

※(問42)～(問43)までは、(問39)で②とお答えになった方にお伺いいたします。

(問42)

貴学で過去に実施されていた生涯研修の必要性に関する教育の内容はどのようなものですか？ (回答後、問43へ)

[ ]

(問43)

生涯研修の必要性に関する教育を中止した理由はどのようなものですか？

[ ]

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(アンケートは以上です。ご協力ありがとうございました。)

※(問44)～(問45)までは、(問39)で③とお答えになった方にお伺いいたします。

(問44)

貴学で今後実施予定の生涯研修の必要性に関する教育の内容はどのようなものですか？

(回答後、問45へ)

[ ]

(問45)

新たに生涯研修の必要性に関する教育を開始する理由はどのようなものですか？

[ ]

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(アンケートは以上です。ご協力ありがとうございました。)

※（問46）までは、（問39）で④とお答えになった方にお伺いいたします。

（問46）

貴学で生涯研修の必要性に関する教育を予定されていない理由はどのようなものですか？

[ ]

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（アンケートは以上です。ご協力ありがとうございました。）

## II. 研究成果の刊行に関する一覧表

### 書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ
一戸達也	パルスオキシメータの勧め、局所麻酔薬使い分けの勧め、アスピリン喘息の恐ろしさ	一戸達也	院内勉強会のためのワークブック 医療安全ワンポイント31	ヒューロン・パブリッシャーズ	東京	2015	60-63, 72-75, 92-95
一戸達也	肺塞栓症, 慢性呼吸不全	一戸達也, 河合峰雄, 重枝昭広, 片倉 朗	安心・安全な臨床に活かす! 歯科衛生士のための病氣とくすりパーフェクトガイド	医歯薬出版	東京	2015	70-71, 72-73
松木由起子, 一戸達也	糖尿病, 甲状腺機能障害, 関節リウマチ, ベーチェット病	一戸達也, 河合峰雄, 重枝昭広, 片倉 朗	安心・安全な臨床に活かす! 歯科衛生士のための病氣とくすりパーフェクトガイド	医歯薬出版	東京	2015	74-77, 78-79, 80-83, 84-85,
松浦由美子, 一戸達也	HIV感染症, 妊娠	一戸達也, 河合峰雄, 重枝昭広, 片倉 朗	安心・安全な臨床に活かす! 歯科衛生士のための病氣とくすりパーフェクトガイド	医歯薬出版	東京	2015	141-143, 144-145
塩崎恵子, 一戸達也	アレルギー	一戸達也, 河合峰雄, 重枝昭広, 片倉 朗	安心・安全な臨床に活かす! 歯科衛生士のための病氣とくすりパーフェクトガイド	医歯薬出版	東京	2015	146-148
一戸達也	専門医制度	桑田文幸, 葛西一貴, 佐藤裕二, 田口則宏, 田中昭男, 沼部幸博	歯科医学教育白書 2014年版	口腔保健協会	東京	2015	146-150
西原達次、有吉涉	人体の正常フローラ	川端重忠、小松澤均、大原直也、寺尾豊、浜田茂幸	口腔微生物学・免疫学 第4版	医歯薬出版	東京	2016	200-203
西原達次、沖永敏則	口腔細菌叢	川端重忠、小松澤均、大原直也、寺尾豊、浜田茂幸	口腔微生物学・免疫学 第4版	医歯薬出版	東京	2016	204-206
西原達次	口腔免疫学	川端重忠、小松澤均、大原直也、寺尾豊、浜田茂幸	口腔微生物学・免疫学 第4版	医歯薬出版	東京	2016	207-209
西原達次	歯周病の細菌学	川端重忠、小松澤均、大原直也、寺尾豊、浜田茂幸	口腔微生物学・免疫学 第4版	医歯薬出版	東京	2016	256-263
荒木孝二	第4章 歯科医学教育プログラム (学士課程教育) 2. モデル・コア・カリキュラム	日本歯科医学教育学会 白書作成委員会	日本歯科医学教育学会雑誌別冊 歯科医学教育白書 2014年版 (2012~2014年)	日本歯科医学教育学会	東京	2015	26-27
荒木孝二	第5章 共用試験 1. モデル・コア・カリキュラムと共用試験	日本歯科医学教育学会 白書作成委員会	日本歯科医学教育学会雑誌別冊 歯科医学教育白書 2014年版 (2012~2014年)	日本歯科医学教育学会	東京	2015	58-59
荒木孝二	第15章 教育の質保証 2. 日本の対応	日本歯科医学教育学会 白書作成委員会	日本歯科医学教育学会雑誌別冊 歯科医学教育白書 2014年版 (2012~2014年)	日本歯科医学教育学会	東京	2015	160-161

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
奥村暢旦、石崎裕子、伊藤晴江、中村太、塩見晶、中島貴子、藤井規孝.	歯科臨床技術教育における動画教材の効果.	日本歯科医学教育学会雑誌	31(1)	10-15	2015
山口摂崇、福泉隆喜、唐木純一、中原孝洋、日高勝美、西原達次.	在宅高齢者におけるEichner分類による咬合支持域数と健康関連指標との関連.	日本歯科医療管理学会雑誌	50(4)	印刷中	2016
Wakasugi Y, Matsuura N, Ichinohe T	Intraoperative blood loss during orthognathic surgery: A comparison of remifentanil-bassed anesthesia with sevoflurane or isoflurane	Journal of Oral and Maxillofacial Surgery	73(12)	2294-2299	2015
Kawaguchi A, Sato M, Kimura M, Ichinohe T, Tazaki M, Shibukawa Y	Expression and function of purinergic P2Y12 receptors in rat trigeminal ganglion neurons	Neuroscience Research	98	17-27	2015
Okamoto S, Matsuura N, Ichinohe T	Effects of volatile anesthetics on oral tissue blood flow in rabbits: A comparison among isoflurane, sevoflurane, and desflurane	Journal of Oral and Maxillofacial Surgery	73(9)	1714, e1-8	2015
Kanbe H, Matsuura N, Kasahara M, Ichinohe T	Tissue blood flow during remifentanil infusion with carbon dioxide loading	Anesthesia Progress	62(2)	51-56	2015
Kawaguchi A, Sato M, Kimura M, Yamazaki T, Yamamoto H, Tazaki M, Ichinohe T, Shibukawa Y	Functional expression of bradykinin B1 and B2 receptors in neonatal rat trigeminal ganglion neurons	Frontiers in Cellular Neuroscience	9	229, e collection	2015
谷口省吾, 一戸達也, 嶋田昌彦, 城 茂治, 梶山加綱, 丹羽 均, 宮脇卓也, 吉田和希, 小谷順一郎	全国の歯学部・歯科大学における歯科麻酔学卒前教育の実態調査	日本歯科麻酔学会雑誌	43(3)	332-341	2015
Kukidome H, Matsuura N, Kasahara M, Ichinohe T	Continuous postoperative pain control using a multiple-hole catheter after iliac bone grafting: comparison between ropivacaine and levobupivacaine	International Journal of Oral and Maxillofacial Surgery			2016 in press doi: 10.1016/j.ijom.2015.09.010
一戸達也	歯科医学教育認証評価トライアル 修正版における評価項目・規準・観点・視点の紹介	日本歯科医学教育学会雑誌	31(3)	118	2015
Kiyomiya H, Ariyoshi W, Okinaga T, Kaneuji T, Mitsugi S, Sakurai T, Habu M, Yoshioka I, Tominaga K, Nishihara T.	IL-33 inhibits RANKL-induced osteoclast formation through the regulation of Blimp-1 and IRF-8 expression	Biochem Biophys Res Commun	460(2)	320-326	2015
Taniguchi K, Hikiji H, Okinaga T, Hashidate-Yoshida T, Shindou H, Ariyoshi W, Shimizu T, Tominaga K, Nishihara T.	Essential Role of Lysophosphatidylcholine Acyltransferase 3 in the Induction of Macrophage Polarization in PMA-Treated U937 Cells	J Cell Biochem	116(12)	2840-2848	2015
荒木孝二、森尾郁子、一戸達也、北村知昭	歯科医学教育認証制度の構築に向けて	日本歯科医学教育学会雑誌	31(3)	117-119	2015

### Ⅲ. 研究成果の刊行物・別刷

## 研究報告

## 歯科臨床技術教育における動画教材の効果

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塩見 晶<sup>3)</sup> 中島 貴子<sup>1,3)</sup> 藤井 規孝<sup>1,3)</sup>

抄録 歯科医師は技術職であるため、歯学教育のなかにおいて精神運動領域、すなわち治療技術の教育が占める要素は決して少なくない。しかしながら、さまざまな機器や材料を使用する歯科治療には、指導者にとって口頭や文書での説明が難しい技術要素を含む処置があり、経験の浅い学生や研修歯科医が同様の理由で失敗を重ねることに関係していると考えられる。本研究は、処置時にさまざまな注意を要するシリコン材料を用いた印象採得を題材に動画教材を作成し、このような技術要素を学習者に伝えることを試み、その効果を検証した。動画教材には、歯科用チェアーユニットに装着したマネキンに対して補綴専門医が下顎右側6番支台歯のシリコン印象を行うさまを録画したものをを用いた。被験者は研修歯科医とし、この動画教材を視聴した群（実験群）とシリコン印象に関する紙媒体の参考資料を配布した群（対照群）に分け、それぞれに対して教材作成時と同じ環境で支台歯の印象採得を指示した。被験者が採得した印象について画像上で形成限界（マージンライン）の印象再現率、マージンラインの断裂数、混入した気泡の数を計測し、対照群と実験群の比較を行った。その結果、すべての調査項目について対照群と実験群の間に有意差が認められた。以上のことから、動画教材は同時に複数の情報を伝えるために効果的であり、歯科臨床技術教育にとって有用であることが示唆された。

キーワード 歯科臨床技術教育、印象採得、動画教材、術者視点

## 緒言

従来、歯科医師に求められる治療技術の教育は、臨床実習を中心とする各種実習において行われてきた。なかでも、歯学の基礎を学んだ後、現場での実習を行う前に実施される模擬患者を想定したファントム実習の教育効果は高く、学生や研修歯科医は口腔内での処置を行うためだけでなく、患者への配慮なども含めてトレーニングすることができる<sup>1-6)</sup>。また、ファントム実習をさらに実践的にするために、複合的に歯科疾患を再現した総合模型の開発も行われており<sup>7)</sup>、学習者に実際の臨床を詳細にイメージさせるための技術教育方法は着実に進歩していると考えられる。

一方、それらのトレーニングを行っていても、実際の臨床現場においては、経験の浅い学生や研修歯科医が初

めて行う処置には同一の原因による失敗がみられることが少なくない。このことには、歯科材料にはそれぞれさまざまな性質があり、術者にとってそれらの使用時には同時に複数の注意が求められることが関与していると考えられる。そこで、本研究では比較的操作時間が短く、経験の浅い学生や研修歯科医にとって取り扱いが難しいと思われるシリコン材料を用いた印象採得を題材に動画教材を作成し、その効果を検証した。

## 対象および方法

被験者は平成26年度新潟大学医歯学総合病院単独型プログラムで研修中の研修歯科医のうち、研究の趣旨に同意し、実際に協力できた者22名とした。模擬患者には歯科用チェアーユニットに設置したシンプルマネキンと補綴修復用顎模型（ニッシン社）を用い、顎模型には下顎右側6番に支台歯形成実施済みの人工歯を装着して全部鑄造冠の印象採得を行う環境を準備した。顎模型の歯肉には歯肉圧排後の状態を再現するためにあらかじめ調整を行い（図1）、シリコン印象には、本学医歯学総合病院歯科において実際に使用されている材料と器材（シリンジ、トレーなど）を用いた。

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平成26年11月28日受付

平成27年1月19日受理

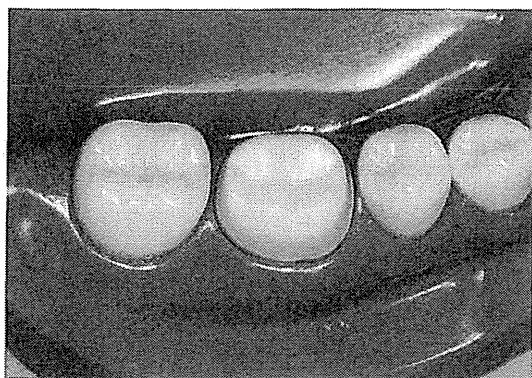


図1 右下6番に支台歯を装着し、歯肉調整を行った模型

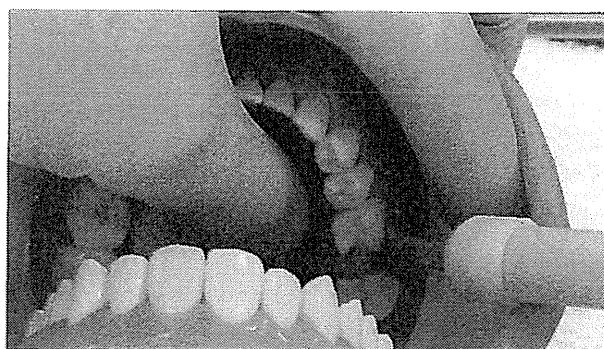


図2 動画教材(タブレット端末での視聴)画面

### 1. 対照群および実験群

実験は8~9月にかけて、研修歯科医を無作為に11名ずつの2グループに分け、一方を対照群、もう一方を実験群として行った。対照群には印象採得時の注意やポイントを記載したプリントを、実験群にはタブレット型端末に保存した動画教材を参考にしながらマネキンの下顎右側6番の印象を行うよう指示を与えた。なお、時間の制限は特に設けず、それぞれの群について配布した参考資料は必要時に自由に閲覧して構わないこととした。

### 2. 実験群用動画教材

実験群に与えた参考資料には、同じ実験環境において指導歯科医(補綴専門医)が印象採得を行う様子を録画した動画教材を用いた。動画教材はできるだけ術者の視野と同じ範囲を画面に収めることを念頭において、最適なカメラの位置を模索したうえで撮影し、印象材注入開始箇所やシリンジの動かし方、印象材の吐出速度について良い例と好ましくない例を作成した(図2)。完成した動画は、実際に印象採得を行った指導歯科医が内容を確認した後に研修歯科医に渡すタブレット型端末に保存した。

### 3. 印象の評価

対照群および実験群が採得した印象は内面の確認を容易にするために4分割し(図3)、倍率や被写体までの距離などを一定に揃え、デジタルカメラで撮影してデータ化した。得られたデータ画像上で印象内面を確認し、①形成限界(マージンライン)の再現率、②マージンラインの断裂箇所数、③軸面および咬合面の気泡数を求めて対照群と実験群の評価および比較を行った。①については、支台歯のマージンライン全周の長さに対する印象内面で確認できるマージンラインの長さの割合(%)を、画像処理ソフト adobe Photoshop を用いて計測・算出した。なお、印象内のマージンラインは印象面にバリとし

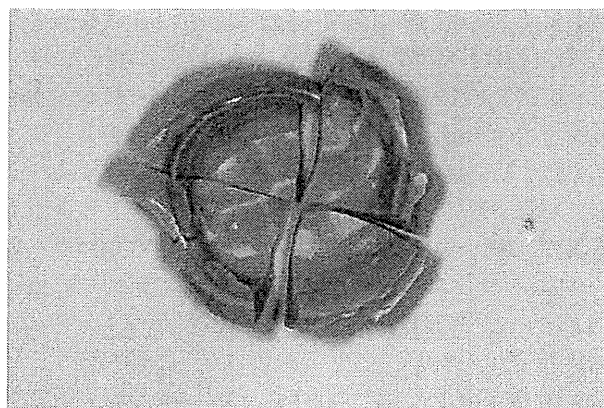


図3 被験者が採得した印象

て現れる歯肉溝の有無ではなく、線状に現れる形成限界のエッジを対象として計測し、得られたデータは Microsoft Excel 2010 を用いて統計学的解析を行い、有意差の有無を確認した。

### 4. アンケート調査

実験終了後、被験者として協力した研修歯科医のうち、同意が得られた者に対してアンケート調査を行った。アンケートは資料のわかりやすさや有用性を問うものとし、それぞれにそう思う理由を記載する欄を設け、対照群と実験群に分けて集計を行った。

なお、本研究は JSPS 科研費 24501178 の助成を受け、本学歯学部倫理委員会の承認(承認番号 26-R22-07-28)を得て行った。

## 結 果

### 1. 印象採得実験

#### 1) マージンラインの再現率

対照群は最小値が63.0%、最大値は82.7%で平均は76.2%であり(表1)、マージンライン全周の3/4強程度を印象することができていた。これに対して実験群では



表 1 対照群, 実験群の結果

対照群			実験群				
マージンライン再現率 (%)	マージン断裂箇所数 (カ所)	気泡数 (個)	マージンライン再現率 (%)	マージン断裂箇所数 (カ所)	気泡数 (個)		
72.39	3	2	80.90	1	0		
78.46	2	2	92.24	1	1		
81.21	2	2	87.84	4	3		
70.46	4	3	92.94	1	1		
81.21	3	3	99.68	1	2		
80.62	2	1	88.29	1	1		
63.04	2	1	81.93	2	1		
80.91	4	4	74.70	3	2		
74.39	2	2	91.64	3	1		
73.11	2	4	93.66	1	2		
82.68	4	3	82.83	2	2		
平均	76.23	2.73	2.45	平均	87.88	1.82	1.45

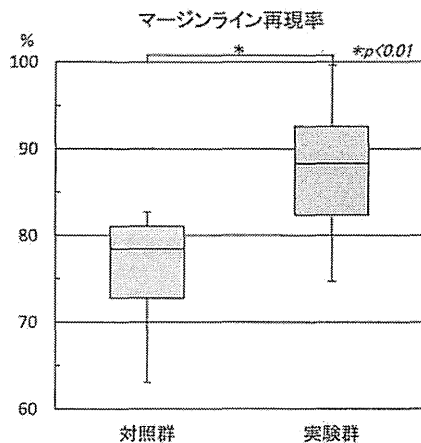


図 4 マージンライン再現率比較

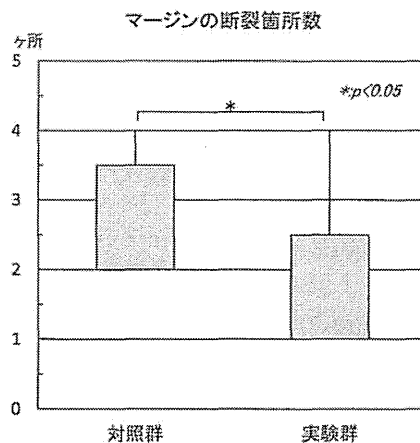


図 5 マージンラインの断裂箇所数比較

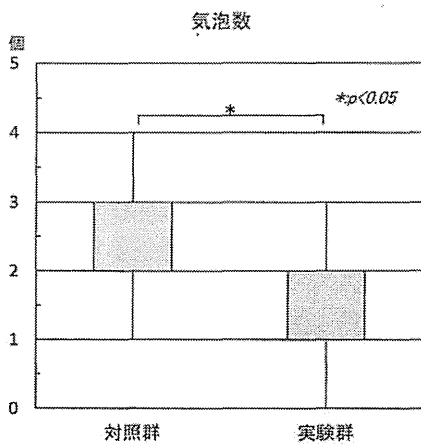


図 6 軸面および咬合面の気泡数

最小値 74.7%, 最大値 99.7%, 平均は 87.9% であり, マン・ホイットニーの U 検定において対照群との間に有意差を認めた ( $p=0.0012 < 0.01$ ) (図 4). また, 実験群では再現率 90% を超えた者が 5 名みられたのに対し, 対照群では 90% 以上の再現率は認められなかった.

#### 2) マージンラインの断裂箇所数

マージンラインの断裂は, 対照群において 2~4 カ所, 実験群においては 1~3 カ所に確認され, 平均はそれぞれ 2.7 カ所, 1.8 カ所であった (図 5). 両群間にはマン・ホイットニーの U 検定において有意差が認められた ( $p=0.0340 < 0.05$ ).

#### 3) 軸面および咬合面の気泡数

対照群には最小 1~最大 4 つの気泡混入がみられた. 一方実験群では 0~3 つまでであり, 平均はそれぞれ 2.5 個, 1.5 個であった (図 6). 気泡混入数についてもマン・ホイットニーの U 検定において両群間に有意差が認め

表 2 アンケート結果

質問 1 用意した資料はわかりやすかったか?	質問 2 再度行えばさらにうまくできるか?				
	対照群	実験群	対照群	実験群	
はい	1	11	はい	4	7
いいえ	5	0	いいえ	2	3
わからない	5	0	わからない	5	1

られた ( $p=0.0280<0.05$ )。気泡のなかには印象内面に溝状に存在する比較的大きなものも認められた。

## 2. アンケート調査

アンケート調査には協力者全員から回答を得た (表 2)。「はい」「いいえ」「わからない」で回答を求めた質問 1: 用意した資料はわかりやすかったか?, 質問 2: 同じ資料を参考にして再度印象採得を行えばさらにうまくできると思うか?, に対する回答を表 2 に示す。質問 1 については、対照群では「いいえ」「わからない」が同数でほぼ半数を占めたのに対し、実験群では全員が「はい」と答えていた。理由欄には、対照群では特に記載がみられなかったのに対し、実験群では「一連の流れを確認することができたため」「言葉での説明よりわかりやすかった」などの記載がみられた。質問 2 については、対照群において「わからない」が多くみられ、実験群では「はい」と回答する者が半数以上を占めていた。理由欄の記載には、対照群において「何もないよりは見た方が良いと思う」「方法を知らずに行ってもうまく出来なと思う」などのコメントが、実験群においては「繰り返してみることができるのでポイントをつかめると思う」「1 度目に自分で行った処置と比較し、相違点を見つけることによって上手くなると思う」などが寄せられた。質問 3 には、さらに上達するためにはどのような資料が必要と思うか? に対する自由記載欄を設けた。回答には対照群、実験群の双方から「的確にフィードバックを得られる資料」「視覚素材を多用したもの」などが寄せられ、特に対照群では動画教材を挙げる意見がいくつかみられた。

## 考 察

シリコン印象材は、ベースとキャタリストを混和した後、比較的すみやかに硬化を開始するため、術者はわずかな時間にさまざまなことを確認しながら処置を行わなければならない。このため、動画教材によって伝わる情報量の多さを確認するために適切な題材であったと思われる。

今回は、研修期間中盤に差しかかった時期の研修歯科

医を対象として実験を行った。このため、頻度的にはまだ高くはないものの、ほとんどの研修歯科医はシリコン印象を見学あるいは自験しており、シリコン印象の手順や術中の注意点についてはそれぞれに理解していると考えられた。そこで、シリコン印象に関する基本的な注意点を統一的に再認識させるために、実験への協力を賛同した研修歯科医にはなんらかの参考資料を与えることとした。その結果、いずれの群においても臨床的に全部鑄造冠を製作することが可能と思われる印象採得を行うことができていた。しかしながら、印象の評価を行った結果、対照群と実験群のマージンラインの再現率、断裂箇所数、気泡の数にはすべて有意差が認められた。特にマージンラインの再現率については、対照群の平均約 76% に対して実験群では平均約 88% であり、両群に比較的大きな差が生じた。さらに、対照群では 1 名も再現率 90% を超えることができなかったのに対し、実験群では半数近くが 90% を超えていた。また、断裂箇所は隣接面部に比較的多く発生する傾向がみられたことから、実験群では正確に印象を採得するために必要なシリンジの動かし方を理解していたことがうかがわれた。今回の実験では、印象材はミキシングチップを使ってシリンジに填入した。このため、印象内面にみられた気泡については、シリンジに印象材を填入する際に混入した可能性も考えられた。しかし、実際には溝状の気泡が多く、印象時の操作速度にも原因があることが疑われた。

今回得られた結果を全体的に確認してみると、臨床的に評価を行った場合にも明らかに両群の差を判断することができると思われた。さらに、このような結果には、術者の診療目線を意識して動画教材を作成したことが影響しているように思われた。シリコン印象採得を正確に行うためには、支台歯周囲に印象材を送るシリンジの移動および印象材の吐出速度をコントロールしながら印象材の流動状況に注意を払い、一般的な水平診療位では重力によって印象材が遠心方向へ流れてしまうことにも配慮する必要がある<sup>8)</sup>。これらの理想的な様相を言葉や文章で説明することは困難であるため、本動画教材は意図したとおりに教育効果を発揮したことを示唆していると考えられた。また、被験者に行ったアンケート調査では、質問 1, 2 の理由欄に記載されたコメントや質問 3 への

意見から、技術教育における視覚素材の効果の大きさが示された。特に実験群において、被験者全員より仕様のわかりやすさや、繰り返し参考にした場合の効果を肯定する意見がみられたことから、動画教材には文書や写真以上の情報が含まれていることが明らかになった。また、被験者は用意された良い例と好ましくない例を見比べることにより、視覚的および感覚的にシリコン印象時の注意点を理解できていたことを示していると思われた。

以上のことから、歯科治療の技術教育においては、口頭や文書で説明しにくい技術要素を的確に学習者に伝えるために、動画教材は有効であることが示された。

### 結 論

本研究では、情報伝達量が豊富な動画教材を歯科臨床技術教育に利用し、その効果を検証した。その結果、動画教材は処置時の複雑な注意点を伝えるために有用であることが示された。

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## The Effectiveness of Video Teaching Materials for Dentists in Clinical Dental Education

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**Abstract** Dentistry is considered to be a technical job. Therefore, clinical education training to improve the treatment skills of dental students and dental trainees is one of the most important factors in dental education. However, there are some points that are difficult to explain with words, even though they are essential in order to perform dental treatment successfully. Such points may be related to errors common among inexperienced dental students and dental trainees. The purpose of this study was to investigate the usefulness of video teaching materials from the viewpoint of dentists. A video recording of the impression procedure of the right-side lower first molar with a silicone material performed by a prosthodontist was prepared. The dental trainees were divided into two groups : the control group received the text while the experimental group was given the video, then the same procedure was performed. The impressions taken by the two groups were analyzed using photographic data, and the length of the abutment margin, the number of torn points and the number of entrained bubbles were measured. As a result, there were statistically significant differences between the two groups in all of the measurements. It was suggested that video teaching materials from the viewpoint of experienced dentists were effective and helpful for dental students or dental trainees to learn about dental treatments.

**Key words** clinical dental education, impression of abutment, video teaching material, viewpoint of dentist

# Intraoperative Blood Loss During Orthognathic Surgery: A Comparison of Remifentanil-Based Anesthesia With Sevoflurane or Isoflurane

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and Tatsuya Ichinobe, DDS, PhD ‡

**Purpose:** The aim of the present study was to compare the blood loss with remifentanil-based anesthesia with sevoflurane or isoflurane during orthognathic surgery.

**Patients and Methods:** In this randomized controlled clinical trial, the patients who were scheduled for orthognathic surgery were divided into 2 groups: the sevoflurane (Sevo) group and isoflurane (Iso) group. Anesthesia was maintained using end-tidal concentrations of 1.4% sevoflurane or 0.9% isoflurane. Remifentanil was continuously infused at 0.05 to 0.5  $\mu\text{g}/\text{kg}/\text{min}$  to maintain the mean blood pressure (MBP) at 60 to 65 mm Hg. The intraoperative blood loss was compared between the 2 groups. The Student *t* test for unpaired samples was used for statistical analysis.  $P < .05$  was considered statistically significant.

**Results:** The study sample included 19 men and 45 women ( $n = 64$ ). The mean age was 25 years (range 16 to 50). The intraoperative blood loss tended to be greater in the Iso group ( $n = 32$ ;  $4.79 \pm 3.22$  mL/kg) than in the Sevo group ( $n = 32$ ;  $4.00 \pm 1.98$  mL/kg). However, the difference between the 2 groups was not significant.

**Conclusion:** In a comparison of intraoperative blood loss during remifentanil-based anesthesia with sevoflurane or isoflurane during orthognathic surgery, no difference was observed between the 2 groups.

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Oral and maxillofacial surgery (OMS), in particular, orthognathic surgery, is performed in areas of high blood flow, including the oral mucosa and bone marrow. Blood loss during surgery can obstruct the visual field, prolong the operating time, and increase the risk of a blood transfusion.<sup>1,2</sup> Blood loss needs to be controlled during surgery to ensure the operation proceeds smoothly, to avoid the risks with blood transfusion, and to reduce postoperative complications.<sup>3</sup>

Volatile anesthetics such as isoflurane and sevoflurane are now widely used in OMS. Previous research in rabbits showed that isoflurane increased blood flow in the tongue mucosa.<sup>4</sup> Another study showed that isoflurane increased blood flow more than

sevoflurane in the mandibular bone marrow and other oral tissues in rabbits.<sup>5</sup> Clinical research has shown that isoflurane increases blood flow in the oral mucosa<sup>6</sup> and sevoflurane reduces microcirculation under the tongue.<sup>7</sup> Narcotic analgesics such as fentanyl and remifentanil are also widely used for pain relief during OMS under general anesthesia. Recent research has shown that, just as for the volatile anesthetics, these narcotic analgesics also affect the oral tissue blood flow. Research in rabbits showed that fentanyl reduced the blood flow in the oral mucosa,<sup>4</sup> and other studies have suggested that remifentanil might be useful for OMS because it reduces the blood flow in the mandibular bone marrow without markedly reducing the

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blood pressure.<sup>8,9</sup> Clinical research has shown that compared with fentanyl, remifentanyl reduces blood loss and allows intraoperative hypotension without serious adverse events.<sup>10-12</sup> One study reported that during nasal septal surgery without a concomitant use of remifentanyl, blood loss was greater with isoflurane than with sevoflurane.<sup>13</sup> Few studies have compared the blood loss during OMS when remifentanyl is used as an adjunct to inhalation anesthesia.<sup>14,15</sup> Also, it is unclear what effects the combined use of isoflurane with its strong vasodilating potency and remifentanyl have on blood loss.

The purpose of the present study was to compare the blood loss during remifentanyl-based anesthesia with inhalation anesthetics during orthognathic surgery. We hypothesized that the blood loss with remifentanyl-based anesthesia might be affected because of the differences in vasodilative activities of inhalation anesthetics. Our specific aim was to compare the blood loss with remifentanyl-based anesthesia with sevoflurane versus with isoflurane during orthognathic surgery.

## Patients and Methods

### STUDY DESIGN AND SAMPLE

The present study was designed and implemented as a randomized controlled trial. The Tokyo Dental College Ethics Committee approved the study (approval no. 541). We enrolled patients with an American Society of Anesthesiologists classification of I or II, aged 16 to 50 years, who were scheduled for Le Fort I osteotomy and sagittal split ramus osteotomy of the mandible at the Chiba Hospital of Tokyo Dental College. All the patients or their guardians provided written informed consent. Patients with severe heart disease, liver or kidney disease, or muscle disease were excluded from the study.

### DATA COLLECTION METHODS

The enrolled patients were randomized using a randomization table before surgery to the sevoflurane (Sevo) group or isoflurane (Iso) group. After transfer to the operating room, the patients were placed in the horizontal position, and venous access was secured using a 20-gauge catheter inserted into the forearm cephalic vein. Anesthesia was induced using 0.01 mg/kg atropine sulfate ( $\leq 0.5$  mg/kg), 2  $\mu$ g/kg fentanyl citrate, and 2 mg/kg propofol. Muscle relaxation was achieved using 0.6 mg/kg rocuronium bromide before nasotracheal intubation was performed. Anesthesia was maintained using 3 L/min air, 1 L/min oxygen, and end-tidal concentrations of 1.4% sevoflurane or 0.9% isoflurane (0.8 minimum alveolar concentration [MAC] for both groups). Muscle relaxation was achieved by continuous

administration of 5  $\mu$ g/kg/min rocuronium bromide. Remifentanyl was continuously infused at 0.05 to 0.5  $\mu$ g/kg/min to maintain a mean blood pressure (MBP) during anesthesia of 60 to 65 mm Hg. When the MBP was lower than 60 mm Hg for more than 5 minutes, 2 to 8 mg of ephedrine hydrochloride was administered, depending on the MBP. A 1% lidocaine solution with 1:100,000 epinephrine was administered to the surgical field. The end-tidal carbon dioxide concentration was maintained during anesthesia in both groups at 35 to 45 mm Hg using controlled mechanical ventilation. Acetated Ringer's solution was administered at 8 mL/kg/hr for fluid infusion during surgery. Continuous monitoring while the patient was under anesthesia involved pulse oximetry, noninvasive blood pressure measurement, electrocardiogram, invasive arterial pressure measurement by cannulation of the radial artery, bispectral index (BIS) measurement, and end-tidal anesthetic gas concentrations. The systolic blood pressure (SBP), MBP, diastolic blood pressure (DBP), heart rate (HR), and BIS values were recorded every 5 minutes, and the final blood loss was measured at the end of surgery. The duration of surgery, duration of anesthesia, fluid infusion volume, and mean remifentanyl infusion rate were also recorded. When the blood hemoglobin concentration at suturing was less than 10 g/dL, an autologous blood transfusion was started after the measurement of the final blood loss.<sup>16</sup>

### STUDY VARIABLES

The primary outcome variable was blood loss. The demographic variables included age, gender, height, weight, and body mass index (BMI). The time variables were the duration of surgery and the duration of anesthesia. The surgery- and anesthesia-related variables were the HR, SBP, MBP, coefficient of variation in MBP (CVMBP), DBP, remifentanyl infusion rate, and BIS value. The other variables of interest were the total volume of local anesthetic, total amount of epinephrine contained in the local anesthetic solution, and total amount of ephedrine hydrochloride to improve hypotension. The HR, SBP, MBP, DBP, and BIS were calculated as the mean values during surgery. The values are presented as the mean  $\pm$  standard deviation.

### STATISTICAL ANALYSIS

From the results of a previous study comparing blood loss during orthognathic surgery between sevoflurane and desflurane anesthesia,<sup>14</sup> and using an  $\alpha$  of 0.05 and a  $\beta$  of 0.2 for a study design incorporating 2 groups of equal size, a sample size of 31 patients per group was estimated. The Student *t* test for unpaired samples was used for statistical analysis.  $P < .05$  was considered statistically significant. Statistical analysis was performed using PASW statistics, version 18 (SPSS Inc, Chicago, IL).

## Results

The present study included 64 patients. The mean age was 25 years (range 17 to 50) in the Sevo group and 26 years (range 16 to 47) in the Iso group. The Sevo and Iso groups included 10 men and 22 women and 9 men and 23 women, respectively. No differences were observed between the 2 groups in terms of gender, age, height, weight, BMI, duration of surgery, duration of anesthesia, total volume of local anesthetics, or total amount of epinephrine (Tables 1 and 2). The operations were performed by 5 surgeons, all of whom had adequate experience with at least 50 cases of orthognathic surgery. The distribution of the surgeons in the 2 groups was similar. The remifentanyl infusion rate was significantly lower in the Iso group than in the Sevo group ( $P = .004$ ). The total amount of ephedrine hydrochloride administered was significantly greater in the Iso group than in the Sevo group ( $P = .008$ ; Table 2). The BIS was significantly lower in the Iso group than in the Sevo group ( $P < .001$ ; Table 3). Although no significant differences were observed between the 2 groups in the intraoperative HR, SBP, MBP, or DBP, the CVMBP was significantly greater in the Iso group than in the Sevo group ( $P = .04$ ; Table 3).

The intraoperative blood loss was  $4.00 \pm 1.98$  mL/kg in the Sevo group and  $4.79 \pm 3.22$  mL/kg in the Iso group. Although the blood loss tended to be greater in the Iso group, the difference between the 2 groups was not significant (Fig 1). No intraoperative adverse events were observed in our study.

## Discussion

The purpose of the present study was to compare the blood loss during remifentanyl-based anesthesia with sevoflurane and with isoflurane during orthognathic surgery. Our results showed that although the

blood loss tended to be greater in the Iso group than in the Sevo group, the difference was not significant. The BIS value during anesthesia was lower in the Iso group than in the Sevo group. The total amount of ephedrine hydrochloride was greater in the Iso group than in the Sevo group. The mean remifentanyl infusion rate was less in the Iso group than in the Sevo group. However, the CVMBP was greater in the Iso group than in the Sevo group.

The combined use of remifentanyl with inhalation anesthetics allows sufficient pain relief and sedation at a low MAC because of drug interactions.<sup>17-19</sup> In the clinical setting, 1 study reported that anesthesia can be maintained appropriately with continuous infusion of remifentanyl at 0.1 to 0.3  $\mu\text{g}/\text{kg}/\text{min}$  and the inhalation anesthetic maintained at 0.5 to 0.8 MAC.<sup>20</sup> Rossi et al<sup>14</sup> investigated desflurane or sevoflurane maintained at 0.8 MAC with concomitant use of 0.5  $\mu\text{g}/\text{kg}/\text{min}$  remifentanyl during orthognathic surgery and reported that the blood loss was lower with desflurane anesthesia. We drew on their research in our study to maintain anesthesia with the inhalation anesthetic at 0.8 MAC and remifentanyl at 0.05 to 0.5  $\mu\text{g}/\text{kg}/\text{min}$ . The blood loss was lower in the patients with the MBP maintained at 55 to 65 mm Hg compared with those with the MBP maintained at 75 to 85 mm Hg.<sup>21</sup> Maintaining the MBP at 65 mm Hg provides appropriate conditions for surgical procedures and reduces blood loss.<sup>22</sup> The circulation in vital organs can be maintained safely if the MBP is 55 mm Hg or greater.<sup>23</sup> We assumed that blood loss could be reduced and the systemic circulation safely maintained if the anesthesia were maintained with the MBP at around 65 mm Hg. Therefore, we maintained the MBP at 60 to 65 mm Hg in the present study.

Research has shown that volatile anesthetics exhibit different BIS values when maintained at the same MAC.<sup>24,25</sup> Olofsen and Dahan<sup>26</sup> investigated the relationships of the isoflurane and sevoflurane concentrations to the BIS value and reported that the BIS value declined in a concentration-dependent manner to a certain concentration of anesthetic (isoflurane, 0.75% or approximately 0.65 MAC; sevoflurane, 1.5% or approximately 0.9 MAC) and then plateaued at about 40 for higher concentrations. The lower BIS value in the Iso group in our study might reflect the change in brainwaves caused by the inhalation anesthetic. However, although the BIS value was within the 40 to 60 range at the optimal depth of anesthesia, we could not rule out the possibility that the level of anesthesia was deeper in the Iso group than in the Sevo group. However, because the objective of our study was to compare blood loss when the inhalation anesthetics were maintained at the same MAC and the MBP was maintained at around 60 mm Hg, we did not correct for differences in the BIS value. If we assume

**Table 1. DEMOGRAPHIC VARIABLES**

Variable	Sevo Group	Iso Group	P Value
Sample size (n)	32	32	NA
Age (yr)	24.9 $\pm$ 8.4	25.5 $\pm$ 8.3	.76
Male gender (n)	10 (31.3)	9 (28.1)	
Height (cm)	164.0 $\pm$ 6.7	163.4 $\pm$ 9.1	.76
Weight (kg)	56.7 $\pm$ 8.2	58.1 $\pm$ 12.0	.58
BMI (kg/m <sup>2</sup> )	21.1 $\pm$ 2.3	21.6 $\pm$ 2.8	.41

Data are presented as n, mean  $\pm$  standard deviation, or n (%).

Student's *t* test for unpaired samples was used for statistical analysis.

Abbreviations: BMI, body mass index; Iso, isoflurane; NA, not applicable; Sevo, sevoflurane.

Wakasugi, Matsuura, and Ichinobe. Intraoperative Blood Loss During Orthognathic Surgery. *J Oral Maxillofac Surg* 2015.



**Table 2. STUDY VARIABLES**

Variable	Sevo Group	Iso Group	P Value
Duration of surgery (min)	329.6 ± 70.2	341.3 ± 60.2	.47
Duration of anesthesia (min)	386.3 ± 66.0	401.5 ± 60.2	.34
Blood loss (mL/kg)	4.00 ± 1.98	4.79 ± 3.22	.24
Total local anesthetic volume (mL)	24.4 ± 4.9	24.1 ± 5.2	.82
Total epinephrine amount (mg)	0.24 ± 0.49	0.24 ± 0.52	.82
Additional use of ephedrine (n)	12 (38)	18 (56)	.21
Total ephedrine amount (mg)	1.9 ± 2.9	4.8 ± 5.2	.008*

Data presented as mean ± standard deviation or n (%).

Student's *t* test for unpaired samples was used for statistical analysis.

Abbreviations: Iso, isoflurane; Sevo, sevoflurane.

\* Statistically significant difference between the 2 groups (*P* < .05).

Wakasugi, Matsuura, and Ichinobe. Intraoperative Blood Loss During Orthognathic Surgery. *J Oral Maxillofac Surg* 2015.

that this difference in BIS value affected blood loss, we might expect the blood loss to be greater in the Sevo group in whom the depth of anesthesia was light. However, no difference was observed between the 2 groups in the present study. Therefore, we believe that differences in the BIS value did not have a major effect in our study.

In addition, the infusion rate of remifentanyl in the present study might have been less in the Iso group than in the Sevo group, because isoflurane dilates the blood vessels in a concentration-dependent manner<sup>27</sup> and renders patients more likely to develop intraoperative hypotension. Accordingly, the blood pressure might have been lower during the noninva-

sive procedures in the Iso group, and the anesthesiologist might have reduced the infusion rate of remifentanyl. Thus, more ephedrine hydrochloride was required in the Iso group. The remifentanyl dose might then have been insufficient for subsequent invasive surgical procedures, which might explain why the CVMBP was larger in the Iso group than in the Sevo group.

**Table 3. SURGERY- AND ANESTHESIA-RELATED VARIABLES**

Variable	Sevo Group	Iso Group	P Value
HR (beats/min)	77.2 ± 9.3	80.2 ± 9.6	.22
SBP (mm Hg)	99.8 ± 5.8	97.3 ± 5.9	.1
MBP (mm Hg)	64.0 ± 4.2	62.3 ± 4.2	.1
CVMBP (%)	9.6 ± 2.2	11.2 ± 3.6	.04*
DBP (mm Hg)	49.3 ± 3.8	47.5 ± 3.6	.06
Remifentanyl infusion rate (µg/kg/min)	0.19 ± 0.06	0.14 ± 0.06	.004*
BIS value	50.5 ± 6.8	44.1 ± 6.4	<.001*

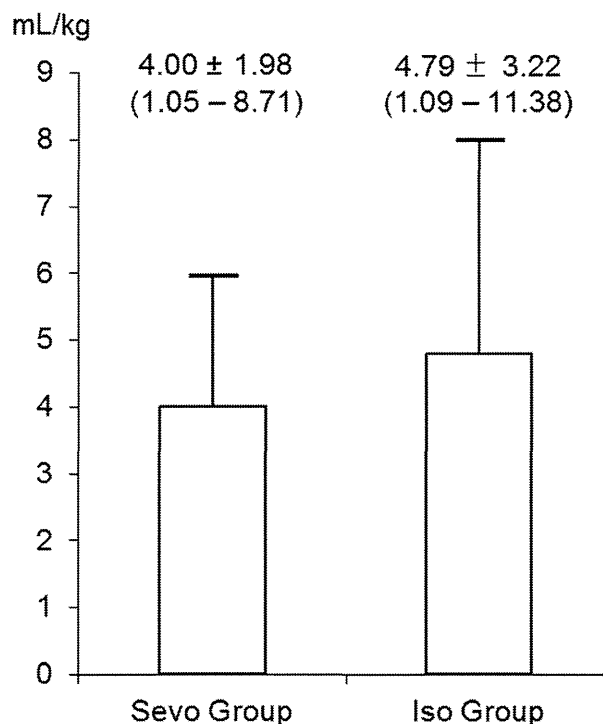
Data are presented as mean ± standard deviation.

Student's *t* test for unpaired samples was used for statistical analysis.

Abbreviations: BIS, bispectral index; CVMBP, coefficient of variation in mean blood pressure; DBP, diastolic blood pressure; HR, heart rate; Iso, isoflurane; MBP, mean blood pressure; SBP, systolic blood pressure; Sevo, sevoflurane.

\* Statistically significant difference between the 2 groups (*P* < .05).

Wakasugi, Matsuura, and Ichinobe. Intraoperative Blood Loss During Orthognathic Surgery. *J Oral Maxillofac Surg* 2015.



**FIGURE 1.** Comparison of blood loss. The mean blood loss in the sevoflurane (Sevo) group was 4.00 ± 1.98 mL/kg and in the isoflurane (Iso) group was 4.79 ± 3.22 mL/kg. No statistically significant differences were found between the 2 groups. Data presented as mean ± standard deviation.

Wakasugi, Matsuura, and Ichinobe. Intraoperative Blood Loss During Orthognathic Surgery. *J Oral Maxillofac Surg* 2015.



Research in rabbits has shown that isoflurane increased tissue blood flow in a concentration-dependent manner in the head and neck region, including the bone marrow and alveolar tissues in the lower jaw. In contrast, sevoflurane produced no such changes in mandibular bone marrow tissue.<sup>5</sup> Özkiris et al<sup>13</sup> compared blood loss with the use of sevoflurane and isoflurane anesthesia in nasal septal surgery and reported greater blood loss with isoflurane. They explained that this occurs because isoflurane is a vasodilator that lowers the arterial blood pressure in a concentration-dependent manner, and the tissue blood flow increases because of vasodilation, regardless of a low perfusion pressure.<sup>13</sup> From these studies, we had expected our comparison of anesthesia maintained with sevoflurane or isoflurane during surgery in the head and neck region to show greater blood loss with isoflurane because of its potent vasodilatory effect. However, no difference in blood loss was seen between the 2 groups in our study. The published data include research on remifentanil-based anesthesia with desflurane or sevoflurane during orthognathic surgery and remifentanil-based anesthesia with desflurane or isoflurane during ear, nose, and throat surgery.<sup>15,28</sup> Remifentanil acts to reduce blood flow in an infusion rate-dependent manner at the site involved, such as in mandibular bone marrow tissue.<sup>8,9</sup> From the previous research in rabbits,<sup>5,9</sup> we estimated the blood flow in the mandibular bone marrow tissue and expected the blood flow to be around 10% greater in the Iso group than in the Sevo group. Although we did find a tendency toward greater blood loss in the Iso group, the difference between the 2 groups was not significant. This suggests that the decrease in blood flow in the mandibular bone marrow tissue induced by remifentanil was greater than the increase in blood flow induced by isoflurane, such that any increase in tissue blood flow was suppressed and blood loss was not affected. However, the CVMBP was higher in the Iso group than in the Sevo group in our study, suggesting that the intraoperative MBP was unstable. This might have been involved, at least in part, in the large variability in the blood loss.

We did not directly measure intraoperative blood flow in the head and neck region in our study. Hence, we could not determine whether changes in the local tissue blood flow were involved in the blood loss variability. These issues need to be investigated from multifaceted perspectives, because it is extremely difficult to measure tissue blood flow in the operative field in humans.

The results from the present study suggest that both anesthetics can be safely used for orthognathic surgery. Clinically, it would be better to use isoflurane, considering the low metabolism rate and its cost

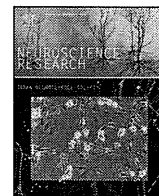
effectiveness. However, it would be better to use sevoflurane because of the emergence profile. Additional studies are required to compare intraoperative blood loss among the 3 groups of inhalational anesthetics, including desflurane.

In conclusion, in a comparison of intraoperative blood loss with remifentanil-based anesthesia with sevoflurane or isoflurane during orthognathic surgery, no difference was observed between the 2 groups. Future research of larger patient populations to compare blood loss during surgery among isoflurane, sevoflurane, and desflurane is required.

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## Expression and function of purinergic P2Y<sub>12</sub> receptors in rat trigeminal ganglion neurons



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### ABSTRACT

Purinergic receptors play key signaling roles in neuropathic pain in the orofacial region, which is innervated by trigeminal ganglion (TG) neurons. The neuropathology of purinergic P2Y<sub>12</sub> receptors is well characterized in glia; however, their physiological role in TG neurons remains to be fully elucidated. The present study investigated the expression and function of P2Y<sub>12</sub> receptors in rat TG neurons. P2Y<sub>12</sub> receptor immunoreactivity was intense in the soma, dendrites, and axons, and colocalized with a pan-neuronal marker, neurofilament H, isolectin B4, and substance P. In the presence of extracellular Ca<sup>2+</sup>, 2-methylthio-ADP (an agonist of P2Y<sub>1, 12, 13</sub> receptors) transiently increased intracellular free Ca<sup>2+</sup> concentrations ([Ca<sup>2+</sup>]<sub>i</sub>), an effect that was abolished by P2Y<sub>12</sub> receptor antagonists. In the absence of extracellular Ca<sup>2+</sup>, ryanodine receptor/channel inhibitors diminished the 2-methylthio-ADP-induced increases in [Ca<sup>2+</sup>]<sub>i</sub>. A sarcoplasmic reticulum Ca<sup>2+</sup>-ATPase (SERCA) inhibitor gradually increased [Ca<sup>2+</sup>]<sub>i</sub>, and after a plateau, application of 2-MeS-ADP induced a rapid and transient, but additive increase in [Ca<sup>2+</sup>]<sub>i</sub>. An adenylate cyclase inhibitor transiently increased [Ca<sup>2+</sup>]<sub>i</sub>, while a phosphodiesterase inhibitor prevented the 2-methylthio-ADP-induced increase in [Ca<sup>2+</sup>]<sub>i</sub>. Our study shows that P2Y<sub>12</sub> receptors are expressed in TG neurons, and act via a cAMP-dependent pathway to release intracellular Ca<sup>2+</sup> from ryanodine-sensitive Ca<sup>2+</sup> stores.

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### 1. Introduction

Extracellular nucleotides, which are important for the transmission and/or integration of pain sensations (Burnstock, 2013), activate the P2 family of receptors, which includes ATP-gated ion channels (i.e., P2X purinergic receptors, subdivided in P2X<sub>1</sub> to P2X<sub>7</sub>), and G protein-coupled receptors (i.e., P2Y receptors, subdivided in P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub>, P2Y<sub>6</sub>, and P2Y<sub>11</sub> to P2Y<sub>14</sub>). The P2Y<sub>12</sub> receptor has seven hydrophobic transmembrane regions linked by extracellular/intracellular loops and is activated by adenine and uracil nucleotides (ADP, UTP, UDP, and UDP-glucose). The P2Y<sub>12</sub> receptor generally couples to the Gi alpha subunit of the G protein, and reduces intracellular cAMP production (Abbracchio et al., 2006; Dussor et al., 2009; Moheimani and Jackson, 2012).

The relationship between P2Y<sub>12</sub> receptors and pain mechanisms has been mostly described in the spinal cord (Fried et al., 2001). After spinal nerve injury resulting in allodynia, the expression of P2Y<sub>12</sub> receptor mRNA increases in the dorsal horn of the spinal cord; however, its expression is highly restricted to microglia (Kobayashi et al., 2008; Tozaki-Saitoh et al., 2008). In the trigeminal ganglion (TG), an increased expression of P2Y<sub>12</sub> receptors in satellite glial cells (SGCs) has also been described following lingual nerve injury; this suggests that in the orofacial region, the activation of P2Y<sub>12</sub> receptors is involved in neuropathic pain induced by a peripheral nerve injury (i.e., hyperalgesia) (Katagiri et al., 2012). Thus, the functional role of P2Y<sub>12</sub> receptors is well described in glial cells. Although allodynia and hyperalgesia are associated with peripheral sensitization that originates from primary afferent neurons (Basbaum et al., 2009; Cervero and Laird, 1996; Ochoa, 2009; Scholz and Woolf, 2002), the expression and function of P2Y<sub>12</sub> receptors in the TG neurons remains to be fully elucidated.

In the present study, we analyzed the expression, localization, and physiological and pharmacological properties of P2Y<sub>12</sub> receptors in primary cultured TG neurons.

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## 2. Materials and methods

### 2.1. Ethical approval

All the animals were treated in accordance with the “Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences.” approved by the Council of the Physiological Society of Japan, and by the American Physiological Society. This study also followed the guidelines established by the National Institutes of Health (USA) regarding the care and use of animals for experimental procedures. The “Animal Research Ethical Committee” of the Tokyo Dental College (approval No. 252502) approved all experimental procedures in this study.

### 2.2. Cell culture

Trigeminal ganglions (TGs) were rapidly excised from 7-day-old Wistar rats under sodium pentobarbital anesthesia (50 mg/kg) following administration of isoflurane (3.0 vol%). Cells in TGs were dissociated by enzymatic treatment with Hank's balanced salt solution (Invitrogen, Carlsbad, CA, USA) containing 20 U/mL papain (Worthington, Lakewood, NJ, USA), for 20 min at 37 °C, followed by dissociation by trituration. Dissociated TG cells were plated onto poly-L-lysine-coated 35 mm diameter culture dishes (Corning, Corning, NY, USA). The primary cultures were performed using Leibovitz's L-15 medium (Invitrogen) containing 10% fetal bovine serum (FBS), 1% penicillin-streptomycin (Invitrogen), 1% fungizone (Invitrogen), 26 mM NaHCO<sub>3</sub>, and 30 mM glucose (pH 7.4). Cells were maintained in culture for 48 h at 37 °C in a humidified atmosphere containing 95% air and 5% CO<sub>2</sub> to allow cell attachment to the bottom of dishes. Recording solutions and drugs were applied to cells by superfusion using a pressurized (driven by N<sub>2</sub> gas) perfusion system (Automate Scientific, Berkeley, CA, USA) that allows a steady flow, thus avoiding unpredicted shear stress in the recording environment. Changes of solution were completed within 20 ms, using the pressurized perfusion system together with a multi-valve perfusion system (Warner Instruments, Hamden, CT, USA) and an aspirator (K.T. Labs, Saitama, Japan). When measuring [Ca<sup>2+</sup>]<sub>i</sub> (see below), the temperature of solutions was maintained at 32 °C (Warner Instruments) to avoid unexpected thermal stimulation of the cells.

### 2.3. Immunocytochemistry

Primary TG cells were seeded and cultured on poly-L-lysine-coated coverslips (Matsunami, Osaka, Japan). TGs excised from 7-day-old Wistar rats were immersed in “optimal cutting temperature” (OCT) compound, and rapidly frozen in liquid nitrogen. These tissues were sectioned at 10-μm thickness and mounted on slides. After fixation with a mixture of 50% ethanol and 50% acetone at -20 °C for 30 min, cultured cells and cryosections were treated with 10% donkey serum at room temperature for 20 min, and then incubated overnight at 4 °C with primary antibodies (Kuroda et al., 2013). A cocktail of primary antibodies (Neuro-Chrom™ pan-neuronal marker, Millipore, Billerica, MA, USA; diluted 1:50), which contains mouse anti-neuronal nuclei (NeuN), anti-microtubule-associated protein 2 (MAP2), anti-βIII tubulin, and anti-neurofilament H (NF-H) antibodies, was used as a neuronal marker. TG cells were also incubated with either rabbit anti-NF-H antibody (Millipore; 1:200 dilution) as an A-neuron marker, FITC-conjugated isolectin B4 antibody (IB4; 1:200 dilution) as a non-peptidergic C-neuron marker, mouse anti-substance P (SP; Alomone Labs; Jerusalem, Israel; 1:50 dilution) as a peptidergic C-neuron marker, and a rabbit anti-P2Y<sub>12</sub> receptor antibody (against amino acid residues 125–142 of the human P2Y<sub>12</sub>-receptor) (Alomone Labs, Jerusalem, Israel; diluted 1:50)

(Carrasquero et al., 2005; Giachini et al., 2014; Pinheiro et al., 2013). Cells and tissues were washed, and then incubated with a secondary antibody for 30 min at room temperature. The secondary antibodies included Alexa Fluor 488 donkey anti-rabbit IgG, Alexa Fluor 488 donkey anti-mouse IgG, Alexa Fluor 568 donkey anti-mouse IgG, and Alexa Fluor 568 donkey anti-rabbit IgG (Molecular Probes, Eugene, OR, USA; both diluted 1:50). In addition, 4',6-diamino 2-phenylindole dihydrochloride (Invitrogen) was applied for 5 min at room temperature as a nuclear staining. Cells and tissues were examined under fluorescence microscopes (Zeiss, Jena, Germany).

### 2.4. Solutions and reagents

A standard solution containing 137 mM NaCl, 5.0 mM KCl, 2.0 mM CaCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub>, 0.44 mM KH<sub>2</sub>PO<sub>4</sub>, 0.34 mM Na<sub>2</sub>HPO<sub>4</sub>, 4.17 mM NaHCO<sub>3</sub>, and 5.55 mM glucose (pH 7.4) was used as an extracellular solution. A high-K<sup>+</sup> solution (91 mM NaCl, 50 mM KCl, 2.0 mM CaCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub>, 0.44 mM KH<sub>2</sub>PO<sub>4</sub>, 0.34 mM Na<sub>2</sub>HPO<sub>4</sub>, 4.17 mM NaHCO<sub>3</sub>, and 5.55 mM glucose, pH 7.4) was used to discern TG neurons from glial cells by the activation of depolarization-induced increases in the concentration of intracellular free Ca<sup>2+</sup> in the neurons. The P2Y<sub>1,12,13</sub> receptor agonist 2-methylthioadenosine diphosphate trisodium salt (2-MeS-ADP), potent and selective P2Y<sub>12</sub> receptor antagonists AR-C66096 and PSB0739, sarcoplasmic reticulum Ca<sup>2+</sup>-ATPase (SERCA) inhibitor cyclopiazonic acid (CPA), ryanodine receptor/channel inhibitor dantrolene (sodium salt), adenylate cyclase (AC) inhibitor 9-(Tetrahydro-2-furanyl)-9H-purin-6-amine (SQ22536), and phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) were obtained from Tocris Bioscience (Bristol, UK). All the other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA), except where indicated.

### 2.5. Measurement of intracellular free Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>)

Primary cultured TG cells were loaded with 10 μM fura-2 acetoxymethyl ester (Dojindo, Kumamoto Japan) and 0.1% (w/v) pluronic acid F-127 (Invitrogen) in Hank's solution, for 90 min at 37 °C. Then, the cultured TG cells were rinsed with fresh Hank's solution, and mounted on a microscope stage (Olympus, Tokyo, Japan). The emission of the fura-2 fluorescence was measured at 510 nm in response to alternating excitation wavelengths of 340 (F340) and 380 (F380) nm using an Aquacosmos system and software (Hamamatsu Photonics, Shizuoka, Japan), which controls an excitation wavelength selector, and an intensified charge-coupled device camera system (Hamamatsu Photonics). The intracellular free Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>) was measured as the fluorescence ratio of F340 and F380 (R<sub>F340/F380</sub>), and expressed as F/F<sub>0</sub> units; the R<sub>F340/F380</sub> value (F) was normalized to the resting value (F<sub>0</sub>).

### 2.6. Statistical and offline analysis

Data were expressed as the mean ± standard error (S.E.) or standard deviation (S.D.) of the mean of *N* observations, where *N* represents the number of independent experiments or cells, respectively. The data were analyzed using the following nonparametric tests: the Wilcoxon signed-rank test, the Kruskal–Wallis one-way analysis of variance followed by a Dunn's post hoc test, or the Mann–Whitney *U*-test. A *P* value of less than 0.05 was considered significant. The statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA).