รายงานวิจัยฉบับสมบูรณ์

โครงการวิจัยเรื่องการปวดฟันและการไหลเวียนเลือด
ของเนื้อเยื่อในโพรงฟันในภาวะที่เนื้อเยื่อในโพรงฟัน
ปกติและอักเสบในมนุษย์

โดย

รองศาสตราจารย์ ทพ.ดร.นพคุณ วงษ์สวรรค์และคณะ
ภาควิชาชีววิทยาช่องปาก
คณะทันตแพทยศาสตร์ มหาวิทยาลัยมหิดล

กรกฎาคม 2555

กิตติกรรมประกาศ

ผู้วิจัยใคร่ขอขอบคุณสำนักงานกองทุนสนับสนุนการวิจัยเป็นอย่างยิ่งที่ให้ทุนสนับสนุนโครงการวิจัยนี้ ขอกราบขอบพระคุณศาสตราจารย์เกียรติคุณนายแพทย์วิจารณ์ พานิช ท่านผู้อำนวยการกองทุนสนับสนุนการวิจัยท่านแรกที่แนะนำและให้โอกาสผู้วิจัยได้รับทุนจากสำนักงานกองทุนสนับสนุนการวิจัยเป็นครั้งแรกตั้งแต่ปี 2539 ขอกราบขอบพระคุณศาสตราจารย์เกียรติคุณ ดร. วิชัย บุญแสง ที่เมตตาและให้การสนับสนุนผู้วิจัยได้ทำงานวิจัยด้านกลไกความเจ็บปวดจากฟันได้ทำงานวิจัยในเวลาต่อมา ขอกราบขอบพระคุณ ศาสตราจารย์ ดร. สวัสดิ์ ตันตระรัตน์ ท่านผู้อำนวยการกองทุนสนับสนุนการวิจัยท่านปัจจุบันที่ให้การสนับสนุนผู้วิจัยได้ทำงานวิจัยต่อเนื่อง ขอขอบคุณ คุณสุจารี สอนง่ายเจ้าหน้าที่ฝ่ายวิชาการของสกว. ที่ช่วยเหลือเป็นอย่างมาก

ขอกราบขอบพระคุณ ศาสตราจารย์เกียรติคุณ ทันตแพทย์ ดร. สถิตย์ สิริสิงห ที่เมตตาและให้การแนะนำด้านการวิจัยและวิชาการ ขอกราบขอบพระคุณ ศาสตราจารย์เกียรติคุณ ทันตแพทย์สมศักดิ์ จักรไพวงศ์ อดีตคณบดีคณะทันตแพทยศาสตร์ มหาวิทยาลัยมหิดล ที่เมตตาและให้การสนับสนุนการวิจัยและสนับสนุนอย่างมากด้านเครื่องมือและอุปกรณ์การวิจัย ขอกราบขอบพระคุณ รองศาสตราจารย์ ทันตแพทย์ ดร. สุขุม ธีรดิลก อดีตหัวหน้าภาควิชาสรีรวิทยาและชีวเคมีและอดีตคณบดีคณะทันตแพทยศาสตร์ มหาวิทยาลัยมหิดล ที่เมตตาและให้การแนะนำด้านการวิจัยและวิชาการตั้งแต่ผู้วิจัยยังเป็นนักศึกษาทันตแพทยศาสตร์ปีที่ 4 เมื่อปีพ.ศ. 2520 และเรื่อยมาจนถึงปัจจุบัน ขอกราบขอบพระคุณ รองศาสตราจารย์ ทันตแพทย์หญิงวรรณะ อภัย รองศาสตราจารย์ทันตแพทย์ชูโชติ ธนะภูมิ ผู้ช่วยศาสตราจารย์ ทันตแพทย์หญิงอิงบุญ เทียนศิริ อดีตหัวหน้าภาควิชาสรีรวิทยาและชีวเคมี ที่เมตตาและให้ให้การสนับสนุน ขอกราบขอบพระคุณ รองศาสตราจารย์ ทันตแพทย์หญิง ดร. ธีรลักษณ์ สุทธเสถียร คณบดีคณะทันตแพทยศาสตร์ มหาวิทยาลัยมหิดล และรองศาสตราจารย์ ทันตแพทย์สุรินทร์ สูอำพัน รองคณบดีฝ่ายโครงการพิเศษที่ให้การสนับสนุน

ในระดับนานาชาติขอกราบขอบพระคุณ Professor Bruce Matthews แห่ง University of Bristol UK อาจารย์ที่ปรึกษาระดับปริญญาเอกที่ยังคงร่วมมือในการศึกษาวิจัยด้านกลไกความเจ็บปวดจากฟัน ต่อเนื่องมากว่า 20 ปี ขอกราบขอบพระคุณ Professor Rex Holland แห่ง University of Michigan Ann Arbor USA, Professor Hideaki Suda แห่ง Tokyo Medical and Dental University, Japan ที่ให้การสนับสนุนด้านต่างๆ

ขอขอบคุณอดีตนักศึกษาปริญญาเอกสาขาชีววิทยาช่องปาก คณะทันตแพทยศาสตร์ มหาวิทยาลัยมหิดล รองศาสตราจารย์ ทันตแพทย์ ดร. สิทธิชัย วนจันทรารักษ์ ผู้ช่วยศาสตราจารย์ทันตแพทย์หญิง ดร. ปานตา เจริญลาภ ผู้ช่วยศาสตราจารย์ ทันตแพทย์หญิง ดร. วรางคณา ชิดช่วง-ชัย อาจารย์ทันตแพทย์หญิง ดร. กนิษฐา กิจสมานมิตร อาจารย์ทันตแพทย์หญิง ดร. อรพินทร์ อัจฉรานุกูล และขอขอบคุณ คุณศิริรัตน์ วัชรอุดมปัญญา คุณฉลองรัตน์ หมื่นขวา คุณศิรินทิพย์ ชูเนตร นักวิทยาศาสตร์ของภาควิชาฯ

ท้ายที่สุดขอขอบคุณ รองศาสตราจารย์ ทันตแพทย์หญิงคัดเค้า วงษ์สวรรค์ ภรรยาของผู้วิจัย ที่เป็นกำลังใจ และช่วยเหลือในทุกๆเรื่องให้โอกาสผู้วิจัยได้ศึกษาวิจัยด้านกลไกความเจ็บปวดจากฟัน ทั้งต่างประเทศและในประเทศต่อเนื่องมากว่า 20 ปี

บทคัดย่อ

รหัสโครงการ: BRG5280009

ชื่อโครงการ:อาการปวดฟันและการไหลเวียนเลือดของเนื้อเยื่อในโพรงฟันในภาวะที่เนื้อเยื่อในโพรงฟันปกติและอักเสบในมนุษย์

ชื่อนักวิจัย: รองศาสตราจารย์ ทพ. ดร. นพคุณ วงษ์สวรรค์

E-mail Address: noppakun.von@mahidol.ac.th

ระยะเวลาโครงการ: วันที่ 15 กรกฎาคม 2552 ถึงวันที่ 14 กรกฎาคม 2555

การศึกษาวิจัยในการศึกษาแรกและที่สองเพื่อหาสัดส่วนของสัญญานเลเซอร์ดอลปเปอร์ในการวัดปริมาณเลือดที่มาเลี้ยงเนื้อเยื่อในโพรงฟันเมื่อใช้แสงเลเซอร์ต่างชนิดกันระหว่างแสงอินฟราเรดและแสงสีแดง ผลการวิจัยพบว่าการใช้แสงอินฟราเรดและแสงสีแดงมีสัดส่วนของสัญญานที่มาจากในโพรงฟันไม่ต่างกันคือประมาณร้อยละ 50 การศึกษาวิจัยในการศึกษาที่สามมีวัตถุประสงค์เพื่อศึกษาผลการอักเสบของเนื้อเยื่อในโพรงฟันต่อภาวะเสียวฟันเมื่อเนื้อฟันสำผัสกับน้ำเย็นอุณหภูมิ 5°C และกระตุ้นด้วยความดัน 400 มม ของปรอทต่ำกว่าความดันบรรยากาศเป็นเวลา 5 วินาที ให้อาสาสมัครประเมินความรู้สึกเสียวฟันด้วยวิชวยอนาลอกสเกล (วีเอเอส) การศึกษาทำในฟันกรามน้อยที่จะถอนออกเพื่อจัดฟัน การศึกษาได้รับอนุมัติจากคณะกรรมการจริยธรรมในคนของมหาวิทยาลัยมหิดล ผลการศึกษาพบว่าเนื้อเยื่อในโพรงฟันเมื่ออักเสบอาสาสมัครรู้สึกภาวะเสียวฟันมากขึ้นต่อน้ำเย็นอุณหภูมิ 5°C แต่กลับรู้สึกภาวะเสียวฟันน้อยลงต่อการกระตุ้นด้วยความดัน 400 มม ของปรอทต่ำกว่าความดันบรรยากาศ การศึกษาวิจัยในการศึกษาที่สี่มีวัตถุประสงค์เพื่อศึกษาผลการอักเสบขอเนื้อเยื่อในโพรงฟันต่อระดับของพรอสตาแกรนดินอีทู (PGE_2) ในน้ำของเนื้อเยื่อในโพรงฟัน น้ำในท่อเนื้อฟัน ปริมาณของเลือดที่มาเลี้ยงเนื้อเยื่อในโพรงฟันและระดับของภาวะเสียวฟันเมื่อเนื้อฟันสำผัสกับน้ำเย็นอุณหภูมิ 10°C และน้ำอุ่นอุณหภูมิ 60°C ให้อาสาสมัครประเมินความรู้สึกเสียวฟันด้วยวิชวยอนาลอกสเกล (วีเอเอส) การศึกษาพบว่า การอักเสบขอเนื้อเยื่อในโพรงฟันต่อระดับของพรอสตาแกรนดินอีทู (PGE_2) ในน้ำของเนื้อเยื่อในโพรงฟันจาก 186.3$\pm$47.9 เป็น 494.9$\pm$106.7 ng/ml การอักเสบขอเนื้อเยื่อในโพรงฟัน ยังเพิ่มปริมาณของเลือดที่มาเลี้ยงเนื้อเยื่อในโพรงฟัน และมีความรู้สึกภาวะเสียวฟันมากขึ้นเมื่อเนื้อฟันสำผัสกับน้ำเย็นอุณหภูมิ 10°C และน้ำอุ่นอุณหภูมิ 60°C โดยสรุปการอักเสบขอเนื้อเยื่อในโพรงฟัน ทำให้ระดับของพรอสตาแกรนดินอีทู (PGE_2)ในน้ำของเนื้อเยื่อในโพรงฟันสูงขึ้น เกิดการขยายตัวของหลอดเลือดที่มาเลี้ยงเนื้อเยื่อในโพรงฟัน และเกิดภาวะเสียวฟันไวเกินในมนุษย์

คำสำคัญ: อาการเจ็บปวด ภาวะเสียวฟัน ปริมาณการไหลของเลือดที่ไปเลี้ยงเนื้อเยื่อในโพรงฟัน การอักเสบของเนื้อเยื่อในโพรงฟัน พรอสตาแกรนดินอีทู ความเย็น ฟันมนุษย์

Abstract

Project Code: BRG5280009

Project Title: Dental pain and pulpal microcirculation in normal and inflamed pulp in man

Investigator: Dr. Noppakun Vongsavan

E-mail Address: dtnvs@mahidol.ac.th

Project Peroid: 15th July 2009- 14th July 2012

The first and second series of experiments aim to compare red (635 nm) and infrared (780 nm) light for recording pulpal blood flow from human anterior teeth with a laser Doppler flow meter. Recordings were made from 7 healthy teeth in 5 subjects (aged 22-55 yr.) using a laser Doppler flow meter (Periflux 4001) equipped with both red and infrared lasers. In conclusion, using dam, there is no advantage to using red rather than infrared light and in each case the pulp contributes less than 50% to the blood flow signal.

The third series of experiments aim to investrigate the effects of pulpal inflammation on the sensitivity of dentin to cold (5°C) and negative hydrostatic pressure (- 300 mm Hg) stimuli in man were compared since recent evidence suggests that these stimuli excite different classes of sensory receptor. Dentin was exposed in premolars in 14 participants aged 15 – 25 years. Stimuli were applied to etched dentin immediately after cavity preparation and after the cavity had been filled with gutta percha for 7 days. *Results*: This treatment increased significantly the intensity of pain produced by cold, and at the same time decreased that evoked by negative pressure stimuli. Pulpal blood flow was increased in the treated teeth, indicating that their pulps were inflamed. It is concluded that the sensory receptors responsible for the response to cold were probably sensitive to some change other than an outward flow of fluid in dentinal tubules, which would be caused by both forms of stimulus. The fourth series of experiments aim to investigate the effects of pulpal inflammation on prostaglandin E_2 (PGE_2) levels, pulpal blood flow and pain sensation evoked by thermal stimulation in human subjects. The experiments were done on 14 healthy premolars. Dentine was exposed at the tip of the buccal cusp. Pain evoked by thermal stimulation (water at 10 and 60°C) for 5 seconds and was scored by the subject on a visual analog scale (VAS). The cavity was then filled with either gutta percha alone (GP) or, on the contra-lateral side, GP sealed with glass ionomer material (GI). After one week, the fillings were removed, the cavities were cleaned and the measurements and stimuli repeated. The teeth were extracted and fractured longitudinally. Pulpal tissue fluid was collected and its PGE_2 level determined using enzyme-immunoassay. *Results*: Treatment with GP alone increased both mean pulpal blood flow and mean VAS scores at 10 and 60°C, respectively. PGE_2 levels were significantly higher after filling with GP than GP+GI (494.9±106.7 and 186.3±47.9 ng/ml, respectively; $p<0.01$, *Student t-test*,). It was concluded that the unsealed GP filling caused an increase in the PGE_2 level of pulpal fluid, vasodilatation and hyperalgesia of dentine

Key Words: Pain, Dentine Sensitivity, Pulpal blood flow, Pulp inflammation, PGE_2, Cold Stimulation, Human Teeth

Research project

Dental pain and pulpal microcirculation in normal and inflamed pulp in man
Project no. BRG5280009

This project was composed of four series of experiments. In series I experiments entitled “A comparison between red and infrared light for recording pulpal blood flow from human anterior teeth with a laser Doppler flow meter”. In series II experiments entitled “Pulpal blood flow recorded from human premolar teeth with a laser Doppler flow meter using either red or infrared light”. These two series of experiments investigated the source of laser Doppler signals which recorded from anterior and premolar teeth with different laser light (infrared and red light). Both papers were published in *Archives of Oral Biology* in 2011. (impact factor = 1.603, From Journal Citation Reports®, 2011) In series III experiments entitled “Sensory transduction in human teeth with inflamed pulps”. These series of experiments investigated effect of mild inflammation of the dental pulp the sensitivity of dentin to cold (5°C) and negative hydrostatic pressure (- 300 mm Hg) stimuli in man. The papers were published in *Journal of Dental Research* in 2011. (impact factor = 3.486, From Journal Citation Reports®, 2011) (cited up to 2012 = 3).) In the series IV experiments entitled “Prostaglandin E_2 levels blood flow and pain sensation inhuman teeth with inflamed pulps”. The objective of this study was to investigate the effects of pulpal inflammation on prostaglandin E_2 (PGE_2) levels, pulpal blood flow and pain sensation evoked by thermal stimulation in human subjects which the manuscript was in preparation.

Series I experiments

A comparison between red and infrared light for recording pulpal blood flow from human anterior teeth with a laser Doppler flow meter

Abstract

***Objective*:** To compare red (635 nm) and infrared (780 nm) light for recording pulpal blood flow from human anterior teeth with a laser Doppler flow meter.

Design: Recordings were made from 7 healthy teeth in 5 subjects (aged 22-55 yr.) using a laser Doppler flow meter (Periflux 4001) equipped with both red and infrared lasers. Average blood flow signals were obtained with both light sources alternately from each tooth under five conditions: intact tooth without opaque rubber dam, intact tooth with dam, after injecting local anaesthetic (3% Mepivacaine) (LA) over the apex of the tooth and cavity preparation to almost expose the pulp, after removal and replacement of the pulp, and with the root canal empty.

Results: With infrared light, because of technical limitations, data were obtained for the first three conditions only. The dam significantly decreased the mean blood flow by 82%. Injecting LA and cavity preparation had no significant effect. With red light, dam produced a decrease of 56%, and the resulting signal was reduced by 33% after LA and cavity preparation. The remaining signal fell by 46% after pulp removal and replacement. This contribution of the pulp is similar to that recorded previously with infrared light. There was no significant change when the root canal was empty.

Conclusions: The importance of using opaque rubber dam is confirmed. Using dam, there is no advantage to using red rather than infrared light and in each case the pulp contributes less than 50% to the blood flow signal.

Introduction

Laser Doppler flow meters have been used in many studies to record blood flow in teeth since the original studies of Gazelius *et al.* (1986, 1988) and Olgart *et al*. (1988.) This is potentially a valuable technique both for research on pulpal blood flow and for clinical diagnosis, but a limitation of the technique is that the records obtained are derived from tissues outside the tooth (Hartmann *et al.*,1996; Soo-ampon *et al*., 2003), such as the gingiva and periodontal ligament, as well as the pulp. The contamination of the records from non-pulpal tissues can be reduced by covering the adjacent gingiva with rubber dam or periodontal paste (Akpinar *et al*., 2004). Soo-ampon *et al*., (2003) found that opaque rubber dam reduced the blood flow signal recorded from an intact, anterior tooth in man by an average of 73%. They also showed that the signal remaining after applying the rubber dam was further reduced by 57% when the pulp was removed and replaced in the root canal. Thus only approximately 10% of the signal recorded from a tooth without dam, and 43% of that recorded with dam can be attributed to blood flow in the pulp.

The laser Doppler record obtained from a tooth will depend on the wavelength of the light employed. Infrared light (780 nm) penetrates deeper into tissues than red light (635 nm) (Bonner *et al*., 1990), thus a record obtained with infrared might be expected to include a greater contribution from tissues outside the pulp than one obtained with red light. Soo-ampon *et al*., (2003) used infrared light. The present experiments were carried using a similar protocol to the one they used but with red light to determine if this resulted in a significantly smaller contribution from tissues outside the pulp.

Materials and methods

The experiments were done on 7 healthy, anterior teeth in 5 subjects (aged 22-55 yr.). These teeth were to have root canal treatment before the construction of a fixed prosthesis. All the teeth were caries-free and were either intact or had only a small restoration. Radiographic examination and electrical pulp stimulation confirmed that they were vital and healthy. The study was approved by the Ethics Committee on Human Rights Related to Human Experimentation of Mahidol University, and complied with the principles of the Declaration of Helsinki. Informed consent was obtained from each subject.

Recordings were made using a Periflux system 4001 two-channel, laser Doppler flow meter (Perimed AB, Järfälla, Sweden). One channel was equipped with an infrared (780 nm) laser and the other, a red (635 nm) laser. The probe (type 407: ext. diam. 1.0 mm; optical fibre diam. 0.125 mm, fibre separation 0.25 mm) was supported in a mini probe holder (type PH 07-5) that was incorporated into a removable acrylic splint. The probe

holder was positioned so that the probe was perpendicular to the labial enamel surface on the labial surface of the tooth, with its tip over the central long axis of the crown, and with its centre 2 mm from the gingival margin (Fig. 1). The rotation of the probe around its long axis was kept constant between trials by aligning marks on the probe and the probe holder. Recordings were made with the probe connected alternately to each of the two channels of the flow meter.

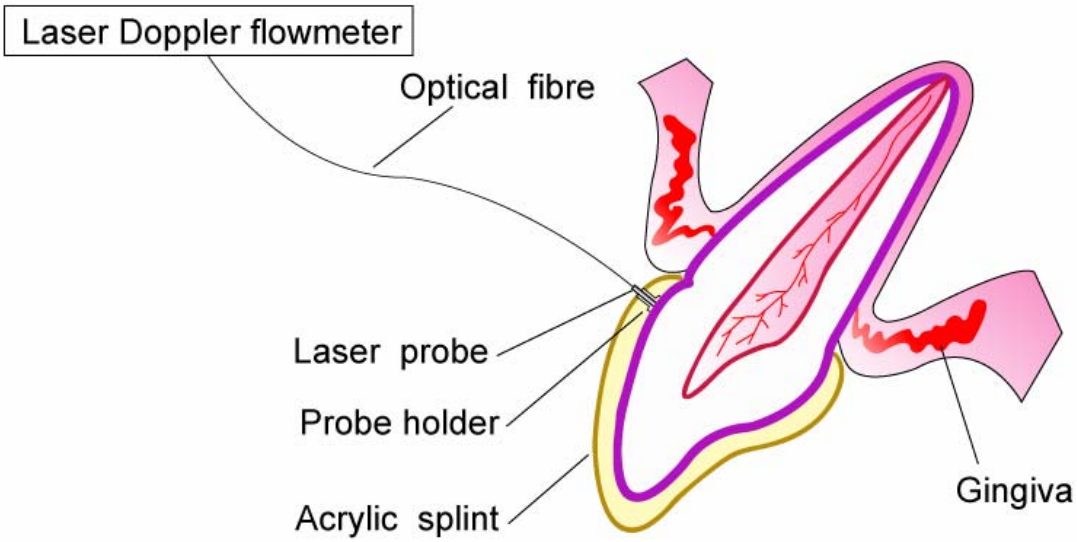

Figure 1. Diagram of the experimental set up.

The probe was zeroed separately with infrared and red light and calibrated according the manufacturer's instructions so that the Brownian motion of a standard suspension of latex particles (Vongsavan and Matthews, 1993a) gave a reading of 250 arbitrary perfusion units (P.U.). An upper bandwidth setting of 12 kHz and a time constant of 0.2 s were used throughout. The data were recorded from the digital output of the flow meter with a computer running the PeriSoft (version 1.13) software program.

Recordings were made from each tooth under five conditions: (1) the intact tooth without rubber dam, (2) after applying opaque black rubber dam (Four D Rubber Co. Ltd., Heanor, England), (3) after local anaesthetic (1 ml, Mepivacaine 3%) had been injected near the apex of the root and a cavity cut in the incisal third of the labial surface until the pulp was nearly exposed, (4) after removal of the pulp with a broach, arrest of bleeding with paper points and replacement of the pulp in the root canal. and (5) with the canal empty after removal of the pulp. At each stage, the blood flow signal was allowed to stabilize for several minutes before measurements were made. These conditions are the same as those used for the Series 2 experiments of Soo-ampon et al.[5], except that the rubber dam remained in place after its initial application at stage 2. The additional time required to make measurements with both infrared and red light in the present study meant that measurements could not be made under all conditions both with and without the dam.

After each experiment, recordings were also made at different light intensities from a stationary reflector (white card). These data were used to calculate the offset of the blood flow signal that would have been present while recording from the teeth due to noise in the detection system (Vongsavan and Matthews, 1993b). For each set of blood flow values recorded from a tooth during the experiment, the mean and standard deviation were calculated and the offset, determined as described above, appropriate for the intensity of backscattered light present was subtracted from the mean.

Comparisons between the overall mean blood flow values recorded under each of the different conditions were made using one–way, repeated measures analysis of variance. Where this showed that there were significant differences between the means, the Tukey test was used to make multiple comparisons between them. Student's paired t-test was used to compare % changes in mean blood flow with the two light sources under otherwise the same conditions. P values of less than 0.05 were considered significant.

Results

Examples of records obtained with infrared and red light under the different conditions of the experiment are shown in Fig. 2. The records with infrared light illustrate a limitation of the Periflux system 4001. Under conditions when the blood flow signal was very low, when the pulp was cut and replaced (Pulp cut) and when it was removed (Pulp removed), the record was clipped when it fell to zero, that is the value estimated to be equivalent to zero blood flow during the calibration procedure. As the records obtained under these conditions were clearly different from those obtained from the white card at the same intensity of back-scattered light, some blood flow was detected; but average values of the clipped blood flow signals would overestimate the true values. For this reason it was not possible to measure the difference between the mean blood flow signals recorded after local anaesthesia and cavity preparation (LA prep) and after the pulp had been cut and replaced (Pulp cut) to estimate the contribution of the pulp to the original blood flow records obtained with infrared light.

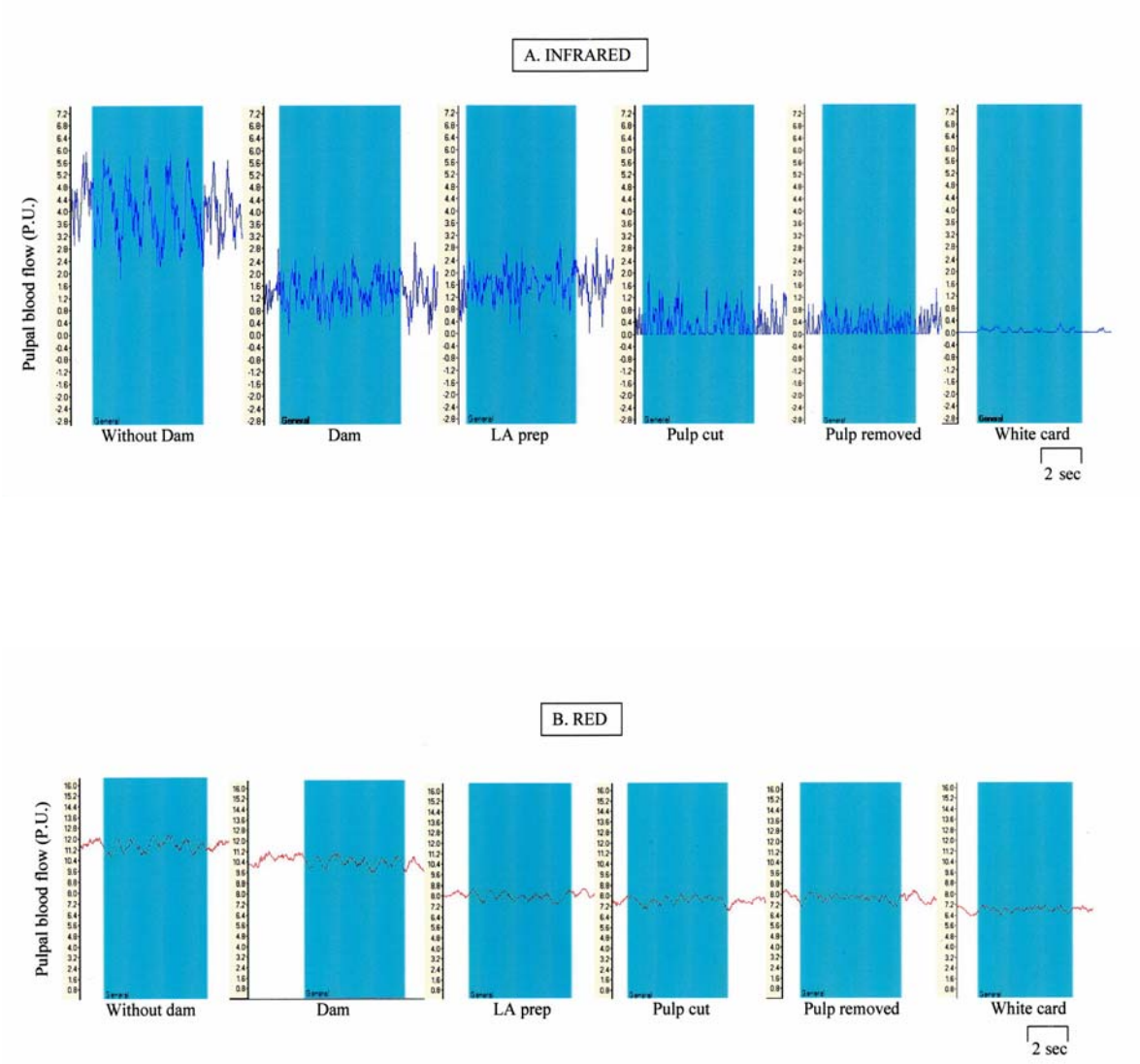

Figure 2. Laser Doppler records of blood flow obtained under different conditions from an upper central incisor in one subject, and from a white card at the same intensity of back-scattered light, with A) infrared light and B) red light. The shaded areas indicate the parts of the records that were used to calculate the mean blood flow.

The limited data available for infrared light showed that the application of the rubber dam and the preparation of the cavity under local anaesthesia produced significant changes in the overall mean blood flow values (One-way, RM ANOVA; $p<0.001$). Application of the rubber dam produced a large and significant ($p<0.001$,Tukey Test) fall in the mean by 82% from 8.23 (S.D. 3.71, n=7) P.U. to 1.50 (S.D, 0.69) P.U, and after administration of the local anaesthetic and cavity preparation there was a further fall in the mean to 1.13 (S.D, 0.56) but this change was not significant.

Satisfactory records were obtained under all the experimental conditions with the red light. The differences between the overall mean blood flow values were significant ($p<0.001$; One-way, RM ANOVA). The mean for the intact teeth before the application of rubber dam was 6.61 (s.d. 2.23, n=7) P.U. This decreased significantly ($p<0.001$, Tukey Test) by 56% to 2.93 (S.D. 0.62) P.U. when the dam was applied. There was a further significant fall by 33% ($p<0.002$) to 1.95 (S.D. 0.75) P.U. after administration of the local anaesthetic and cavity preparation. Removal and replacement of the pulp reduced the latter figure by 46% to 1.05 (S.D. 0.42) P.U., a change which again was significant ($p<0.003$). Finally removing the pulp from root canal resulted in no significant change (mean 1.10, S.D. 0.46 P.U.).

A summary of the data is shown in Fig.3.

The percentage reduction in the blood flow signal produced by the rubber dam was significantly greater ($p<0.001$, Student's paired t-test) with infrared compared with red light.

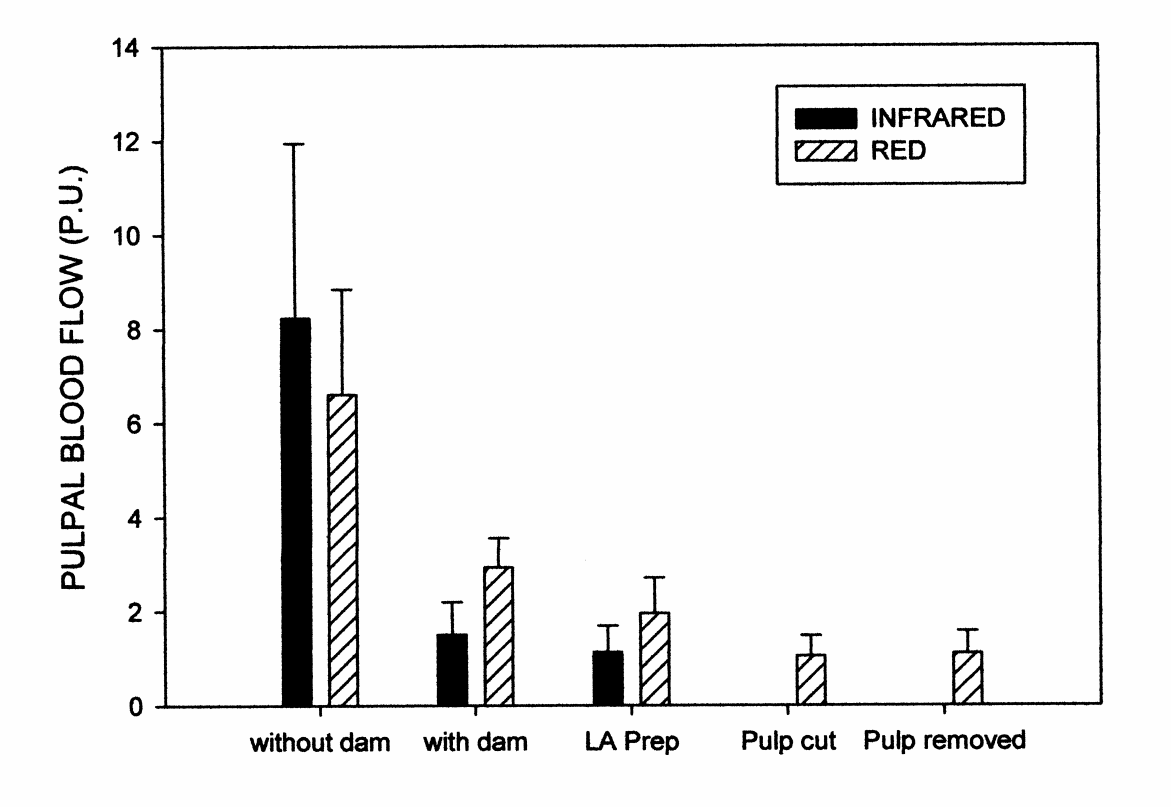

Figure 3. Mean values from 7 teeth of the pulpal blood flow signal recorded under different conditions with infrared (solid columns) and red (hatched columns) light. In each case, the value was corrected by subtraction of the reading from the white card. The error bar indicates one S.D.

Discussion

The results confirm earlier findings that, with infrared light, covering the gingiva with opaque rubber dam produces a very large reduction in the blood flow signal recorded from anterior teeth in man. In the present study, the reduction was 82%, and in the study of Soo-ampon *et al*., (2003) it was 73%. With red light in the present study the dam also reduced signal significantly: the corresponding figure was 56%. This effect of rubber dam has been attributed to screening of light from periodontal, gingival and other surrounding tissues, and to a reduction in gingival blood flow due to compression of the gingival tissue.[5] The observation that the dam produced a significantly greater effect with infrared than with red light can be accounted for by the deeper penetration of the infrared light into the surrounding tissues.

The combined effect of the local anaesthesia and cavity preparation was to reduce the blood flow signal recorded with the rubber dam using red light by 33%, but with infrared light there was no significant change. Soo-ampon *et al*., (2003) found that with infrared light under the same conditions the blood flow signal fell by 20%. No explanation can be given for this difference, apart from the fact that data were collected from more teeth in the earlier study (14 compared with 7). The reduction with red light was probably due mainly to a vasoconstriction produced by the Mepivacaine local anaesthetic. Also injecting 1ml. of local anaesthetic solution will have increased the interstitial fluid pressure in the gingival and periodontal tissues, which will have tended to compress the vessels in these tissues.

Removing and replacing the pulp reduced the signal recorded with the dam and red light by 46%. This is very similar to the result obtained under similar conditions but with infrared light by Soo-ampon *et al*., (2003). This proportion of the signal, which can be attributed to blood flow in the pulp, does not appear therefore to be affected by the wavelength of the light employed. For the reasons given above, it was not possible to make a direct comparison between infrared and red light in the same teeth in the present study.

When the pulp was finally removed, leaving the root canal empty, there was no further reduction in the signal obtained with red light. The same procedure produced a significant fall in the signal in the earlier study by Soo-ampon *et al*., (2003), which was attributed to an effect of the pulp tissue on the distribution of light within the tooth and

supporting tissues and on Brownian motion of particles in the replaced pulp (Polat *et al*., (2004).

Polat *et al*., (2004). also concluded that laser Doppler signals from anterior teeth in adult humans include a large contribution from tissues other than the pulp. They also used a Periflux 4001 system with infrared light. Their conclusion was based on the effects of removing the pulp and then replacing it with a root filling. They did not take precautions, such as the use of opaque rubber dam, to limit the contributions of tissues other than pulp to the recorded signal. Also, some of the effects they observed could have been the result of changes in the distribution of light in the tooth and surrounding tissues when the pulp was removed and replaced by a root filling. This would explain their observation that there was no difference between the blood flow recorded from an intact tooth and from the same tooth after it had been root filled. Their data cannot therefore provide a reliable guide to the contribution of the pulp to the signal recorded from an intact tooth.

The problem we encountered with clipping of the records obtained with infrared light occurred under conditions when the blood flow would have fallen to very low levels close to the limit of resolution of the instrument. The problem did not arise with red light; probably because, in zeroing and calibrating the probe, the backscattered light intensity was different from that present when recording from the teeth. Consistent with this, the recordings from the white card with red light were significantly above zero (e.g. Fig. 2B). Soo-ampon *et al*., (2003) used a different flow recorder, which also used infrared light (Moor, type MBF3D/42), and were able to detect blood flow in teeth after applying a rubber dam and after removing and replacing the pulp, but the instrument was operating near the limit of its resolution.

In conclusion, laser Doppler flow meters work at the limit of their resolution when recording pulpal blood flow from anterior teeth in adult humans, particularly when the contributions of non-pulpal tissues are minimised by the use of opaque rubber dam. Even under these conditions, the pulp contributes less than 50% to the recorded signal, irrespective of whether the recording is made with infrared or red light.

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Series II experiments

Pulpal blood flow recorded from human premolar teeth with a laser Doppler flow meter using either red or infrared light

Abstract

***Objective*:** To compare red (635 nm) and infrared (780 nm) light for recording pulpal blood flow from human premolar teeth.

Design: Recordings were made from 11 healthy teeth in 9 subjects (aged 16-30 yr.) using a laser Doppler flow meter (Periflux 4001) equipped with both red and infrared lasers. Average blood flow signals were obtained with both light sources alternately from each tooth under five conditions: intact tooth without opaque rubber dam, intact tooth with dam, after injecting local anaesthetic (3% Mepivacaine) (LA) over the apex of the tooth and cavity preparation to almost expose the pulp, after removal and replacement of the pulp, and with the root canal empty.

Results: With infrared light, the dam significantly decreased the mean blood flow by 80%. Injecting LA and cavity preparation had no significant effect. Removal and replacement of the pulp reduced the mean blood flow by 58%. There was no further change when the pulp was removed. With red light, the dam reduced the signal from intact teeth by of 60%. Injecting LA and cavity preparation had no significant effect. The signal fell by 67% after pulp removal and replacement and did not change significantly when the pulp was removed.

Conclusions: Opaque rubber dam minimises the contribution of non-pulpal tissues to the laser Doppler signal recorded from premolars. Using dam, the pulp contributed about 60% to the blood flow signal with both red and infrared light. The difference between them in this respect was not significant.

Introduction

Laser Doppler flow meters have been used extensively in attempts to record pulpal blood flow in human anterior teeth (Jafarzadeh, 2009), but the technique has been used less frequently with posterior teeth. Odor *et al.* 1994 a & b investigated the effects of local anaesthesia of the inferior alveolar nerve on molar blood flow, Mensdorff-Pouilly *et al.* (1995) used a laser Doppler flow meter to monitor changes in blood flow in auto-transplanted molars as a method of assessing the progress of pulpal revascularization, Meseros *et al.* (1997) recorded from premolars before and re-implantation of the teeth, Norer *et al.* (1999) investigated the differences in blood flow between different teeth, and Chandler *et al*. (2010) studied the effects of restorations on blood flow in molars. In none of these studies has it been possible to determine what proportion of the recorded signal originated from the pulp. It has been shown that less then 50% of the signal obtained from anterior teeth is from the pulp, even when precautions are taken to minimise the contribution of periodontal and gingival tissues by covering the gingiva with opaque rubber dam (Soo-ampon *et al*., 2003; Kijsamanmith *et al*., 2011). The methods used in those studies have been employed in the present experiments to estimate the proportion of the signal recorded from premolar teeth is derived from the pulp.

The effect of the wavelength of the light used for recording pulpal blood flow has also been investigated. Infrared light (780 nm) penetrates deeper into tissues than red light (635 nm) (Bonner *et al*., 1990), thus a record obtained with infrared might be expected to include a greater contribution from tissues outside the pulp than one obtained with red light. Both wavelengths were investigated in the earlier study on anterior teeth (Kijsamanmith *et al*., 2011) but, for technical reasons, the recordings obtained with infrared light under conditions in which the blood flow was very low were unsatisfactory. A further attempt was made to compare recordings made from the same tooth with both infrared and red light in the present study

Materials and methods

The experiments were done on 11 healthy premolar teeth in 9 human subjects (aged 16-30 yr.). These teeth were scheduled to be extracted for orthodontic purposes. All the teeth were intact. Radiographic examination and electrical pulp stimulation confirmed that they were vital and healthy. The study was approved by the Ethics Committee on Human Rights Related to Human Experimentation of Mahidol University, and complied with the

principles of the Declaration of Helsinki. Informed consent was obtained from each subject, or for those under 18 years, a parent or guardian.

Recordings were made using a Periflux system 4001 two-channel, laser Doppler flow meter(Perimed AB, Järfälla, Sweden). One channel was equipped with an infrared (780 nm) laser and the other, a red (635 nm) laser. The probe (type 415-159: ext. diam. 1.0 mm; optical fibre diam. 0.125 mm, fibre separation 0.25 mm) was supported in a mini probe holder (type PH 07-5) that was incorporated into a removable acrylic splint. The probe holder was positioned so that the probe was perpendicular to the enamel surface on the buccal surface of the tooth, with its tip over the central long axis of the crown, and with its centre 2 mm from the gingival margin (Fig. 1). The rotation of the probe around its long axis was kept constant between trials by aligning marks on the probe and the probe holder. Recordings were made with the probe connected alternately to each of the two channels of the flow meter

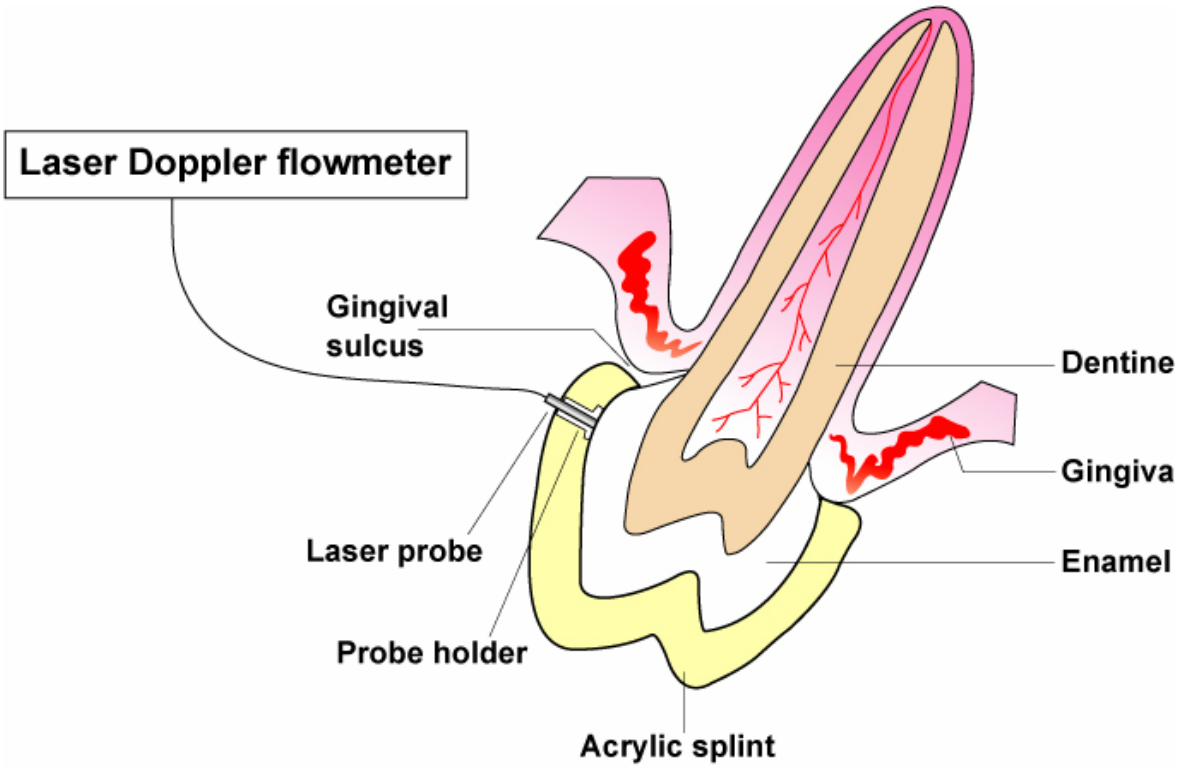

Figure 1. Diagram of the experimental set up.

The probe was zeroed separately with infrared and red light and calibrated according the manufacturer's instructions so that the Brownian motion of a standard suspension of latex particles (Vongsavan and Matthews, 1993a) gave a reading of 250 arbitrary perfusion units (P.U.). An upper bandwidth setting of 12 kHz and a time constant of 0.2 s were used throughout. The data were recorded from the digital output of the flow meter with a computer running the PeriSoft (version 1.13) software program.

Recordings were made from each tooth under five conditions (Fig. 2) as in the earlier study (Kijsamanmith *et al*., 2011): (1) the intact tooth without rubber dam, (2) after applying opaque black rubber dam (Four D Rubber Co. Ltd., Heanor, England), (3) after local anaesthetic (1 ml, Mepivacaine 3%) had been injected sub-mucosally on the buccal side of the test tooth and an oval access cavity cut in the occlusal surface until the pulp was nearly exposed, (4) after removal of the pulp with a broach, arrest of bleeding with paper points and replacement of the pulp. and (5) with the pulp cavity empty after removal of the pulp. At each stage, the blood flow signal was allowed to stabilize for several minutes before measurements were made

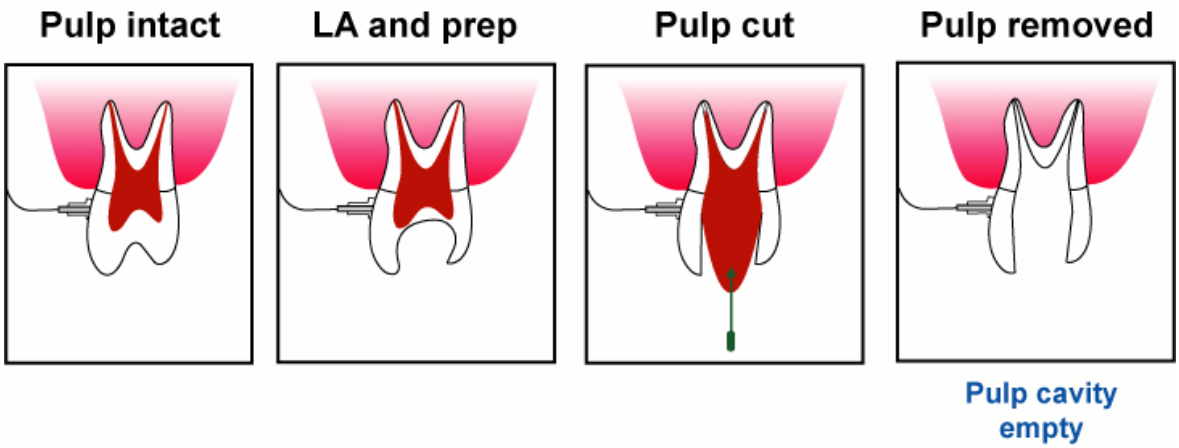

Figure 2. Laser Doppler records of blood flow obtained under different conditions from a premolar tooth in one subject, and from a white card at the same intensity of back-scattered light, with A) infrared light and B) red light. The shaded areas indicate the parts of the records that were used to calculate the mean blood flow.

After each experiment, recordings were also made at different light intensities from a stationary reflector (white card). These data were used to calculate the offset of the blood flow signal that would have been present while recording from the teeth due to noise in the detection system (Vongsavan and Matthews, 1993b). For each set of blood flow values recorded from a tooth during the experiment, the mean and standard deviation were calculated and the offset, determined as described above, appropriate for the intensity of backscattered light present was subtracted from the mean.

Comparisons between the overall mean blood flow values recorded under each of the different conditions were made using one–way, repeated measures analysis of variance. Where this showed that there were significant differences between the means, the Tukey test was used to make multiple comparisons between them. Percentage changes in mean blood flow with the two light sources under otherwise the same conditions were compared with either Student's paired t-test or the Wilcoxon Signed Rank test, depending on whether the data satisfied a normality test. P values of less than 0.05 were considered significant.

Results

Examples of records obtained with infrared and red light under the different conditions of the experiment are shown in Fig. 3. The problem we experienced when recording with infrared light under conditions of very low blood flow in the earlier study[9] was not encountered in the present experiments, and a full set of data was obtained with both infrared and red light.

With infrared light, the differences between the overall mean blood flow values recorded under the different conditions were significant ($p<0.001$; One-way, RM ANOVA). The mean for the intact teeth before the application of rubber dam was 9.52 (s.d. 5.52, n=11) P.U. This decreased significantly ($p<0.001$,Tukey Test) by 80% to 1.84 (S.D. 0.96) P.U. when the dam was applied. The administration of the local anaesthetic and cavity preparation resulted in a further fall in the mean to 1.34 (S.D. 0.80) P.U. but this change was not significant. Removal and replacement of the pulp reduced this latter figure significantly ($p<0.002$) by 58% to 0.55 (S.D. 0.27) P.U. There was no significant change in the mean (mean 0.46, S.D. 0.30 P.U.) when the pulp chamber was left empty.

With red light the differences between the overall mean blood flow values were also significant ($p<0.001$; One-way, RM ANOVA). The mean for the intact teeth before the application of rubber dam was 5.40 (s.d. 2.73, n=11) P.U. This decreased significantly ($p< 0.001$,Tukey Test) by 60% to 2.13 (S.D. 0.77) P.U. when the dam was applied. There was a further fall in the mean to 1.80 (S.D. 0.74) P.U. after the administration of local anaesthetic and cavity preparation, but this change was not significant. Removal and replacement of the pulp reduced the latter figure by 67% to 0.60 (S.D. 0.30) P.U., a change which was significant ($p<0.001$). Removing the pulp from the pulp chamber produced no significant change in the mean blood flow value (mean 0.45, S.D. 0.23 P.U.).

A summary of the data is shown in Fig.4.

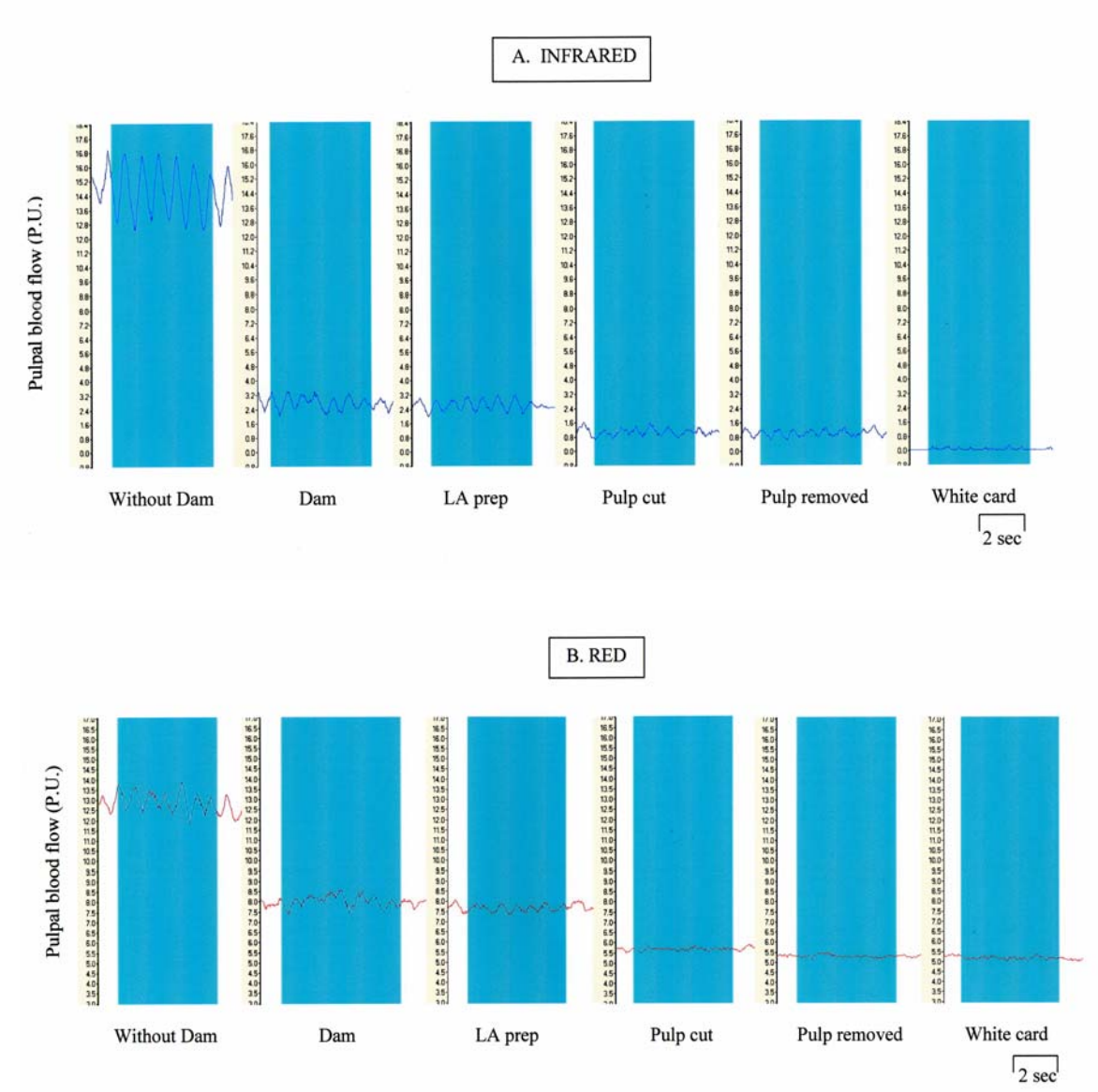

Figure 3. Laser Doppler records of blood flow obtained under different conditions from a premolar tooth in one subject, and from a white card at the same intensity of back-scattered light, with A) infrared light and B) red light. The shaded areas indicate the parts of the records that were used to calculate the mean blood flow.

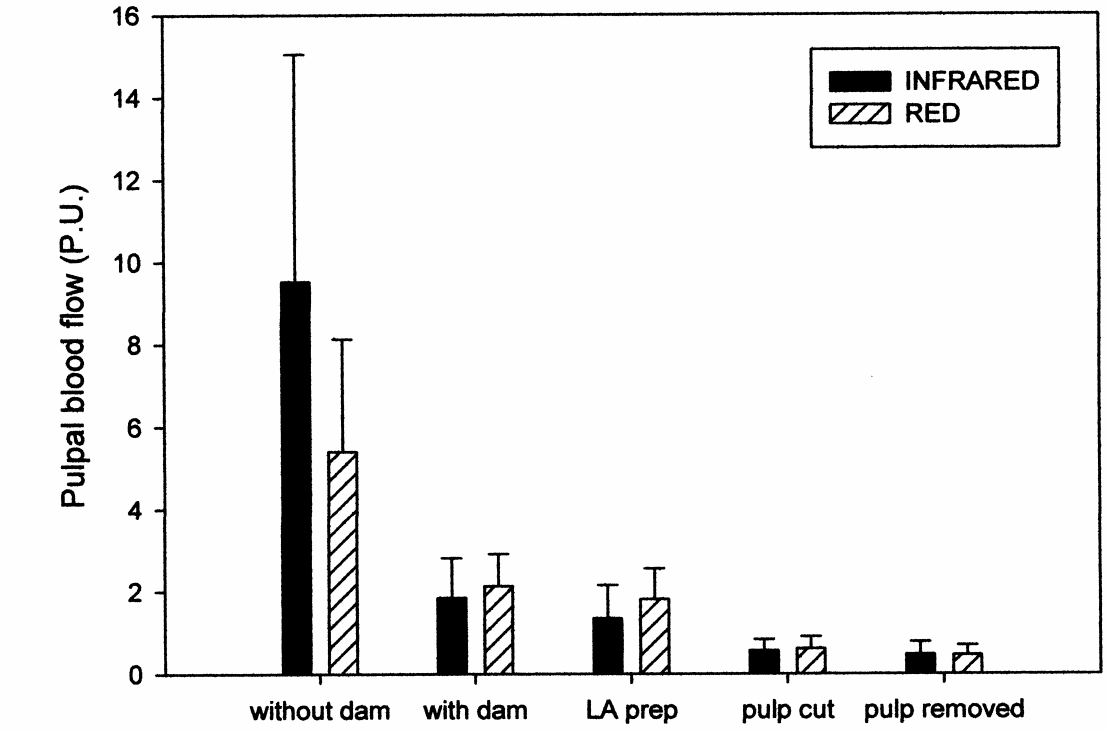

Figure 4. Mean values from 11 teeth of the pulpal blood flow signal recorded under different conditions with infrared (solid columns) and red (hatched columns) light. In each case, the value was corrected by subtraction of the reading from the white card. The error bar indicates one S.D.

The percentage reduction in the blood flow signal produced by the rubber dam was significantly greater ($p<0.003$, Student's paired t-test) with infrared compared with red light. The corresponding reductions following removal and replacement of the pulp, which provide an estimate of the contribution of blood flow in the pulp to the records obtained after LA and cavity preparation, (58% with infrared and 67% with red light) were not significantly different ($p=0.147$, Wilcoxon Signed Rank Test).

Discussion

Without dam, the laser Doppler blood flow record obtained from a premolar included a much greater contribution from non-pulpal tissues when the instrument employed an infrared laser (at least 80% on average) than when it employed a red one (60%). These figures are very similar to the results obtained from anterior teeth (Soo-ampon *et al*., 2003; Kijsamanmith *et al*., 2011). This difference between the two wavelengths can be attributed to the greater penetration of infrared light into the surrounding tissues.

However, the contribution of the pulp to the signals obtained with dam was not affected significantly by the wavelength of the light. In both cases the contribution of pulpal blood flow to the record obtained after LA and cavity preparation was approximately 60%. This corresponds closely with the result (57%) obtained with infrared light in premolar teeth by Tongsupan *et al.* (1998). This is the first time that it has been possible to compare the results obtained with the two wavelengths in the same teeth. The fact that they were so similar indicates that the penetration of the red light was adequate to detect blood flow through most of the pulp and that the opaque rubber dam was largely successful in restricting what otherwise would have been the greater penetration of the infrared light.

Using a similar protocol with anterior teeth, Soo-ampon *et al*., (2003) found that 43% of the signal recorded with infrared light after LA and cavity preparation could be attributed to the pulp and the corresponding figure obtained with red light by Kijsamanmith *et al*., 2011 was 46%. The difference between the two groups of teeth is probably due mainly to the greater volume of pulp in the premolars.

The blood flow signal that remained after removing and replacing the pulp when using opaque dam, can be attributed mainly to the spread of light through the root to

adjacent tissues. Although this spread would have been greater with infrared than red light, the difference was not sufficient to have a significant effect on the records obtained.

Replacing the extirpated pulp into the pulp chamber had no significant effect on the blood flow signal from the premolar teeth. This indicates that the distribution of light to the adjacent tissues when the pulp chamber was filled with air was similar to that when the pulp was replaced. This was not the case with infrared light in incisors in the study of Soo-ampon *et al*., 2003.

Unlike the previous study on anterior teeth Kijsamanmith *et al*., 2011, in which the same flow meter was used, the blood flows recorded with infrared light from premolars after extirpating the pulp were sufficiently above zero to prevent the records being clipped. The reason the signals were larger is probably because they were derived from a greater volume of tissue around the teeth.

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Series III experiments
Sensory Transduction in Human Teeth with Inflamed Pulps

Abstract

***Objective*:** The effects of pulpal inflammation on the sensitivity of dentin to cold (5°C) and negative hydrostatic pressure (- 300 mm Hg) stimuli in man were compared since recent evidence suggests that these stimuli excite different classes of sensory receptor.

***Design*:** Dentin was exposed in premolars in 14 participants aged 15 – 25 years. Stimuli were applied to etched dentin immediately after cavity preparation and after the cavity had been filled with gutta percha for 7 days.

***Results*:** This treatment increased significantly the intensity of pain produced by cold, and at the same time decreased that evoked by negative pressure stimuli. Pulpal blood flow was increased in the treated teeth, indicating that their pulps were inflamed.

***Conclusion*:** It is concluded that the sensory receptors responsible for the response to cold were probably sensitive to some change other than an outward flow of fluid in dentinal tubules, which would be caused by both forms of stimulus.

Introduction

Much of the available evidence indicates that all the different forms of stimulus that produce pain when they are applied to enamel or dentin generate impulses in intradental nerves via a common sensory transduction mechanism (the hydrodynamic mechanism) that detects movement of the contents of the dentinal tubules through hydrodynamic receptors (Brännström, 1963; Pashley, 1990; Andrew and Matthews, 2000; Orchardson and Cadden, 2001; Vongsavan and Matthews, 2007). However, recent experiments (Chidchuangchai *et al*., 2007) indicate that pain caused by cold stimulation of dentin in man may not always involve hydrodynamic receptors. It was found that etching freshly exposed dentin increased its sensitivity to cold, but decreased the fluid flow through the dentin that was caused by a cold stimulus. Furthermore, oxalate treatment, which re-occluded the tubules of the etched dentin, decreased the sensitivity of the dentin to cold but increased the dentinal fluid flow that was produced by a cold stimulus.

The present experiments were carried out to determine the effects of pulpal inflammation on the sensitivity of dentin to cold and to sub-atmospheric (negative) hydrostatic pressure stimulation of exposed, etched dentin in man. Cold stimuli cause outward flow of fluid through dentin (Andrew and Matthews, 2000), as does the application of negative hydrostatic pressure stimuli. The latter have been used previously to activate hydrodynamic receptors in teeth (Andrew and Matthews, 2000; Vongsavan and Matthews, 2007; Charoenlarp *et al*., 2007).

Pulpal inflammation was induced by filling the teeth with gutta percha for one week (Anderson *et al*., 1958). Clinical experience indicates that carious teeth with inflamed pulps may be very sensitive to cold.

Materials & methods

The experiments were carried out on 14 pairs of healthy premolars in 14 human participants (mean age 20.5 years, range 15 - 25). All teeth were scheduled to be extracted for orthodontic purposes. Radiographic and clinical examinations confirmed that all teeth were fully erupted, vital, free of caries and without restorations.

The experiments were carried out in the Advanced Clinic, Faculty of Dentistry, Mahidol University. The study was approved by the Ethics Committee on the Use of Human Rights Related to Human Experimentation of the Mahidol University and complied with the

principles of the Declaration of Helsinki. An informed consent was obtained from each participant, or for those under 18 years, a parent or guardian.

Experimental design

In each participant, two upper or two lower premolars were used; one on each side. Both hydrostatic pressure and cold stimuli were applied to one of the two teeth (Pressure and Cold Group), which was selected at random; and just cold stimuli were applied to the other (Cold Only Group).

On Day 0, a cavity was prepared in each of the pair of teeth, both cavities were etched, and the test stimuli were applied. Gutta percha temporary fillings were then placed in the teeth for 1 week. On Day 7, the temporary fillings were removed and the teeth were again tested as before. At the end of the experiment, the teeth were extracted under local anesthesia (2% lidocaine HCl with 1:100,000 epinephrine).

Tooth preparation

The preparation was the same as used in previous experiments (Ajcharanukul *et al.* 2007). Without local anesthesia, dentin was exposed at the tip of the buccal cusp by cutting a small cylindrical cavity (approximately 3 mm in diameter and 3 mm in depth) with diamond burs (No. 201, round and No. 204, cylindrical; Intensive®, Viganello-Lugano, Switzerland) in an air-rotor handpiece under a constant stream of water. The smear layer was removed from the exposed dentin by etching with 35% phosphoric acid for 30 s, after which the dentin was rinsed with water for 1 minute. A stainless steel tube (needle gauge 14: o.d. 2.11 mm, i.d. 1.56 mm; length 5 mm), which was used to apply stimuli, was sealed into the cavity with composite resin. Between stimuli, the tube was filled with normal saline at room temperature. The tube was removed by rotating it in the cavity before the gutta percha temporary filling was inserted on Day 0, and replaced after the filling was removed on Day 7. The dentin was not re-etched on day 7.

Test Stimuli

The hydrostatic pressure stimulus was applied by connecting a manometer preset at 300 mmHg below atmospheric to the stainless steel tube attached to the tooth for 5 seconds. This negative pressure stimulus was selected because it evoked pain reliably in a previous study (Charoenlarp *et al.*, 2007).

The cold stimulus was applied by injecting normal saline at 5°C from a syringe into the stainless steel tube for 5 seconds, while removing the overflow by suction. A 5°C

stimulus was chosen rather than the 0°C used previously (Chidchuangchai *et al.*, 2007), because in the earlier experiments the 0°C stimulus evoked a near maximum response in some teeth when it was applied to freshly exposed, etched dentin and we wished to be in a position to record an increase in the response to cold stimuli after the induction of pulpal inflammation. The cavity was dried with a cotton pellet before the cold stimulus was applied.

After each stimulus, the participant indicated the intensity of any pain produced by placing a mark on a simple visual analogue scale calibrated from 0 (no sensation) to 100 mm (the most severe pain one can imagine) (Holland *et al.*, 1997).

Pulpal blood flow

Pulpal blood flow was recorded with a Moor Type MBF3D/42 blood flow monitor (Moor Instruments, Axminster, England). During recording, opaque black rubber dam (Four D Rubber Co. Ltd., Heanor, England) was applied on the tooth in order to reduce to a minimum the contribution of blood flow in tissues outside the tooth (Soo-Ampon *et al.*, 2003). The probe of the instrument (o.d. 1.5 mm) contains two, 0.2 mm diameter optical fibers with their centers separated by 0.5 mm. The probe was fixed to the tooth with a clip-on splint. The splint was constructed from self-curing acrylic resin on a plaster model of the tooth. The probe tip was supported on the tooth surface by a short length of stainless-steel tube (i.d. 1.5 mm) that was incorporated into the splint. The tube was positioned so that it was perpendicular to the enamel surface, with its center 2 mm from gingival margin and over the central long axis of the crown of the tooth. The rotation of the probe around its long axis within the tube was kept constant between trials by aligning marks on the probe and tube. This precaution was necessary to ensure that reproducible results were obtained under each of the experimental conditions.

The flux signal from the blood flow monitor was transferred to a computer and analyzed using the Moorsoft program (Moor Instruments, Axminster, England). The sensitivity of blood flow signal was standardized as described previously and recordings made with an upper bandwidth setting of 14.9 kHz and a time constant of 0.1 s. Blood flow was measured in arbitrary perfusion units (Vongsavan and Matthews, 1993).

Control recordings of pulpal blood flow were made from both teeth on Day 0 approximately 5 min. after cavity preparation but before inserting the stainless steel tube for stimulation. The recording was repeated after stimulation, approximately 5 min. after removing the tube; and at the corresponding stages before and after stimulation on Day 7.

During the recordings on each occasion, the cavity was filled with saline and the mean pulpal blood flow signal was determined over a period of 1 min.

Statistical Analysis

The pain and blood flow data are summarized in box-plots. Each box represents the median (a line through its center) and the 25th and 75th percentiles. Whiskers above and below the box indicate the 90th and 10th percentiles. The significance of changes in the pain scores following gutta percha treatment were determined by comparing corresponding median pain scores using the non-parametric Wilcoxon Signed Rank Test for paired data. This test was used in preference to a parametric test because the pain data, although satisfying tests of normality, were not drawn from a continuous distribution. The pulpal blood flow data were not normally distributed and the effects of the different stimuli and of gutta percha treatment on blood flow were also analyzed using the Wilcoxon Signed Rank Test. P values of less than 0.05 are considered significant.

Results

The VAS pain scores obtained from stimulating both groups of teeth, before and after gutta percha treatment, are summarized in Fig. 1. In the Pressure and Cold Group, the median pain score recorded in response to the –300 mmHg hydrostatic pressure stimulus decreased significantly from 39 mm (range 0 – 98) to 0 mm (range 0 – 50) between Days 0 and 7 (p=0.001, *Signed Rank Test, n*=14). Over the same period and in the same teeth, the median pain score in response to the 5°C stimulus increased significantly from 10.5 mm (range 0 – 42) to 43.5 mm (range 8 – 82), (p=<0.001, *n*=14). In the Cold Only Group, the median pain score evoked by the 5°C stimulus increased significantly from 11 mm (range 0 – 67) on Day 0 to 34.5 mm (range 4 – 66) on Day 7 (p=0.017, *n*=14).

Examples of the blood flow records obtained from both teeth in one participant before and after stimulation, both before and after gutta percha treatment, are shown in Fig. 2.

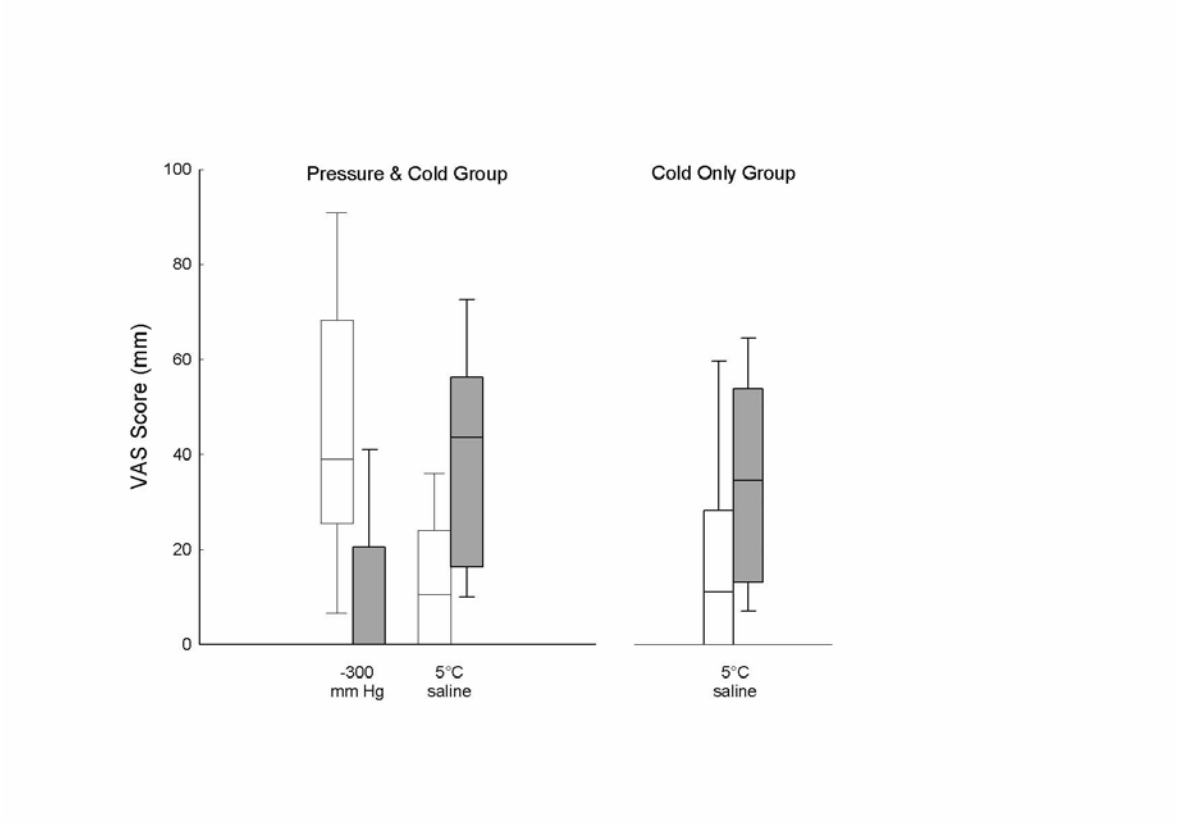

Figure 1. Pain scores recorded after the application of cold (5°C normal saline) and sub-atmospheric hydrostatic pressure (-300 mm Hg) stimuli to both groups of teeth in 14 participants on Day 0 (open boxes) and on Day 7, after gutta percha treatment (shaded boxes). The line through the center of each box indicates the median and the lower and upper limits of the box, the 25th and 75th percentiles respectively. The bars below and above each box indicate the 10th and 90th percentiles.

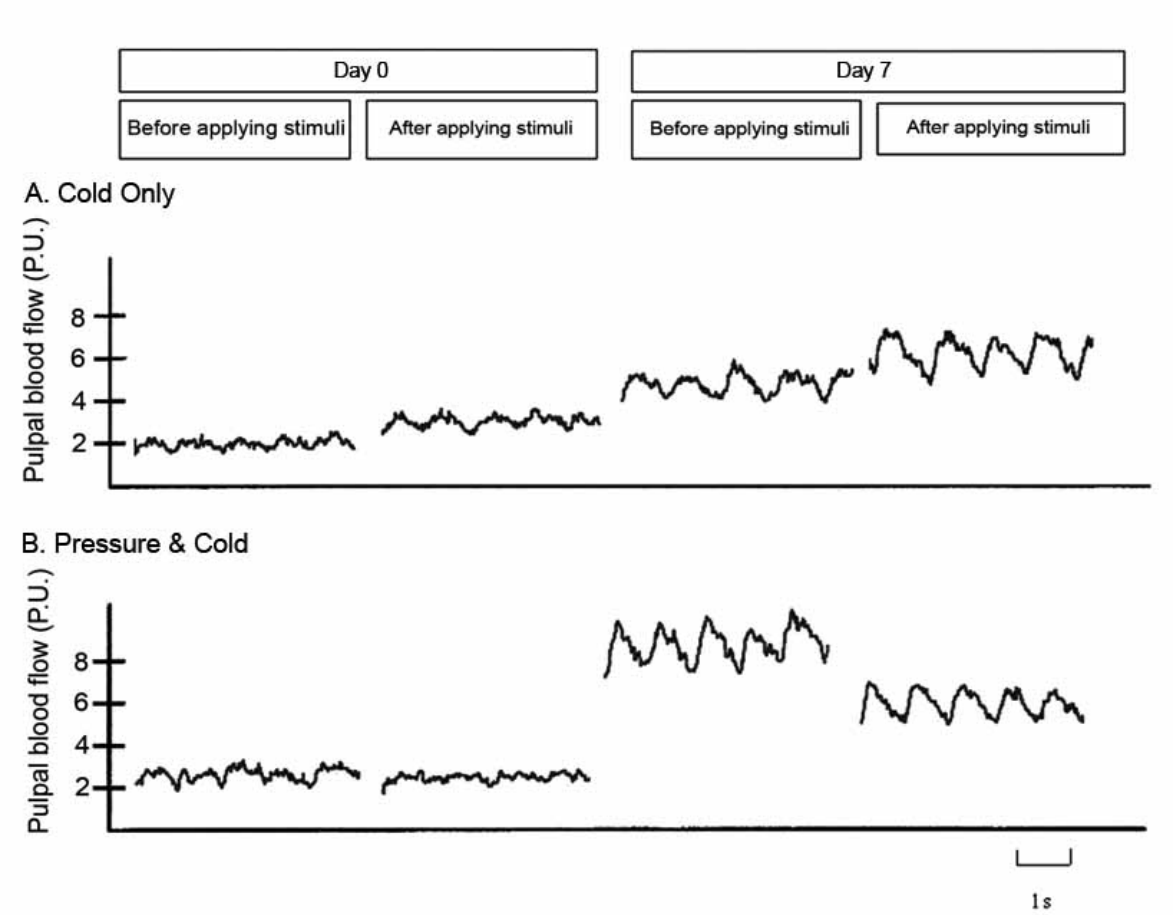

Figure 2. Records of pulpal blood flow from teeth tested with pressure and cold stimuli (A) and with cold stimuli only (B) in one participant before and after stimulation, both before and after gutta percha treatment.

The topical application of 500, 250 mmol/l KCl or 500 mmol/l NaCl in Ringer's solution for 10 min at atmospheric pressure did not produced pain in any of the subjects.

Before treatment, both air blast and probing stimuli produced pain from all the teeth. The mean VAS score with air blast stimuli (88.0, S.D. 14.8, n=24) was significantly greater than that with probing (59.1, S.D. 20.1) (paired t-test, $P<0.001$).

The effects of the 3 forms of treatment on the VAS scores are summarized in Fig. 2. In each case, there was a tendency for the mean responses to decrease from the baseline values for up to 10 min. after treatment, and then to recover during the subsequent 10 min. The only treatments that produced significant changes were the 500 mmol/l KCl solution, after both 5 and 10 min. with air blast stimuli, and after 10 min. with probing; and the 250 mmol/l KCl solution, after both 5 and 10 min. with air blast stimuli. Five min. after treatment with 500 mmol/l KCl, the fall in the mean VAS score with air blast stimuli was from a baseline of 88.1 mm (S.D. 9.1) to 54.6 mm (S.D. 26.9) (one way repeated-measures ANOVA, Tukey test, $p<0.05$); and after 10 min. it had fallen further to 44.3 mm (S.D. 32.0) ($p<0.05$). The corresponding mean values for 250 mmol/l KCl were a fall from a baseline of 79.1 mm (S.D. 21.1) to 63.9 mm (S.D. 26.3) ($p<0.05$) after 5 min. and to 57.4 mm (S.D. 28.0) ($p<0.05$) after 10 min. With probing, the mean VAS score 10 min. after treatment with 500 mmol/l KCl had fallen from a baseline of 64.4 mm (S.D. 19.0) to 41.6 mm (S.D. 21.0) ($p<0.05$). The effect on responses to probing 5 min. after 500 mmol/l KCl was not significant (one way repeated-measures ANOVA, Tukey test, $p>0.05$), nor were the responses at either 5 or 10 min. after 250 mmol/l KCl (Friedman repeated-measures ANOVA on ranks , $p>0.05$). With 250 mmol/l KCl, the median VAS score to probing was 54.5 mm before treatment and 45.5 and 37.5 mm, respectively, 5 and 10 min. after treatment. None of the changes from baseline after 500 mmol/l NaCl were significant.

The overall mean area of dentine exposed in the floor of the cavity was 7.30 mm^2 (S.D. 1.06), and the mean remaining dentine thickness was 1.19 mm (S.D. 0.25). In neither case were there significant differences between the mean values for the groups of teeth used for each of the different test solutions (one way ANOVA, $p>0.05$). There was no significant correlation between the baseline VAS scores with air blast or probing stimuli, and either the remaining dentine thickness or the exposed dentine area (Pearson product moment correlation coefficient). The same results were obtained when the

The pulpal blood flow values recorded from both groups of teeth, before and after gutta percha treatment, are summarized in Fig. 3. In this Figure, the data have been normalized by expressing the values recorded after stimulation on Day 0 and those

recorded both before and after stimulation on Day 7, as percentages of the corresponding control value obtained before stimulation on Day 0. Pulpal blood flow increased significantly between Days 0 and 7, but stimulation produced no significant change on Day 0 or on Day 7 in either group. In the pooled data from both groups, the median value on Day 0 was 1.6 (range 0.1 – 68) perfusion units and this increased to 2.7 (range 0.5 – 111) perfusion units on day 7 ($p<0.001$, Wilcoxon Signed Rank Test, $n=56$).

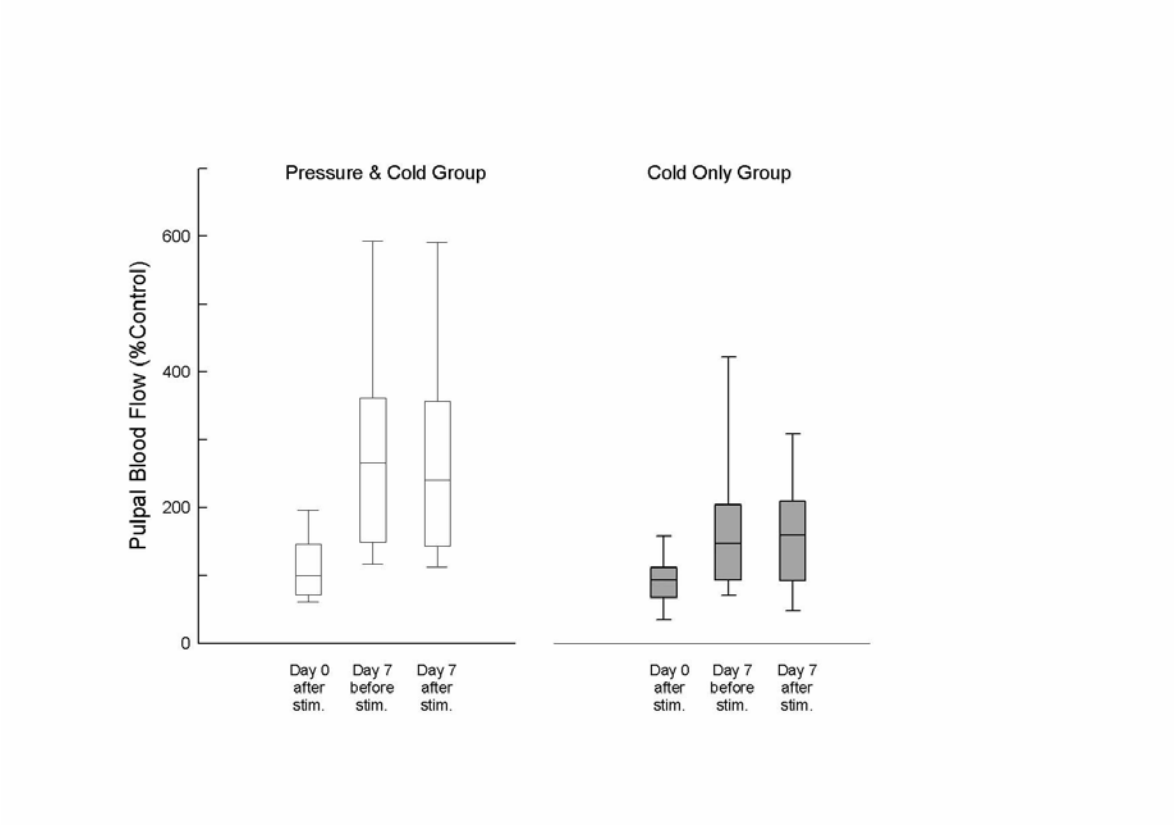

Figure 3. Values of the pulpal blood flow signal recorded from both groups of teeth in 14 participants on Day 0, and on Day 7 after gutta percha treatment. The data have been normalized by expressing the values recorded after stimulation on Day 0 and those recorded both before and after stimulation on Day 7, as percentages of the corresponding control value obtained before stimulation on Day 0. Each box shows the median and the 10^{th}, 25^{th}, 75^{th} and 90th percentiles, as in Fig.1.

Discussion

This study has shown that gutta percha treatment increases the sensitivity of human dentin to cold stimuli at the same time as it decreases its sensitivity to negative hydrostatic pressure stimuli. The increase in the response to cold in the Pressure and Cold Group was not due to an effect of the preceding hydrostatic pressure stimulus since the same effect was seen in the Cold Only Group.

Since both the cold and negative pressure stimuli would have caused outward flow through the dentin, these observations provide further evidence that the pain produced by cold stimuli in man is not due to hydrodynamic receptors; but to some other type, such as specific cold receptors (Chidchuangchai *et al*., 2007). Naylor (1963) showed that the reaction times to cold stimulation of dentin in man were too short to be due to activation of cold-sensitive receptors at the pulp-dentin junction. The involvement of hydrodynamic receptors at the pulp-dentin junction could account for this short latency; but the proposed specific cold receptors would have to be located more peripherally, in the dentinal tubules. Since nerve terminals are confined to the inner 100 μm of the dentin (Lilja, 1979, Holland *et al*, 1987), this raises the possibility that the odontoblasts, with cold-sensitive membrane ion channels, may be involved in the sensory transduction mechanism (Magloire *et al*., 2009). However, Son *et al*.,(2009) found no evidence that the specific cold receptors TRPM8 and TRPA1 were expressed in odontoblasts in neonatal mouse incisors.

The gutta percha treatment also produced a significant increase in pulpal blood flow. This can be attributed to inflammation of the pulp, which is known to occur under this type of filling (James *et al.*; 1954; Massler, 1956). An increase in pulpal blood flow, with histological evidence of inflammation, has been observed previously with preparations similar to that used in the present experiments (Veerayutthwilai, 2001).

Although the test stimuli evoked pain, they did not produce any significant increase in pulpal blood flow. This lack of evidence for neurogenic inflammation may be because only the large diameter pulpal afferents were stimulated (Andrew and Matthews, 2002), or, in the gutta percha-treated teeth, because the blood flow had already increased maximally. Also, a transient increase in blood flow may have been missed due to the delay of *c*. 5 mins while the laser Doppler recording system was set up after the stimulation was completed.

There are several possible explanations for the changes in sensitivity of the dentin that were observed following gutta percha treatment. For example, the increase in response to cold may have been due either to an increase in the sensitivity of the sensory receptors in the teeth (peripheral sensitization) or to changes in the pain pathways in the brain that are

activated by the discharge evoked in the primary afferent nerve fibers (central sensitization). An increased sensitivity of pulpal afferents to cold following exposure of the dentin for one week has been reported in the dog (Hirvonen *et al*. 1992), although, in other studies in experimental animals, little evidence was found of peripheral sensitization in inflamed teeth (Andrew, Sirimaharaj and Matthews, unpublished observations). The decrease in response to a negative pressure stimulus we observed could have been due to a decrease in hydraulic conductance of the dentin, resulting in less flow through dentinal tubules and a reduced response from hydrodynamic receptors. Such a change might result from the polymerization of fibrinogen that leaked into the tubular fluid from the pulp (Pashley et al., 1984). If there was a decrease in conductance that was confined to the peripheral ends of the tubules, the outward flow produced at the pulpal ends of the tubules by the application of a cold stimulus to the cavity may have been increased compared with that in freshly exposed dentin (see Chidchuangchai *et al*., 2007). This is a possible alternative explanation for the observed difference in the effects of gutta percha treatment on responses to negative pressure and cold stimuli.

Evidence for central sensitization in trigeminal nociceptive pathways has been obtained in experimental animals (Sessle, 2000). Also, Veerayutthwilai *et al*. (2002) showed that the sensory thresholds to electrical stimulation of tooth pulp in human participants were decreased following gutta percha-treatment, an effect that is likely to have been due to central sensitization.

Our finding that gutta percha treatment reduced the sensitivity of dentin to negative pressure stimuli appears to contradict the result of Anderson and Matthews (1967), who showed that the same treatment increased the sensitivity of dentin to osmotic stimuli. However, in those experiments no allowance was made for a smear layer. A smear layer would have been present on the exposed dentin surface immediately after cavity preparation, and this may have been etched away during the ensuing week by acids that accumulated under the leaky gutta percha filling. Removal of the smear layer in this way could account for the observed increase in sensitivity of the dentin. No data are available on the effects of smear layer removal on the sensitivity of dentin to osmotic stimuli.

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Series IV experiments

Prostaglandin E_2 Levels Blood Flow and Pain Sensation in Human Teeth with Inflamed Pulps

Abstract

***Objective*:** The objective of this study was to investigate the effects of pulpal inflammation on prostaglandin E_2 (PGE_2) levels, pulpal blood flow and pain sensation evoked by thermal stimulation in human subjects.

***Design*:** The experiments were done on 14 healthy premolars. Dentine was exposed at the tip of the buccal cusp. Pain evoked by thermal stimulation (water at 10 and 60°C) for 5 seconds and was scored by the subject on a visual analog scale (VAS). The cavity was then filled with either gutta percha alone (GP) or, on the contra-lateral side, GP sealed with glass ionomer material (GI). After one week, the fillings were removed, the cavities were cleaned and the measurements and stimuli repeated. The teeth were extracted and fractured longitudinally. Pulpal tissue fluid was collected and its PGE_2 level determined using enzyme-immunoassay.

***Results*:** Treatment with GP alone increased both mean pulpal blood flow and mean VAS scores at 10 and 60°C, respectively. PGE_2 levels were significantly higher after filling with GP than GP+GI (494.9±106.7 and 186.3±47.9 ng/ml, respectively; $p<0.01$, *Student t-test*,).

***Conclusion*:** The unsealed GP filling caused an increase in the PGE_2 level of pulpal fluid, vasodilatation and hyperalgesia of dentine.

Introduction

It has been demonstrated that the exposed dentine was permeable to Evans blue dye both *in vivo* and *in vitro* study (Vongsavan and Matthews, 1991; Vongsavan *et al*., 2000). Generally, enamel loss, gingival recession, dental caries, and unsealed margin of restoration can cause bacteria in oral cavity and their toxins diffusing through exposed dentine into the dental pulp and producing pulpal inflammation. Bergenholt and Lindhe (1975; Bergenholtz, 1977) showed inflammatory reactions of the pulp tissue responding to topical application of a plaque extract on class V cavities. Some unsealed filling materials, for instance gutta percha (GP) produced inflammatory changes in the pulps (Jame *et al*., 1954; Massler, 1956; Mjör and Tronstad, 1972) and also increased pain sensation to osmotic excitants (Anderson and Ronning, 1962). Moreover, in experimentally induced pulpitis, the pulpal tissue fluid pressure increased significantly (Tønder and Kvinnsland, 1983) and the mechanism of these may involve the alteration of pulpal blood flow. Recently, in human study Veerayuthwilai *et al* (2001) demonstrated that leaky filling materials also produced pulpal vasodilatation and decreased pain threshold to electrical stimulator.

Prostaglandin E_2 (PGE_2) is an important mediator in the pathological diseases including dental pulp diseases (Torabinejad and Bakland, 1980a; 1980b). PGE_2 initiates and induces many inflammatory events such as vasodilatation, increased vascular permeability, chemotaxis and pain. Previous evidences of PGE_2 in dental pulps were studied in animals (Türker and Türker, 1974; Ahlberg, 1978; Hirafuji and Ogura, 1983; Okiji *et al.*, 1987; 1989). Türker and Türker (1974) used a bioassay to show PGE-like activity in the effluent from electrically stimulated teeth in dogs. Ahlberg (1978) demonstrated the indirect evidences that the pre-medication with prostaglandin-inhibiting drugs could block induced nerve impulses in cat teeth. Hirafuji and Ogura (1983) measured PGE_2 concentration in rat dental pulps with radioimmunoassay. In a later series of investigations by Okiji *et al.* (1987; 1989) in rat dental pulp showed that when the pulp was inflamed by applying bacterial lipopolysaccharide (Okiji *et al.,* 1987). PGE_2 was increased approximately 9 times compared with normal and vascular permeability in the markedly increased inflamed pulp (Okiji *et al.,* 1989). Recently, immunohistological studies showed that fibroblasts and macrophages mainly participate in the production of PGE_2 (Miyauchi *et al*., 1996; Nakanishi *et al*., 2001).

Cohen *et al*. (1985) firstly demonstrated that the painful human dental pulps contain a significantly higher level of PGE_2 than the asymptomatic pulps. Recently,

Nakanishi *et al*. (1995) and Waterhouse *et al*. (1999; 2002) detected and quantified PGE_2 levels in pulpal blood from exposed pulp (range 1-2,641 ng/ml).

However, there is no evidence to show the presence of PGE_2 in dentinal fluid and pulpal tissue fluid in human teeth with inflamed pulp. We, therefore, purpose that detection and quantity of this mediator in dentinal and pulpal tissue fluid will help to evaluate the progression of the inflammation in the dental pulp. Moreover, there is no data available on the results of inflammation in the levels of PGE_2 relation to pulpal blood flow, sensory threshold and pain sensation produced by thermal stimulation in well-control study in human subjects. The aims of this study were to, firstly, to develop method to measure prostaglandin E_2 (PGE_2) levels in dentinal fluid and pulpal tissue fluid and secondly, to Investigate the effects of pulpal inflammation on pain sensation evoked by cold stimulation of dentine, pulpal blood flow, sensory threshold, and PGE_2 levels in human subjects.

Materials & methods

Subjects

The experiments were performed on 20 healthy permanent premolar teeth from 10 human subjects (aged 17-30 years, mean 20.7 years). The teeth were scheduled for extraction as part of orthodontic treatment. All the teeth were fully erupted, completely formed root, free of dental caries, without restorations and intact. Radiographic and clinical examinations confirmed that they were vital and healthy. The study was approved by the Ethics Committee on human rights related to human experimentation at Mahidol University. All voluntary subjects who participated in this experiment had been described the experimental procedures and the possible discomforts. The informed consents were obtained from each subject or, for those under 18 years, a parent or guardian.

Experimental Design and Procedure

The experiment was designed to use either right and left upper premolars or right and left lower premolars in each subject. One tooth was randomized to be the experimental tooth and another contralateral tooth was the sham-control tooth. Data of age, number of tooth, preoperative sign and symptom of each subject were taken. Prior to starting the experimental procedure, impression was taken to build the cast model of teeth and then the acrylic splints of the premolar teeth were made by self-curing acrylic on the model to stabilize a laser Doppler probe in the proper position.

Pulpal blood flow, the pain evoked by thermal stimulation and sensory threshold to electrical stimulation were recorded preoperatively as baseline values (see detailed below). After tooth preparation and smear layer removal by acid etching (35% phosphoric acid; Scotchbond™, 3M Dental Products, ST. Paul, MN, USA; for 30 seconds and rinsing with water for 1 minute), the measurements and stimuli were repeated. Dentinal fluid effluent was also collected for PGE_2 determination as baseline. The cavity was , then, filled with either gutta percha (GP) alone (the experimental group) or, on the contralateral side, GP sealed with glass ionomer material (GI, Vitremer®, 3M Dental Products, St. Paul, MN, USA) (the sham-control tooth).

After one week, the fillings were removed, the cavities cleaned with distilled water, and the measurements and stimuli repeated again. Dentinal fluid effluent was also collected again. Then, the teeth were carefully extracted either with forceps and elevator under local anesthesia (2% lidocaine HCl with 1 : 100,000 epinephrine) and fractured longitudinally. Pulpal tissue fluid was collected and its PGE_2 level determined using enzyme immunoassay.

Tooth Preparation and Filling

Without local anesthesia, dentine was exposed at the tip of the buccal cusp by cutting a small cylindrical cavity (approximately 3 mm in diameter and 3 mm in depth) with two diamond bur (round and cylinder diamond burs; Intensive® No. 201 and No. 204, Viganello-Lugano, Switzerland) in an airotor handpiece under a constant stream of water. The exposed dentine was prevented from drying with filling distilled water in the cavity.

The experimental tooth was filled with GP to allow bacterial leakage via dentinal tubules through dental pulp. Whereas the sham-control tooth was filled with GP about 1 mm thickness and sealed with resin-modified glass-ionomer cement (GI) on the top of the cavity in order to prevent the bacterial leakage (Figure 1)

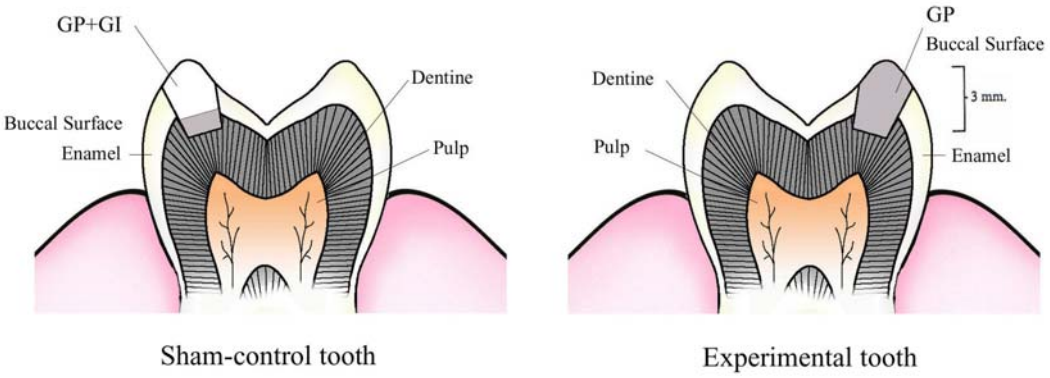

Figure 1. Diagram of the tooth preparations and fillings

Cavities were prepared at the tip of buccal cusps (approximately 3 mm in diameter and 3 mm in depth). The sham-control tooth was filled with gutta percha (GP) about 1 mm thickness and sealed with resin-modified glass-ionomer cement (GI) on the top of the cavity whereas the experimental tooth was filled with gutta percha alone.

Applications of Thermal Stimuli and Pain Assessments

The applications of thermal stimulation were performed with ice stick (3 mm in diameter), cool water (10^{o}C) and warm water (60^{o}C) to each tooth for 5 seconds. The subject indicated the intensity of any pain produced by placing a mark on a visual analog scale (VAS) in each stage.

Before tooth preparation, only ice stick was applied by placing the tip of an ice stick on the tip of buccal cusp. Whereas, after tooth preparation, cold and hot stimuli were placed both in the un-etched and etched exposed dentine cavity. The ice stick was also placing on the middle of buccal surface

. The exposed dentine was prevented from drying with distilled water droppings. Each of thermal stimulation was applied at 2-minute intervals throughout the experiment. Before stimulation of cool and hot water, the cavity was gently dried by cotton pellet.

Sensory Threshold to Electrical Stimulation

The sensory threshold was determined with a monopolar electrical pulp tester (EPT, 1 ms, constant current). The teeth were stimulated with the same electrical probe applied on the middle part of buccal surfaces while the subject was holding the electrode in his/her hand. The stimulating current initially caused pain was recorded as a value of sensory threshold.

Measurement of Pulpal Blood Flow

The pulpal blood flow was monitored throughout the experiment with a laser Doppler flowmetry (Moor type MBF3D/42, Moor Instruments Ltd., Axminster, Devon, England) which was configured in the standard way (Vongsavan and Matthews, 1993) after isolating the tooth with opaque black rubber dam (Four D Rubber Co. Ltd., Heanor, England). The probe of the instrument (external diameter. 1.5 mm) contained two, 0.2 mm diameter optical fibres with their centers separated by 0.5 mm. The probe was fixed to each tooth with a clip-on splint that covered the crown of the test tooth. The splint was constructed from self-curing acrylic resin on a plaster model of the teeth. The probe tip was inserted into a stainless-steel tube (internal diameter 1.5 mm) which was incorporated into the splint over the central long axis of the crown of the test tooth, perpendicular to the enamel surface and with its center 2 mm from the gingival margin. The alignment of the probe around its long axis was kept constant between trials by aligning marks on the probe and the tube. This precaution was necessary to ensure that reproducible results were obtained under each of the experimental conditions. The data were recorded with the

Moorsoft (version 4.3) software programme. Recordings of pulpal blood flow were made for approximately 1 minute for each time measurement period. The pulpal blood flow values recorded from both sham-control and experimental teeth were corrected by subtracting the values made at the same light intensities from a stationary reflector.

Measurement of Prostaglandin E_2 Levels

Sample Collection

- Dentinal Fluid

Dentinal fluid was collected by placing 10 μl phosphate-buffered saline (PBS) in the etched cavity for 5 minutes and then collecting by microsyringe. The volume and weight of the samples were recorded. The samples were stored at -80^{o}C until PGE_2 determination.

- Pulpal Fluid

After extraction, the tooth was grooved along buccal, lingual and occlusal surfaces with thin diamond disc under a constant stream of water, and then fractured longitudinally with chisel and hammer within 5 minutes. Pulpal tissue fluid was collected from the buccal horn with a microsyringe. The volume and weight of the samples were also recorded. The samples were stored at -80^{o}C until PGE_2 determination.

The histological evaluation of dental pulp obtained from some teeth of the subjects was also done to confirm that there were the evidences of inflammation in dental pulps.
After extraction, the tooth was fractured longitudinally and pulpal tissue fluid was collected from the buccal horn with a microsyringe. The teeth were placed immediately in fixative agent for short period of time and measured for remaining dentine thickness. Then, the teeth were fixed in neutral buffered 10% formalin (fixative agent) for 7 days. Specimens were decalcified in 10% neutral ethylene diamine tetraacetic acid (EDTA) solution about 4-5 months depending on the size of the tooth, then were dehydrated in the ascending order of alcoholic concentrations in order to prepare for paraffin embedding with a tissue changer machine (Fisher Tissuematon®, Model No.15-182-205, Fisher Scientific Company, USA). After the process was completed, specimens were embeded in paraffin, melted in a parraffin dispenser (Model No. 222 CY 50-60, Lipshaw MFG Company, Michigan, USA). Then, paraffin block was mounted on a hand cutting microtome (R Jung AG Heidelberg, Geramany) and serial sections through the entire pulp

were cut at a thickness of 6 μm. The general condition of the pulp was evaluated with hematoxylin and eosin staining under the microscope (CH2, Olympus, Japan).

Measurement of remaining dentine thickness

After all teeth were fractured longitudinally through the cusps and processed for histological assessment. The remaining dentine thickness (RDT) of all teeth were also measured between the highest of pulpal horn and floor of the cavity along the dentinal tubules by using a measuring microscope.

Statistic Analysis

Comparisons between the overall mean values of VAS, pulpal blood flow, and sensory threshold at each stage in each group of the experiment were analyzed statistically using one-way repeated-measures analysis of variance (one-way RM ANOVA). Where this shows that there were significant differences between the means, the Tukey test was used to make multiple comparisons between them. The mean values of PGE_2 levels in dentinal fluid and pulpal fluid were analyzed using *Paired* t-test and *Student's* t-test, respectively. Data were reported as means ± 1 S.D. and P values of less than 0.05 were considered significant. The correlation between overall data and RDT, were also evaluated.

Results

Pain Sensation produced by Placing Water at 10°C on exposed dentine

Before treatment, when water at 10°C was placing into exposed dentine cavities under different conditions in sham-control and experimental teeth, the mean VAS scores were not significant differences in each group. The mean VAS scores in sham-control group were 2.00 mm ± 3.50 (before etching) and 3.50 ± 4.53 mm (after etching) (Figure 2); and in experimental group were 2.60 ± 3.34 mm and 3.30 ±4.08 mm, respectively (Figure 2).

Treatment for 1 week with GP + GI had no significant effect on mean VAS scores (3.70 ± 6.24 mm). While treatment with GP alone increased mean VAS score to18.55 ± 11.48 mm. There was significantly different between the mean VAS scores in sham-control and experimental groups ($P<0.05$) as shown in Figure 2.

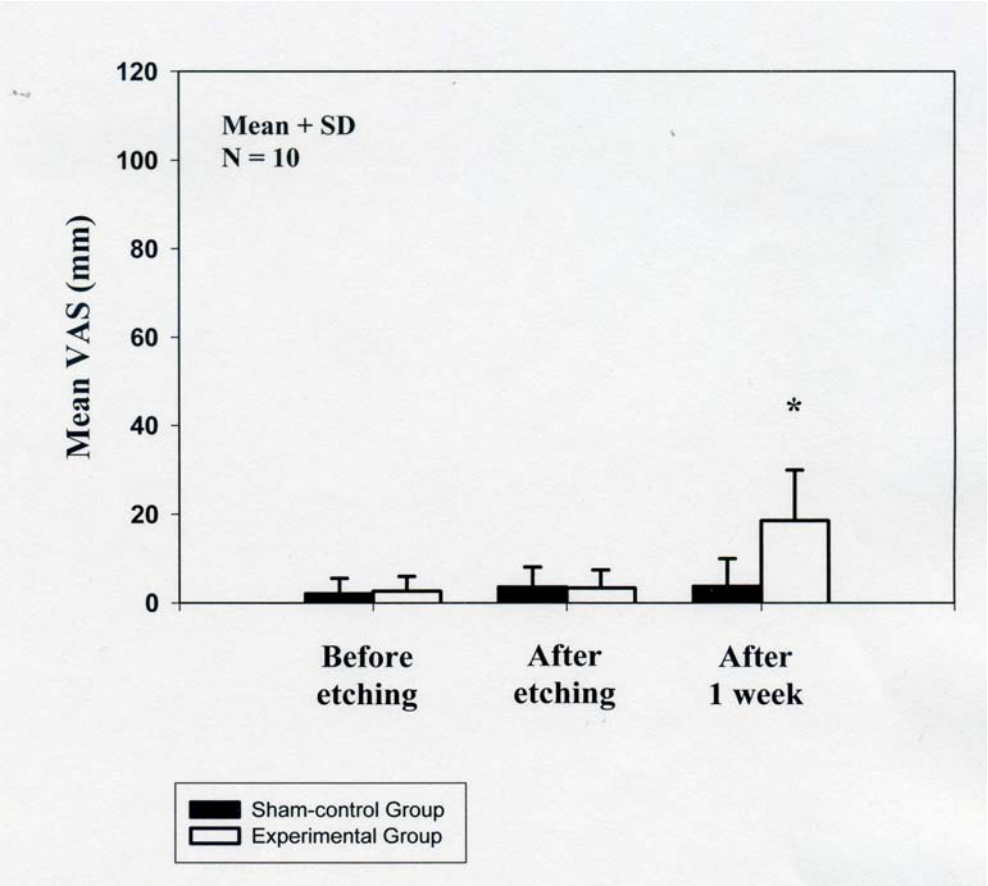

Figure 2. Effect of pulpal inflammation on pain sensation produced by cold stimulation (cool water: 10 ^{o}C) of exposed dentine in human teeth.The summary of the mean VAS score produced by placing cool water : 10 ^{o}C in the prepared cavity under different conditions in vivo from 10 pairs of sham-control and experimental teeth in 10 human subjects was shown. The error bars represent +1 SD. After 1 week, treatment with GP alone in experimental group increased the mean VAS score significantly when compare to treatment with GP+GI in the sham-control group (*: $P<0.05$.

Effect of Pulpal Inflammation on Pulpal Blood Flow

Treatment for 1 week with GP+GI in sham-control teeth had no significant effect on pulpal blood flow values (baseline; mean 3.22 ± 1.05 perfusion units (P.U.), after preparation; 3.67 ± 1.40 P.U., and after 1 week; 4.34 ± 2.71 P.U.; $P>0.05$, One-way repeated measures ANOVA). In contrast, treatment with GP alone for 1 week in experimental teeth caused a significant increase in pulpal blood flow (baseline; 3.42 ± 1.15 P.U., after preparation, 4.06 ± 1.63 P.U., and after 1 week; 9.9 ± 2.4 P.U.; $P<0.05$, One-way repeated measures ANOVA, Tukey test). Even though pulpal blood flow values were slightly increased after preparation in some teeth from both groups (5 of 10 teeth in sham-control group and 4 of 10 teeth in experimental group). The mean pulpal blood flow values obtained from sham-control and experimental groups at each stage are summarized in Figure 3.

Furthermore, the percentages of increased pulpal blood flow (IPBF) in sham-control and experimental groups were calculated from the following equation:

$$\text{Percentage of IPBF} = \frac{\text{IPBF}}{\text{BPBF}} \times 100$$

When IPBF = increased pulpal blood flow (PBF after 1 week – BPBF), and BPBF = baseline pulpal blood flow.

Similar to the mean pulpal blood flow values, percentage of IPBF was significantly higher after filling with GP than GP + GI (198.94 ± 249.27 and 41.10 ± 68.519 respectively; $P<0.05$, *Student's t-test*).

Almost inflamed teeth (9 of 10 teeth in experimental group, pulpal blood flow values were increased more than 50% (range 61.54 – 883.33). There was only one case in experimental group that pulpal blood flow value was comparable or slightly increased. While 7 of 10 teeth in sham-control teeth, pulpal blood flow values were decreased or comparable or increased less than 50%.

Effect of Pulpal Inflammation on Prostaglandin E_2 Levels

Prostaglandin E_2 levels both in dentinal and pulpal fluid in human subjects was firstly detectable.

Prostaglandin E_2 Level in Dentinal Fluid

After placing 10 μl phosphate-buffered saline (PBS) in the cavity for 5 minutes to dissolve or mix PGE_2 in dentinal fluid into the solvent and then collecting the mixture by microsyringe, the volumes of the dentinal fluid samples were ranged 10-11 μl. Because of the very small amount of PGE_2 in some dentinal fluid samples, which could not be

precisely detected by EIA technique, the concentration of PGE_2 in dentinal fluid sample was calculated form the minimum value, which this technique could measure. At baseline, 9 of the 10 samples in sham-control group and 7 of the 10 samples in experimental group contained undetectable amount of PGE_2.

After 1 week, PGE_2 concentrations in dentinal fluid from teeth treated with GP+GI in sham-control group range 0.25-2.02 ng/ml. While those from teeth treated with GP alone in experimental group range 0.28-3.83 ng/ml (Figure 4).

Treatment with GP alone and GP + GI increased the production of PGE_2 in dentinal fluid significantly (mean from 0.33 ± 0.26 ng/ml to 1.32 ± 1.26 ng/ml, and from 0.35 ± 0.26 ng/ml to 0.67 ± 0.56 ng/ml, respectively, $P<0.01$ and $P<0.05$, Paired t-test). However, the mean values of PGE_2 levels in dentinal fluid from teeth treated with GP was significant higher than that from teeth treated with GP+GI ($P<0.05$, *Student's* t-test) as shown in Figure 4.

Furthermore, the percentages of increased dentinal fluid PGE_2 levels ($IdPGE_2$) in sham-control and experimental groups were calculated from the following equation:

$$\text{Percentage of } IdPGE_2 = \frac{IdPGE_2}{BdPGE_2} \times 100$$

When $IdPGE_2$ = increased dentinal fluid PGE_2 levels (dentinal fluid PGE_2 levels after 1 week – $BdPGE_2$), and $BdPGE_2$ = baseline dentinal fluid PGE_2 levels.

Similar to the mean PGE_2 levels in dentinal fluid, percentage of $IdPGE_2$ was higher after filling with GP than GP + GI (329.52 ± 465.46 and 135.05 ± 214.18 respectively) as shown in Figure 4.

Prostaglandin E_2 Level in Pulpal Fluid

After the teeth were extracted and fractured longitudinally, pulpal tissue fluid was collected from the buccal horn with a microsyringe. The volumes of the pulpal fluid samples were ranged 0.25-0.75 µl. However, the small volume of the pulpal fluid samples contained high PGE_2 concentration enough to determine the level using EIA technique. All teeth both in the sham-control and experimental groups contained a detectable amount of PGE_2. The concentrations of pulpal fluid PGE_2 in teeth treated with GP+GI in sham-control group ranged 16.73 - 537.39 ng/ml (mean 150.38 ng/ml, S.D. 164.70 ng/ml). While

those in teeth treated with GP alone in experimental group varied from 126.53 to 1189.54 ng/ml (mean 424.44 ± 377.06 ng/ml) as shown in Figure 5

PGE_2 levels in pulpal fluid were significantly higher after filling with GP than GP + GI ($p<0.05$, *Student t-test*) as shown in Figure 5. Moreover, in the same subjects, all of teeth in experimental group had pulpal fluid PGE_2 levels higher than those in sham-control group had.

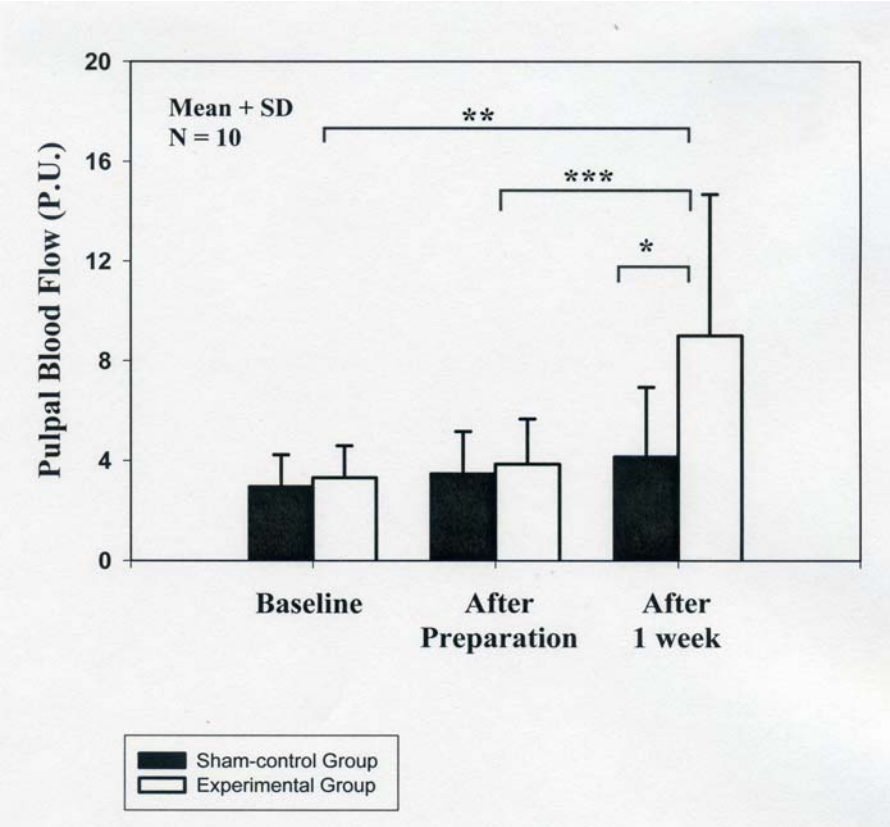

Figure 3. Effect of pulpal inflammation on pulpal blood flow in human teeth. The summary of the mean pulpal blood flow values from 10 pairs of sham-control group and experimental group in 10 human subjects was shown. The error bars represent +1 SD. After 1 week, treatment with GP alone in experimental group increased pulpal blood flow significantly when compare to treatment with GP+GI in the sham-control group (*: $P<0.05$), and when compare to the baseline (**: $P<0.05$) and after preparation values (***: $P<0.05$) The error bars represent 1 standard deviation.

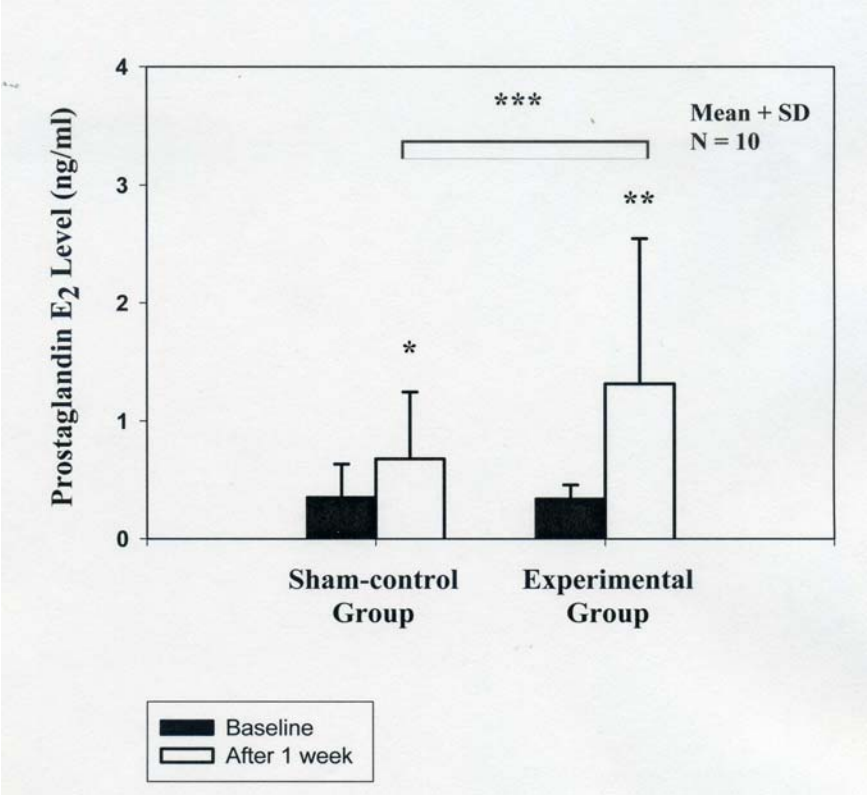

Figure 4. Effect of pulpal inflammation on prostaglandin E_2 levels in dentinal fluid in human teeth. The summary of the mean PGE_2 levels from duplicate determinations in dentinal fluid from 10 pairs of sham-control group and experimental group in 10 human subjects was shown. The error bars represent +1 SD. Treatments with GP alone in experimental group and with GP+GI in sham-control group increased the production of PGE_2 in dentinal fluid significantly when compare to the baseline (*: $P<0.05$, **: $P<0.01$). GP alone caused an increase in PGE_2 level of dentinal fluid significantly when compare to GP+GI (***: $P<0.05$).

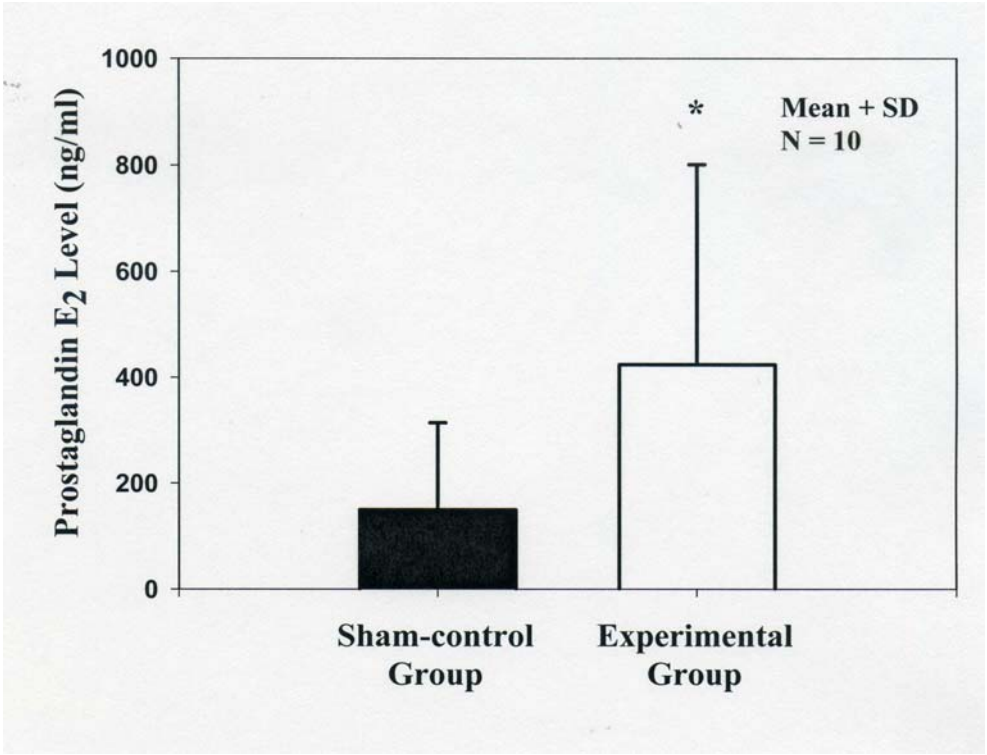

Figure 5. Effect of pulpal inflammation on prostaglandin E_2 levels in pulpal fluid in human teeth. The summary of the mean PGE_2 levels from duplicate determinations in pulpal fluid from 10 pairs of sham-control group and experimental group in 10 human subjects was shown. The error bars represent +1 SD. Treatment with GP alone in experimental group increased the production of PGE_2 in pulpal fluid significantly when compare to treatment with GP+GI in the sham-control group (*: $P<0.05$)..

Discussion

The present study showed that unsealed gutta percha (GP) filling produces pulpal inflammation and causes an increase in the PGE_2 levels of dentinal and pulpal fluid. GP also increases pulpal vasodilatation and hyperalgesia of dentine to thermal stimulation. In contrast, GP can cause decrease in sensory threshold to electrical stimulation. The present work was the first time to show that PGE_2 could be detected in dentinal fluid from teeth with inflamed pulps.

In present study, thermal stimuli (water 10^oC and 60^oC) were found to produce hyperalgesia in teeth treated with GP for 1 week. The experiments in animals indicate that there are two main groups of sensory receptor in teeth. The first is that responds to movement of the contents of the dentinal tubules, the so-called hydrodynamic receptors, and another that are sensitive to heat and chemical stimuli, some of which may express the VR1 receptor. Cold stimuli that have been shown to cause pain from dentine in man cause movement of the dentinal tubule contents and/or stimulate cold fibers, have been shown to excite hydrodynamic receptors in animals. Andrew and Matthews (1996), studied the effects of acute inflammation on intradental nerves in the cats. In his experiments, acute pulpal inflammation was induced by placing dental caries in deep cavities and studied the properties of the sensory receptors in the teeth for 7 days. Interestingly, he found only minor changes in the properties of the receptors. There was no evidence that the receptors were producing a lot of spontaneous discharge, and they were not a lot more sensitive to hot and cold or other stimuli that would cause pain in man. This suggested that the pain in man might not be due to an increased sensitivity of the receptors in the teeth, as has always been assumed, but to the central pathways responsible for the pain becoming more sensitive to input from the afferent nerves supplying the teeth. This phenomenon was called “central sensitisation”.

Previous studied (Veerayuthwilai *et al*.,2001) on lowering sensory threshold in teeth treated with GP for 1 week, similar results were found in this study. These suggested that the decrease of sensory thresholds to electrical stimulation detected from teeth with inflamed pulps could be due to either the sprouting of nerve terminals in the tooth or the central sensitization in the pain pathways from the pulps. Rodd and Boissonade (2001) also demonstrated the increase of innervation density of intradental nerve in inflamed human teeth. These changes including the sprouting of nerve terminals could result in the increase in size of receptive field and consequently lowering of sensory threshold to electrical stimulation (Rodd and Boissonade, 2001). Recently, some studies (Chiang *et al*., 1998;

Mannion *et al*., 1999; Katakura, 2001; Wakisawa *et al*., 1992) have shown that injury and inflammation occurring in the peripheral tissues could lead to the changes in the central nervous system. Moreover, Gillam *et. al*. (2000) suggested that electrical stimulating of the teeth did not rely on only the receptors at the pulp-dentine junction but the activation possibly involved in more central components of the axons in the pain pathway from the pulp (Gillam *et al*., 2000).

In this study, it was found that the increase of pulpal blood flow concurring with the lowering of electrical thresholds after inflammation existed in the pulp. These evidences strongly correlated to the previous findings in rat teeth (Byers *et al*., 1990; Byers, 1994) which demonstrated the sprouting of calcitonin gene-related peptide (CGRP) immunoreactive nerve fibers during inflammation in pulp and dentine near the injury. CGRP that is a sensory neuropeptide provides a potent vasodilatory effect (Brain *et. al*. 1985).

When inflammation caused by injury or bacterial infection occurs in dental pulps, cells in the tissues including odontoblasts, fibroblasts and nerve fibers produce PGE_2, which induced many inflammatory effects, including pain, vascular dilatation, increased vascular permeability and leukocyte migration. PGE_2 concentrations in dental pulp were determined in various samples in human (Cohen *et al*., 1985; Nakanishi *et al.*, 1995; Waterhouse *et al*., 1999, 2002). Cohen *et al*. (1985) determined PGE_2 levels in extracts of human dental pulp from subjects experiencing pain and from asymptomatic subjects, using a radioimmunoassay. They showed that the painful pulps had much higher PGE_2 levels than the non-painful pulps. The mean PGE_2 value for pulp from subjects experiencing pain was 139.25 ± 167.67 pg/ml of wet tissue. Nakanishi *et al.* (1995) established a method for collecting pulpal blood samples from exposed pulpal sites. They used pellets of absorptive material that were easy to handle when approaching the exposed pulp site and determined PGE_2 levels in blood of human dental pulp from inflamed and normal permanent teeth, using a PGE_2 enzyme immunoassay. They found that the mean PGE_2 value of blood of inflamed pulp significantly increased than that in normal pulps. These levels of the eicosanoid PGE_2 correlate well with pulpal inflammation in permanent teeth. Waterhouse *et al*. (1999, 2001) also detected and quantified PGE_2 in small blood sample of primary molar pulps. They found that the amount of PGE_2 varied widely, from 1.0 to 2,641 ng/ml (the majority of samples were below 100 ng/ml). Although the sample materials were different, PGE_2 levels in inflamed pulp or those from subjects experiencing pulpal pain varied widely, and levels in normal pulp were virtually undetectable in those studies.

Therefore, in this study the method to collect and quantify PGE_2 levels in dentinal and pulpal fluid was developed to provide the information about pulpal status. Interestingly, the results of well-control study are consistent with those previous studies (Cohen *et. al*., 1985; Nakanishi *et al.*, 1995; Waterhouse *et al*., 1999, 2002). At baseline, most of dentinal fluid samples contained undetectable PGE_2 levels. Although some of the samples (only 4 of 20 samples) contained a detectable amount of PGE_2 levels but they were very low. Treatment for 1 week with unsealed GP alone and with GP+GI caused an increased PGE_2 level in pulpal tissue fluid, which could outward flow through dentinal tubules from pulps to dentine exposed due to increased pulpal tissue fluid pressure. Thus, the increased PGE_2 levels could be detectable in dentinal fluid. The concentration of PGE_2 in pulpal fluid was more than 200-fold of that in dentinal fluid because of the dilution of dentinal fluid PGE_2 concentration with PBS during the sample collecting procedure. In some cases, even if in inflamed pulps, the PGE_2 level in dentinal fluid could not be detected. It may be that the diameters of dentinal tubules at the exposed area are too small to yield dentinal fluid volume enough to quantify PGE_2 level. To solve this problem, the time during collecting the dentinal fluid samples should be more than that used in the present study. However, this results revealed that in inflamed pulps, the PGE_2 concentrations in small volumes of pulpal and dentinal fluids did not varied widely as the concentrations in blood samples reported in the studies of Waterhouse *et.al*. (1999, 2001) and Nakanishi *et.al*. (1995). This findings suggest that the quantification of the eicosanoid PGE_2 in dentinal fluid samples could possibly serve as indicators or marker of the inflammatory state of pulpitis. Its non-invasive technique seems to be useful for diagnosis of early pulpal inflammation.

In comparison, the blood samples in previous studies (Waterhouse *et.al*.,1999, 2001; Nakanishi *et.al*., 1995) were collected by using absorbent materials such as paper point and nylon fiber. In this study, microsyringe was used to collect the dentinal fluid. Using absorbent materials can cause the rapidly outward movement of fluid content in dentinal tubules resulting in nerve endings stimulating and then pain, which effected on the pain assessment in the present study. Moreover, a matrix effect may occur if the test medium from which the PGE_2 is extracted interacts with the assay.

Production of PGE_2 which sensitize peripheral nociceptor terminal and produce localized pain hypersensitivity is initiated by the oxidation of arachidonic acid via one of three isoforms of cyclooxygenase (COX), COX-1, COX-2 and COX-3. COX-1 is constitutively expressed in various tissues and involves in mediating many physiologic functions. COX-2 is an inducible enzyme believed to be responsible for PG synthesis at

sites of inflammation. Recent discovery, COX-3 is present in brain, heart and muscle (Chandrasekharan *et. al.*, 2002). At the moment, the functions of COX-3 are still unknown. Nakanishi *et al*. (2001) revealed the localization of COX-2 mainly in fibroblasts in inflamed pulp by using an immunohistological method. Lin *et. al*. (2002) firstly demonstrated that COX-2 gene expression in dental pulp fibroblasts (cell culture) after found in inflamed periodontal tissues (Morton and Dongari-Bagtzoglou, 2001) and suggested that the fibroblasts involved in the development of pulpitis and related painful sensation. Moreover, increased synthesis of PG is known to increase neuronal excitability and may play a role in CNS. Samad and colleagues (2001) have demonstrated significant upregulation of COX-2 in CNS parenchyma in response to acute peripheral inflammatory pain. COX-2 inhibitor, rofecoxib, has been shown to have excellent CNS penetration and reduce CNS responses to locally synthesized PG (Buvanendran *et. al.* 2001). Based on these findings, the use of selective COX-2 inhibitors may provide a valuable tool in control of pulpal inflammation without the side effects of the present range of conventional NSAIDs i.e. gastrointestinal ulceration, renal damage and platelet dysfunctions. Clinical studies with selective COX-2 inhibitors are needed to further elucidate the potential of these agents as therapeutic adjuncts in the treatment of pulpal diseases.

Remaining dentine thickness is one of the most important factors to determine pulpal response (Stanley, 1970; Rayner and Southam, 1979). It was shown that the less RDT the more pulpal response or damage. In the present study, the means of RDT were around 1.6 mm in both groups. This study suggested that not only the RDT but the sealability of filling materials also provided much more effect on the pulps (Lundy and Stanley, 1969; Bergenholtz, 1977; Massler, 1956; Anderson and Ronning, 1962; Mjör and Tronstad, 1972). However, I found that the pulpal level of PGE_2 tend to more amount when the remaining dentine thickness was less. Thus, the remaining dentine thickness should be kept in mind as an important factor to produce pulpal inflammation.

This study has shown that gutta percha treatment increases the sensitivity of human dentin to cold stimuli at the same time as it decreases its sensitivity to negative hydrostatic pressure stimuli. The increase in the response to cold in the Pressure and Cold Group was not due to an effect of the preceding hydrostatic pressure stimulus since the same effect was seen in the Cold Only Group. Since both the cold and negative pressure stimuli would have caused outward flow through the dentin, these observations provide further evidence that the pain produced by cold stimuli in man is not due to hydrodynamic receptors; but to some other type, such as specific cold receptors (Chidchuangchai *et al*., 2007). Naylor

(1963) showed that the reaction times to cold stimulation of dentin in man were too short to be due to activation of cold-sensitive receptors at the pulp-dentin junction. The involvement of hydrodynamic receptors at the pulp-dentin junction could account for this short latency; but the proposed specific cold receptors would have to be located more peripherally, in the dentinal tubules. Since nerve terminals are confined to the inner 100 μm of the dentin (Lilja, 1979, Holland *et al*, 1987), this raises the possibility that the odontoblasts, with cold-sensitive membrane ion channels, may be involved in the sensory transduction mechanism (Magloire *et al*., 2009). However, Son *et al*.,(2009) found no evidence that the specific cold receptors TRPM8 and TRPA1 were expressed in odontoblasts in neonatal mouse incisors.

There are several possible explanations for the changes in sensitivity of the dentin that were observed following gutta percha treatment. For example, the increase in response to cold may have been due either to an increase in the sensitivity of the sensory receptors in the teeth (peripheral sensitization) or to changes in the pain pathways in the brain that are activated by the discharge evoked in the primary afferent nerve fibers (central sensitization). An increased sensitivity of pulpal afferents to cold following exposure of the dentin for one week has been reported in the dog (Hirvonen *et al*. 1992), although, in other studies in experimental animals, little evidence was found of peripheral sensitization in inflamed teeth (Andrew, Sirimaharaj and Matthews, unpublished observations). The decrease in response to a negative pressure stimulus we observed could have been due to a decrease in hydraulic conductance of the dentin, resulting in less flow through dentinal tubules and a reduced response from hydrodynamic receptors. Such a change might result from the polymerization of fibrinogen that leaked into the tubular fluid from the pulp (Pashley et al., 1984). If there was a decrease in conductance that was confined to the peripheral ends of the tubules, the outward flow produced at the pulpal ends of the tubules by the application of a cold stimulus to the cavity may have been increased compared with that in freshly exposed dentin (Chidchuangchai *et al*., 2007). This is a possible alternative explanation for the observed difference in the effects of gutta percha treatment on responses to negative pressure and cold stimuli.

Evidence for central sensitization in trigeminal nociceptive pathways has been obtained in experimental animals (Sessle, 2000). Also, Veerayutthwilai *et al*. (2002) showed that the sensory thresholds to electrical stimulation of tooth pulp in human participants were decreased following gutta percha-treatment, an effect that is likely to have been due to central sensitization. Our finding that gutta percha treatment reduced the

sensitivity of dentin to negative pressure stimuli appears to contradict the result of Anderson and Matthews (1967), who showed that the same treatment increased the sensitivity of dentin to osmotic stimuli. However, in those experiments no allowance was made for a smear layer. A smear layer would have been present on the exposed dentin surface immediately after cavity preparation, and this may have been etched away during the ensuing week by acids that accumulated under the leaky gutta percha filling. Removal of the smear layer in this way could account for the observed increase in sensitivity of the dentin. No data are available on the effects of smear layer removal on the sensitivity of dentin to osmotic stimuli.

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Outputs จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานที่ตีพิมพ์ในวารสารระดับนานาชาติ

1.1 ผลงานที่ได้รับการตีพิมพ์ในวารสารระดับนานาชาติแล้ว

1. Kijsamanmith, K., Timpawat, S., Vongsavan, N., Matthews, B. A comparison between red and infrared light for recording pulpal blood flow from human anterior teeth with a laser Doppler flow meter. *Archives of Oral Biology* 2011;56 (6), pp. 614-618.
(impact factor = 1.603, From Journal Citation Reports®, 2011)

2. Kijsamanmith, K., Timpawat, S., Vongsavan, N., Matthews, B. Pulpal blood flow recorded from human premolar teeth with a laser Doppler flow meter using either red or infrared light**.** *Archives of Oral Biology* 2011;56 (6), pp. 629-633
(impact factor = 1.603, From Journal Citation Reports®, 2011)

3. Ajcharanukul, O., Chidchuangchai, W., Charoenlarp, P., Vongsavan, N., Matthews, B. Sensory transduction in human teeth with inflamed pulps**.** *Journal of Dental Research* 2011;90 (5), pp. 678-682
(impact factor = 3.486, From Journal Citation Reports®, 2011)

1.2 ผลงานที่กำลังเตรียมส่งเพื่อตีพิมพ์ในวารสารระดับนานาชาติ

Chidchuangchai, W., Akarasereenont P., Junyaprasert, K. Vongsavan, N., Matthews, B. Prostaglandin E_2 Levels Blood Flow and Pain Sensation in Human Teeth with Inflamed Pulps. (manuscript inpreparation)

2. การนำผลงานวิจัยไปใช้ประโยชน์

ได้รับการอ้างอิงระดับนานาชาติ (cited)

Kijsamanmith, K., Timpawat, S., Vongsavan, N., Matthews, B. A comparison between red and infrared light for recording pulpal blood flow from human anterior teeth with a laser Doppler flow meter. *Archives of Oral Biology* 2011;56 (6), pp. 614-618.
ได้รับการอ้างอิงใน Scopus® (cited) = 1 ครั้ง

Ajcharanukul, O., Chidchuangchai, W., Charoenlarp, P., Vongsavan, N., Matthews, B. Sensory transduction in human teeth with inflamed pulps**.** *Journal of Dental Research* 2011;90 (5), pp. 678-682
ได้รับการอ้างอิงใน Scopus® (cited) = 3 ครั้ง

ภาคผนวก

1. Reprints ของ

Kijsamanmith, K., Timpawat, S., Vongsavan, N., Matthews, B. A comparison between red and infrared light for recording pulpal blood flow from human anterior teeth with a laser Doppler flow meter. *Archives of Oral Biology* 2011;56 (6), pp. 614-618.
(impact factor = 1.603, From Journal Citation Reports®, 2011)

Kijsamanmith, K., Timpawat, S., Vongsavan, N., Matthews, B. Pulpal blood flow recorded from human premolar teeth with a laser Doppler flow meter using either red or infrared light**.** *Archives of Oral Biology* 2011;56 (6), pp. 629-633
(impact factor = 1.603, From Journal Citation Reports®, 2011)

Ajcharanukul, O., Chidchuangchai, W., Charoenlarp, P., Vongsavan, N., Matthews, B. Sensory transduction in human teeth with inflamed pulps**.** *Journal of Dental Research* 2011;90 (5), pp. 678-682
(impact factor = 3.486, From Journal Citation Reports®, 2011)

ภาคผนวก

2. ได้รับการอ้างอิงใน Scopus® (cited)

Kijsamanmith, K., Timpawat, S., Vongsavan, N., Matthews, B. A comparison between red and infrared light for recording pulpal blood flow from human anterior teeth with a laser Doppler flow meter. *Archives of Oral Biology* 2011;56 (6), pp. 614-618.

ได้รับการอ้างอิงใน Scopus® (cited) = 1 ครั้ง

Ajcharanukul, O., Chidchuangchai, W., Charoenlarp, P., Vongsavan, N., Matthews, B. Sensory transduction in human teeth with inflamed pulps**.** *Journal of Dental Research* 2011;90 (5), pp. 678-682

ได้รับการอ้างอิงใน Scopus® (cited) = 3 ครั้ง