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RESEARCH ARTICLE

PLASMA PROCESSES AND POLYMERS

Cold atmospheric plasma does not affect the regenerative potential of the pulp in rats

Madline P. Gund[1] | **Matthias Hannig**[1] | **Matthias W. Laschke**[2] | **Antje Lehmann**[3] | **Axel Schindler**[3,4] | **Stefan Rupf**[1,5]

[1]Clinic of Operative Dentistry, Periodontology and Preventive Dentistry, Saarland University, Homburg, Germany

[2]Institute for Clinical and Experimental Surgery, Saarland University, Homburg, Germany

[3]Leibniz Institute of Surface Modification (IOM), Leipzig, Germany

[4]Piloto Consulting Ion Beam and Plasma Technologies, Grimma, Germany

[5]Chair of Synoptic Dentistry, Saarland University, Homburg, Germany

Correspondence
Madline P. Gund, Clinic of Operative Dentistry, Periodontology and Preventive Dentistry, Saarland University Hospital, Kirrberger Str 100, Bldg 73, D-66421 Homburg, Germany.
Email: madline.gund@uks.eu

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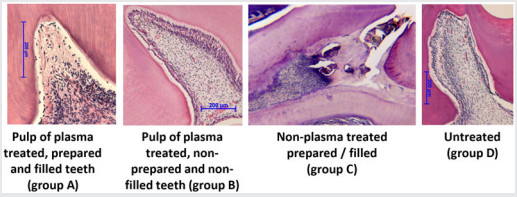
Abstract

The aim of this study was to investigate the effect of cold atmospheric plasma (CAP) treatment combined with adhesive filling therapy on rat dental pulps. Cavities were prepared in the first maxillary molars of 20 Sprague Dawley rats. The first molar and the unprepared second molar of one randomly selected maxillary quadrant were treated with CAP. The prepared cavities were filled with composite. After 24 h and 28 days, 10 rats each were killed. Teeth were demineralized and embedded in paraffin and histological sections were stained with hematoxylin–eosin and chloracetatesterase. None of the pulps displayed necrosis. Plasma treatment caused no additional alteration to the dental pulp in combination with adhesive filling therapy. These findings indicate that plasma treatment is compatible with the regenerative potential of the pulp.



KEYWORDS
cold atmospheric plasma, composite filling, mucosa, pulp, rat, tooth cavity preparation

1 | INTRODUCTION

The use of physical plasma has become an important field of research in medicine in recent years. In fact, plasmas are considered to have great application potential in the medical industry and medical technology. Used for more than 20 years in medicine, physical plasmas are gaining increasing importance in dentistry.[1–4] Examples of proposed applications in dentistry are: implant modification to prevent periimplantitis,[5,6] antibacterial applications to reduce microbial load,[7] decontamination,[8–11] disinfection in endodontic therapy,[12] tooth bleaching in

combination with bleaching agents,[13] soft tissue application in periodontology (periodontology is the study of the periodontium [the periodontium consists of cementum, bone, gum, and root membrane and is the functional anchoring system of the tooth]),[14,15] support of wound healing[16] and improvement of bonding interfaces.[17] When modifying bonding interfaces, cold atmospheric plasma (CAP) applied to adhesive systems helps monomers to better convert than conventional photopolymerization does.[18] CAP combined with silanization or sandblasting of water-aged restorative resin composite improves the reactive level of the aged surfaces.[19] Regarding bonding agents, CAP chemically modifies the adhesive, increases surface energy and water wettability of the tooth substance.[20–23] Hydrophilic components better penetrate into the dentinal tubes with more and longer resin tags, improving bonding strength[21–26] and long-term durability of bonding.[17,22,27]

The dental pulp fills the inner part of the tooth, the pulp cavity (pulp cavum), which is enveloped by tooth hard tissues. The pulp cavity extends from the crown of the tooth to the tip of the tooth root(s). The pulp consists of connective tissue with blood and lymph vessels as well as nerve fibers. Although the effects of CAP on bonding interfaces are well studied, undesirable side effects, such as possible damage to the pulp, remained unexplored so far.

Therefore, the aim of the present study was to investigate the influence of CAP treatment on the rat dental pulp in combination with an adhesive filling therapy. Our hypothesis was CAP has no harmful effect on the pulp.

2 | EXPERIMENTAL SECTION

2.1 | CAP source

The CAP source used was developed at the Leibniz Institute for Surface Modification in Leipzig. This microwave-excited, miniaturized plasma source consisted of an electrode coaxial system operating with helium gas. The inner conductor consisted of a 0.3 mm thick steel tube representing the inner electrode. The process gases were added through this inner conductor. The outer body of the plasma source formed the second electrode consisting of 3.4 mm thick steel, shielding the generated microwaves. A pulsed microwave generator generating a frequency of 2.45 GHz was used to excite the plasma source, the generator allowing a peak power of 100–300 W, an average power of 1–9 W, and a pulse width of 1–10 μs. The flow rate of the gases was regulated by a mass flow controller. For this study, the plasma source, which was originally mounted on a computer-controlled 3-axis stage system, was modified to allow simplified handling in the oral cavity of the experimental animals (Figure 1).

2.2 | Treatment parameters

Constant treatment parameters were maintained on all samples with the microwave pulse length being 2 μs at a peak power of 250 W, a helium gas flow of 2.0 L/min, and average power of 2.4 W. The generated plasma jet was gaseous, approximately 8 mm long and had a half-width (HBW) of 0.5 mm. The temperature of the tooth surface at the beam impingement point was 40°C. The temperature was controlled by infrared thermography (Optis PI infrared camera, PI Connect software; Optiris GmbH) and the treatment time was set up to 5s per tooth.

2.3 | Rat molars

The anesthesia of rats was carried out in accordance with the animal welfare application. For induction of anesthesia, animals were placed in a tight Plexiglas box with isoflurane. The subsequent injection anesthesia was performed with administration of 80 mg/kg ketamine and 5 mg/kg xylazine. Subsequent injections were based on body weight as needed.

The first molar of both maxillary quadrants was prepared for a composite filling in a split-mouth design on 20 Sprague Dawley rats. The occlusal cavities had a diameter of 1 × 1.5 mm and a depth of 0.5 mm with a residual dentin thickness of 0–0.3 mm. Cavity preparation was performed with a spherical preparation diamond ISO 008 (Komet Dental, Gebr. Brassler GmbH & Co., KG). To minimize heat generation, 6000 rpm was used with constant air and water cooling. The second molars of both upper quadrants were left unprepared.

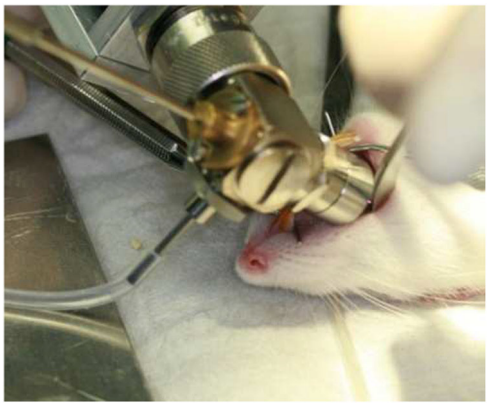


FIGURE 1 Irradiation of a laboratory animal. The distance of the jet to the tooth was 4 mm.

Animals were numbered and marked on the ears for recognition.

Both, the prepared first molar (Group A) and the unprepared second molar (Group B) of a randomly selected maxillary quadrant, which was treated with cold plasma, formed the test groups (plasma-treated). The prepared cavities on both halves of the jaw were then filled in an identical manner with the self-etching adhesive AdheSE One (Ivoclar Vivadent GmbH, Principality of Liechtenstein) and a flow composite Tetric EvoFlow (Ivoclar Vivadent GmbH, Principality of Liechtenstein). The prepared and filled, nonplasma-treated first molars (Group C) and the intact, nonplasma-treated second molars (Group D) formed the nonplasma-treated control groups.

2.4 | Oral mucosa

When treating dental hard tissues with cold plasma, accidental treatment of the oral mucosa is possible. To assess the possible effects of the plasma jet on the mucosa, an approximately 5×5 mm mucosal area in the buccal region of the maxillary incisors was treated with the above parameters in addition to the teeth.

2.5 | Preparation of the teeth for histological examination

After 24 h and 28 days, 10 rats each were killed with 200 mg/kg body weight of pentobarbital, and both upper molar segments were dissected. The segments were freed from soft tissue and fixed in 4% formalin for 24 h. Teeth were demineralized by 10% ethylenediaminetetraacetic acid for 4 weeks, allowing the preparation of fine histological sections. Molar segments were embedded in paraffin and jaw segments were placed in a certain way obtaining mesiodistal (sagittal) sections of the teeth.

2.6 | Histology

Histological sections closest to the treated region of the jaw segments with a slice thickness of 6 µm were prepared using a microtome. Attention was paid to filling residues, cavities, or distinct histological changes. Histological sections (including those of the oral mucosa) were stained with hematoxylin–eosin (HE) and chloracetatesterase (CAE). Finding a suitable section in the HE stain, the section before or after was additionally stained with CAE. The sections were viewed with a light microscope (Olympus BX 50; Olympus Corporation) at magnifications of ×1.25, ×5, ×10, ×20, and ×40. Images of the sections were captured using the camera incorporated on the microscope (AxioCam; Carl-Zeiss) and the associated program AxioVision (Carl-Zeiss) for Microsoft Windows documentation and assessment. Further processing of the images for brightness and contrast was performed using Microsoft Picture Manager for Microsoft Office.

In the histological preparations, the odontoblast layer, appearance of inflammatory cells, necrosis, and pre and secondary dentin formation were assessed and the Wilcoxon signed-rank test was used for statistical validation.

2.7 | Scoring system

A simple scoring system (Figures 2 and 3) was used to evaluate the results in each case (molars/mouth mucosa). Two blinded examiners assessed the sections independently.

3 | RESULTS

3.1 | Molars

3.1.1 | Results after 24 h

Light microscopic analyses demonstrated a clear inflammatory reaction in all plasma-treated and filled molars (Group A). The reaction was characterized by pulp necrosis in the pulp grains near the cavity with the destruction of the odontoblast layer and leukocytic demarcation of the lesion. In all sections of this group, CAE staining confirmed the prominent presence of neutrophilic granulocytes. No reaction in terms of the formation of pre- or secondary dentin could be detected. All specimens received a score of 3.

The specimens of Group B, consisting of teeth provided with a composite filling but without plasma treatment, showed necrosis zones in the area of the pulpal recesses close to the cavity. The odontoblast layer was partially or completely destroyed in these areas with increasing immigration of leukocytes in both HE and CAE staining. Pre- or secondary dentin was not present. All specimens also received a score of 3.

The plasma-treated, unprepared second molars of Group C as well as all specimens from Group D showed no pathological changes, receiving a score of 1 after 24 h (Figure 4).

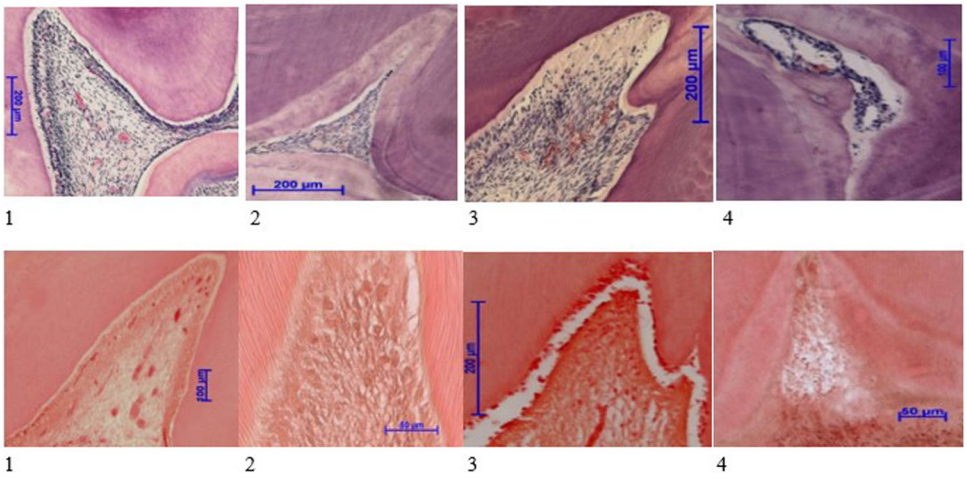


FIGURE 2 Presentation of typical histological findings of pulp sections describing the scoring used. The upper row shows corresponding preparations of HE staining, and the lower row of CAE staining. Score 1: no inflammation, no secondary dentin 2: no inflammation, secondary dentin visible 3: partial pulp necrosis, inflammation, reduced odontoblast layer, no secondary dentin 4: partial pulp necrosis, inflammation, reduced odontoblast layer, secondary dentin. CAE, chloracetatesterase; HE, hematoxylin–eosin.

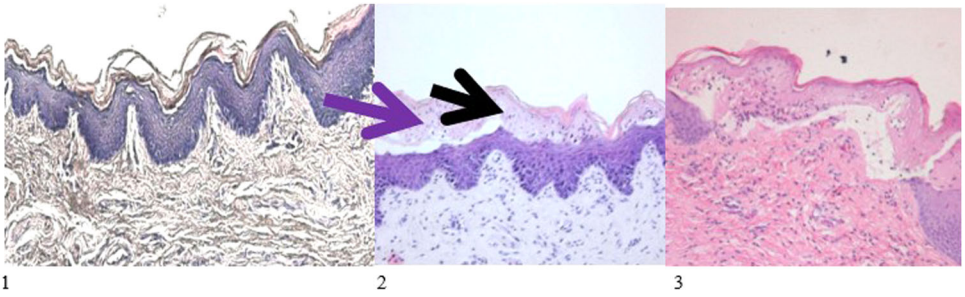


FIGURE 3 Typical histological findings for the oral mucosa, representing the used scoring. Score 1: no pathological change, undamaged epithelium of oral mucosa, Score 2: necrosis/detachment of epithelial cells without crossing the basement membrane, purple arrow: epithelium changed by plasma treatment, black arrow: Gap formed between damaged and healthy tissue, Score 3: necrosis/detachment of epithelial cells beyond the basement membrane, black arrow: gap between healthy and necrotic tissue, purple arrow: necrotic tissue with the immigration of inflammatory cells.

3.1.2 | Results after 28 days

About half of the teeth in Groups A and B showed decreasing signs of inflammation and presence of reparative dentin 28 days after cavity preparation, plasma treatment, and restoration with a composite filling. These preparations received a score of 2 (pre- and secondary dentin formation, no increased signs of inflammation). In one preparation, no change was evident, receiving a score of 1. In the other half of the teeth, a strong inflammatory reaction was observed in both Groups A and B. Significant intrapulpal dentin formation was observed in all specimens with 50% of the specimens in both groups receiving a score of 4 (partial pulp necrosis and/or odontoblast layer disrupted, pre- and secondary dentin formation, inflammation). Definite complete necrosis of the dental pulp was not observed in any of the teeth (Figure 5).

The plasma-treated, unprepared second molars (Group C) and the untreated molars (Group D) received Score 1 ratings after 28 days (Figure 5).

3.2 | Plasma-treated mucosal areas

3.2.1 | Results after 24 h

Necrosis with the detachment of epithelial cells was observed in the plasma-treated mucosa after 24 h. These changes were characterized by a marked widening of the intercellular gap and loss of nuclear markings. In some cases, whole-cell clusters were

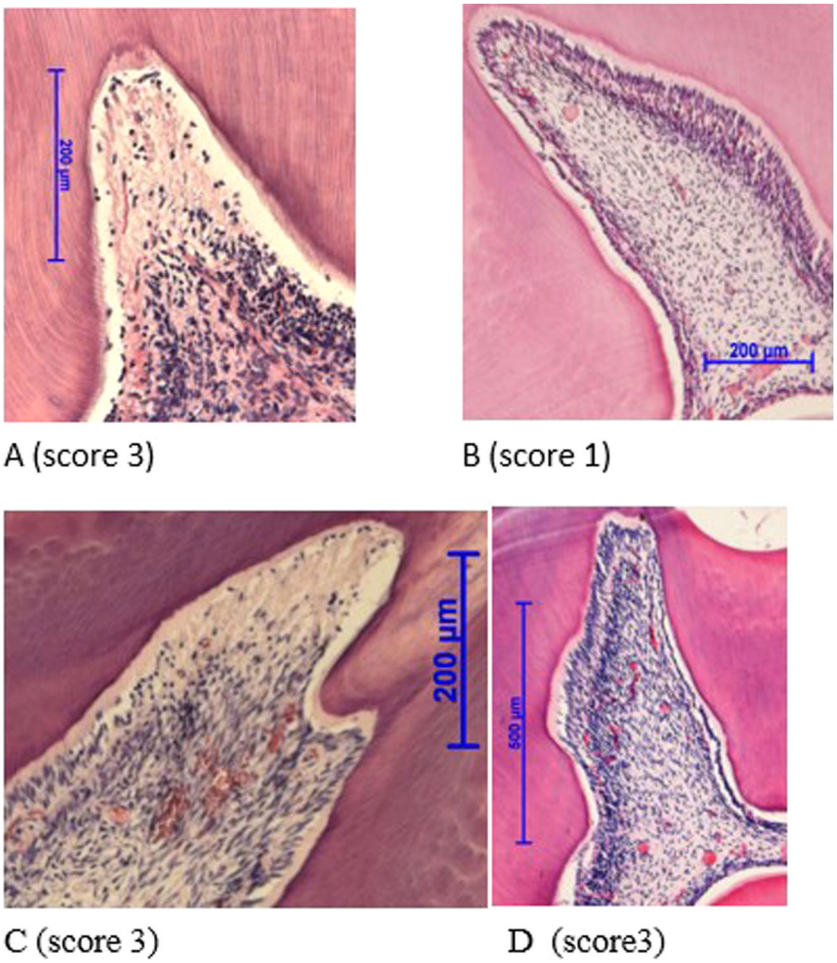


FIGURE 4 Representative findings of pulps of plasma-treated, prepared/filled teeth (Group A) in comparison to plasma-treated, nonprepared/nonfilled teeth (Group B) and nonplasma-treated prepared/filled (Group C) and untreated (Group D) teeth after 24 h. A: The prepared/filled and plasma-treated molars displayed inflamed pulps (Score 3). B: In contrast, plasma-treated, nonprepared/nonfilled molars presented unaltered pulps (Score 1). C: The prepared/filled but nonplasma-treated molars displayed inflamed pulps (Score 3). D: Pulps of nontreated and nonprepared/nonfilled teeth presented unaltered pulps (Score 1).

completely separated from the main tissue. A gap was present between normal and altered epithelium in all specimens and minor inflammatory reactions were noted. In two of eight specimens, the pathologic change remained confined to a few cell layers. In four other preparations, the epithelium was affected up to or beyond the basement membrane with the inflammation being more pronounced in these preparations. In three preparations, no changes were detected. One mucosal preparation was not assessable due to insufficient epithelium on the specimen (Figure 3, Table 1)

3.2.2 | Results after 28 days

Twenty-eight days after treatment with CAP, no pathological changes were observed in the epithelium of the oral mucosa with all specimens receiving a score of 1 after the light microscopic examination (Figure 3, Table 1).

4 | DISCUSSION AND CONCLUSION

This is the first study investigating the effects of CAP treatment on dental pulps in rats. It could be demonstrated that plasma treatment had no additional destructive effect on filling therapy on the pulp. The influences of restorative therapy on the pulp mainly resulted from the preparation of the dentin and the filling system. The pulps of teeth that were not prepared showed no inflammatory changes, regardless of the application of cold plasma. Therefore, this study provides promising results for the application of CAP in the context of restorative dental therapy.

The advantages of using CAP in this context are clear: The ability to disinfect and modify the wetting behavior of dental surfaces,[28,29] prevention of formation of marginal gaps at the filling margin, and the development of secondary caries. Therefore, fillings are achieving a longer life with the general long-term damage to the dental pulp being reduced as a result.

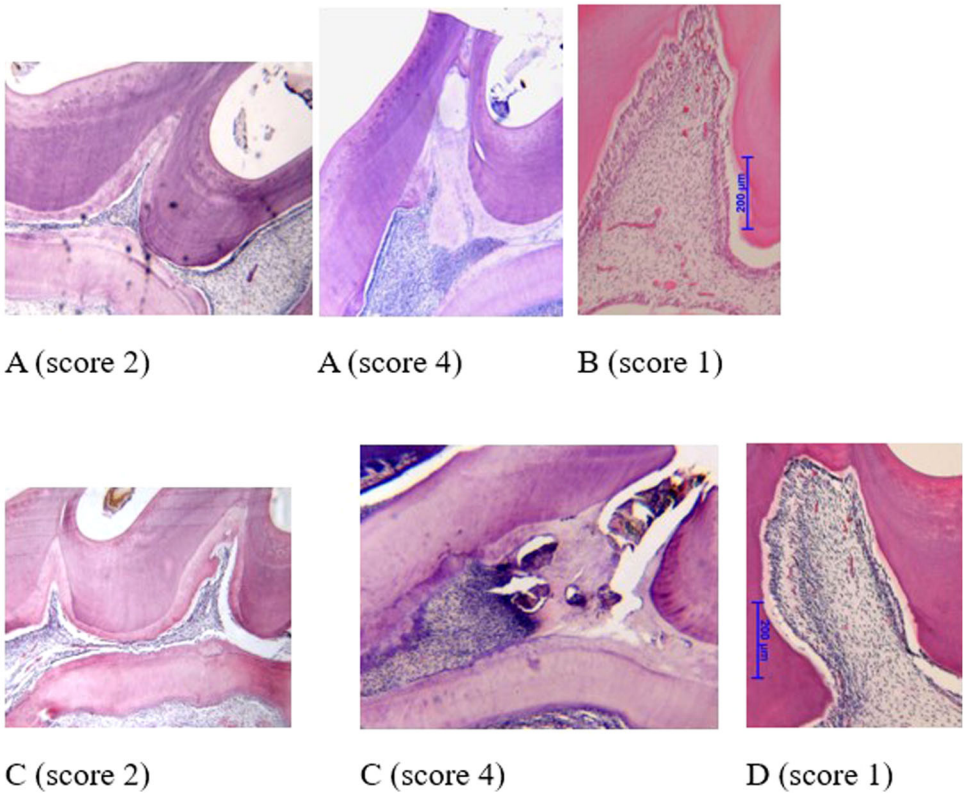


FIGURE 5 Representative findings of pulps of plasma-treated, prepared/filled teeth (Group A) in comparison to plasma-treated, nonprepared/nonfilled teeth (Group B) and nonplasma-treated prepared/filled (Group C) and untreated (Group D) teeth after 28 days. A: The prepared/filled and plasma-treated molars displayed results from Score 1 to Score 4. B: In contrast, plasma-treated, nonprepared/nonfilled molars presented unaltered pulps (Score 1). C: The prepared/filled but nonplasma-treated molars displayed pulps with Scores 2 and 4. D: Pulps of nontreated and nonprepared/nonfilled teeth presented unaltered pulps (Score 1).

TABLE 1 Histological scores obtained for the pulps of rat molars and oral mucosa after 24 h and 28 days

Pulps teeth prepared/filled				Pulps teeth unprepared/nonfilled				Mucosa			
24 h		28 days		24 h		28 days		24 h		28 days	
P+ (A)	P− (B)	P+ (A)	P− (B)	P+ (C)	P− (D)	P+ (C)	P− (D)	P+	P−	P+	P−
10 × 3	10 × 3	1 × 1 4 × 2 4 × 4	5 × 2 4 × 4	10 × 1	10 × 1	10 × 1	10 × 1	3 × 1 2 × 2 3 × 3	10 × 1	10 × 1	10 × 1

Note: Groups A–D indicated in branches. Scores pulps: see Figure 2, scores mucosa: see Figure 3.
Abbreviations: P+, plasma-treated; P−, nonplasma-treated.

During our study, mucosal damage (24 h) was observed as a result of plasma treatment, drawing the conclusion that physical plasma cannot be used in the oral cavity without side effects. Therefore, protective measures, such as the use of rubber dams, should be taken into account when applying physical plasmas in the oral cavity.

Lehmann et al.[30] tested a microwave-driven atmospheric plasma jet with regard to possible health risks for humans (including the effect of gas temperature, UV, and ozone emission) in dental applications. The study concluded that plasma jets possess high potential in this field of application without health risks. According to the authors, the observed changes may also suggest therapeutic applications of atmospheric plasma to the mucosa-in particular, the therapy of oral leukoplakia (premalignant lesions). Studies in this direction are to be carried out in the future. Hasse et al.[31] also reported modulating effects on tissue regeneration of the oral mucosa.

Nevertheless, considering the great benefit and involving the high regenerative capacity (no changes

after 28 days) of the oral mucosa, the use of plasma is justifiable in the context of restorative dental therapy. Jablonowski et al.[32] confirm our observations. Liu et al.[33] were able to clarify in an animal experiment on six white Japanese rabbits that plasma irradiation is not producing pathological changes in the mucosa. Delben et al.[34] also found no toxic effects on the oral mucosa when treated with CAP. Evert et al.[35] investigated the long-term effects of CAP on the oral mucosa and found that 1 year of repeated CAP treatment does no harm and does not initiate carcinogenesis.

Rat molars can be considered anatomically, histologically, biologically, and physiologically similar to human teeth. The biological reactions and processes during the different stages of wound healing of the rat tooth pulp are comparable with those of other mammals. Rat teeth have been used in dental research for a long time, serving as a model for research of pulpal reactions after pulpal openings, direct capping, pulpotomies,[36] and for testing the biocompatibility of various restorative materials.[37] Therefore, rat molars are a valid study model for investigating pulpal reactions and allow research with higher mammals to be dispensed with.[36] Therefore, they should be considered for future studies on CAP.

Further studies on the adjunctive use of cold plasma in filling therapy appear useful to discuss the results of this study in more detail. In addition, further studies should investigate the long-term damage of plasma irradiation, the quantity of formation of reactive species, and mutagenicity.

AUTHOR CONTRIBUTIONS

The study was planned by Stefan Rupf, Matthias Hannig, Matthias W. Laschke, and Axel Schindler. The experimental work was conducted by Stefan Rupf, Matthias W. Laschke, and Antje Lehmann. The data analysis and interpretation were conducted by Madline P. Gund, Stefan Rupf, Matthias Hannig, Matthias W. Laschke, Antje Lehmann, and Axel Schindler. The manuscript draft was written by Madline P. Gund and Stefan Rupf. The manuscript revision was conducted by Stefan Rupf, Madline P. Gund, Matthias Hannig, Matthias W. Laschke, Antje Lehmann, and Axel Schindler.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Madline P. Gund http://orcid.org/0000-0001-9053-8864

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