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Interstitial fluid drainage from rat apical area takes place via vessels in the mandibular canal

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We sought to investigate the transport route for protein-rich fluid from the apical area towards the draining lymph nodes. The first mandibular molar root canals in 24 female Wistar rats were instrumented and filled with radioactive-labelled human serum albumin. The rats were sacrificed at different intervals beginning after 10 min (time 0) and continuing up to 72 h. Three jaw segments, gingiva around the first molar, blood samples, submandibular and cervical lymph nodes were collected and analyzed for radioactivity. The starting volume of tracer (control) for all experiments was calculated from measurements at time 0. At time 0, radioactivity was only detected in the jaw segments. Within lymph nodes and serum, the tracer was found after 4 h, with the highest amount recorded in serum up to 24 h. Lymphatics were found within the mandibular canal along blood vessels and nerves and exiting via foramen mandibularis, after immunohistochemical staining in four untreated rats. Our results show tracer distribution from the apical area towards the mandibular canal in a posterior direction. The tracer washout rate was low, and the fluid was mainly absorbed into blood vessels. The lymphatics in the mandibular canal may be more important for immune cell transport than for fluid drainage.

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Lymphatic vessels drain excess protein-rich fluid from the interstitial space and also transport immune cells for antigen presentation in regional lymph nodes. The dental pulp is frequently challenged by pathogens, which can result in pulpitis and, if left untreated, the infection can spread outside the pulpal compartment and promote apical periodontitis. In an acute phase of apical periodontitis, the patients can experience swollen regional lymph nodes due to the transport of bacterial products or inflammatory mediators from the inflamed periapical area (1,2). During inflammation, plasma-derived proteins leak out of blood vessels into the interstitial compartment. As uptake of fluid containing larger proteins rarely takes place in blood vessels, protein-rich fluid transport from the inflamed area must occur in lymphatic vessels.

Several recent studies have shown that lymphatic vessels are not found in the dental pulp or in the apical periodontal ligament (3-6), whereas gingiva is well supplied with lymphatic vessels (7). The teeth and surrounding periodontal ligament represent one of few areas in the body without lymphatic vessels. Other exceptions are the bone marrow, bone and, central parts of the brain. In an elegant study on mice, Bhingare *et al.* (8) demonstrated that it took 16 h for dendritic dental pulp cells to move to regional lymph nodes after dentin exposure. The questions we seek to answer here are how protein-rich fluid is transported from the apical area and where the lymphatics that drain excess fluid from the pulp and apical periodontal ligament are located. We also aim to measure the washout rate of fluid from the apical area and test the hypothesis that drainage, at least in part, takes place via gingival lymphatics.

Material and methods

Experimental animals

The research protocol comprised details on background, planned sample size, study design, and experimental outcomes; it was approved by the Norwegian Food Safety Authority and conducted under supervision of the local Animal Welfare Body at the Laboratory Animal Facility, University of Bergen, Norway. The ARRIVE guidelines were followed in the report of the animals used (9). We used a total of 34 female Wistar rats (Mol:WIST Han) weighing 206–230 g. The animal sample size was determined in accordance with animal welfare concerns and for the need of a pilot study for optimization of the procedure, as well as for a minimum number that would provide reproducibility and validation of the observations. As

such, the aim was to observe distribution of protein-rich fluid in a minimum of 6 jaws per observation time by use of a radioactive tracer. Details on the distribution of used animals and experimental outcomes are provided in the text under each procedure. All rats were housed in polycarbonate cages (2–3 rats per cage) and fed a standard pellet diet (801157W Expanded Pellets; Stepfield, Witham, Essex, UK) with tap water ad libitum.

Radioactive protein-rich fluid distribution in the lower jaw

Following a 7–10 d period of acclimatization, the rats were anesthetized with a mixture of medetomidine (Domitor 1 mg mL^{-1}; Orion Pharma, Espoo, Finland), 0.4 mg kg^{-1} body weight, and ketamine (Ketalar 50 mg mL^{-1}; Pfizer, Sollentuna, Sweden), 60 mg kg^{-1} body weight.

Dental pulps of bilateral mandibular first molars were exposed and removed with 0.25 mm carbide burs. In order to establish the working length, one rat was sacrificed by overdose of anesthesia after pulp exposure and the lower jaws were removed. The bone surrounding the first molar was scraped off and K-files #0.6 to #15 were inserted in the mesial and distal canals. Once the first endodontic file tip was protruding the apex, the length was measured in each canal. We first established a working length using mesial and distal coronal cusps as references with the file at the apical constriction (4.5 mm length) in three rats, but due to no tracer distribution outside the first molar up to 4 h after application, we had to try another approach. We therefore extended the working length by 0.5 mm and opened the apical constriction in all remaining experiments for draining purposes.

The mesial and distal canals were instrumented with K-files #0.6 to #15 in increasing order, followed by SX rotary file, ProTaper Universal (Dentsply Sirona, York, PA, USA) to the established working length of 5 mm.

Following instrumentation, the canals were dried with paperpoints. To avoid radioactive contamination of surrounding tissues, OpalDam (Ultradent, South Jordan, UT, USA) was applied around the first molar. The canals and pulp chambers were filled thereafter with an average of 4–6 μl ^{125}I labelled human serum albumin (^{125}I-HSA, average dose of 922.944 counts per μl) and sealed with Cavit (3M; ESPE, Minneapolis, MN, USA). The HSA was labelled with ^{125}I using iodogen as previously described in detail (10). In summary, a solution of 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril (product number T0656; Sigma-Aldrich, St Louis, MO, USA) in chloroform (1:1) was prepared and transferred into a 1.8-ml Nunc vial (Nunc-Kamstrup, Roskilde, Denmark). Chloroform was evaporated under nitrogen forming water-insoluble Iodo-Gen in the Nunc vial. Next, 1.5 mg of compound was added to 1 ml of 0.05 M PBS with 15 µl of 0.01 MNaI and 10 MBq ^{125}I (PerkinElmer, Waltham, MA, USA). The tracer solution was dialyzed against 1 l of 0.9% of saline and 0.02% azide to remove unincorporated isotopes. The labelled probes were additionally purified using a 40 kDa cut-off spin filter (Amicon, Danvers, MA, USA).

The rats included for analysis were sacrificed after 10 min (control), and at 4, 6, 8, 24, 48, or 72 h by an overdose of anesthesia (Pentobarbital/Mebumal 50 mg mL^{-1}; Svaneapoteket, Bergen, Norway). Atipamezole (Antisedan; 0.5 mg kg^{-1} body weight; Orion Pharma, Oslo, Norway) and pain relief medication Buprenorfin (Vetergesic; 0.05 mg kg^{-1} body weight; Orion Pharma) were administered to all rats except controls. Awake animals exhibited normal behavioral habits during the observation time.

At the end of the experiments, heart blood samples, submandibular and cervical lymph nodes, and lower jaws were collected. In six rats, a unilateral endodontic file separation took place. The unilateral jaw and corresponding lymph nodes were excluded from further analysis, which led to the use of three additional rats and a total of twenty-four animals in seven groups in order to reach reproducibility and equal numbers of observations for each time point. The jaws were separated in three parts comprising: first molar and bone (M1), second and third molar and bone (M2 + M3), and posterior part of corpus and ramus mandibularis (posterior tissue). Gingiva around the first molar was also removed. The blood was centrifuged and serum collected. All samples (jaw parts, gingiva, lymph nodes, and serum) were analyzed for radioactive tracer in a gamma counter (LKB-Wallac; model 1282 Compugamma; Wallac, Turku, Finland). Two μl ^{125}I-HSA were also counted for each separate experiment to measure radioactivity per μl. To estimate the starting volume of tracer for all experiments (control), we harvested all tissue from three rats immediately after completion of the above-described procedure [all tissues were harvested within 10 min (time 0) after application of ^{125}I-HSA]. Radioactivity was detected in all three parts of the jaw, but not observed in the blood or in the cervical lymph nodes. The total amount of tracer in the three jaw parts related to the concentration of radioactivity in 1 μl ^{125}I-HSA was used for calculation of starting volume. The average starting volume (results from three rats) was set to 100%, and the results for each jaw part in all experiments are presented in percentage of starting volume. The counts of radioactive tracer within the lymph nodes and serum were calculated as a percentage of that in 1 μl ^{125}I-HSA measured for each experiment, which was set to 100%.

In order to visualize how fluid distributed from the root canals, the lower first molars of two rats were injected with India ink instead of ^{125}I-HSA and the tissues immediately harvested for histological evaluation by routine hematoxylin-eosin (H&E) staining (Fig. 1).

Immunohistochemistry

Lower jaws from four untreated rats were subjected to immunohistochemical analysis for the presence of lymphatics using previously described protocols (11). In short, the jaws were fixed in 4% paraformaldehyde (PFA), decalcified in 10% ethylenediaminetetraacetic acid (EDTA) and either embedded in paraffin or frozen. Parasagittal and sagittal sections of 10–20 μm thickness were used for single staining with the avidin-biotin peroxidase (ABC) method and 3,3′-diaminobenzidine (DAB) as the chromogen. Primary antibodies against podoplanin (mouse anti-rat; 1:600; Acris/OriGene, Herford, Germany/USA) and lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1; dilution 1:500; Abcam, Cambridge, UK) were used. The specificity of all immune reactions was tested by omission of the primary antibodies or substitution with isotype controls. Gingiva from the sectioned jaws known for the presence of lymphatics (7) served as positive control.

All sections were visualized with a photomicroscope (Nikon Eclipse E600; Nikon, Kanagawa, Japan) connected

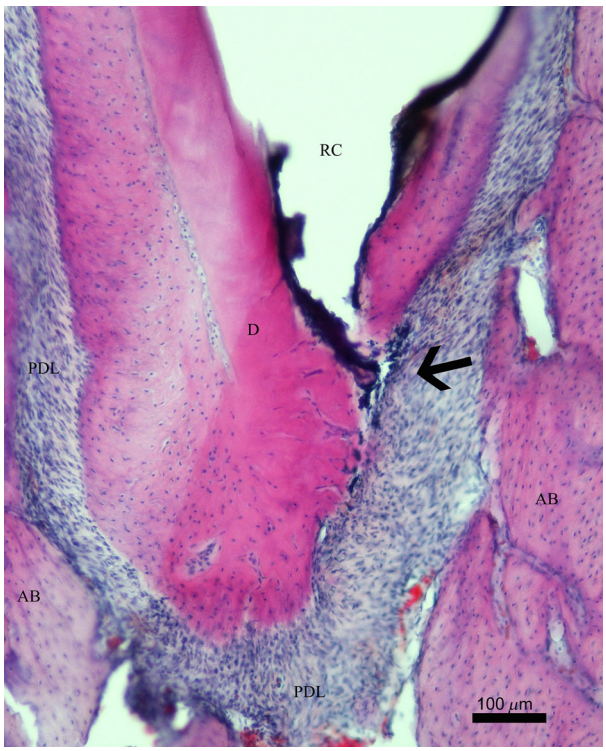

Fig. 1. Representative image of the apical root canal (RC) in a first mandibular molar following instrumentation and application of India ink. The dye is seen in the canal leaving through the apical foramen and in the periodontal ligament (arrow). AB, alveolar bone; D, dentin; PDL, periodontal ligament.

to a digital camera employing Lucia imaging software (Lucia, v. 480; Laboratory Imaging, Hostivař, Czech Republic).

Statistical analyses

Statistical analysis was performed using the GRAPHPAD PRISM 5 software (GraphPad, San Diego, CA, USA). The radioactivity in each sample at each time point was analyzed by multiple group comparisons using one-way or two-way ANOVA and Tukey's post-hoc test; a P value of <0.05 was considered statistically significant.

Results

When we extended the working length and opened the apical constriction, part of the applied ^{125}I-HSA tracer left the canal through the apical foramen (illustrated with India ink, see Fig. 1) and was immediately distributed (time 0) to the two posterior parts of the jaw (M2 + 3 and posterior tissue) within the mandibular canal. At time 0 we observed no radioactivity in the serum, lymph nodes or in gingiva. The highest amount of radioactive tracer was found within M1 at all time points (Fig. 2A). In the two posterior parts of the jaw (M2 + M3 and posterior tissue), the highest level of tracer was also observed at time 0 and declined thereafter to lower, and relatively even, levels for up to 24 h (Fig. 2B). From 24 h there was a further decline, and at 72 h only 0.3% and 0.1% was found in M2 + M3 and posterior tissue, respectively (Fig. 2B).

Within the lymph nodes and serum, evidence of the radioactive tracer was found after 4 h. While still minimal, the highest amount of tracer was recorded after 8 h in the lymph nodes, declining to negligible values thereafter. The amount of radioactive tracer in the serum was highest after 24 h ($P < 0.05$) and significantly decreased to undetectable levels after 3 d (Fig. 3). In the gingiva, negligible levels of tracer were detected from 4 h up to 72 h (data not shown).

Immunohistochemical analysis of the jaws revealed LYVE-1$^+$ and podoplanin$^+$ lymphatic vessels within the mandibular canal in close proximity to blood vessels and nerve bundles, exemplified in Fig. 4A,B. Lymphatic vessels were also seen in the posterior part of the mandibular canal exiting via the mandibular foramen. No lymphatic vessels were observed in the periodontal ligament, but lymphatic vessels were present underneath the apical periodontal ligament, towards the mandibular canal (Fig. 4C).

Discussion

In this study, we followed the distribution of radio-labelled albumin from the apical area of the first molar towards the posterior parts of mandibular jaws, as well as its dispersal in cervical lymph nodes and the blood stream over a 3 d period.

In cases of routinely performed endodontic treatment, the apical constriction is not enlarged during instrumentation. However, under the current experimental setup, following the initial observations from three animals for a period of up to 4 h, there was no distribution of the tracer beyond the apical constriction of the first mandibular molars. Since the main scope of these experiments was to determine how protein-rich fluid is transported from the periapical area, we were forced to use a different approach, and we decided to instrument the canals beyond the apical constriction by 0.5 mm. Nevertheless, the maximal apical diameter of instruments employed in these experiments did not exceed 0.19 mm, corresponding to the last used ProTaper Sx file. In jaw sections, a minimal enlargement of the apical constriction in first mandibular molars was observed, as seen in Fig. 1.

Following our extension of the working length and opening of the apical constriction in the first molar, the radioactive tracer was immediately distributed to the posterior parts of the lower jaw. At this time point (time 0, up to 10 min), no radioactivity was observed in serum, lymph nodes, or in gingiva. The protein-rich fluid with tracer must therefore have been transported in the interstitial tissue from the apical area into the mandibular canal. Probably, the interstitial tissue in the mandibular canal has a high conductance that allows fast transport of the tracer to the posterior parts. Nevertheless, a decline of radioactive tracer occurred after 4 h, stayed relatively stable up to 24 h, and further

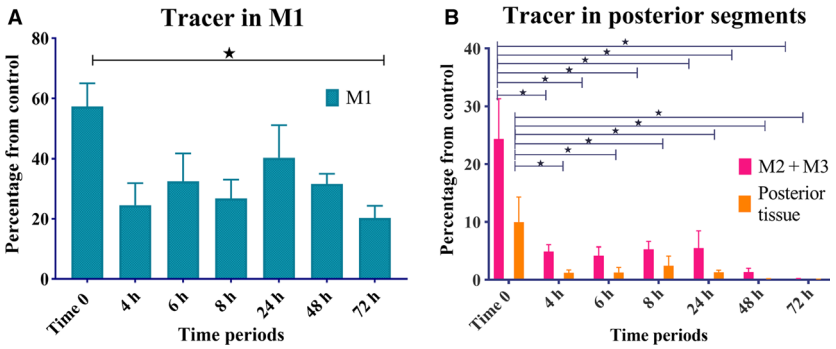

Fig. 2. Presence of radioactive tracer within the jaws calculated as percentage from control (time 0). (A) The highest amount of ^{125}I-HSA is seen in the first molar and bone (M1) at time 0 with a decline thereafter. (B) In the two posterior segments, the peak amount of tracer is seen at time 0, with a stable lower level up to 24 h, before a substantial decline is found after 72 h. The bars represent means with SEM. (*$P < 0.05$. One-way and two-way ANOVA; Tukey's post-hoc test).

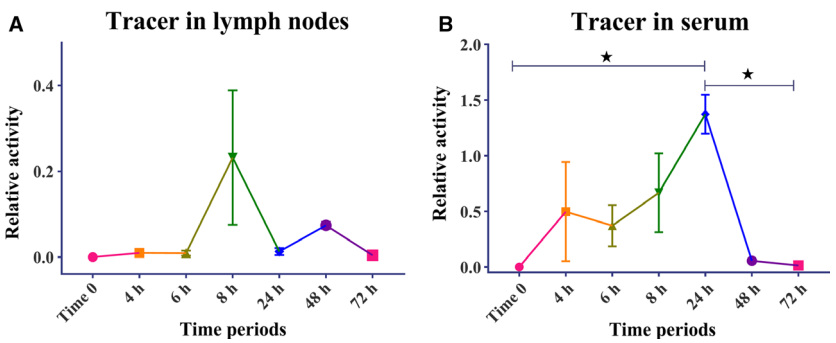

Fig. 3. Relative activity of ^{125}I-HSA within lymph nodes (A) and serum (B) calculated as percentage of that in 1 µl isotope (100%). The graphs represent mean values with SEM. Percentage values were multiplied with 10^2 (*$P < 0.05$. One-way ANOVA; Tukey's post-hoc tests).

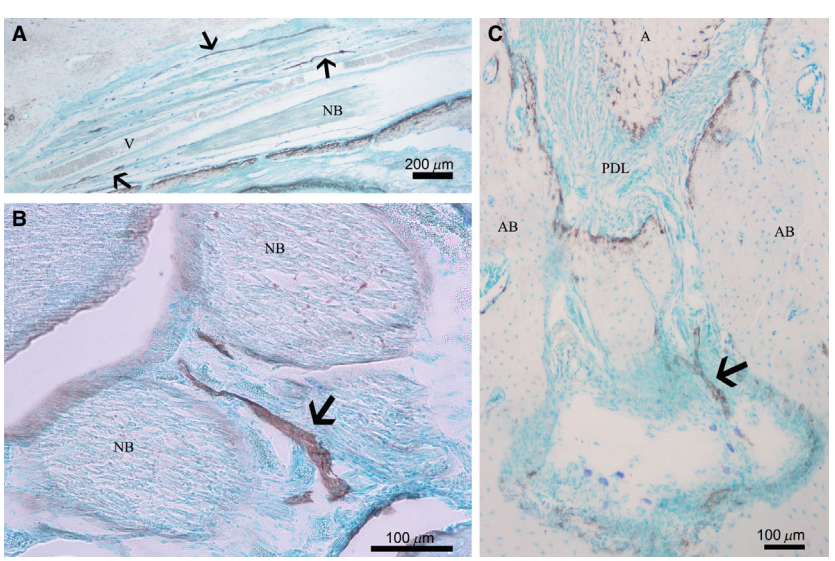

Fig. 4. Representative images of podoplanin$^+$ lymphatic vessels (arrows) within mandibular jaws. Lymphatics are seen on sagittal and transverse sections, along blood vessels (V) and nerve bundles (NB) within the mandibular canal (A and B). No lymphatic vessels are seen within the periodontal ligament; however, a vessel can be seen underneath the apical periodontal ligament (arrow), towards the mandibular canal (C). A, apex; AB, alveolar bone.

dropped until the 72-h observation time in the posterior parts of the jaws. At the same time, a substantial amount of tracer remained inside the first molar during all time periods and was never transported out.

Transport of protein-rich fluid inside and out of the mandibular canal can take place via blood vessels, lymphatics, or within the interstitium. The reason for this slow washout of tracer from the canal is likely due to small changes in interstitial fluid pressure in this bone-embedded tissue. Tissue pressure increase is a prerequisite for opening up lymphatic vessels, and for fluid transport in the interstitial tissue, a pressure gradient must exist (12). A combination of both small pressure changes and little driving forces are likely the reasons for the slow washout observed. It is possible that chewing promotes tracer washout by creating small tissue pressure increases in the periodontal ligament that can be transferred into the canal. The relatively stable levels of tracer up to 24 h may be explained by moderate chewing activity in this timeframe after induction of anesthesia and operative procedures. Some protein-rich fluid in the interstitium may also exit the canal via foramen mandibularis.

In the lymph nodes and serum, radioactivity was observed from 4 h after tracer application. Nevertheless, the level of radioactive tracer within the lymph nodes remained very low, whereas higher amounts were detected in serum (Fig. 3). This may imply that interstitial fluid from the apical area is mainly absorbed into blood capillaries and transported out of the jaw by blood vessels, and that the lymphatic vessels play little part in this transport. A similar observation was done in the kidney, where infusion of ^{125}I-HSA tracer in the interstitium in the medulla gave a rise in plasma tracer level, demonstrating absorption of tracer into local capillaries (13). Interestingly, there appeared to be a substantial increase of tracer transport 8 h following injection, dropping again at 24 h post injection, suggesting a high lymphatic clearance lagging after the clearance to plasma.

After 72 h, a dramatic drop in radioactivity was observed in all compartments, likely due to degradation of the tracer in the liver followed by clearance of the radioactive component out of the body. One disadvantage of the method used is that it does not allow us to observe tracer uptake in lymphatic vessels. The bone embedment makes it difficult to visualize the uptake in lymphatics by imaging techniques in the mandibular canal. However, we do show podoplanin^{+} lymphatic vessels in all parts of the mandibular canal, and vessels were observed below the apical periodontal ligament of the first molar. Our observation is in line with those in humans and mice where lymphatic vessels have been observed in the mandibular canal in close proximity to nerve bundles and blood vessels (6,14). In a study on sheep injected with radioactive albumin in gingiva, as much as 64.7% of the injected protein was recovered in the prescapular and cervical lymph vessels 7.5 h after injection (15). The gingiva is well supplied with lymphatic vessels (7,16,17), and when lymph is formed, muscle movements and soft tissue deformation may promote a rapid transport of fluid via the lymphatic system. This is in contrast to the bone-embedded interstitial tissue in the mandibular canal, and may explain why we observed a much slower washout rate from this compartment. However, it is likely that when excess fluid is drained out of the pulp to the apical periodontal ligament, tooth movement during chewing will compress the periodontal ligament and promote movement of the fluid into the mandibular canal. It is unlikely that the fluid will be drained upwards into the cervical periodontal ligament due to simultaneous compression and pressure increase in all parts of the periodontal ligament. We observed negligibly low levels of tracer from 4 h observation time in our gingival samples in awake rats. This finding supports the above-mentioned hypothesis that fluid from the apical periodontal ligament is not drained via gingival lymphatics. If drainage was to take place via gingiva, it would have opened the possibility for spreading of inflammatory products from the apical area to the gingiva. Our results indicate that this does not occur and that inflammatory products reach the gingiva via blood vessels (systemic effects). This is important information for the clinician and means that a patient with apical periodontitis is not at risk for developing gingival inflammation as long as the lesion is limited to the apical area. However, whether immune cell transport from the pulp and apical area to the lymph nodes only takes place via lymphatic vessels in the mandibular canal is unknown and requires further investigation.

To summarize, we have shown that protein-rich fluid from the apical periodontal ligament is transported into the mandibular canal where it rapidly distributes in its full canal length. It is likely that most protein-rich fluid left the canal via blood vessels, due to higher levels of radioactive tracer in serum than in the lymph nodes. The lymphatics within the mandibular canal are probably more important for immune cell transport than for interstitial fluid uptake from the periapical area.

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Conflicts of interest – The authors state no conflicts of interest.

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