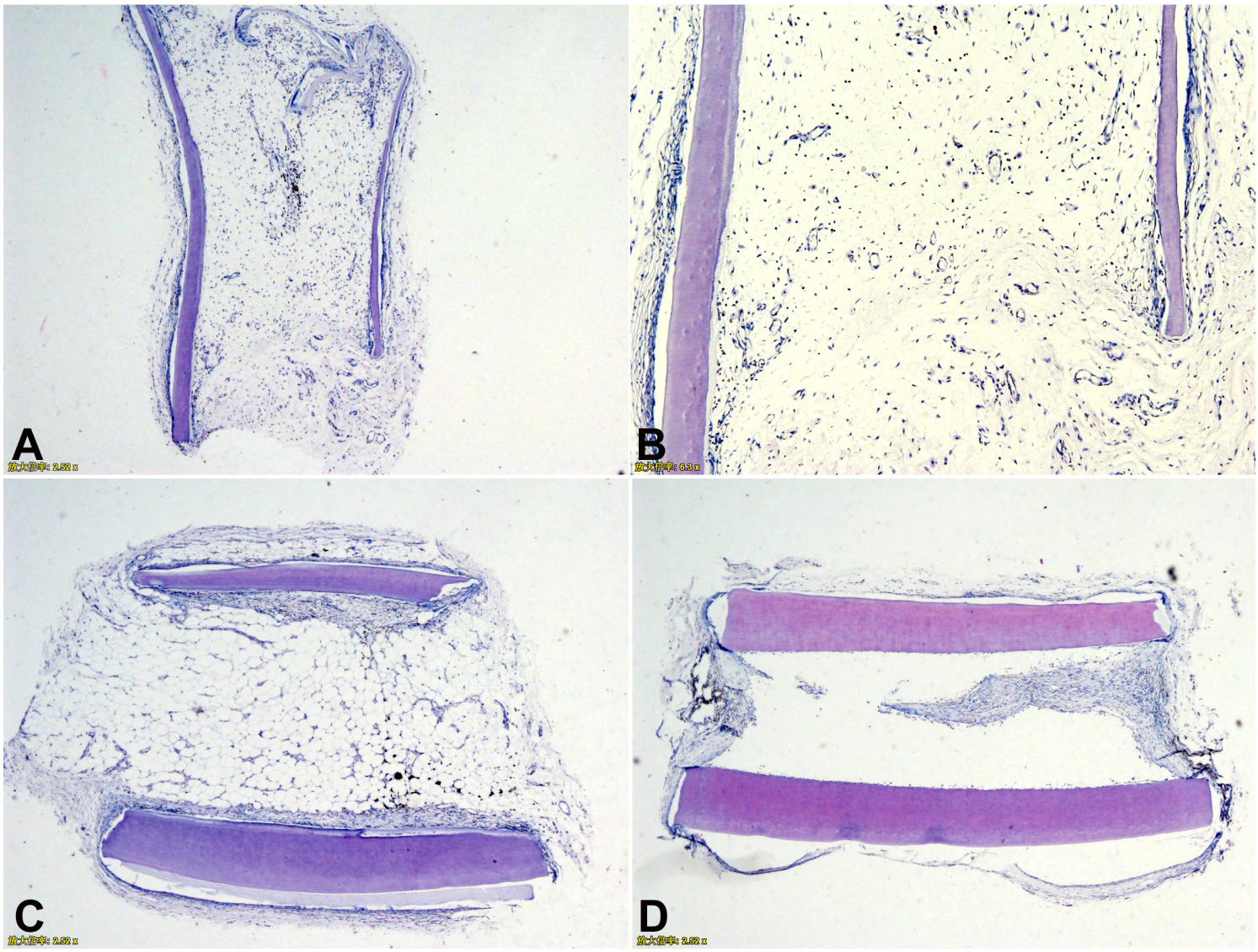

Figure 6

Histological analysis of control group. A, B: In the rBMSC group, pulp-like tissue filled the lumen and new blood vessels were seen at the opening. C: Incomplete absorption of collagen membrane was observed in the lumen. D: In the blank group, there was fibrous tissue growing into the opening on both sides of the root.

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