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ORIGINAL ARTICLE

Proliferation of rat molar pulp cells after direct pulp capping with dentine adhesive and calcium hydroxide

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Abstract The aim was to evaluate the proliferation of pulp cells 1, 3 and 7 days after direct pulp capping with the dentine adhesive Gluma Comfort Bond (GCB) and to compare it with calcium hydroxide ($Ca(OH)_2$). An occlusal cavity was prepared in 72 molar teeth of 36 Wistar rats. Then GCB or $Ca(OH)_2$ was placed on the exposed pulp. All cavities were restored with composite. After 1, 3 and 7 days, the animals were sacrificed. One hour prior sacrification, 5-bromo-2′-desoxyuridine (BrdU) was injected into the intraperitoneal cavity for immunohistological analysis of 18 animals. BrdU was incorporated into the DNA to tag proliferating cells using an antibody staining. Three animals served as controls and were not further treated. The number of the tagged cells was statistically analysed by comparing the results of the three groups. In 18 rats, routine histological analysis was performed in order to evaluate the pulp tissue for bacterial infection, inflammatory cells and necrosis. The marked cells were identified as fibroblasts, endothelial cells (after 1, 3 and 7 days) and Höhl cells (after 7 days). One day after capping, significantly more cells were stained in the GCB than in the $Ca(OH)_2$ group ($p<0.05$). After 3 days, significantly more cells were stained in the GCB than in the $Ca(OH)_2$ and the control group ($p<0.016$). Direct contact of GCB with pulp tissue leads to an increased formation of granulation tissue (fibroblasts, endothelial cells) because of an inflammatory reaction. This may be explained by missing antibacterial effect and foreign body reactions. Also, GCB may have a negative effect on Höhl cells.

Keywords Calcium hydroxide · Dentine adhesive · Direct pulp capping · Pulp tissue · Rat · Immunohistology

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Introduction

Although good clinical long-term results can be achieved in direct pulp capping with calcium hydroxide ($Ca(OH)_2$) [1], alternative materials—e.g., dentine adhesives (DA)—have been examined in recent years. Several histological studies conducted in direct pulp capping with DA in monkeys [2–5] and dogs [6] reported similar results for pulp healing using DA and $Ca(OH)_2$. In contrast, studies in human pulp tissue showed unsatisfactory results. The infiltration with inflammatory cells, neutrophilic granulocytes and resin particles into pulp tissue were clearly visible without

evidence for any cellular repair [7–13]. It is possible that ingredients of composites may have a negative effect on pulp tissue [14, 15]. It has been suggested that non-polymerised, polymerised and aged DA may release components, which are cytotoxic [15, 16]. This cytotoxicity may explain the poor results in direct pulp capping for DA in humans [11]. Bis-GMA, HEMA, UDMA, TEGDMA, initiators and their dissolutions have shown a cytotoxic effect on fibroblast and odontoblast-like cells in vitro [17–21]. In addition, DA has been shown to be clearly cytotoxic when in direct contact with human pulp cells in vitro [15, 16, 22]. Methacrylates, which are components of DA and composites, may interact with cellular phospholipids and cholesterol and therefore may cause changes in membrane-linked cell functions. Furthermore, mitochondrial membrane structures may be altered and the ATP synthesis may be inhibited. Single adhesive components may reduce enzymatic cell activity [18, 22]. Results of an in vitro study in pulp cells showed that after direct contact with DA or composite components the cell division rate decreased due to damage of DNA. Due to apoptosis (programmed cell death), a cell decline may have occurred [23].

Hence, the hypothesis of the present animal study was that direct pulp capping with DA will lead to a decrease in the cell division rate in vivo because of its cytotoxic effect on pulp cells which is in contrast to $Ca(OH)_2$. For this purpose the DA Gluma Comfort Bond (GCB; Table 1) has been compared with $Ca(OH)_2$ by examining the proliferation of pulp cells 1, 3 and 7 days after direct pulp capping of rat molar teeth. In order to quantify the ratio of cells undergoing mitosis by enumeration and calculation of a mitosis index (MI), an immunohistological study was performed by vital staining using 5-bromo-2′-desoxyuridine (BrdU). Thymidin is a nucleoside and a typical component of the DNA in the cell nucleolus. Therefore, it can be assumed that thymidin is specific for DNA. BrdU is an analogue to thymidin, i.e., thymidin can be replaced by BrdU via incorporating it into the cell nucleus during the S-phase of the cell cycle. Using a specific antibody staining with alkaline phosphatase and anti-alkaline phophatase antibody (APAAP), the BrdU can be tagged and counted in proliferating cells [24]. A cell that contains BrdU underwent cell division at the moment when BrdU was applied exogenously because BrdU has only a short bioavailability [25, 26]. These tagged cells are cells of the S, G_2 and M phases of the cell cycle. The amount of incorporated BrdU into the cell DNA can be determined with a MI, which provides information on cell activity. The MI is a quotient of the amount of tagged cell nuclei divided by the amount of all cell nuclei examined [24].

Materials and methods

Operative procedure

Thirty-six male and female Wistar rats (= 72 molar teeth) with an age of 3 months and a body weight between 250 and 300 g were used for this study. This animal research project was reviewed and licenced by the regional government administration (Münster, Germany) with the registration no. G39/99. For anaesthesia, a combination of 1 mL ketamine 10% (CEVA, Düsseldorf, Germany) and 0.2 mL xylazine 2% (CEVA, Düsseldorf, Germany) was added to 3.8 mL isotonic saline solution. The rats were anaesthetized with an intramuscular injection (0.1 mL per 50 g body weight). Because of the small size of rat molar teeth, the use of rubber dam was not possible. Prior to cavity preparation, the teeth were cleaned mechanically with a small brush, chemically with NaOCl (5%) and disinfected with chlorhexidine digluconate (0.1%, Chlorhexamed-Fluid, GlaxoSmithKline, Bühl, Germany). Aided with magnifying glasses (magnification: ×4.5, Zeiss, Aalen, Germany) occlusal cavities were prepared in the first upper, caries-free right and left molar teeth with a micromotor handpiece and a cylindrical diamond bur (ISO 008, NTI, Kahla, Germany) running at max. 3,000 min^{-1} up to the vicinity of the pulp chamber. The cavities were prepared under permanent cooling with water spray. Subsequently, the roofs of the pulp chambers were perforated with a sharp probe (EXD2H, Hu-Friedy, Chicago, IL, USA) in a slot-like shape. Pulpal blood was removed from the cavities with sterile paper points (Roeko, Langenau, Germany). In order to remove all blood remnants from the cavity walls, the cavities were rinsed carefully with saline solution and then air-dried. The pulp tissue of upper right molar teeth was directly capped with the DA GCB (Heraeus, Hanau, Germany; LOT 020023). GCB is an ethanol containing, fifth generation one-bottle-adhesive-system. The applica-

Table 1 Ingredients of the dentine adhesive Gluma Comfort Bond (Heraeus, Hanau, Germany) according to the manufacturer

Gluma Comfort Bond	
Conditioner	20% Phosphoric acid
Adhesive	HEMA (hydroxyethylmethacrylate), UDMA (urethane dimethacrylate), 4-META (4-methacryloxyethyl-trimelitt acid), malein acid, polycarboxylic acid ester, ethanol, water, photoinitiators

tion followed the manufacturer's specifications. All cavities were subsequently restored with a flowable composite restoration material (Venus flow, Heraeus, Hanau, Germany). The pulps of upper left molar teeth were capped with a $Ca(OH)_2$ paste. The $Ca(OH)_2$ paste was freshly prepared from Calciumhydroxid pro analysi (Merck, Darmstadt, Germany) and isotonic saline solution. After acid etching, all cavities were then restored with Venus flow in combination with GCB as DA as well.

Immunohistology

After defined postoperative time intervals of 1, 3 and 7 days, an intravital injection of 1 mL of a BrdU solution (Sigma-Aldrich, Munich, Germany) was applied intraperitoneally in order to quantify the rate of pulp cells capable for mitosis after direct pulp capping by enumeration and calculation of a MI. Six animals (= 12 molar teeth) were treated in each time interval. For this purpose, 50 mg BrdU powder was solubilise in 5 mL saline solution (the applied quantity was 50 mg/kg body weight and 10 mg BrdU for an average body weight of 200 g/animal, i.e., resulting in 1 mL mixed solution). The animals were sacrificed by the use of CO_2 inhalation after an exposure period to BrdU solution for 1 h. A group of three animals (= 6 molar teeth) served as controls and were not treated, but solely received an intravital injection with BrdU.

The maxillary molar teeth and the surrounding bone were dissected, fixed in formalin (2.5%), demineralised for 8 weeks with EDTA and embedded in paraffin. All specimens were divided into 13 sections with a layer thickness of 8 μm using a microtome (Ultracut, Reichert-Jung, Vienna, Austria) and positioned on glass slides (Superfrost, Menzel-Gläser, Braunschweig, Germany). To expose the antigen, the paraffin sections must be completely deparaffinated and rehydrated. For this reason, the sections were incubated twice in xylol for 10 min. The sections were subsequently incubated in descending ethanol series (100%, 96%, 70%) and washed with distilled water (aqua dest.). In order to improve immune reactivity, the sections were proteolytically digested with 0.05% protease for 6 min. This resulted in a demasking of the antigen and an exposure of hidden determinants. The specimens were subsequently rinsed with Tris-Tween buffer (Tween 20; Sigma-Aldrich, Munich, Germany). After this the sections were incubated for 60 min with monoclonal mouse anti-bromodeoxyuridine (M 0744, DakoCytomation, Hamburg, Germany), the so-called "primary anti-body", at a concentration of 1:20. The sections were then rinsed and incubated for 30 min with polyclonal rabbit anti-mouse immunoglobulin (Z 0259, DakoCytomation, Hamburg, Germany), the so-called "bridge antibody" (40 μL antibody + 200 μL human serum + 760 μL Tris). After another irrigation with Tris-Tween, the sections were incubated with an APAAP antibody immune complex (D 0651, DakoCytomation, Hamburg, Germany) for 30 min (20 μL + 980 μL Tris). The two last steps (incubation with bridge antibody and immuno-enzyme complex) were then repeated with an incubation time of 10 min. During the second incubation with the rabbit anti-mouse antibody the human serum was omitted but instead 960 μl Tris added. The glass slides were subsequently rinsed with Tris-Tween. The sections were covered with a liquid permanent red solution (LPR; K 0640; DakoCytomation, Hamburg, Germany), prepared according to manufacturer's specification (20 μL LPR chromogen to 3 mL substrate buffer), incubated for 9 min and rinsed with distilled water. Then staining with haematoxylin was performed in order to present the cell nuclei. This effectuates a contrast to the antigen staining. Subsequently, the specimens were washed with water, dried and incubated with xylol. All sections were then covered with resin (DPX; BDH Ltd., Pool, Great Britain) and a coverslip.

Three sections with completely visible crown pulp were selected from each treated molar tooth. For digital image evaluation, i.e., count of red tagged cells capable of mitosis with a picture analysis programme, stained specimens were photographed with a photo microscope (Phomi III, Zeiss, Oberkochen, Germany) and a camera (XP 900, Sony, Tokyo, Japan) and processed with a picture imaging programme (Lucia Net, Nikon, Kingston Surrey, UK) at a 250-fold magnification. In order to cover the complete extension of the crown pulp, approximately 15–20 photos were taken for each section. All pictures that cover the complete crown were combined together. The image analysis was conducted with the computer programme UTHSCSA ImageTool (University of Texas, Health Science Centre, San Antonio, USA). The tagged and untagged cells were counted with this programme and the quantities recorded. The counting process and identification of cells was performed by an experienced oral anatomist in a blind scoring modus and without knowing how the specimens were treated.

The numbers of the red tagged dividable cells per tooth were summated and in order to generate a MI, divided by the number of all cells and then multiplied with 100:

$$\mathrm{MI} = \mathrm{taggedcells}/\mathrm{numberofallcells} \times 100.$$

A statistical evaluation of the MI of the two used materials, GCB and $Ca(OH)_2$, was performed with the univariable, non-parametric Mann–Whitney U test and a multivariate variance analysis.

Furthermore, the tagged cells of the S-phase were determined with regard to their type and quantitative distribution for

each section. The statistical evaluation of the counts and distribution of the different cell types was performed with the univariable, non-parametric Mann–Whitney *U* test as well.

Light microscopic evaluation

For routine histological analysis, the second half of the treated animals (= 18 rats) were sacrificed by the use of CO_2 inhalation 1, 3 and 7 days, after direct pulp capping. Six of these animals (= 12 molar teeth) were treated in each time interval. The maxillary segments were dissected and chemically fixed with immersion in glutaraldehyde (2.5% in phosphate buffer, pH 7.4). After a demineralization period of 8 weeks with EDTA the specimens were embedded in epoxy-resin (Epon 812; Serva, Heidelberg, Germany) for light microscopical (LM) evaluation. Semithin serial sections with a thickness of 1 µm were cut throughout the complete extent of the exposed pulp and the adjoined regions of the cavity floor and then stained with toluidine blue in order to estimate the three-dimensional extension of the pulp damage.

Crown pulp and root pulp tissue were histologically evaluated and the findings recorded separately according to the following criterions: bacterial infection, inflammatory cells and necrosis. The evaluation was performed by an experienced oral anatomist who compared sections of the complete extension of the exposed pulp from the treatment group with appropriate sections from the control group in a blind scoring modus and without knowing how the specimens were treated. The criteria for intensity of tissue changes were the following ordinal scaled variables:

1 = absent
2 = slight/superficial (i.e., up to one third of the crown or root pulp tissue are affected)
3 = moderate (i.e., one third to two thirds of the crown or root pulp tissue are affected)
4 = severe/profound (i.e., two thirds—all of the crown or root pulp tissue are affected)

All data were separately recorded and statistically analysed. Owing to the ordinal nature of the scores, the data were subjected to the non-parametric Kruskal–Wallis test ($p<0.05$) in order to compare the frequency in which a particular score was given to a treatment group.

Bacteria staining

One central criterion in the histological evaluation was the occurrence of bacteria. In the present study, toluidine blue staining of Epon sections was used to assess the bacterial infection. As previously described, the spread of a bacterial infection in the pulp tissue was estimated in scores from 1 to 4.

Leakage test

In order to ensure that the coronal restorations were adequate and did not leak in the 7-day period, the sealing ability of the composite resin restorations was evaluated in a pilot study using a standard leakage test. Access cavities were prepared in four maxillary molar teeth of one rat until the pulp was exposed. The coronal cavity was then sealed with resin composite as described above. After 7 days, the rat was sacrificed, and the molar teeth and the surrounding bone were dissected. The teeth were then immersed in new fuchsine for 24 h at 37°C, washed in water and sectioned longitudinally with a diamond saw in a bucco-palatal direction. The sections were positioned in the middle of the restoration and evaluated for leakage under a stereomicroscope (Wild M400, Wild Heerbrugg, Heerbrugg, Switzerland) at a ×32 magnification. In addition, the interface between composite and tooth was assessed in all histological sections, and the composite restorations were investigated for marginal gaps.

Results

Immunohistology (Table 2)

Marking index of all tagged pulp cells

The MI had its maximum 3 days after direct pulp capping in both capping materials (GCB and $Ca(OH)_2$). After the 3-day period, the MI decreased in the two groups. Therefore, the data of the control group were compared with these maximum values (Table 2). A multivariate variant analysis was performed in order to provide in-depth comparison between the two treatment groups (GCB and $Ca(OH)_2$). Dependent variable was “rate of tagged cells”; independent variables were “time interval” and “treatment group”. The analysis demonstrated a significant dependence of the tagged cells to “time period” ($p=0.02$) and “treatment group” ($p=0.045$).

Results 1 day after direct pulp capping

The statistical evaluation with the Mann–Whitney *U* test showed that 1 day after capping significantly more cells were stained in the GCB than in the $Ca(OH)_2$ group ($p<0.05$).

Results 3 days after direct pulp capping

Because the data of the $Ca(OH)_2$ group was used three times for pairwise comparison, a Bonferroni correction was performed. Normally α is set at 0.05. In this case, α had to

Table 2 Results of the statistical evaluation of the immunohistology: mean values and standard deviation of the marking index of cells in the S-phase marked with BrdU (5-bromo-2′-desoxyuridine)

Period (day)	Without operation (mean ± SD)	$Ca(OH)_2$ (mean ± SD)	GCB (mean ± SD)
0	0.53±0.18		
1		0.52±0.46	1.06±0.26*
3		1.64±0.81***	3.48±1.39**·***
7		0.96±0.64	0.84±0.36

*$p<0.05$ significant difference compared to the appropriate $Ca(OH)_2$ group; **$p<0.016$ significant difference compared to the appropriate $Ca(OH)_2$ group; ***$p<0.016$ significant difference compared to the control group without treatment

be divided by the number of performed tests (i.e., three). Therefore, α had to be set at 0.016. The MI comparison of the group of animals without any treatment with the $Ca(OH)_2$ group showed no significant difference between them ($p>0.016$), whereas the MI was significantly increased when compared with the GCB group ($p<0.016$). The MI in the GCB group was significantly higher than in the $Ca(OH)_2$ group ($p<0.016$). Hence, after 3 days significantly more cells were stained in the GCB than in the $Ca(OH)_2$ group ($p<0.016$)

Results 7 days after direct pulp capping

After 7 days, no significant difference between the $Ca(OH)_2$ and the CGB group was detected ($p>0.05$).

Distribution of the tagged cells (Table 3)

The majority of cells marked with BrdU during the S-phase were fibroblasts and at lower rate endothelial cells in the control group 1 and 3 days after direct pulp capping. Höhl cells were visible in both treatment groups (GCB and $Ca(OH)_2$) after 7 days after direct pulp capping. Additional staining was omitted since these cell types (fibroblasts, endothelial cells and Höhl cells) can be easily distinguished even in light micrographs due to their specific morphology and location.

The data of the control group were compared with the results for the treatment groups 7 days after direct pulp capping because at this point the differences to the controls were most prominent. No other cells than fibroblasts, endothelial cells and Höhl cells could be tagged in any section. Figures 1 and 2 show an exemplary illustration of the immune histological-tagged cells 3 days and Figs. 3 and 4, 7 days after direct pulp capping with $Ca(OH)_2$ and GCB, respectively.

Results 1 day after direct pulp capping

Regarding the distribution between fibroblasts and endothelial cells, no statistically significant differences could be observed between the two treatment groups ($p>0.025$). Höhl cells could not be marked.

Results 3 days after direct pulp capping

Comparison between the $Ca(OH)_2$ and the GCB group showed significantly more fibroblasts and significantly less endothelial cells in the GCB group ($p<0.05$).

Results 7 days after direct pulp capping

For statistical evaluation of the distribution of tagged cells, the Bonferroni correction was performed and α was set at

Table 3 Results of the statistical evaluation of the immunohistology: percentile distribution (mean values and standard deviation) of the different cells marked with BrdU (5-bromo-2′-desoxyuridine) during the S-phase

Material	Period (day)	Fibroblasts (mean ± SD)	Endothelial cells (mean ± SD)	Höhl cells (mean ± SD)
Without operation	0	90.75±5.52%	9.24±5.52%	0%
$Ca(OH)_2$	1	94.02±7.30%	5.97±7.30%	0%
GCB	1	87.23±5.83%	12.76±5.83%	0%
$Ca(OH)_2$	3	68.49±10.41%	31.50±10.41%	0%
GCB	3	79.17±8.04%*	20.83±8.04%*	0%
$Ca(OH)_2$	7	63.33±7.17%***	26.15±5.54%***	10.51±4.98%***
GCB	7	73.04±17.95%	24.32±16.65%	2.62±3.77%**·***

*$p<0.05$ significant difference compared to the appropriate $Ca(OH)_2$ group; **$p<0.016$ significant difference compared to the appropriate $Ca(OH)_2$ group; ***$p<0.016$ significant difference compared to the control group without treatment

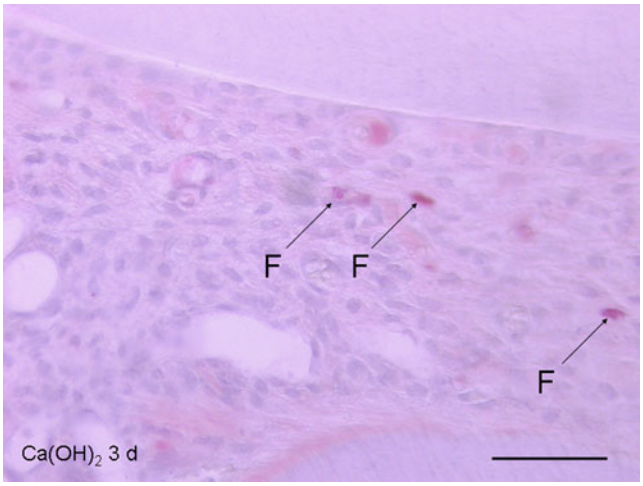

Fig. 1 Light micrograph of an *upper* rat molar tooth after pulp exposure and 3 days contact with $Ca(OH)_2$. *F* fibroblast. Bar=50 µm, original magnification ×250 (immunohistological staining)

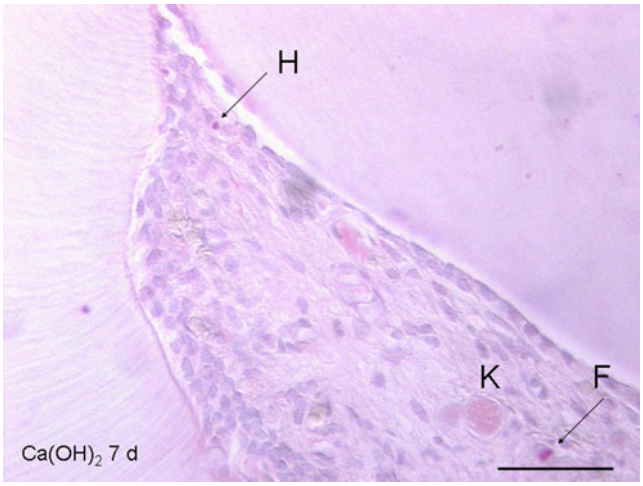

Fig. 3 Light micrograph of an *upper* rat molar tooth after pulp exposure and 7 days contact with $Ca(OH)_2$. *H* Höhl cell, *F* fibroblast, *K* capillary. Bar=50 µm, original magnification ×250 (immunohistological staining)

0.016 ($\alpha/3=0.016$), because the data of the controls were used twice for the paired test. The results for the $Ca(OH)_2$ group showed that significantly fewer fibroblasts and significantly more endothelial cells were tagged when compared with the control group ($p<0.016$). Comparison between the GCB group and the control group showed no significant difference ($p>0.016$). The distribution of fibroblasts and endothelial cells showed no significant difference between the $Ca(OH)_2$ and the GCB group ($p>0.016$). A significant difference between the two treatment groups and the control group was found in the rate of tagged Höhl cells because no Höhl cells were tagged in untreated teeth ($p<0.016$). Furthermore, the $Ca(OH)_2$ group showed significantly more Höhl cells in the S-phase than the GCB group ($p<0.016$).

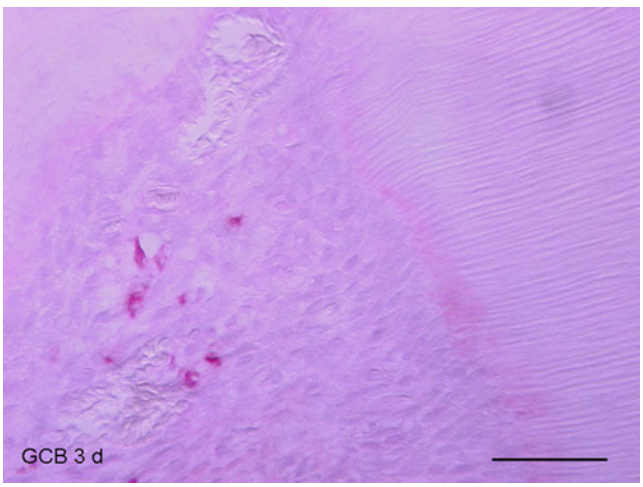

Fig. 2 Light micrograph of an *upper* rat molar tooth after pulp exposure and 3 days contact with GCB. All *red tagged cells* were identified as fibroblasts. Bar=50 µm, original magnification ×250 (immunohistological staining)

Evaluation of routine histological analysis (Table 4)

One day after direct pulp capping teeth treated with GCB showed significantly more bacterial infection and inflammatory cells in the crown pulp than teeth treated with $Ca(OH)_2$ ($p<0.05$).

Three days after the operative procedure a similar picture was found: significantly higher levels of bacterial infections of crown pulp tissue were found in the GCB group ($p<0.05$). However, no statistically significant difference with regard to infiltration with inflammatory cells was found between these two groups ($p>0.05$).

Seven days after direct pulp capping, the GCB group showed again significantly higher levels of bacterial infection when compared with the $Ca(OH)_2$ group ($p<0.05$). With regard to the extension of the necrosis, no significant differences between the different groups were detected ($p>0.05$). Figures 5 and 6 show an exemplary illustration of the routine histological analysis 7 days after direct pulp capping with $Ca(OH)_2$ and GCB, respectively.

Leakage test

All specimens revealed no dye penetration and no voids or porosities. All histological sections allowed an assessment of the margins, which were found to be composed of tight sealed layers without any indication of microleakage towards the oral cavity.

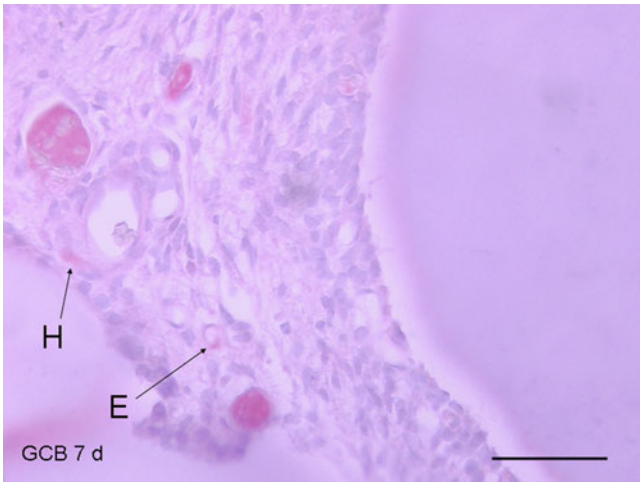

Fig. 4 Light micrograph of an *upper* rat molar tooth after pulp exposure and 7 days contact with GCB. *H* Höhl cell, *E* endothelial cell. Bar=50 µm, original magnification ×250 (immunohistological staining)

Discussion

Bacteria staining

Bacterial staining is needed to assess the presence of any bacteria in each molar tooth in order to decide if the pulp tissue response is caused by contamination with microorganisms (due to microleakage) or by the capping material. Histobacteriological assessments are usually undertaken by the use of specific staining such as Brown and Brenn or Brown and Hopps and performed on paraffin sections. The present study used toluidine blue staining of Epon sections in order to assess the bacterial infection. This was done because previous research revealed that the bacteria detection rate in semi-thin sections (thickness, 1 µm) after toluidine blue staining is superior to that of paraffin sections due to the superposition of image planes lying out of the focus in paraffin sections with a thickness of 8 µm [27].

Leakage test

The leakage test used is a common procedure for evaluating the sealing ability of restorations, allowing the observation of dye that penetrates into gaps between cavity walls and restoration [28]. It is commonly accepted that if no dye penetration occurred, the restoration will prevent microleakage of microorganisms between the cavity walls and the restorative material [29]. Marginal gaps of approximately 0.5–1.0 µm or more may allow leakage of bacteria [29], and it is reasonable to assume that the dye would have gained access to such gaps. The present results confirm that the used composite resin material will prevent the penetration of bacteria into the pulp.

Histological evaluation

It was surprising to find that although all teeth were treated identically (preparation, restoration) and all tooth surfaces were cleaned and disinfected before preparation, a bacterial contamination of the pulp tissue occurred statistically significantly more often in teeth treated with GCB than treated with $Ca(OH)_2$. The leakage test and the histological evaluation proofed bacteria-tight restorations. For this reason, it is quite plausible that an infiltration of microorganisms from the oral cavity and saliva during preparation and restoration of the cavities occurred. The application of rubber dam was not feasible in the present study. Nevertheless, rat molar teeth are a valid model to evaluate histologically the outcome of direct pulp capping [30].

In addition, in order to avoid any interference with the pulp reaction, it was decided not to use any disincentive agents (such as chlorhexidine or sodium hypochlorite) for cavity cleaning.

Therefore, experience from similar clinical conditions in humans may explain the underlying mechanism. The test condition in this study was similar to a clinical situation where the application of rubber dam is difficult or not feasible, e.g., a deep carious lesion or a fractured crown

Table 4 Results of the statistical evaluation of the routine histology; mean values and standard deviation are given

Crown pulp	Period (day)	$Ca(OH)_2$ (mean ± SD)	GCB (mean ± SD)
Bacterial infection	1	1.0±0	2.0±0*
Inflammatory cells	1	1.25±0.5	2.0±0*
Necrosis	1	2.0±0	2.0±0
Bacterial infection	3	1.0±0	2.0±0*
Inflammatory cells	3	1.5±0.57	2.0±0
Necrosis	3	2.0±0	2.0±0
Bacterial infection	7	1.0±0	2.5±0.57*
Inflammatory cells	7	1.66±0.57	2.25±0.5
Necrosis	7	2.0±0	2.5±0.57

1 absent, *2* slight, *3* moderate, *4* profound

*$p<0.05$ significant difference compared to the $Ca(OH)_2$ group

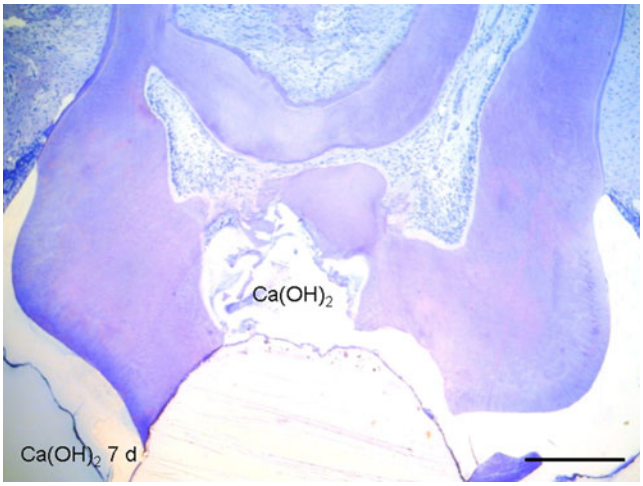

Fig. 5 Light micrograph of an *upper* rat molar tooth after pulp exposure and 7 days contact with $Ca(OH)_2$. *$Ca(OH)_2$* calcium hydroxide remnants at the cavity floor. Bar=250 µm, original magnification ×40 (toluidine blue staining)

after trauma. Furthermore, even if the application of rubber dam is possible, a bacterial invasion during cavity preparation and restorative procedures can never be fully excluded [31]. Most likely and in contrast to $Ca(OH)_2$, GCB was not able to eliminate microorganisms, which invaded the exposed pulp tissue during the operative procedure.

Because of the cytotoxicity of DA, the hypothesis of this animal study was that direct pulp capping with GCB in vivo will lead to a decrease in the cell division rate. However, this was not the case. Quite the contrary became apparent: 3 days after direct pulp capping with GCB the MI was significantly increased when compared to the control and the $Ca(OH)_2$ group. Using immunohistological analysis techniques, it could be demonstrated that the turnover rate

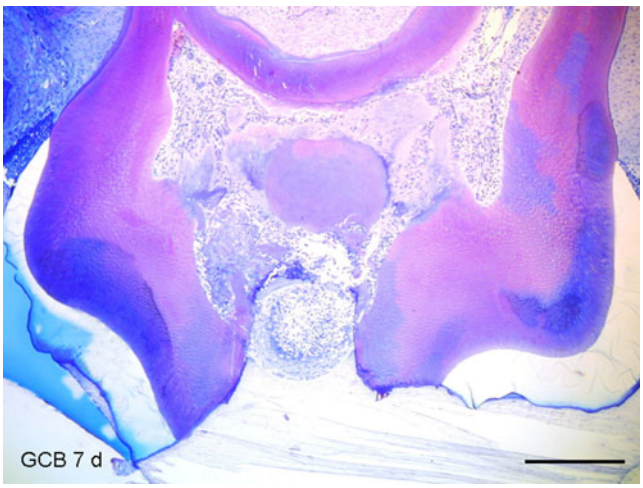

Fig. 6 Light micrograph of an *upper* rat molar tooth after pulp exposure and 7 days contact with GCB. Bar=250 µm, original magnification ×40 (toluidine blue staining)

of cells in healthy pulp tissue is low [32, 33], e.g., odontoblasts did not undergo mitosis [34, 35]. A low proliferation rate was also found in the control group. Cavity preparation alone leads to an increase of the mitosis rate, which resulted in cell proliferation in direction towards the damaged pulp tissue [34]. If rat molar teeth are ground occlusally, the mitosis activity of pulp cells will increase within 24 h with a maximum after 48 h [36].

The present study found evidence for the development of granulation tissue after direct pulp capping with $Ca(OH)_2$ and GCB. Initially, a distinct superficial necrosis occurred due to pulp tissue exposure and direct pulp capping, regardless which of the two materials were used. The necrosis induced an inflammation because necrotic tissue acts as a foreign body in a healthy environment. This necrotic tissue will soon be metabolised and replaced by granulation tissue due to a cellular reaction of the surrounding connective tissue. The formation of granulation tissue through the proliferation of capillaries and fibroblasts initiates the reparation of pulp tissue. Thus, the inflammatory reaction is an essential part of the wound healing process [37, 38]. If the underlying cause for the necrosis has been eliminated the inflammation will heal fast. If the reason for the necrosis persists over a longer period of time, an inflammation with severe granulation and poor healing will occur [39]. Existing necrotic tissue will prolong the inflammatory reaction, which will subsequently delay the progress of the reparation process [40]. The invasion of bacteria into the necrotic tissue will lead to an infected necrosis. The inflammatory reaction itself may also cause tissue damage [39]. The inflammation of a wound can be supported by the persistence of a bacterial contamination or by foreign bodies [37, 41]. Both factors impede reparation and healing can be obviated or delayed. In addition, granulation and scar tissue formation will be enhanced [39]. Therefore, these causes must be eliminated before the inflammation can subside [37].

Bacterial infections are the most frequent complication in wound healing [42]. If microorganisms invade a wound, the healing process will depend on the capability of the tissue to establish a successful defence in the initial phase of the inflammation [35]. If this defence mechanism is not successful, tissue necrosis will inevitably result [43, 44].

If a foreign body has been implanted into healthy tissue, an inflammatory reaction will result, which will subside after 3 days and result in regular wound healing, as long as no bacterial infection occurs [45]. However, a foreign body may delay wound healing. One reason for this is that foreign bodies may stimulate the growth of invading bacteria, which may induce a purulent infection [35]. Furthermore, biomedical polymers may also cause tissue inflammation (without any bacterial infection). When biomedical polymers get in contact with healthy tissues,

they will activate macrophages. The macrophages will then secrete cytokines, like interleukin 1. This growth factor will induce the proliferation of fibroblasts and endothelial cells [46–48]. This proliferation mechanism occurs in addition to the earlier described regular wound healing. The additional enzyme activity of macrophages at the tissue polymer interface may cause damage and/or loss of connective tissue [45]. Therefore, macrophages appear to be the critical cells in disturbed wound healing because they trigger an inflammatory reaction [49]. This overshooting cell activity induced by polymers may lead to an excessive inflammation and a failure of wound healing [48]. Therefore, macrophages may be activated by polymers [46–48], but will be inhibited by $Ca(OH)_2$, which will then benefit the healing process [50].

The present study demonstrated that teeth treated with $Ca(OH)_2$ showed a chemically induced necrosis of pulp tissue due to the release of OH^- ions (in addition to the physical damage caused by the cavity preparation and pulp tissue exposure). In teeth where the pulp tissue was capped with DA, the chemical stimulus and the foreign body reaction (phosphoric acid, dentine adhesive and restoration material components, respectively) were followed by an additional bacterial infection.

Hence, it became apparent that the significant increase of the MI and reduction in cell division rate 3 days after pulp capping with $Ca(OH)_2$ can be explained by the regular healing processes of pulp tissue, i.e., granulation tissue had been formed. This view has been vindicated by the observation that only fibroblasts and endothelia cells (i.e., typical cells of granulation tissue) were tagged after 3 days.

In contrast, the cell reaction differs considerably after direct pulp capping with GCB. Already after 1 day a statistically significant increase in tagged cells was observed when compared with $Ca(OH)_2$. Furthermore, after 3 days the MI was significantly higher when compared to the control group. Thus, the formation of granulation tissue was much more distinct. It can be assumed that this is caused by the material's properties. The LM evaluation demonstrated that after direct pulp capping with GCB a bacterial infection with the occurrence of inflammatory cells (granulocytes and macrophages) was significantly more often observed, when compared to the $Ca(OH)_2$ group. This bacterial infection may lead to an increased tissue reaction with the formation of granulation tissue due to a foreign body reaction in combination with an infection of the wound area. Previous histological studies have shown an invasion of immune defence cells within a chronically inflammatory reaction after direct pulp capping with dentin adhesives [7–9]. Because polymer particles and dentin adhesive components invaded the pulp tissue, a foreign body reaction occurred. Signs of any cellular repair process were not detectable [7–9]. Furthermore, components of the composite restoration material may cause a suppression of immune competent cells, which increases the risk of a bacterial infection of the pulp [14, 15, 17] and results in an increased inflammatory reaction.

Dentin adhesives may cause distinct alterations of pulp tissue when in direct contact with pulp cells (anything between chronically inflammatory reactions to necrosis) [11, 13, 18, 19, 21]. Therefore, persisting inflammation of pulp tissue has been described after direct pulp capping with dentin adhesives in humans [7–13].

Monomers from restoration materials (DA/composites) may interfere with the immune system of the pulp and impair the body defence in case of an invasion with microorganisms [14, 15]. The immune defence of the pulp tissue is reduced, when in direct contact with DA and composites, due to their cytotoxicity or specific changes in the immune response [17]. A chemical-induced immune suppression often correlates with a reduced resistance to infections [51]. Thus, components of DA inhibit the proliferation of immune competent cells and cause a chemical immune suppression, which will increase the development of pathogenic changes in the pulp tissue [14].

Beside fibroblasts and endothelial cells, Höhl cells were found 7 days after direct pulp capping. In case of an injury or a decline of primary odontoblast, these Höhl cells may have the potency to differentiate into odontoblast-like cells and then produce reparative dentine [52–55]. The present study suggests that GCB had a negative effect on Höhl cells because the MI for tagged Höhl cells was significantly lower after direct pulp capping with GCB when compared to $Ca(OH)_2$. This may have an influence on the formation of reparative dentine. Seventy days after direct pulp capping, teeth treated with GCB showed a significantly lower amount of reparative dentine when compared to the $Ca(OH)_2$ group [56]. This suggestion has been supported by in vitro observations. About et al. brought human pulp tissue in contact with composite monomers in a non-toxic concentration. They demonstrated that this suppressed the differentiation of pulp cells into replacement odontoblasts. In contrast to the untreated pulp tissue, the synthesis of mineralised tissue was completely inhibited [57]. Contrary observations concerning cell proliferation were found for $Ca(OH)_2$. $Ca(OH)_2$ promotes the differentiation of odontoblasts or odontoblast-like cells, which will form hard tissue bridges in the pulp. Therefore, $Ca(OH)_2$ contributes actively to the formation of new hard tissue by induction and upregulation of the differentiation of odontoblast-like cells [58].

Conclusion

The hypothesis of this study could not be confirmed. The total number of proliferating cells was significantly in-

creased after direct pulp capping with GCB. Direct contact of GCB with vital pulp tissue leads to an increased formation of granulation tissue because of an inflammatory reaction. Hence, significantly more granulation tissue was formed after pulp capping with GCB when compared to $Ca(OH)_2$. This may be explained by missing antibacterial effect and foreign body reactions caused by the DA.

In contrast to the period between day 1 and 3, Höhl cells could be tagged after 1 week. This is an indication for the formation of reparative dentine. Significantly less Höhl cells were tagged in the GCB group. This leads to the conclusion that after direct pulp capping with GCB a significantly lower formation of reparative dentine occurred, when compared with $Ca(OH)_2$. This finding is in accordance with the LM findings after 70 days [56]. Therefore, GCB may have a negative effect on the activation of Höhl cells and subsequently of reparative dentine formation.

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Conflict of interest The authors declare that they have no conflict of interest.

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