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Cytotoxicity and Bone Biocompatibility of the C-Root SP Experimental Root Canal Sealer

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ABSTRACT

This study evaluated the cytotoxicity and biocompatibility of a new strontium silicate-based root canal sealer (C-Root SP), in comparison with those of iRoot SP and AH plus. The sealer extract was diluted to the concentrations of 100%, 75%, 50%, and 25%. L929 cells were cultured for 24 h, and the absorbance value was determined. Two-way analysis of variance (two-way ANOVA) and sealers were implanted in the tibia of Sprague–Dawley (SD) rats, At 2, 6, and 12 weeks, rats were euthanised. The tissue reaction was evaluated by HE staining. The least significant difference (LSD) *t*-test and Kruskal–Wallis test were used. The cytotoxicity of C-Root SP and iRoot SP was found to be less than that of AH plus. At 12 weeks, new bone formation was induced around C-Root SP and iRoot SP sealer, but minimal evidence of bone formation was found in AH plus. C-Root SP has low cytotoxicity and superior biocompatibility.

1 | Introduction

Root canal therapy (RCT) is a common method for clinical treatment of dental pulp and periapical diseases, including preparation, disinfection, and filling in clinical operation [1]. In RCT, the performance of root canal sealer plays a decisive role in the well three-dimensional filling effect, and directly affects the success rate of treatment. In the process of filling, the root canal sealer may be squeezed into the periapical tissue [2]. The sealer will have direct physical contact with the surrounding tissue or indirect contact with the tissue through channels such as the apical foramen or lateral branches of the root canal [3]. Therefore, the sealer should be mild and non-irritating. In addition, chronic periapical periodontitis is often accompanied by periapical bone loss; the process of apical tissue repair is closely related to the proliferation and differentiation of osteoblasts, and the root canal sealer with osteogenic activity may help to promote tissue repair [4]. Therefore, the sealer with favourable biocompatibility and osteogenic potential can improve the long-term success rate of root canal therapy to a great extent.

The development of sealers that can interact with complex root canal systems and have a favourable effect has played a key role in the development of endodontics. There are many types of root canal sealers, and bioceramic sealers are the focus of dental pulp material research, which has been widely concerned by scholars and has been widely studied. In 1984 [5], researchers from the American Dental Association Health Foundation developed a calcium phosphate bone cement (CPC) and applied it to the sensitive root surface of exposed dentin. It was found that CPC can intercalate and penetrate the open dentinal tubules on the root surface, and ultimately promote the formation of hydroxyapatite. At the same time, it was found that the CPC had a similar function in the root canal. Šimundić Munitić et al. [6] think that this is the earliest study on bioceramic root canal sealers.

C-Root SP is a new bioceramic root canal sealer which is immediately used by injection, and its effective composition is similar to that of iRoot SP. The difference is that C-Root SP replaced calcium silicate ($CaSiO_3$) with strontium silicate

($SrSiO_3$). Many studies had shown that strontium (Sr) was a trace element in human bone, which can effectively promote bone tissue repair and stimulate angiogenesis by increasing the expression of angiogenic factors in cells [7–9]. $SrSiO_3$ biological scaffolds replaced by Sr. can stimulate the proliferation, osteogenesis, and blood angiogenesis of bone marrow stromal cells [10]. Strontium-containing silicate materials have great application potential in tissue engineering because of their favourable biological activity. In recent years, there have been endless reports on its application in the field of biomaterials [11–14]. As an implant material, the biocompatibility of root canal sealer is an important consideration. In the field of stomatology, there are few reports on the application of Sr. in root canal sealer. Despite the favourable application prospect, the laboratory comparative study on the biocompatibility of C-Root SP is still insufficient.

Thus, the aim of this study was to evaluate the cytotoxicity and biocompatibility of C-Root SP experimental sealer in comparison with iRoot SP and AH plus, so as to provide a theoretical basis for the selection of clinical root canal sealers. The null hypothesis was adopted, and it is expected that the sealers do not exhibit significant differences in terms of biocompatibility and cytotoxicity.

2 | Materials and Methods

2.1 | Sealers Extract

The composition of the materials is shown in Table 1. Each sealer was injected into a cylindrical polyethylene mould (diameter: 5 mm; length: 2 mm). Subsequently, the discs were kept in an incubator at 37°C for 72 h in a humid atmosphere. After freezed, the material was then removed from the mould and sterilised via ultraviolet irradiation for 1 h. The extracts were prepared according to ISO Standards 10993-5 [15] (surface/medium rate, 1.5 cm^2/mL, extraction time, 72 h, temperature, 37°C). The Dulbecco's Modified Eagle Medium (DMEM) with 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin was used as the extraction vehicle. The extracts were filtered by a 0.22 μm filter. Then the extract of complete medium was diluted in gradient, and the concentrations were 100%, 75%, 50%, and 25%.

2.2 | Cell Viability Assay

L929 cells were seeded into a 96-well plate at 100 μL per well (3000 cells/well) and four multiple holes. The cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. After 24 h, the extract of different concentration gradients was added to each experimental group, and the complete medium without extract was used in the control group. After 24 h, the extract was replaced by 100 μL complete medium and 10 μL Cell Counting Kit-8 (CCK-8) (Beijing Zhuangmeng Biotechnology Co. Ltd., China), and the plate was incubated at 37°C. After 4 h, the absorbance (*A* value) was measured at a 490 nm wavelength. Each condition was analysed in triplicate.

TABLE 1 | Composition of the sealers.

Material	Composition
C-Root SP	Zirconium oxide, strontium silicate, calcium phosphate, magnesium phosphate, and calcium hydroxide
iRoot SP	Zirconium oxide, calcium silicate, calcium phosphate, and calcium hydroxide
AH plus	A paste: Zirconium oxide, diepoxy, calcium tungstate, and dye B paste: Zirconium oxide, N,N′-dibenzyl-5 oxanonandiamine-1,9, 1-adamantane amine, TCD-diamine, calcium tungstate, and silicon oil

2.3 | Animals

Male adult Sprague–Dawley (SD) rats from Liaoning Changsheng Biotechnology Co. Ltd., China (age, 6 months; weight, 250 ± 30 g). The rats were raised in the centre of the experimental animals of NCST and all procedures were carried out in accordance with institutional guidelines for animal care and use.

2.4 | Bone Implantation Experiment

A total of 36 male SD rats were randomly divided into four groups with nine rats in each group. 2% pentobarbital sodium (Wuhan Dongkangyuan Technology Co. Ltd., China) was anaesthetised by intraperitoneal injection according to the dosage of 50 mg/kg. A small incision was made in the skin over the proximal tibia of the rat to expose the bone surface. This step was performed under aseptic conditions to prevent any contamination. We used a high-speed dental drill with a sterilised 2 mm diameter round bur to create the cavity. The drill was operated with a constant irrigation to prevent overheating and damage to the bone tissue. One cavity was prepared in both femurs of each animal on the bone plane about 1 cm from the joint head of the proximal tibia, for a total of 72 cavity (diameter: 2 mm; depth: 2 mm). The drilling was performed using an electric motor with a controlled speed setting to ensure precision and to avoid any unnecessary trauma to the surrounding bone. Sealers were injected into the cavity in the experimental group, and bone wax was used instead of sealer in the control group. The skin incision was closed using 4-0 sutures in a simple interrupted pattern. The animals were housed in a temperature controlled-environment (22 ± 1°C, 70% humidity) with a 12-h light–dark cycle and received water and food ad libitum. The wound was cleaned every day.

2.5 | Histologic Analysis

The animals were euthanised in each group at 2, 6, and 12 weeks, and anaesthetic overdose by intraperitoneal injection of pentobarbital. After separation of soft tissue, bilateral tibia specimens were removed and fixed in 4% formaldehyde solution (Beijing Solarbio Technology Co. Ltd., China) for 48 h. After fixation, the

samples were decalcified for a week at room temperature using 10% nitric acid (Beijing Solarbio Technology Co. Ltd., China). Haematoxylin and eosin (H&E, Beijing Solarbio Technology Co. Ltd., China) staining was applied to transversely cut sections of the femur that had a thickness of 5 μm.

The histological analysis was conducted using an optical microscope, the analysis was performed at a magnification of 100× and 250×, which allowed for a detailed examination of the bone tissue's response to the root canal sealer. A total of five slides were evaluated from each experimental group. The analysis was performed by a trained, calibrated operator who was blinded to the experimental conditions. The field of analysis was selected based on the presence of the material-tissue interface, where interaction was expected to be most pronounced.

The presence of neutrophils, lymphocytes, and eosinophils identified the cellular inflammatory component. According to Tavares et al. [16], these cellular events were categorised using the following scale: (0) Absent: inflammation was either absent or within blood vessels; (1) Mild: cells were present although sparse or in reduced clusters; (2) Moderate: cells were present but did not dominate the microscopic field; and (3) Intense: cells were present in the form of an infiltrate within the surgical cavity. The criteria for hard tissue barrier formation were modified from Assmann et al. [17]: (I) absence: no hard tissue deposition on the cavity region; (II) formation of immature bone tissue, beginning the process of linear closure of the experimental defect; (III) partial: partial close of cavity by hard tissue deposition; and (IV) complete: total close of cavity by hard tissue deposition.

2.6 | Statistical Analysis

The data were analysed by SPSS software (version 27.0; SPSS Inc., Chicago, USA). For in vitro cytotoxicity test, two-way analysis of variance (two-way ANOVA) and least significant difference (LSD) *t*-test were used. For animal experiments, a non-parametric test was used to compare the differences between groups, and Kruskal–Wallis test was used to compare multiple groups, with a Bonferroni correction at $p = 0.05$.

3 | Results

3.1 | Cell Viability

The data of L929 cell viability are shown in Figure 1. The cell viability of the C-Root SP group was always higher than that of the AH plus group, and the cell viability of iRoot SP group was higher than that of the C-Root SP group in undiluted solution ($p < 0.05$). In addition, the 25% dilution of the C-Root SP group extract exhibited similar results to that of the iRoot SP and control groups ($p > 0.05$).

3.2 | Animal Samples

The tissue responses of each time are shown in Figure 2. At 2 weeks, sealers were in favourable position, and there was no swelling, suppuration, and rejection in the tibial operation area. While at 6 weeks, the bone cavity in the control group tended to heal, but no healing tendency was found in experimental groups. In addition, there is no redness, suppuration, and rejection seen, and no enlargement was found in the defect area of each group. At 12 weeks, the bone of the control group healed completely; the surface of sealer in C-Root SP and iRoot SP groups was covered by tissue. However, the boundary between sealer and bone defect was clear in the AH plus group.

3.3 | Histologic Analysis

The scores of all histopathological responses evaluated in each time interval are presented in Tables 2 and 3. And the representative images of the tissue responses of each time are shown in Figure 3.

At 2 weeks, there were small amounts of lymphocytes in the cavity of control group, and more lymphocyte could be seen in each experimental group ($p > 0.05$). The bone defect was manifest, and the edge of the bone defect was irregular; the scattered trabeculae were interspersed in the bone defect area, and there was no fibrous connective tissue cover in each group ($p > 0.05$). The boundary between sealers and bone defect was clear.

At 6 weeks, there were small new trabeculae, fibrous connective tissue, and few lymphocytes filled in the defect area of the control group. We can see few lymphocytes in the cavity, and the bone repairs were active in C-Root SP and iRoot SP groups. However, there were irregular bone trabeculae and more lymphocytes in the AH plus group ($p < 0.05$).

At 12 weeks, the bone defect was filled with mature bone, and the edge of the defect was unclear in the control group. There was more bone tissue around the sealer, a small amount of sealer embedded in the bone tissue and closely combined with the bone tissue in the C-Root SP and iRoot SP groups. In the AH plus group, we can see that the defect was connected by bone tissue; there was fibrous connective tissue between the material and bone tissue ($p < 0.05$).

4 | Discussion

The study aimed to evaluate the cytotoxicity of the root canal sealer C Root SP compared to AH plus and iRoot SP. The results indicated that C Root SP demonstrated lower cytotoxicity than AH plus and was found to have comparable biocompatibility to iRoot SP, suggesting its potential as a safer alternative in endodontic treatments. Consequently, the null hypothesis, stating that there would be no difference in biocompatibility and cytotoxicity among the materials, is rejected.

As an implant material, the biocompatibility of root canal sealer cannot be ignored. The experiments related to the biological evaluation of root canal sealer include cell, animal, and clinical application levels [18]. Among them, the operation of the cell experiment is relatively simple, and the result is intuitive. So it is generally used as a preliminary screening experiment for biocompatibility evaluation [18, 19]. However, in vitro

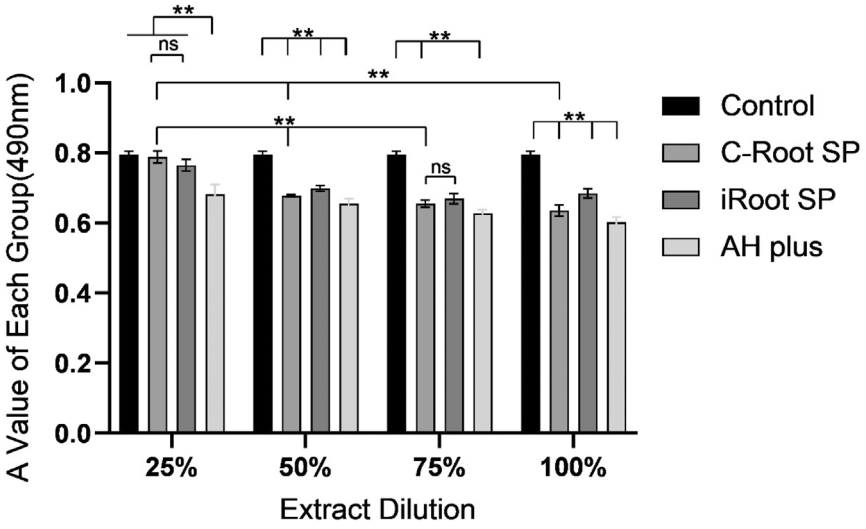

FIGURE 1 | Average *A* value of each group at 24 h (**$p < 0.01$; ns, $p > 0.05$).

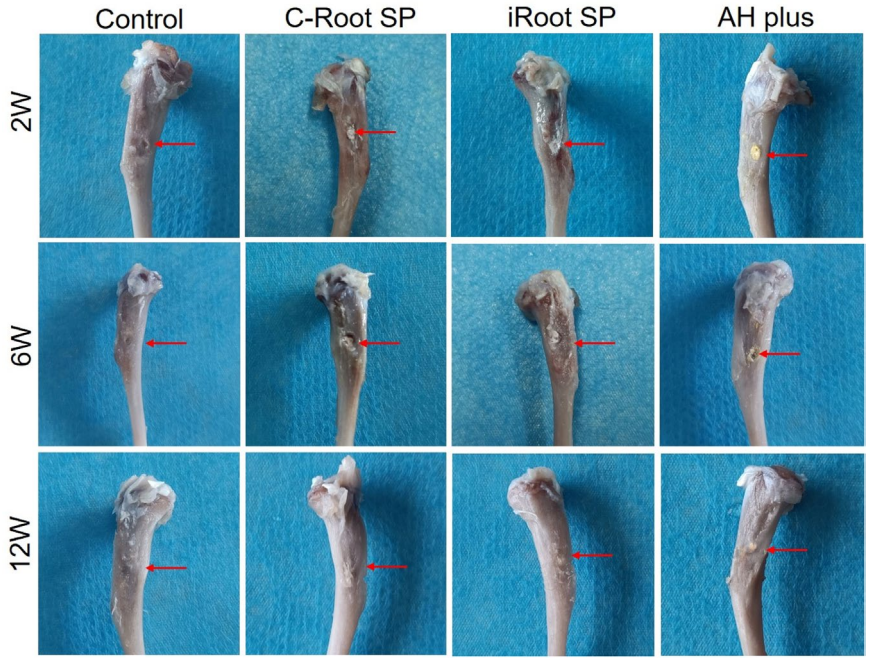

FIGURE 2 | Tibia specimens of each group (the red arrow indicates the location of sealers).

cytocompatibility tests can only reflect the effect of materials on specific cells, but cannot fully reflect the complex host response [20]. Implantation experiments in vivo are recognised as an effective method to evaluate the biocompatibility of materials, and their results are more reliable than cell experiments and more time-saving than clinical trials. In addition, the tissue implantation experiment can simulate the direct contact between the material and the periapical tissue, and can reflect the tissue reaction to a certain extent, so it is closer to the clinical application than in vitro cytotoxicity experiments.

In the cytotoxicity test, we selected the L929 cell line because it is sensitive to material toxicity. According to the ISO 10993-5 Vol 2009 standard [15], this cell was usually used to detect the cytocompatibility of materials in vitro [18]. In addition, rats' bone tissue mimicked the human periapical tissue. In this study, we have selected SD rats to detect the bone tissue response of root canal sealer in vivo. As a typical bioceramic sealer, iRoot SP is widely used in clinics [21, 22], while epoxy resin-based sealer AH plus has been set as a control in many studies, which is considered to be the gold standard for evaluating root canal sealers [23–25].

The results of the cell experiment showed that the relative proliferation rate of cells in the low concentration group was higher than that in the low concentration group at the same time, which indicated that the low concentration extract had less negative effect on the L929 cells, which may be due to the dilution of irritating components. The main active components of C-Root SP include strontium silicate, calcium phosphate, and calcium hydroxide. Strontium silicate can release free Sr^{2+} into the solution; hence the material shows favourable

TABLE 2 | Absolute frequencies for cellular events of histological reaction according to different groups and periods of evaluation.

		Neutrophils				Lymphocytes				Eosinophils			
Time	**Group**	**Absent**	**Mild**	**Moderate**	**Intense**	**Absent**	**Mild**	**Moderate**	**Intense**	**Absent**	**Mild**	**Moderate**	**Intense**
2W	CO	0	5	0	0	0	4	1	0	5	0	0	0
	CR	0	2	2	1	0	3	1	1	5	0	0	0
	IR	0	2	3	0	0	2	2	1	5	0	0	0
	AH	0	2	2	1	0	0	4	1	4	1	0	0
	p-value			0.174				0.136				0.392	
6W	CO	5	0	0	0	5	0	0	0	5	0	0	0
	CR	4	1	0	0	5	0	0	0	5	0	0	0
	IR	4	1	0	0	5	0	0	0	5	0	0	0
	AH	0	3	2	0	2	2	1	0	5	0	0	0
	p-value			0.004				0.019				1	
12W	CO	5	0	0	0	5	0	0	0	5	0	0	0
	CR	5	0	0	0	5	0	0	0	5	0	0	0
	IR	5	0	0	0	5	0	0	0	5	0	0	0
	AH	5	0	0	0	4	1	0	0	5	0	0	0
	p-value			1				0.392				1	

TABLE 3 | Absolute frequencies for hard tissue deposition according to different groups and periods of evaluation.

Time	Group	Absent	Partial	Complete	Intense immature hard tissue
2W	CO	0	1	0	4
	CR	0	0	0	5
	IR	0	0	0	5
	AH	0	0	0	5
	p-value	0.392			
6W	CO	0	1	4	0
	CR	0	5	0	0
	IR	0	5	0	0
	AH	0	4	0	1
	p-value	0.022			
12W	CO	0	0	5	0
	CR	0	0	5	0
	IR	0	0	5	0
	AH	0	3	2	0
	p-value	0.018			

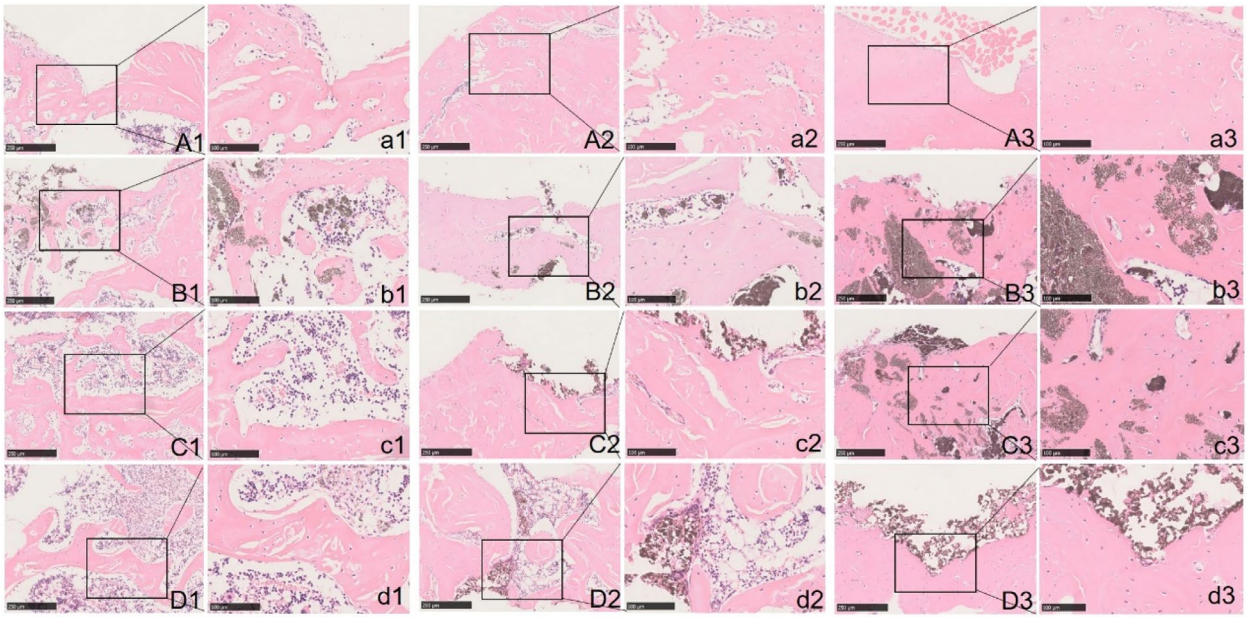

FIGURE 3 | Tissue reaction of each group at 2, 6, and 12 weeks. (A1, a1) There was small amount of lymphocytes in the cavity of control group at 2 weeks. (B1, b1, C1, c1, D1, d1) More lymphocyte could be seen in each experimental group. (A2, a2) There were small new trabeculae, fibrous connective tissue, and few lymphocytes filled in the defect area of the control group. (B2, b2, C2, c2) The bone repair were active in C-Root SP and iRoot SP groups. (D2, d2) There were irregular bone trabeculae and more lymphocyte in the AH plus group. (A3, a3) The edge of the defect was unclear in the control group. (B3, b3, C3, c3) A small amount of sealer embedded in the bone tissue and closely combined with the bone tissue in C-Root SP and iRoot SP groups. (D3, d3) There was fibrous connective tissue between the material and bone tissue in AH plus group (1-4 of A, B & C) 100×, (1-4 of a, b & c) 250×.

cytocompatibility [8]. Khashaba, Chutkan, and Borke [26] synthesised calcium phosphate cement and determined its cytotoxicity in vitro; it showed qualified cytotoxicity to human gingival fibroblasts and mouse fibroblasts. In addition, the active ingredient of calcium hydroxide in C-Root SP material is also the basis of its favourable biocompatibility. Jamshidi, Ansari, and Gheibi [27] determined the cytotoxicity of calcium hydroxide to human apical papilla stem cells in vitro. The results showed that calcium hydroxide had almost no toxic effect on the cells.

iRoot SP is widely used in the treatment of clinical dental pulp diseases, which is composed of calcium silicate, zirconia, calcium phosphate, and calcium hydroxide. The cytotoxic results of iRoot SP in this experiment are similar to those of previous studies. Nair et al. [28] evaluated the toxic effect of iRoot SP on L929 cells, which was low cytotoxicity and genotoxicity. AH plus sealers release formaldehyde and amines [29], which may have negative effects on cells, thus affecting cell proliferation. Epoxy resin has been shown to be mutagenic and cytotoxic [30, 31].

The results of animal experiments showed that there was no irritant reaction in the sample of each group at 2, 6, and 12 weeks. iRoot SP and AH plus have been verified by a large number of experiments for many years, and they are relatively qualified root-filling materials. From the specimens, we can see that the C-Root SP was also biocompatible. At 12 weeks, the bone defect area of the control group had healed completely, while that of the experimental groups had not healed completely, which may be caused by the physical occupation of the sealer. Malagnino et al. [32] show that it takes a long time for the sealer to be absorbed by the body tissue, and there is no absorption of the sealer in the limited observation time in this experiment.

The pathological results showed that there were a large number of inflammatory cells in each sealer group at 2 weeks after implantation, which may be related to the traumatic stress response of the body. The number of lymphocytes in the C-Root SP and iRoot SP groups decreased significantly at 6 weeks, and the inflammatory reaction tended to reduce, which means that the materials were biocompatible in vivo. At 12 weeks, there was conspicuous new bone formation around C-Root SP and iRoot SP sealer; the bond between the material and new bone was tight, the interface was smooth, and no inflammatory cells were found, so it can be considered that the sealer were biocompatible and has the ability to induce new bone formation. While there was no evident new bone trabecula around AH plus material, and fibrous connective tissue formed between material and bone tissue, which indicated that AH plus had poor osteogenic ability in vivo.

Studies on iRoot-SP indicated that the release of silicate, phosphate, and calcium ions synergically promoted the proliferation and mineralisation process of osteoblasts [33–36]. C-Root-SP and iRoot-SP have highly similar chemical components, so we speculate that in vivo implantation experiments, the blocking agent can also slowly release active ingredients in the complex body environment, so that osteoblasts become active and coordinate to promote bone tissue repair. Lea et al. [37] implanted calcium hydroxide into an animal periapical periodontitis model. Four months after implantation, the root tip tissue healing effect of the calcium hydroxide group was apparent, which indicated that calcium hydroxide could positively promote the repair of bone tissue after adding calcium hydroxide to the sealer. Xu et al. [38] found that strontium silicate significantly increased the osteogenic effect of calcium phosphate cement in vitro and in vivo by adding different proportions of strontium silicate to calcium phosphate cement, and the osteogenic effect was positively correlated with the proportion of strontium silicate. Wang et al. [39] reported that strontium-doped compounds can promote bone function in vivo.

According to the above studies, a variety of active components contained in C-Root SP can promote osteogenesis in vivo, we can speculated that, the sealer can effectively promote the healing of chronic periapical periodontitis through direct or indirect contact with periapical tissue when used in root canal filling, but the overall outcome is influenced by a combination of factors, such as the role of inflammation in the healing process. Reducing bacterial load and modulating the inflammatory response, which are critical for periodontal healing. The mechanical stability provided by the root canal sealer, along with the local microenvironment, is also essential for the healing process. The material's ability to adapt to the root canal anatomy and provide a seal against microbial recontamination is highlighted.

While our findings provide valuable insights, it is important to acknowledge the limitations of our study. For example, the cytotoxicity assessment was conducted using an in vitro cell culture model, which may not fully replicate the complex biological environment within the human body. This could potentially limit the extrapolation of these findings to clinical settings. In addition, the study utilised a Sprague–Dawley rat model for the in vivo assessment, which may not perfectly represent the human response to the root canal sealers. Different species may exhibit varying degrees of tissue compatibility and healing processes. The findings from the rat tibial defect model may not be directly applicable to other bone sites or defect types. The root canal sealer, once implanted, did not disperse uniformly within the bone defect. This variability made it challenging to standardise the histological analysis, as the sealer's interaction with the bone tissue varied across the defect site. We have also encountered the challenges during the implantation process, such as maintaining sterility, ensuring accurate placement of the material, and the potential for postoperative complications that could affect the histological analysis.

5 | Conclusion

Our findings indicate that the cytocompatibility and biocompatibility of C-Root SP and iRoot SP were superior then that of AH plus, such that C-Root SP was similar to iRoot SP, and the results of in vitro and in vivo experiments are mutually verified.

Author Contributions

Xiliang Yang: conception and design of study. **Xiliang Yang** and **Lingyun Xia:** acquisition of data. **Yongji Chen:** analysis and/or interpretation of data. **Lei Jiang:** drafting the manuscript. **Tianxia Zheng** and **Yuhong Bai:** revising the manuscript critically for important intellectual content. All authors have contributed significantly, and all authors are in agreement with the manuscript.

Ethics Statement

The Animal Ethics Review Committee of North China University of Science and Technology (NCST) approved the study (approval number: 2022-SY-007).

Conflicts of Interest

The authors declare no conflicts of interest.

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