Dental Traumatology 2008; **24**: 38–42; doi: 10.1111/j.1600-9657.2005.00400.x

Expression of proliferating cell nuclear antigen in pulp cells of extracted immature teeth preserved in two different storage media

Uğur Tekin[1], Andreas Filippi[2], Yango Pohl[3], Horst Kirschner[4]

[1]Department of Oral and Maxillofacial Surgery, School of Dentistry, Bornova-Izmir, Turkey; [2]Department of Oral Surgery, Oral Radiology and Oral Medicine, University of Basle, Basle, Switzerland; [3]Department of Oral Surgery, University of Bonn, Bonn, Germany; [4]Department of Oral Surgery, Justus-Liebig-University, Giessen, Germany

Corresondence to: Dr Uğur Tekin, Ege Üniversitesi Dişhekimliği Fakültesi, Ağız-Diş-Çene Hastalıkları ve Cerrahisi Anabilim Dalı, Bornova Kampüsü 35100 İzmir, Türkiey
Tel.: +90232 388 11 08
Fax: +90232 388 03 25, +90232 339 82 89
e-mail: utekin@yahoo.com

Accepted 28 June, 2005

Abstract – A specially composed medium for storing avulsed teeth has been developed. In experimental and clinical studies it could be shown that PDL cells could be kept viable during storage in the medium for up to 53 h. In the present study the medium was tested on pulp cells. A total of 40 immature unerupted third molars with open apices were removed surgically and the teeth were stored in a special cell culture medium (SCCM) or in Hank's balanced salt solution (HBSS) at room temperature for 6, 12, 18 or 24 h. Five teeth were assigned to each group. A total of seven consecutive pulp cross-sections per tooth were examined, resulting in a total of 280 specimens. Viable cells were marked using proliferating cell nuclear antigen (PCNA). The pulp was divided in three regions: apical region (0–0.5 mm), middle region (>0.5–1.5 mm) and coronal region (>1.5 mm). The labelling index (LI) was calculated for the whole cut (regions 1, 2 and 3) and for each region separately. The statistical evaluation was made using the One-way ANOVA and Mann–Whitney Test. Pulp cells of all teeth expressed PCNA. About 110 of 140 specimens in the SCCM and 101 of 140 specimens in the HBSS group showed PCNA-positive cells. The highest LI was observed within the apical region and decreased with increased distance from the medium. No marked cells were observed at a distance of more than 1.5 mm. The LI for both media showed a significant increase with storage intervals ($P < 0.05$). The pulp cells of teeth stored in SCCM showed a LI nearly twice as high compared to pulp cells of teeth stored in HBSS for the apical and middle region (time interval 6, 18 and 24 h: $P < 0.05$). The LI for the apical region was found to be 8.43% for the SCCM and 4.50% for the HBSS after 24 h. For the middle region the LI was found to be 2.02% for the SCCM and 0.81% for the HBSS after 24 h. Within the parameters of this study, it appears that the SCCM is able to maintain pulp cell viability better than HBSS. The use of special cell culture media in case of tooth avulsion may be beneficial.

Tooth avulsion accounts for 0.5–16% of traumatic injuries in the permanent dentition (1) and for 7–21% of injuries in the primary dentition (2). Avulsions of permanent teeth occur most often in 7–10-year old children, an age when the relatively resilient alveolar bone provides only minimal resistance to extrusive forces (1, 2). Tooth avulsion represents severe pulpal and periodontal injuries (3). The most common complications after avulsions are necrosis of the pulp and root resorption (1). The status of the pulp is one decisive object for periodontal healing. Pulp necrosis and consecutive infection cause infection related resorption, which is related to early tooth loss (4, 5). Pulp necrosis is predictable in mature teeth, and an endodontic treatment is strongly recommended. In immature teeth there is a chance of revascularization. Revascularization is dependent on the absence of an infection (6–9), on the diameter of the apical foramen and the length of the root (10), and on the extraoral storage conditions: The chance is about 60% after replantation within 1 min after avulsion but only around 10–30% when the avulsed teeth are stored for just 5 min in saliva or saline or in dry conditions before replantation (10).

Avulsed teeth are normally not replanted at the site of the accident (11). A tooth rescue box had been developed which contains a special cell culture medium (SCCM). It is commercially available in Europe since 1995 and in the USA (Dentosafe®, Dentosafe GmbH, Iserlohn, Germany; EMT Tooth Saver, SmartPractice.com, Phoenix, AZ, USA). According to *in vitro* investigations the medium maintains vitality and viability of PDL cells (cementoblasts, fibroblasts) for at least 48 h at room temperature (12, 13). In a clinical long-term study all avulsed and replanted teeth that had been stored in the medium within several minutes after avulsion showed healing with physiologic function (functional healing,

also named: favourite healing, normal healing). In that study all teeth had been endodontically treated before replantation (5, 14, 15). No data are available on the viability of pulp cells after storage of immature teeth in the special tissue culture medium.

The aim of the present study was an immunohistochemical evaluation of cell proliferation, assessed by proliferating cell nuclear antigen (PCNA) and in the healthy dental pulp from extracted immature human teeth.

Materials and methods

A total of 40 immature unerupted third molars with open apices and free of caries were removed surgically for prophylactic reasons in patients between 12 and 17 years of age at the Department of Oral Surgery, University of Giessen, Germany. This provided the investigation of healthy dental pulps. Immediately after extraction, the teeth were individually stored in 50 ml tubes containing 30 ml of a cell culture medium, either a specially prepared cell culture medium (Dentosafe; Dentosafe GmbH, Iserlohn, Germany, hereafter named SCCM) or Hank's balanced salt solution (HBSS). The teeth were stored at room temperature for 6, 12, 18 or 24 h. Thus eight groups were established. Five teeth were assigned to each group by drawing lots after tooth removal. Following fixation with formalin (4%) for 24 h, the width of the apical foramen was measured. The teeth were then decalcified over a period of 12 weeks using EDTA. All specimens underwent routine histologic processing (paraffin embedding), and 7-μ thick longitudinal serial cuts of the teeth were produced. A total of seven consecutive pulp cross-sections per tooth were examined, resulting in a total of 280 specimens. The specimens were stained immunohistochemically and the evaluation was performed with a light microscope at magnifications of 40, 160 and 400. The visualization of proliferating cells was obtained with the help of proliferating cell nuclear antigen (PCNA). The immunohistochemical preparation was performed according to the APAAP-method (Dianova, Hamburg, Germany). The monoclonal PC10-antibody (Dianova, Hamburg, Germany), defining an epitope of PCNA, served as the primary antibody. Development was performed with neofuchsine, which produces a dark red colour when reacting.

In every cut, the number of marked cells and the number of all cells were determined. Quantification was done by calculating the percentual labelling index (LI), the relation between the number of marked cells and the total number of cells. Starting from the tissue present most apically the pulp was divided in three regions: apical region (0–0.5 mm), middle region (> 0.5–1.5 mm) and coronal region (> 1.5 mm). The LI was calculated for the whole cut (regions 1, 2 and 3) and for each region separately. Mean values for each tooth (seven cuts) were determined and then for each of the eight groups. The distance from the apex to the marked cell situated most coronally was measured. The statistical evaluation was performed at the Institute of Statistics, Ege University, Izmir, Turkey, using the One-way ANOVA and Mann–Whitney Test.

Results

In all teeth marked cells were observed. About 110 of 140 specimens in the SCCM group and 101 of 140 specimens in the Hank's balanced salt solution (HBSS) group showed PCNA-positive cells. There was an increase of PCNA-positive specimens with time in the different media groups (Table 1). Statistics revealed no significant differences between the storage groups and time intervals ($P > 0.5$).

For both media, the PCNA-marked cells were mostly seen in the apical region (Figs 1–3). In the middle region the number of marked cells decreased, and in the coronal region no marked cells were visible. Independent from the region investigated no marked cells were observed in the odontoblastic layer.

Table 1. PCNA-positive specimens for both groups (all time intervals)

	PCNA-positive specimens	
Storage time (h)	SCCM	HBSS
6	24 (35)	22 (35)
12	26 (35)	24 (35)
18	29 (35)	26 (35)
24	31 (35)	28 (35)
Total	110 (140)	101 (140)

PCNA, proliferating cell nuclear antigen; SCCM, special cell culture medium; HBSS, Hank's balanced salt solution.

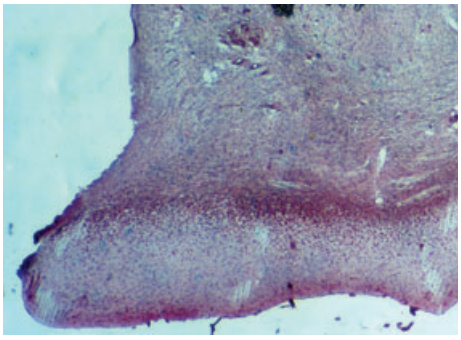

Fig. 1. PCNA-positive cell in the apical region of the pulp. Storage in SCCM for 12 h at room temperature (Original magnification 10×).

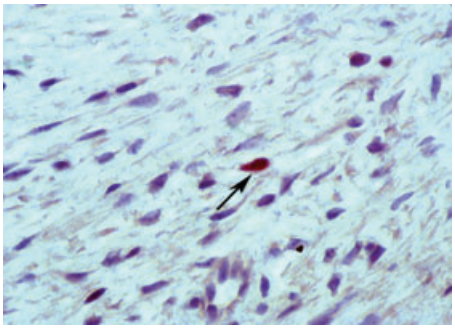

Fig. 2. PCNA-positive cell in the apical region of the pulp. Storage in HBSS for 6 h at room temperature (Original magnification 160×).

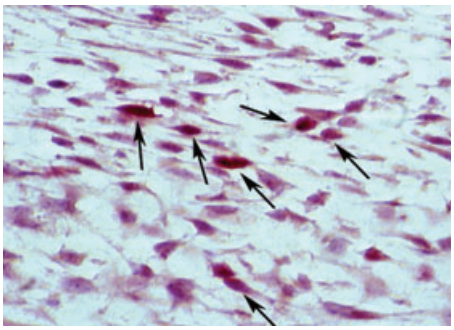

Fig. 3. Proliferating cells at the apical pulp wound. Storage in SCCM for 24 h at room temperature. Immunohistochemical staining with PCNA (Original magnification 160×).

The maximal distance of marked cells from the apex was 1.5 mm after storage in SCCM and 1.40 mm after storage in HBSS. Statistics revealed no significant differences between the storage groups and time intervals ($P > 0.5$).

There was an increase in the LI with time, in the different media groups (SCCM, HBSS) and in the different regions (apical, middle). The LI approximately doubled every 6 h. The differences between the time groups were significant (One-way ANOVA-Test, $P < 0.05$). No statistical significance was found in the relation 6–12 h in the SCCM group for the middle region ($P = 0.212$).

The pulp cells of teeth stored in the SCCM showed an IL nearly twice as high compared to pulp cells of teeth stored in HBSS in all time intervals and regions. The geometrical mean values and their corresponding 95% confidence intervals are shown in (Figs 4 and 5, Tables 2 and 3). The differences were significant (ANOVA-Test, apical region, for 6-, 18- and 24-h time intervals: $P < 0.01$; middle region, for 6-, 18- and 24-h time intervals: $P < 0.05$). Statistics revealed no significant differences for the 12-h time interval for both regions ($P > 0.1$).

The minimal width of the apical foramen was 1.6 mm in all teeth, the median was 2.3 mm in teeth stored in SCCM and 2.4 mm in teeth stored in HBSS. Statistics revealed no significant differences between the storage groups ($P > 0.5$).

Discussion

Avulsion is a serious injury to teeth. The PDL is damaged, predominantly due to inadequate storage until replantation. The pulp tissues undergo necrosis. In replanted immature teeth with a wide-open apical foramen and short roots, there is a chance of revascularization (1, 4, 10, 16). However the risk of a persistend pulp necrosis and a consecutive infection is high. Endodontic treatment is necessary but very demanding especially in immature teeth. Apexification with long-term use of calcium hydroxide is related to a very high incidence of cervical root fractures (17, 18). Infection related resorption – an endodontic problem – is observed in 37% of replanted immature teeth (19). An extraoral

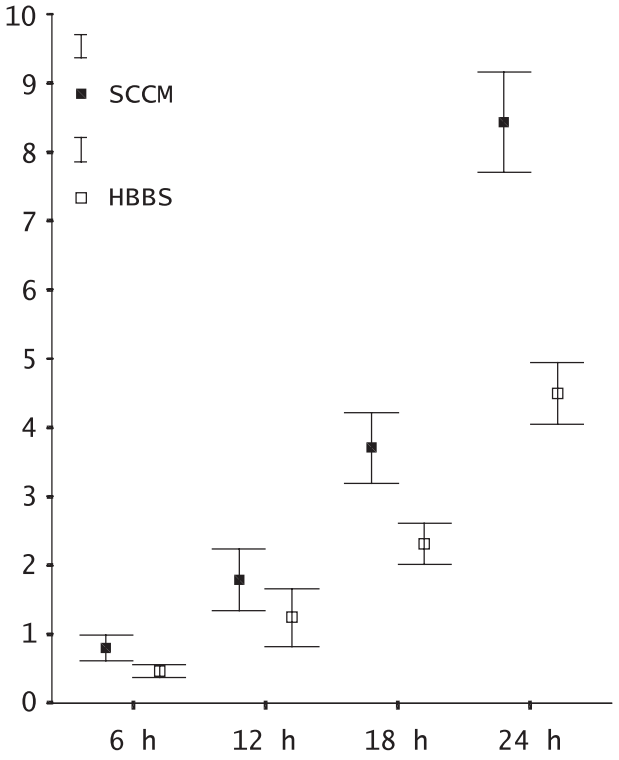

Fig. 4. Boxplot of LI values for apical region (Geometrical mean values and their corresponding 95% confidence intervals for apical region).

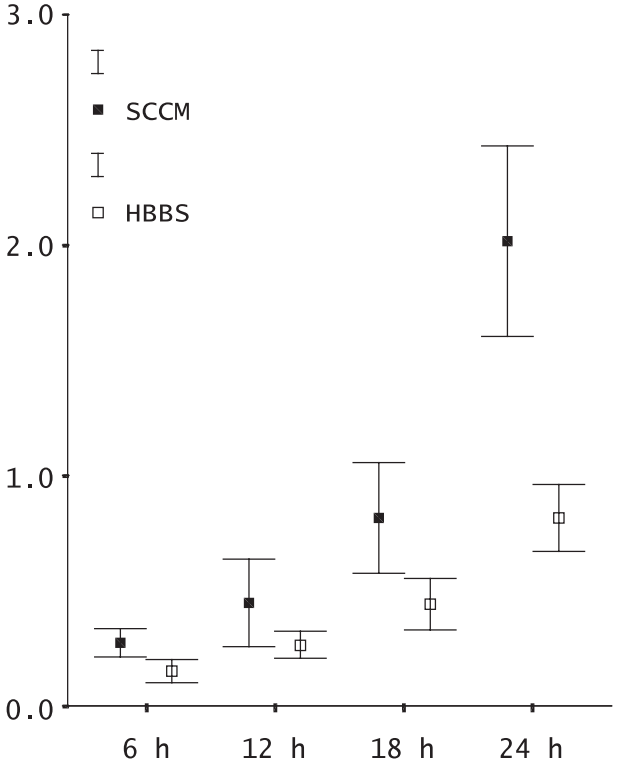

Fig. 5. Boxplot of LI values for middle region (Geometrical mean values and their corresponding 95% confidence intervals for middle region).

Table 2. LI values for both groups for the apical region (all time intervals)

	Storage time (h)	*n*	Mean	SD	SE	95% confidence interval for mean		Minimum	Maximum
						Lower bound	Upper bound		
SCCM group	6	5	0.79	0.14	0.06	0.61	0.98	0.67	1.04
	12	5	1.79	0.36	0.16	1.33	2.24	1.48	2.29
	18	5	3.70	0.41	0.18	3.18	4.22	3.24	4.16
	24	5	8.42	0.58	0.26	7.69	9.15	7.60	9.23
	Total	5	3.68	3.03	0.67	2.26	5.10	0.67	9.23
HBSS group	6	5	0.46	0.07	0.03	0.36	0.55	0.35	0.56
	12	5	1.24	0.34	0.15	0.81	1.66	0.87	1.63
	18	5	2.31	0.24	0.10	2.01	2.61	2.03	2.60
	24	5	4.49	0.36	0.16	4.04	4.95	4.13	5.06
	Total	5	2.12	1.57	0.35	1.39	2.86	0.35	5.06

SCCM, special cell culture medium; HBSS, Hank's balanced salt solution.

Table 3. LI values for both groups for the middle region (all time intervals)

	Storage time (h)	*n*	Mean	SD	SE	95% confidence interval for mean		Minimum	Maximum
						Lower bound	Upper bound		
SCCM group	6	5	0.27	0.04	0.02	0.21	0.33	0.19	0.32
	12	5	0.44	0.15	0.06	0.25	0.63	0.21	0.63
	18	5	0.81	0.19	0.08	0.57	1.05	0.57	1.10
	24	5	0.01	0.33	0.14	1.60	2.43	1.73	2.42
	Total	5	0.88	0.72	0.16	0.54	1.22	0.19	2.42
HBSS group	6	5	0.15	0.04	0.01	0.10	0.20	0.10	0.21
	12	5	0.26	0.04	0.02	0.20	0.32	0.22	0.33
	18	5	0.44	0.08	0.04	0.33	0.55	0.34	0.59
	24	5	0.81	0.11	0.05	0.66	0.95	0.68	0.96
	Total	5	0.41	0.26	0.05	0.29	0.54	0.10	0.96

endodontic treatment prevents these complications but is limited to teeth with round or oval-round root canals (5). A higher incidence of revascularization in teeth replanted without extraoral endodontic treatment would be beneficial: repair usually occurs by an ingrowth of new blood vessels and the replacement of necrotic pulp tissue by new tissue elements (20). Successful revitalization of teeth may lead to continued root development and usually results in the deposition of calcified tissue within the root canal, which may strengthen the existing root structures (21). This increases the potential for long-term retention of the teeth (6, 9).

Factors influencing the revascularization that can be affected are extraoral storage conditions and the topical use of antibiotics. In animal experiments the rate of revascularization increased from 18 to 41% after storage of the isolated teeth in saline or saliva for 1 h when doxycycline was applicated topically (6). Topical application of minocycline seems to enhance these results (8). In a clinical study even short storage in unphysiologic conditions – i.e. in dry conditions or in saline or saliva – decreased the rate of revascularization to 10–30% while it was 60% after immediate replantation (10). Dry storage and media like saline and saliva are known to be detrimental to vital tissues and result in cell damage and fast cell death. Cell culture media maintain vitality and viability of cells. A medium specially composed for the storage of avulsed teeth had been developed. *In vitro* it maintained the viability of PDL cells as measured by autoradiography and in cell cultures, but pulp cells were not investigated (12, 13, 22). With longer storage periods (from 6 to 48 h) the IL of PDL cells increased (12, 22). In a clinical long-term study all avulsed teeth that were stored within few minutes in the medium exhibited functional healing after replantation. However all the teeth in that study had been immediately treated endodontically (5, 14, 15) and no conclusion can be drawn for the maintenance of dental pulp cells.

This medium was also used for the present investigation. As observed for PDL cells the LI of pulp cells increased with time. Following wounding tissue regeneration is initiated but takes some time for up-regulation. A prerequisite for cell proliferation is that the cells feel 'comfort' and are equipped with nutrients. This could be ensured by the special culture medium and – on a lower level – by HBSS. However this supply can only be afforded by diffusion in isolated teeth and is therefore restricted to areas that have a local relation to the medium. The highest LI was observed within a narrow region of 0.5 mm width and decreased with increased distance from the medium. No marked cells were observed at a distance of more than 1.5 mm. Whether the cells at a higher distance from the medium could be kept vital (without demonstrating viability) was not investigated.

It cannot concluded from this study whether the maintenance of viability (or vitality) of pulp cells is advantageous for a revascularization. However when cells are a vital these have to be actively resorbed before healing can take place. In a clinical study the rate of

revascularization decreased rapidly when the teeth were stored for even very short periods in inadequate media, which are known to be detrimental for cell vitality (23). In drawing a reversal conclusion the maintenance of cell vitality or viability should be helpful for revascularization. On the other hand also microorganisms may profit from a good nutrition as offered by cell culture media. To hinder unlimited growth of microorganisms the SCCM contains a preservative, and no complications related to a bacterial overgrowth were observed in the *periodontal* healing of avulsed teeth that had been stored in the medium for up to 53 h (14). However, after replantation, the PDL and adhering microorganisms can be easily accessed by defence mechanisms while the anatomic situation of the pulp is different and makes a successful defence reaction more difficult.

The depth of diffusion that is obviously achieved has an implication for the maintenance of the PDL that is adhering to the root of an avulsed or otherwise isolated tooth. The PDL has a normal width of 0.2 mm, and it is normally ruptured in the middle by extraction (and presumably by avulsion). Thus it can obviously completely provided with nutrients by diffusion. Accordingly it could be shown that cells in the cementoblastic layer prepared for cell dublication after storage for up to 48 h in the medium (12, 22). Clinically, teeth showed functional healing predictably after storage for up to 53 h in the medium when they were stored in the rescue box soon after avulsion (14). Thus a sufficient long-term maintenance must have been afforded by the medium.

The LI after storage in SCCM was nearly twice as high when compared to HBSS. The special medium seems to better promote the cells with nutrients. HBSS is a pure salt solution while the special medium also contains aminoacids, vitamins, glucosis. Whether this has any implication for revascularization after replantation cannot be concluded from this study.

Acknowledgements

This work was supported by TUBİTAK (The Scientific and Technical Research Council of Turkey).

References

1. Andreasen JO, Andreasen FM. Avulsions. In: Andreasen JO, Andreasen FM, editors. Textbook and colour atlas of traumatic injuries to the teeth. Copenhagen: Munksgaard, 1994; pp. 383–425.
2. Andreasen JO. Etiology and pathogenesis of traumatic dental injuries. A clinical study of 1298 cases. Scand J Dent Res 1970;78:329–42.
3. Berggreen E, Sae-Lim V, Bletsa A, Heyeraas KJ. Effect of denervation on healing after tooth replantation in the ferret. Acta Odontol Scand 2001;59:379–85.
4. Andreasen JO, Hjörting-Hansen E. Replantation of teeth. I. Radiographic and clinical study of 110 human teeth replanted after accidental loss. Acta Odontol Scand 1966;24:263–86.
5. Pohl Y, Filippi A, Kirschner H. Results after replantation of avulsed permanent teeth. I. Endodontic considerations. Dent Traumatol 2005;21:80–92.
6. Cvek M, Cleaton JP, Austin J, Lownie J, Kling M, Fatti P. Effect of topical application of doxycycline on pulp revascularization and periodontal healing in reimplanted monkey incisors. Endod Dent Traumatol 1990;6:170–6.
7. Cvek M, Cleaton JP, Austin J, Lownie J, Kling M, Fatti P. Pulp revascularization in reimplanted immature monkey incisors – predictability and the effect of antibiotic systemic prophylaxis. Endod Dent Traumatol 1990;6:157–69.
8. Ritter AL, Ritter AV, Murrah V, Sigurdsson A, Trope M. Pulp revascularization of replanted immature dog teeth after treatment with minocycline and doxycycline assessed by laser Doppler flowmetry, radiography, and histology. Dent Traumatol 2004;20:75–84.
9. Yanpiset K, Trope M. Pulp revascularization of replanted immature dog teeth after different treatment methods. Endod Dent Traumatol 2000;16:211–7.
10. Andreasen JO, Borum MK, Jacobsen HL, Andreasen FM. Replantation of 400 avulsed permanent incisors. 2. Factors related to pulpal healing. Endod Dent Traumatol 1995;11:59–68.
11. Trope M. Clinical management of the avulsed tooth: present strategies and future directions. Dent Traumatol 2002;18:1–11.
12. Pohl Y, Kirschner H. Autoradiographische Untersuchungen zur Erfassung von Zellneubildung im Restdesmodont isolierter Zähne des Menschen. Dtsch Z Mund Kiefer GesichtsChir 1994;18:224–7.
13. Pohl Y, Tekin U, Boll M, Filippi A, Kirschner H. Investigations on a cell culture medium for storage and transportation of avulsed teeth. Aust Endod J 1999;25:70–5.
14. Pohl Y, Filippi A, Kirschner H. Results after replantation of avulsed permanent teeth. II. Periodontal healing and the role of physiologic storage and antiresorptive-regenerative therapy (ART). Dent Traumatol 2005;21:93–101.
15. Pohl Y, Wahl G, Filippi A, Kirschner H. Results after replantation of avulsed permanent teeth. III. Tooth loss and survival analysis. Dent Traumatol 2005;21:102–10.
16. Kirschner H. Atlas der chirurgischen zahnerhaltung, 2nd edn. München: Hanser, 1996.
17. Andreasen JO, Farik B, Munksgaard EC. Long-term calcium hydroxide as a root canal dressing may increase risk of root fracture. Dent Traumatol 2002;18:134–7.
18. Cvek M. Prognosis of luxated non-vital maxillary incisors treated with calcium hydroxide and filled with gutta-percha. A retrospective clinical study. Endod Dent Traumatol 1992;8:45–55.
19. Andreasen JO, Borum MK, Jacobsen HL, Andreasen FM. Replantation of 400 avulsed permanent incisors. 1. Diagnoses of healing complications. Endod Dent Traumatol 1995;11:51–8.
20. Johnson WT, Goodrich JL, James GA. Replantation of avulsed teeth with immature root development. Oral Surg Oral Med Oral Pathol 1985;60:420–7.
21. Heithersay GS. Replantation of avulsed teeth: a review. Aust Dent J 1975;20:63–72.
22. Pohl Y. Autoradiographische Untersuchungen zur Erfassung von Zellneubildung im Restdesmodont isolierter Zähne des Menschen. Med Diss: Gießen, 1994.
23. Andreasen JO, Borum MK, Jacobsen HL, Andreasen FM. Replantation of 400 avulsed permanent incisors. 4. Factors related to periodontal ligament healing. Endod Dent Traumatol 1995;11:76–89.