Universidade do Estado do Rio de Janeiro
Centro Biomédico
Faculdade de Odontologia

Gabriela Caldeira Andrade Americano

O efeito da febre na mineralização do esmalte dentário: um modelo murino

Rio de Janeiro
2021

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Tese apresentada, como requisito parcial para obtenção do título de Doutor, ao Programa de Pós-Graduação em Odontologia, da Universidade do Estado do Rio de Janeiro. Área de concentração: Odontopediatria.

Orientador (es): Prof.ª Dra. Vera Mendes Soviero

Prof. Dr. José Orivaldo Mengele Júnior

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DEDICATÓRIA

Dedico este trabalho aos meus pais, Liberalina e Élcio, meu esposo Juscelino e meus irmãos Fernandinha e Elcinho, que sempre me apoiaram!

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RESUMO

AMERICANO, Gabriela Caldeira Andrade. **O efeito da febre na mineralização do esmalte dentário**: um modelo murino. 2021. 76 f. Tese (Doutorado em Odontologia) - Faculdade de Odontologia, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, 2021.

A etiologia da Hipomineralização Molar-Incisivo ainda é desconhecida, mas tem sido associada com febre na infância. O objetivo deste estudo foi avaliar o efeito da febre na mineralização do esmalte de incisivos inferiores de camundongos da linhagem C57Bl/6. Os animais foram divididos em dois grupos: 6 no experimental e 6 no controle. Uma dose de 12 ml/ Kg de 15 % p/v de levedura de cerveja e 12 ml/ Kg de salina foi injetada subcutaneamente em cada camundongo do grupo experimental e controle, respectivamente, duas vezes por dia, durante três dias consecutivos. A temperatura corporal foi medida, usando um termômetro digital, imediatamente antes das injeções e 3, 6 e 10 horas depois das injeções. Considerando a taxa de erupção do dente de 0,18 ± 0.05 mm por dia, os animais foram sacrificados 15 dias após a primeira injeção. A análise elementar do esmalte foi feita usando microfluorescência de raio x (μXRF). O mapeamento da superfície do esmalte analisou a distribuição espacial de Ca, P, Fe e Sr. A quantificação de minerais (Ca, P, Sr, Fe, Zn e Ca:P) foi feita em dois pontos posicionados no esmalte completamente formado e dois pontos posicionados no esmalte em maturação. O teste de Mann Whitney foi usado para comparar a temperatura corporal antes e depois das injeções e para comparar a quantidade mineral entre os grupos experimental e controle. A temperatura média corporal dos grupos experimental e controle antes da primeira injeção foi 35,9 ± 0,1 ºC and 35,9 ± 0,2 ºC, respectivamente. A temperatura média corporal dos grupos experimental e controle depois da primeira injeção foram 37,4 ± 0,3 ºC and 36,3 ± 0,2 ºC, respectivamente. No grupo experimental, o Fe estava concentrado no terço incisal, enquanto no grupo controle estava distribuído ao longo dos terços incisal e médio da superfície do esmalte. Nenhuma diferença significativa foi observada nas quantidades de Ca, Fe, Zn e relação Ca:P entre os grupos. O grupo experimental apresentou uma maior concentração de P no esmalte em maturação ($p = 0,02$) e uma menor concentração de Sr no esmalte completamente formado ($p = 0,01$) e no esmalte em maturação ($p = 0.006$). Portanto, este estudo sugere que a febre pode alterar o movimento de íons através das junções dos ameloblastos, modificando a mineralização do esmalte de incisivos inferiores de camundongos da linhagem C57Bl/ 6.

Palavras-chave: Esmalte Dentário. Febre. Hipomineralização Molar-Incisivo. Hipoplasia do Esmalte Dentário. Modelos Animais.

ABSTRACT

AMERICANO, Gabriela Caldeira Andrade. **Fever effect on enamel mineralization**: a murine model*.* 2021. 76 f. Tese (Doutorado em Odontologia) - Faculdade de Odontologia, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, 2021.

Molar-Incisor Hypomineralization aetiology is still unknown, but it has been associated with fever in childhood. This study aimed to evaluate the fever effect on enamel mineralization of lower incisors of C57Bl/ 6 strain mice. Animals were divided into two groups: 6 in the experimental and 6 in the control. A dose of 12 ml/ Kg of 15 % w/v of brewer yeast and 12 ml/ Kg of saline was subcutaneous injected into each experimental and control mouse, respectively, twice a day for three consecutive days. The body temperature was measured, using a digital thermometer, immediately before the injections and 3, 6 and 10 hours after the injections. Considering a tooth eruption rate of 0.18 ± 0.05 mm per day, the animals were sacrificed 15 days after the first injection. Elemental analysis of the enamel was done using Micro X-ray fluorescence (μXRF). The mapping of the enamel surface analyzed the spatial distribution of Ca, P, Fe and Sr. The quantification of minerals (Ca, P, Sr, Fe, Zn, and Ca:P) was done in two points positioned in the mature enamel and two points in the maturing enamel. Mann Whitney test was used to compare the body temperature before and after the injections as well as to compare the mineral quantity between the experimental and control groups. The mean body temperature of the experimental and control groups before the first injection were 35.9 ± 0.1 ºC and 35.9 ± 0.2 ºC, respectively. The average body temperature of the experimental and control groups after the first injection were 37.4 ± 0.3ºC and 36.3 ± 0.2ºC, respectively. In the experimental group, Fe was concentrated in the incisal third, while in the control group it was distributed along the incisal and medium thirds of the enamel surface. No significant difference was observed in the quantities of Ca, Fe, Zn, and Ca:P ratio between groups. The experimental group showed a higher concentration of P in the maturing enamel ($p = 0.02$) and a lower concentration of Sr in the mature ($p = 0.01$) and maturing enamel ($p = 0.006$). Therefore, this study suggests that fever can disturb ion movement through tight junctions of ameloblasts, modifying the enamel mineralization of lower incisors of C57Bl/ 6 strain mice.

Keywords: Dental Enamel. Dental Enamel Hypoplasia. Fever. Models, Animal. Molar Incisor Hypomineralization.

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INTRODUÇÃO

A Hipomineralização Molar-Incisivo (HMI) é um defeito de mineralização do esmalte, caracterizada por opacidade demarcada. Embora os primeiros molares e incisivos permanentes sejam os dentes mais afetados, este defeito pode acometer outros dentes. A etiologia ainda é desconhecida. No entanto, vários fatores tem sido associados à HMI, como: os pré-natais, estresse ou doença na gravidez; os perinatais, parto cesáreo ou complicações no nascimento; e os durante os três primeiros anos de vida da criança, febre, doenças respiratórias ou da infância. A relação dos níveis de vitamina D, uso de medicamentos, presença de Dioxina ou Bisfenol A, com a HMI também tem sido estudada. O fato é que, a maioria dos estudos são transversais ou retrospectivos, introduzindo o viés de memória e limitando as conclusões. Além disso, em humanos não é possível ter certeza se doenças da infância, febre ou o tratamento por si só é a causa ou se todos eles estão envolvidos na etiologia da HMI. Tudo isto dificulta o consenso sobre um único fatore etiológico ou de um conjunto de fatores. Estudos em animais podem contribuir no esclarecimento da etiologia da HMI, uma vez que é possível avaliar os possíveis fatores etiológicos separadamente. Na área odontológica, os incisivos de roedores, que tem contínua erupção, têm sido muito utilizados como modelo pela semelhança global com dentes humanos. A HMI também parece ter contribuição de fatores genéticos. Porém, mais estudos são necessários. Estudos investigando o papel dos fatores epigenéticos na HMI também são sugeridos a fim de entender melhor a relação desta condição com os fatores ambientais. A busca pelo conhecimento da etiologia da HMI acontece pela sua relevante prevalência no mundo todo e pelas suas complicações clínicas. É de senso comum o desafio do manejo clínico de pacientes acometidos por esta condição. Pois, a menor resistência do esmalte hipomineralizado predispõe a ocorrência de fraturas pós-eruptivas, lesões cariosas ou até mesmo a perda dentária. Comumente, estas crianças apresentam problemas de comportamento, medo e ansiedade, pois costumam ter sensibilidade dentária e ser submetidas mais vezes a procedimentos restauradores.

1 **HIPOMINERALIZAÇÃO MOLAR-INCISIVO**

1.1 **Definição**

A Hipomineralização Molar-Incisivo (HMI) é um defeito de mineralização do esmalte, que acomete 1 a 4 primeiros molares permanentes, frequentemente associados com incisivos permanentes afetados(1). Clinicamente, o esmalte hipomineralizado se apresenta como opacidade demarcada, possui espessura normal e coloração branca, amarela ou marrom(2, 3). Os critérios para o diagnóstico da HMI são presença de opacidade demarcada; fratura pós eruptiva do esmalte; restauração atípica e/ou exodontia de molares por HMI(2). A HMI é classificada como leve ou severa. Em casos de HMI leve há opacidades demarcadas sem fraturas do esmalte, ocasional sensibilidade a estímulos externos e leve preocupação com a descoloração dos incisivos. Nos casos severos, há opacidades demarcadas com fraturas de esmalte, lesões cariosas associadas, sensibilidade persistente e espontânea, e forte preocupação com a descoloração dos incisivos(4). A prevalência mundial é de aproximadamente 14,2%, variando de 2,8% em Hong Kong a 40,2% no Rio de Janeiro (5).

1.2 **Etiologia**

A etiologia da HMI ainda é desconhecida, porém a HMI tem sido associada a perturbação sistêmica no período pré-natal, perinatal ou durante os três primeiros anos de vida da criança, momento em que em que as coroas dos dentes mais afetados, primeiros molares permanentes, estão sendo mineralizadas(1, 4). Uma revisão sistemática com metanálise sobre a etiologia mostrou associação de fatores pré-natais, estresse ou doenças na gravidez, com HMI(6). Sobre os fatores perinatais, parto cesáreo e complicações no nascimento foram significativamente associados com HMI(6). Este mesmo estudo mostrou associação de fatores pós natais, doenças respiratórias ou febre, com HMI(6). Outra revisão sistemática sem

metanálise mostrou associação de doenças na gravidez, complicações no nascimento, pneumonia, febre ou doenças da infância com HMI(7). Tung et al(8) induziram febre alta persistente em ratos e observaram uma linha radilúcida na camada mais externa do esmalte, prismas irregulares e aumento do espaço interprismático. Outro estudo cultivou molares de camundongos *in vitro* sob temperatura 39 °C até o estágio precoce de maturação do esmalte e observaram alteração na formação do esmalte. Mas, não investigaram se a temperatura alta interfere na mineralização do esmalte(9). Os resultados dos estudos são divergentes sobre a influência da vitamina D na etiologia da HMI. Dois estudos observaram uma associação inversa entre níveis de vitamina D e a presença de HMI(10, 11). Outros dois estudos não observaram esta associação. Um deles não viu associação dos níveis de vitamina D aos 6 anos de idade e a presença de HMI(12). E o outro, não encontrou associação da HMI com alterações no gene *VDR* (receptor de vitamina D)(13), responsável por mediar os efeitos da vitamina D na mineralização(12).

Sobre o uso de medicamentos na infância, uma revisão sistemática com metanálise sobre a etiologia não mostrou associação do uso de antibióticos com HMI(6). Além disso, materiais arqueológicos mostram a presença de HMI antes da introdução da Amoxicilina, indicando que se ela está envolvida na causa deste defeito, isso explica apenas parte dos casos de HMI(4). Uma revisão sistemática sem metanálise estudou a relação de medicamentos (antineoplásicos, antibióticos, antieplépticos e antiasmáticos) e a etiologia da HMI. Este mesmo estudo concluiu que nenhum medicamento pode ser considerado como fator etiológico da HMI. Novos estudos prospectivos, relatando a idade de consumo dos medicamentos e o tipo de defeito encontrado, são necessários para esclarecer melhor esta relação(14). Outro fato é que, em humanos não é possível ter certeza se doenças da infância, febre ou o tratamento por si só é a causa ou se todos eles estão envolvidos na etiologia da HMI(4).

Em estudos sobre fatores ambientais, a Dioxina presente no leite materno foi associada com Defeitos de desenvolvimento do esmalte, incluindo HMI(15). Posteriormente, o mesmo grupo mostrou que a exposição das criança à Dioxina, através da placenta ou leite materno, não está associada com a HMI(16). A exposição de ratos ao Bisfenol A, revelou um aumento de Albumina exógena no esmalte. Além disso, foi observado aumento da expressão de Enamelina e redução da expressão de Calicreína 4 (KLK-4)(17, 18). Tem sido sugerido que a Abulmina

pode se ligar aos cristais de esmalte e inibir seu crescimento(19). A Enamelina é uma das proteínas secretadas pelos ameloblastos e tem papel no crescimento dos cristais(20). Já a KLK-4 é uma das proteinases secretadas pelos ameloblastos e responsável por degradar e remover as proteínas da matriz do esmalte(21).

A HMI parece ter contribuição de fatores genéticos. Um estrudo mostrou que em 65% dos pares de gêmeos monozigóticos, ambos apresentaram HMI. O mesmo ocorreu em 34,5% dos pares de gêmeos dizigóticos(22). Vários genes envolvidos na formação do esmalte parecem contribuir na etiologia da HMI(23). Posteriormente, outro estudo do mesmo grupo mostrou associação da HMI com variações em genes envolvidos na amelogênese(24).

Ainda outro estudo observou associação da HMI severa com alteração no gene *TGFBR1* (receptor de fator de transformação do crescimento β, TGF-β), sugerindo que alterações em genes relacionados a resposta imune podem influenciar o desenvolvimento do esmalte. Os autores especulam que esta associação pode ser devido o TGF-β modular a expressão de KLK4 e Metaloproteinase 20 (*MMP-20*)(25). A MMP-20 é uma das proteinases secretadas pelos ameloblastos e responsável por degradar as proteínas da matriz do esmalte, o que permite formação e crescimento dos cristais de hidroxiapatita(26). Além disso, o mesmo estudo mostrou que interação entre alterações nos genes relacionados a resposta imune, como Interleucina (*IL*)*-4, IL-17A, IL-10, IL-1A, STAT1* (transdutor de sinal e ativador de transcrição 1) e amelogênese, *AMELX*, tem um efeito adicional na susceptibilidade ao desenvolvimento da HMI. O gene AMELX codifica amelogenina, que é secretada pelos ameloblastos na fase de secreção(27). Interação entre o gene *IL-4* e *TUFT1* (tuftelina 1) bem como *BMP2* (proteína morfogenética óssea 2) também foi encontrada. Sobre interação entre gene e ambiente, foi oservada associação entre *TGFBR1* e pneumonia na infância(25). Por ultimo, um estudo mostrou que os genes *IRF6* (fator regulador de Interferon 6) e *TGFA* (fator de transformação do crescimento A) podem interagir e estar envolvidos na etiologia da HMI. Eles também observaram uma interação destes genes com o uso de medicamentos em torno de 3 anos de idade, embora o uso de medicamentos possa ser um substituto para doenças ocorridas neste período(28).

1.3 **Caracteristicas do esmalte hipomineralizado**

O esmalte defeituoso se extende por toda a espessura do esmalte(29-31). O esmalte hipomineralizado tem sua densidade mineral reduzida a partir da junção amelo-dentinária em direção a subsuperfície(3, 29, 32, 33). A superfície deste esmalte é mais mineralizada, embora não seja tão mineralizada quanto o esmalte normal(3, 32, 34). Quanto mais o esmalte é hipomineralizado, menor o conteúdo de Cálcio (Ca) e Fósforo (P) e maior a concentração de Carbono (C), indicando vestígios de material orgânico neste esmalte(35). Fagrell et al(36) não encontrou diferença nos valores de Ca entre os esmaltes normal e hipomineralizado, mas assim como Bozal et al(33), observou que o conteúdo de C é maior no esmalte hipomineralizado. Bozal et al(33) encontraram menor quantidade de Ca e P apenas nas opacidades de cor amarelo-amarronzada. Um estudo observou menor taxa da relação Ca:P no esmalte hipomineralizado(35), enquanto outros não observaram nenhuma diferença entre os esmaltes hipomineralizado e normal(36, 37). Quanto ao conteúdo de Sr, não há diferença entre os esmaltes normal e hipomineralizado, mas há uma maior concentração de Magnésio (Mg) nas áreas defeituosas(35). Bozal et al(33) observou Mg, Zinco (Zn) e Rubídio (Rb) apenas nas opacidades de cor amarelo-amarronzada. O esmalte hipomineralizado também tem maior conteúdo de proteínas(38-40), sendo que as opacidades amarelas e marrons possuem maior quantidade do que as opacidades brancas. As opacidades amarelas e marrons possuem maior concentração de albumina sérica, alfa-1-antitripsina e antitrombina III. A ameloblastina foi encontrada apenas nas opacidades marrons(38, 39).

As regiões hipomineralizadas são menos duras(29, 36, 37, 41) e possuem menor módulo de elasticidade do que as áreas não afetadas(37, 41). Os valores de micro dureza relativa do esmalte hipomineralizado diminuem a partir da superfície em direção a junção amelo-dentinária, ou seja, quanto menor a taxa Ca:C, menor o valor de micro dureza(36). Outra característica do esmalte hipomineralizado é a porosidade(29, 34, 36, 37, 41). As opacidades de cor amarelo-esbranquiçada são menos porosas, mais duras e possuem maior densidade mineral que as de cor amarelo-amarronzada(29, 33, 34, 42).

No esmalte hipomineralizado, as bordas dos prismas e o espaço interprismático não são distintos. Os prismas de esmalte são finos e irregulares com

espaço interprismático bastante amplo. Os cristais de hidroxiapatita parecem ser agrupados de forma irregular e frouxa (29, 36, 37, 41, 43, 44).

1.4 **Implicações clínicas**

A baixa resistência do esmalte hipomineralizado pode resultar em fraturas pós-eruptivas do esmalte(1, 4). Consequentemente, estas fraturas facilitam o acúmulo de placa e o desenvolvimento de cárie(1, 2, 4). Os dentes hipomineralizados podem ser sensíveis a alimentos frios, e/ou ar quente e frio, mesmo quando não há fraturas(1). O dente com hipersensibilidade frequentemente cria problemas para os pacientes e dentistas. Para os pacientes, a sensibilidade atrapalha a escovação, favorecendo também o acúmulo de placa(1). Para os dentistas, a sensibilidade dificulta o controle da dor (ex.: analgesia)(4). Além da baixa adesão dos materiais restauradores neste esmalte, o controle inapropriado da dor também contribui para as falhas das restaurações, uma vez que dificulta a realização do procedimento(45). Assim, as crianças com HMI são submetidas mais vezes a tratamentos e retratamentos odontológicos do que crianças sem HMI(46).

2 **FEBRE**

O sistema imune é dividido em Inato e Adaptativo. A imunidade inata representa uma resposta rápida e estereotipada durante uma infecção ou lesão, na tentativa de manter a homeostase do tecido. É representada por barreiras físicas, químicas e biológicas, células e moléculas solúveis presentes nos indivíduos independentemente de contato prévio com imunógenos ou agentes agressores. A fagocitose, liberação de mediadores inflamatórios, ativação de proteínas do sistema complemento, bem como síntese de proteínas de fase aguda, citocinas e quimiocinas são os principais mecanismos da imunidade inata ou resposta de fase aguda. Nesta resposta, os macrófagos ao reconhecerem o agente agressor, tornam-se ativados e liberam citocinas e quimiocinas iniciando o processo conhecido como inflamação. A inflamação é representada clinicamente por calor, dor, rubor e inchaço, e pode repercutir apenas no local do dano ou se manifestar de modo sistêmico(47, 48).

A febre é uma consequência clínica importante da resposta de fase aguda. Os pirógenos exógenos são substâncias infeccisas ou não, capazes de induzir febre. Levedura de cerveja, Lipopolissacarídeos (LPS) e Terebintina são exemplos deles. No entanto, os pirógenos exógenos não são os responsáveis diretos pela febre, eles são reconhecidos por células do sistema imune, que liberam citocinas mediadoras da resposta febril. Estas citocinas, conhecidas como pirógenos endógenos, são a IL-1, Fator de Necrose Tumoral (TNF), Interferon (IFN) e IL-6. A febre ocorre pela ação dos pirógenos endógenos sobre os centros termorreguladores do Hipotálamo, elevando o limiar térmico que normalmente é em torno de 37 °C. Estas citocinas ao caírem na corrente sanguínea a partir do foco inflamatório estimulam a produção de Prostaglandina-E2 (PGE2) por várias células na vizinhança do centro termorregulador hipotalâmico. A PGE2 se difunde para o centro termorregulador estimulando a produção de Monofosfato cíclico de Adenosina (AMP cíclico) e inibindo a atividade dos neurônios sensíveis ao calor. Deste modo, o limiar térmico é elevado e as respostas de geração e conservação de calor são acionadas(49).

Na resposta inflamatória, as proteínas de fase aguda exibem alterações na sua concentração sérica. A concentração de Ceruloplasmina, responsável pela inativação de radicais livres é aumentada, por exemplo, em oxidar Fe+2 em Fe+3

para que possa ser transportado pela Transferrrina sérica. No entanto, a Transferrina sérica, responsável pelo transporte de Fe, tem sua concentração reduzida na resposta inflamatória, enquanto há o aumento da afinidade do Fe pela Lactoferrina intracelular. A redução da disponibilidade de Fe limita a proliferação bacteriana, por exemplo(49).

3 **PROPOSIÇÃO**

3.1 **Objetivo geral**

Investigar os efeitos da febre na mineralização do esmalte dentário, utilizando modelo animal.

3.2 **Objetivos específicos**

a) Propor uma metodologia de indução de febre em camundongos da linhagem C57Bl/6 utilizando Levedura de cerveja (*Saccharomyces cerevisiae*).
b) Avaliar a taxa de erupção dos incisivos inferiores de camundongos da linhagem C57Bl/6.
c) Avaliar o efeito da febre no conteúdo mineral do esmalte dentário de incisivos inferiores de camundongos da linhagem C57Bl/6.

4 **DELINEAMENTO DA PESQUISA**

A presente tese é composta por três estudos a fim de responder o objetivo geral.

Todos os experimentos foram aprovados pelo Comitê de Ética no Uso de Animais do Centro Universitário Arthur Sá Earp Neto (UNIFASE) (ANEXO) e realizados de acordo com Conselho Nacional de Controle de Experimentação Animal em Pesquisas (CONCEA – 11.794 de 08 de outubro de 2008).

4.1 **Estudo 1**

4.1.1 Animais

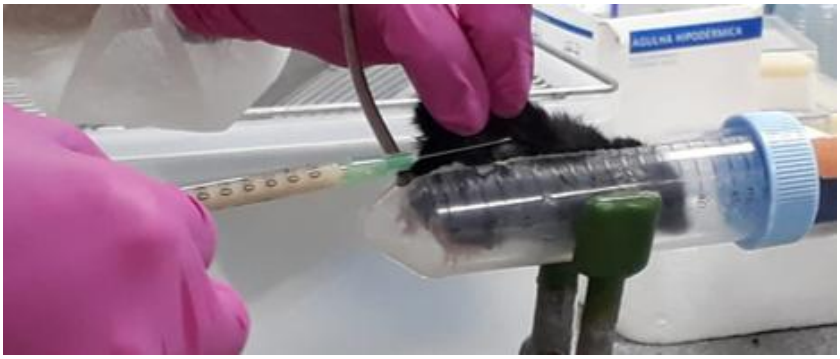
Doze camundongos da linhagem C57Bl/6, criados e mantidos no biotério da UNIFASE foram usados neste estudo. Os animais eram fêmeas, adultos, isogênicos, 3 meses de idade e pesando em média 16,66 ± 1,2 g no início do experimento. Eles foram mantidos em uma temperatura média de 21,2 ± 0,7 °C e com livre acesso a comida e água filtrada durante todo o período do experimento. Os animais foram divididos em 2 grupos, sendo 6 camundongos no grupo experimental e 6 no grupo controle.

4.1.2 Indução da febre

Baseado no estudo de Jan e Khan (2016)(50), uma dose de 12 ml/Kg de suspensão de 15% p/v de Levedura de cerveja foi injetada em cada animal do grupo experimental. A suspensão foi preparada com 15 g de Levedura de cerveja em pó disponível comercialmente (*Saccharomyces cerevisiae*) e 100 ml de solução salina (0,9% p/v de Cloreto de Sódio – NaCl). Cada camundongo do grupo controle

recebeu 12 ml/Kg de solução de 0,9% p/v de Cloreto de Sódio do mesmo modo. Todos os animais receberam injeções subcutâneas duas vezes por dia durante três dias consecutivos. As injeções foram aplicadas às 7 horas da manhã e às 17 horas da tarde depois da medição da temperatura. Para aplicar as injeções, cada animal foi colocado dentro de um tubo de ensaio com um corte longitudinal. Então, a pele foi esticada através do corte e a injeção aplicada como mostra a Figura 1.

Figura 1 – Método para aplicação da injeção subcutânea

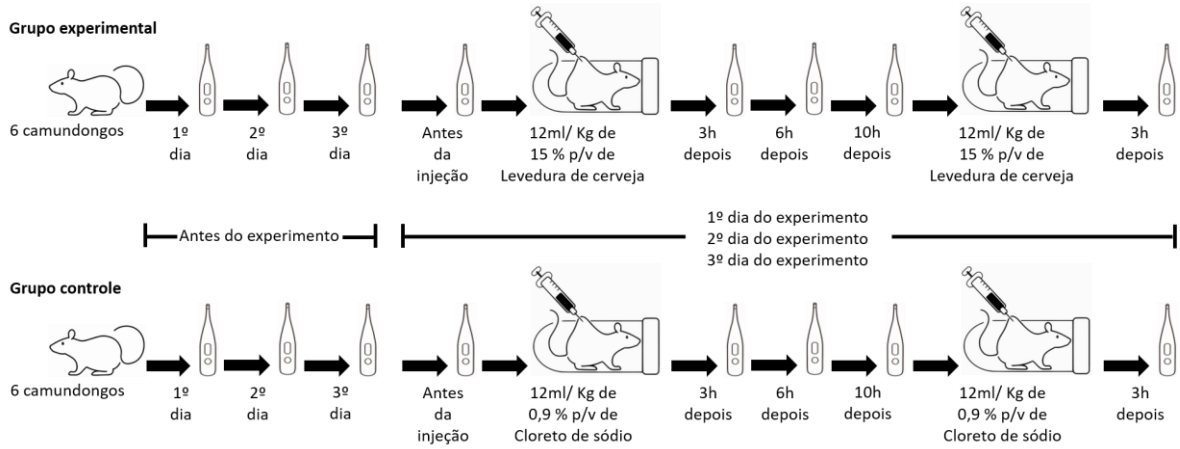


Fonte: O autor, 2021.

4.1.3 Medição da temperatura corporal

As temperaturas corporais dos animais foram medidas usando um termômetro digital com uma sonda retal RET-3 apropriada para camundongos (Physitemp®, Clifton, NJ, USA). A sonda foi introduzida no reto, 2 cm a partir do ânus. Antes do experimento, as temperaturas corporais foram medidas duas vezes por dia por três dias consecutivos. Então, as temperaturas foram registradas imediatamente antes das injeções e, 3, 6 e 10 horas depois das injeções por 3 dias consecutivos. A Figura 2 mostra o diagrama com os procedimentos experimentais.

Figura 2 – Diagrama do experimento



Fonte: O autor, 2021.

4.1.4 Análise estatística

Os dados foram analizados com o programa *Statistical Package for Social Sciences* versão 23.0 (SPSS Inc., IBM, Chicago, Ill., USA). A análise descritiva incluiu o cálculo da média e desvio padrão da temperatura do ambiente onde os animais foram mantidos, do peso dos animais e da temperatura corporal dos animais do grupo controle e experimental. Todas as variáveis avaliadas apresentaram distribuição assimétrica. O teste Mann Whitney U foi usado para comparar temperaturas corporais entre os grupos experimental e controle antes e depois das injeções. O teste de Wilcoxon foi usado para comparar temperaturas corporais antes e depois das injeções de cada grupo. O nível de significância estatísica foi de 5%.

4.2 **Estudo 2**

4.2.1 Animais

Cinco camundongos da linhagem C57Bl/6, criados e mantidos no biotério da UNIFASE foram usados neste estudo. Os animais eram fêmeas, adultos e

isogênicos. Eles foram mantidos com livre acesso a comida e água filtrada durante todo o período do experimento.

4.2.2 Anestesia

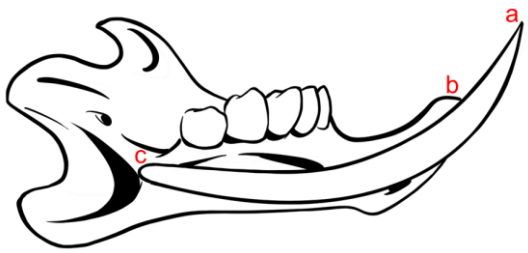
Os camundongos foram anestesiados antes de todos os procedimentos via intraperitoneal com 0,2 ml de solução preparada com 0,9 ml de Cloridrato de Xylazina (Rompun®, Bayer®), 0,45 ml de Cloridrato de Ketamina (Vetanarcol®, König®) e 6 ml de salina (0,9% p/v de Cloreto de Sódio – NaCl).

4.2.3 Medição do tamanho do dente e da taxa de erupção

No primeiro dia, os camundongos tiveram seus dois incisivos inferiores medidos com paquímetro digital (MTX®) desde a borda incisal até a margem gengival (Figura 3). Os incisivos também foram marcados rente a margem gengival na lingual com disco diamantado Micro Dupla Face (KG Sorensen®) montado em Micromotor LB100 (Beltec®). A distância entre a margem gengival na lingual e a marca foi medida com o paquímetro quatro vezes em um intervalo de dez dias (dia 2, dia 4, dia 7 e dia 10).

A eutanásia dos animais foi realizada através da inalação de isofluorano para coleta dos incisivos inferiores. Os incisivos inferiores foram dissecados da mandíbula e medidos com o paquímetro desde a borda incisal até a volta apical (Figura 3).

Figura 3 - Diagrama do incisivo inferior de camundongo



Legenda: ponto ‘a’ ao ponto ‘b’ é a distância da borda incisal até a margem gengival. Ponto ‘a’ ao ponto ‘c’ é a distância curvilínea da borda incisal até a volta apical.
Fonte: O autor, 2021.

4.2.4 Análise estatística

Os dados foram analizados com o programa *Statistical Package for Social Sciences* versão 23.0 (SPSS Inc., IBM, Chicago, Ill., USA). A análise descritiva incluiu o cálculo da média e desvio padrão do comprimento do incisivo inferior da borda incisal até a margem gengival e da borda incisal até a volta apical. Para o cálculo da média e desvio padrão da taxa de erupção foi considerada a medida da distância entre a margem gengival e a marca realizada no 10º dia dividido por 10.

4.3 **Estudo 3**

Este estudo seguiu o estudo 1, descrito anteriormente.

4.3.1 Coleta das amostras

Considerando a taxa de erupção média dos incisivos inferiores igual a 0,18mm por dia (estudo não publicado), a eutanásia dos animais foi realizada através da inalação de isofluorano, 15 dias depois da primeira injeção. Um incisivo

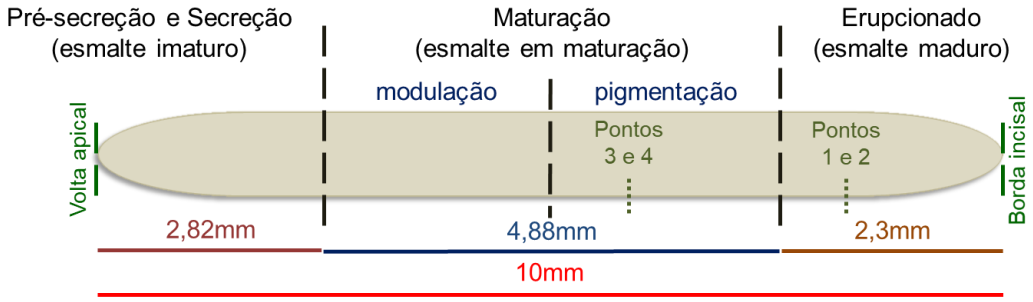
inferior de cada camundongo foi cuidadosamente dissecado da mandíbula e armazenado em água destilada.

4.3.2 Medição do conteúdo mineral do esmalte

A análise de distribuição elementar de todos os dentes foi feita usando a microfluorescência de raios X (μXRF). Esta análise foi realizada com o equipamento M4 Tornado® (Bruker, Berlin, Germany), o qual é composto por um tubo de raios X com anodo de Ródio (Rh), um policapilar para o tamanho de *spot* de 25 μm e um detector SDD (Silicon Drift Detector) com resolução de energia de 142 eV para Mn-Kα. O mapeamento das amostras foi feito com com uma voltagem de 30 kV, corrente de 600 mA, um filtro de Al de 12,5 μm, com um tamanho de passo de 25 μm, durante um tempo de medida de 210 ms por pixel sob condição de vácuo de 20 mbar. A quantificação do espectro foi realizada usando um software integrado com base em uma matriz de Hidroxiapatita [Ca10 (PO4) 6 (OH) 2].

O mapeamento total foi realizado para avaliar a distribuição dos minerais ao longo da superfície vestibular. Além disso, a análise para quantificar os minerais foi realizada em quatro pontos: dois pontos (1 e 2) a 1,3 mm e os outros dois pontos (3 e 4) a 3,8 mm da borda incisal de todos os dentes, como mostra a Figura 4. Considerando o diagrama de desenvolvimento do esmalte de um incisivo inferior de rato (animal com peso igual a 200 g)(51), e que o incisivo de um camundongo (animal com peso igual a 20 g) mede em média 10 mm, os pontos 1 e 2 estão situados no esmalte erupcionado e os pontos 3 e 4 estão no esmalte em maturação (Figura 4).

Figura 4 – Posição dos pontos para análise do conteúdo mineral no esmalte



Legenda: diagrama mostrando a posição dos pontos 1, 2, 3 e 4 no esmalte de incisivo inferior de um camundongo. O presente diagrama foi baseado no diagrama de desenvolvimento do esmalte de um incisivo inferior de rato(51).
Fonte: O autor, 2021.

4.3.3 Análise estatística

Os dados foram analizados com o programa *Statistical Package for Social Sciences* versão 23.0 (SPSS Inc., IBM, Chicago, Ill., USA). A análise descritiva incluiu o cálculo da média, mediana e desvio padrão da quantidade mineral do esmalte nos pontos 1 e 2 bem como nos pontos 3 e 4 de todos os dentes dos animais do grupo controle e experimental. Todas as variáveis avaliadas apresentaram distribuição assimétrica. O teste Mann Whitney U foi usado comparar a quantidade mineral do esmalte nos pontos 1 e 2 bem como nos pontos 3 e 4 entre os grupos experimental e controle. O nível de significância estatísica foi de 5%.

5 **DESENVOLVIMENTO DA PESQUISA**

Os manuscritos serão enviados para publicação em revistas científicas e os termos de autorização para publicação na Biblioteca Digital de Teses e Dissertações (BDTD) estão nos Anexos B, C e D.

Manuscrito 1: Brewer yeast-induced fever: a model for mice.

Manuscrito 2: Eruption rate of mouse lower incisors.

Manuscrito 3: Fever effect on enamel mineralization: a murine model.

5.1 **Brewer yeast-induced fever: a model for mice (Manuscrito)**

Running title: Brewer yeast-induced fever

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Abstract

Fever is a body's defence against an infectious or inflammatory agent, however the extreme temperature rise can be harmful. In order to help studies of fever mechanisms and thermoregulation as well as fever consequences, this study aimed to describe a protocol to induce fever using brewer yeast in mice. Two groups of 6 female mice of the C57Bl/6 strain were injected either with 12 ml/ Kg of 15 % w/v of brewer yeast subcutaneously or with equivalent volume of saline. All animals received subcutaneous injections twice a day for three consecutive days. The body temperatures of animals were recorded before the experiment, immediately before the injections and 3, 6 and 10 hours after the injections, using an appropriate thermometer. The average temperatures before the experiment between the experimental and control groups were not different ($p = 0.6$). The mean temperature of the experimental group during the fever period was significantly higher than the control group ($p = 0.004$). The protocol suggested in this study presented as an effective method and easy to reproduce.
Key words: Brewer Yeast. Fever. Mice. Models, Animal. Saccharomyces cerevisiae.

Introduction

Fever is a temperature rise in response to an infectious or inflammatory agent (1). That is a physiological event involving the immune system and the brain (1). Although the pathways used for signalling to the brain are still not clear, it is known that cytokines released by immune cells interact and signalize to the hypothalamus to increase the thermal set point (1). Fever is well-known for acting as a teratogenic factor during the first trimester of pregnancy. It increases the likelihood of children be born with neural tube and cardiac malformations (2). At moment of the craniofacial development, it can cause cleft and tooth malformations (3, 4). The body temperature rise can lead to deliriums and disorientations (5), and aggravate heart and pulmonary failures (6).

Experimental animal models are useful to induce fever in order to study its mechanisms, thermoregulation and consequences (7). Both endogenous and exogenous pyrogens are able to induce fever in animals. The endogenous ones are the cytokines such as interleukin (IL)-1, IL-6, tumour necrosis factor (TNF) and interferon (IFN)(8). Exogenous pyrogens like turpentine oil, lipopolysaccharides (LPS), and brewer yeast induce fever by acting on macrophages and monocytes to

release those cytokines (9). The magnitude and duration of fever depends on animal species, the pyrogen type and its dose (1).

Turpentine oil and LPS were able to induce fever in mice (10). Brewer yeast caused fever in rats and presented to be easily available, a nontoxic pyrogen and a low cost method (7). Thus, this study aimed to describe a protocol to induce fever using brewer yeast in mice.

Materials and methods

This study was approved by the Animal Use Ethics Committee of the Arthur Sá Earp Neto University Centre (UNIFASE). All experimental were carried out in accordance with the Brazilian Regulatory Council for the use of animal in research (CONCEA – 11.794 of 08 October 2008).

Animals

Twelve mice of the C57Bl/ 6 strain, raised and kept in the vivarium of UNIFASE, were used in this study. The animals were female, adult, isogenic, aged 3 months and weighed on average 16.66 ± 1.2 g at the beginning of the experiment. They were housed at an average temperature of 21.2 ± 0.7 ºC and with free access to lab chow and tap water during the whole period of the experiment. Figure 1 shows a diagram with the experimental procedures. The animals were divided into two groups: 6 mice in the control group and 6 in the experimental group.

Experimental design

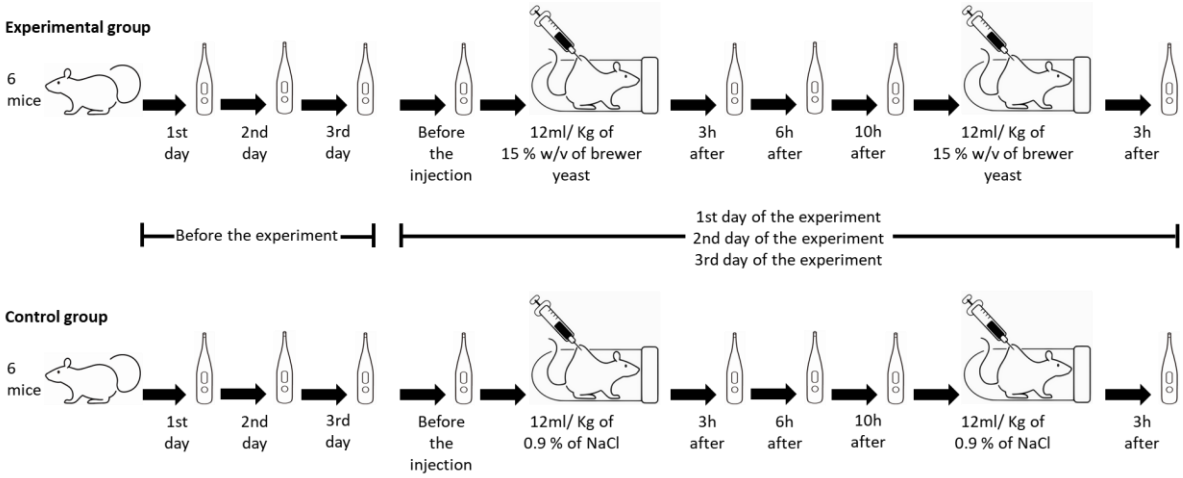
A dose of 12 ml/ Kg of 15 % w/v of brewer yeast was injected into each experimental mouse. The suspension was prepared with 15 g of commercially available dried brewer yeast (*Saccharomyces cerevisiae*) and 100 ml of saline (0.9 % w/v of Sodium chloride - NaCl). Each experimental animal received 1.8 g of brewer yeast per kilo. Each control mouse received 12 ml/ Kg of saline in the same way. All animals received subcutaneous injections twice a day for three consecutive days. The injections were applied at 7 am and 5 pm after the temperature measurement. To apply the injections, each animal was placed into a test tube with a longitudinal cut. The cut was pointing upwards and a mouse was placed into the tube trough the tail. So, the skin was stretched through the cut and the injection applied as shown the Figure 1.

Figure 1 – Application of the subcutaneous injection

Body temperature measurements

The body temperatures of animals were measured using a BAT-7001H digital thermometer with the RET-3 rectal probe appropriate for mice (Physitemp®, Clifton, NJ, USA). The probe was introduced into the colon, 2 cm away from the anus. Before the experiment, the body temperatures were measured twice a day for three consecutive days. Then, the body temperatures were recorded immediately before the injections and 3, 6 and 10 hours after the injections for three consecutive days. Figure 2 shows a diagram with the experimental procedures.

Figure 2 – Diagram of the experiment



Statistical analysis

Data were analyzed with Statistical Package for Social Sciences program version 23.0 (SPSS Inc., IBM, Chicago, Ill., USA). The descriptive analysis included the average calculation and standard deviation of environment temperature where animals were kept, animal weight, and body temperature for experimental and control groups. All evaluated variables presented asymmetrical distribution. The Mann Whitney test was used to compare body temperatures between the experimental and

control groups before and after the injections. Statistical significance level was set at 5%.

Results

Figure 3 shows the average body temperature of the experimental and control groups three days before the injection and immediately before the first injection.

Figure 4 shows the average body temperature of the experimental and control groups immediately before the injections and 3, 6 and 10 hours after the injection for three consecutive days.

The mean body temperature of the experimental and control groups before the first injection were 35.9 ± 0.1 ºC and 35.9 ± 0.2 ºC, respectively. The average temperatures between the groups were not different ($p = 0.6$).

The average body temperature of the experimental and control groups after the first injection were 37.4 ± 0.3 ºC and 36.3 ± 0.2 ºC, respectively. The mean temperature of the experimental group during the fever period was significantly higher than the control group ($p = 0.004$). The mean body temperature of the experimental group was 1.1 ºC higher than the control group, during the fever period. The body temperature of the experimental group reached the maximum after nine hours of the first injection on the first day and after three hours after the injections on the second and third days. The body temperature ranged from a minimum of 37.2 ºC to a maximum of 38.2 ºC and the average of the maximum temperature of each day was 37.8 ± 0.3 ºC. On average, the body temperature of the experimental group was 1.5 ºC higher than the temperature before the experiment. In the control group, a difference of 0.4 ºC in the mean body temperature was observed before and after the experiment.

Figure 3 – Mean body temperature of the experimental and control groups before the experiment

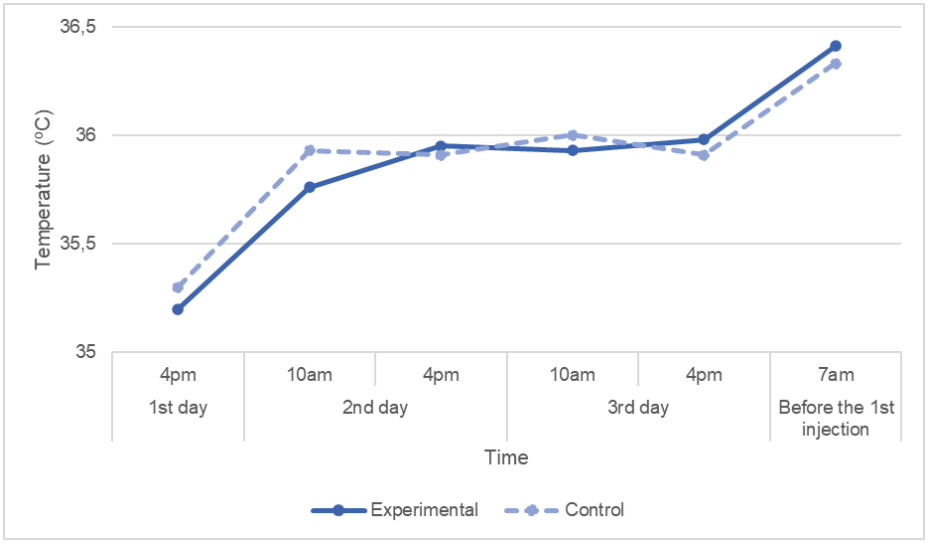
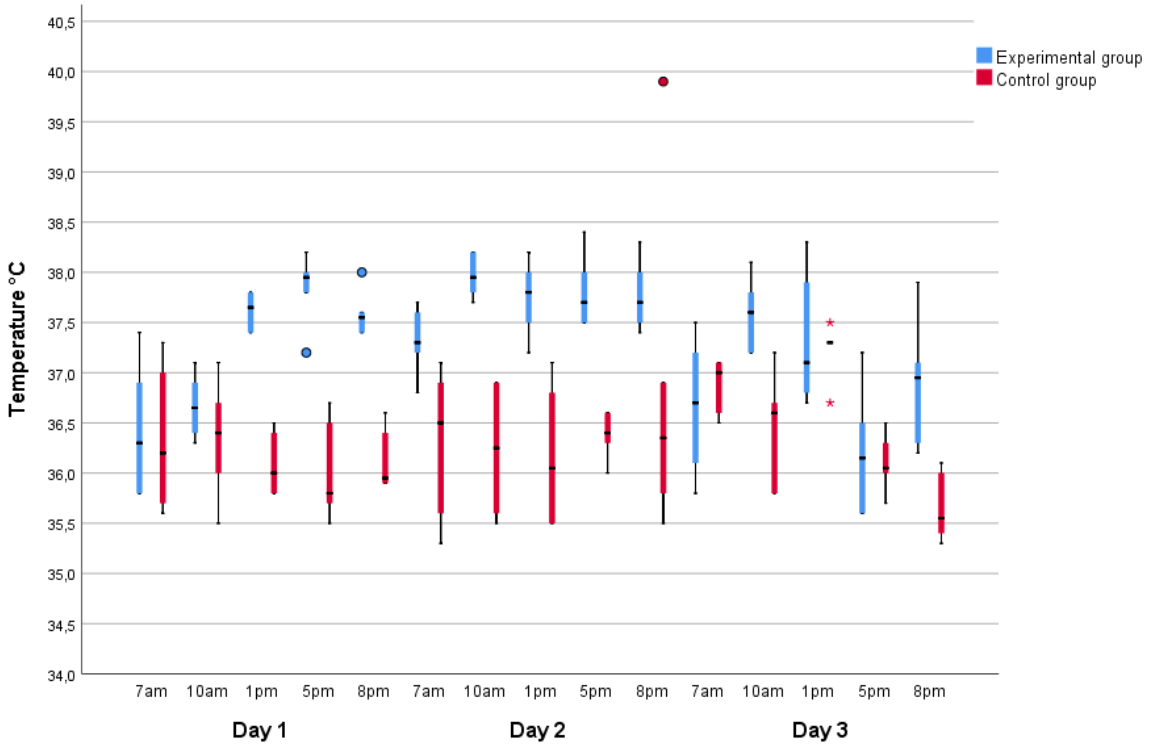


Figure 4 – Mean body temperature of the experimental and control groups immediately before the first injection and 3, 6 and 10 hours after the first, second and third injections during three consecutive days



Discussion

Mice are a good choice of small experimental animals because they are easier to handle, to accommodate due to the minimum space required, and less costly because of low amount of food and drug necessary for tests(7), as examples. Female mice were selected because they are considered as being of comparable size and weight.

The temperature was monitored before the experiment period in order to inure animals to manipulation, because hyperthermia can occur due to stress induction and then mask fever induction with pyrogen (11). In the experimental group, an increase of 1.5 ºC in the body temperature was observed. The normal body temperature of mice is 36.5 ºC, and ~1 °C higher is considered fever (22, 23). In the control group, the body temperature showed an increase of 0.4 ºC probably due to stress from manipulation. The slight increase in the body temperature of the control group was not enough to be considered fever and it was significantly lower than the body temperature increase of the experimental group.

There are some desirable conditions for an animal model of fever induction. Besides complying the ethical aspects, fever should occur in most animals of the research sample, be high enough not to be mistakenly recorded, start within a few hours, and last enough to test antipyretic drugs (7) and to study its consequences without animals acquiring resistance to pyrogens. The present protocol met all those requirements, since all animals had fever and there was a small variability of the response among them, which contributes for reducing the number of animals necessary for tests (7). Secondly, the body temperature of the experimental group was high enough to detect significant different between groups besides the small temperature variability within animals of each group. The normal body temperature of mice is 36.5 ºC, and ~1 °C higher can be considered fever (1, 12). Although the body temperature of the experimental group reached the maximum after nine hours on the first day, fever was already installed after six hours of the injection. A quick start of fever has some advantages such as shorter duration of the experiment and less suffering of animals (7). Finally, fever lasted at least 4 hours, which is rather enough to realize tests. The animals also did not acquire resistance to brewer yeast for at least 3 days because fever remained constant. A study observed that fever response started 4-5 hours after yeast injection, lasted at least 4 hours and the rectal temperature increased a little more than 1 ºC, which were similar to the present

results. However, they used rats, the injection was intraperitoneal and each animal received 0,135 g of brewer yeast per kilo(7). A further three studies also induced fever in rats with subcutaneous injection of yeast, though they used 1.0, 1.5, 2.0 or 2.5 g of brewer yeast per kilo. The vehicle used for injection was saline or 0.5% w/v Carboxymethyl cellulose solution(13-15). Two of them observed that fever began 18 hours after the injection(13, 15). The other one reported that fever started five hours later when the injection was applied in the morning and 11 hours later when the injection was applied at night. As night is the activity period rats, it is believed that rats are better able to adjust to fever induced by exogenous pyrogens during the night than during daytime(14). Those three studies observed that the rectal temperature increased 1.6 ºC or ranged from around 38.5 ºC to 40 °C(13-15).

Studies showed that turpentine oil and LPS were also able to induce fever in mice of the C57Bl/ 6 strain. Fever induced by turpentine injected subcutaneously began 9h after injection and lasted ~32 hours. That fever takes longer to start than brewer yeast-induced fever. While fever induced by LPS injected intraperitoneally began immediately after the injection and lasted up to 7 hours (10). Subcutaneous injection can be more comfortable for the animals and easier for the operators. To apply this injection, it is not necessary to hold the animal with hands. Instead, they can be placed in a container with its tail stabilized and skin stretched as shown the Figure 1. Turpentine oil and LPS have disadvantages in relation to brewer yeast. Lipopolysaccharides depends on the serotype of its source to induce fever (16), can result in hypothermia (17), and animals can acquire tolerance with repeated stimulations (18). Turpentine oil is a tissue irritant, which causes aseptic abscess and tissue necrosis (19), as well as requires anaesthesia before the injection (4, 10). Brewer yeast has some advantages as an exogenous pyrogen such as to be easily available, a nontoxic pyrogen, a low cost method and easy to reproduce (7).

Thus, the protocol suggested in this study presented as an effective method and easy to reproduce.

Acknowledgements

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5.2 **Eruption rate of mouse lower incisors (Manuscrito)**

Running title: Incisor eruption rate

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Abstract

Rodent incisors, which have a continuous eruption and are similar to human teeth, can be widely used to test hypotheses on dental abnormality aetiology. Thus, this study aimed to evaluate the eruption rate of lower incisors of C57Bl/6 strain mice. Five mice of the C57Bl/ 6 strain were used in this study. The animals were female, adult and isogenic. The animals were anesthetised before every procedure. On the first day, mice had their two lower incisors measured with pachymeter from the incisal edge to gingival margin. So, all teeth were marked in the gingival margin using diamond disc. The length between the gingival margin on lingual surface and the mark was measured. The mean length of the erupted tooth is 2.64 ± 0.8 mm. The mean eruption rate of lower incisors per day is 0.18 ± 0.05 mm. Then, a mouse takes on average 14.6 days for an incisor renews in the oral cavity.

Keywords: Eruption Rate. Incisor. Mice. Models, Animal. Tooth Eruption.

Introduction

Studies on aetiology of diseases may start with hypotheses based on clinical findings, and then that hypotheses should be checked with laboratory studies(1). In Dentistry, murine models are of particular interest since rodent incisors have a continuous eruption and are similar to human teeth. Thus, they can be widely used for studying the aetiology of developmental defects of enamel or dental abnormalities(2). Mice can be a good choice of experimental rodents because they are small and easy to handle. Moreover, they require minimum space and are less costly because of low amount of food and substances for tests(3), as examples. A crucial aspect of studying the effect of any insult to tooth development is the definition of the correct area where the analysis must be done. The analysis must correlate the portion that suffered the insult with the time when the insult occurred. This definition is dependent on the tooth eruption rate. Only one study reporting the eruption rate of lower incisors of mice was retrieved from a search in the literature(4). However, this study evaluated a mice strain, called MRCTO, which is no longer available for laboratory experiments. No studies have been found in the literature reporting the tooth eruption rate of any of the mice strains commonly used nowadays. Thus, this study aimed to evaluate the eruption rate of lower incisors of C57Bl/6 strain mice.

Materials and methods

This study was approved by the Animal Use Ethics Committee of the Arthur Sá Earp Neto University Centre (UNIFASE). All experimental were carried out in accordance with the Brazilian Regulatory Council for the use of animal in research (CONCEA – 11.794 of 08 October 2008).

Animals

Five mice of the C57Bl/ 6 strain, raised and kept in the vivarium of UNIFASE, were used in this study. The animals were female, adult, isogenic, and weighted 16.7 g ± 1.2. They had free access to lab chow and tap water.

Anaesthesia

The anaesthesia was prepared with 0.9 ml of Xylazine hydrochloride (Rompun®, Bayer®, Barmen, Germany), 0.45 ml of ketamine hydrochloride (Vetanarcol®, König®, Argentina) and 6 ml of saline (0.9 % w/v of Sodium chloride - NaCl). A dose of 0.2 ml of the solution was injected intraperitoneally into each animal before every procedure.

Tooth size and eruption rate measurement

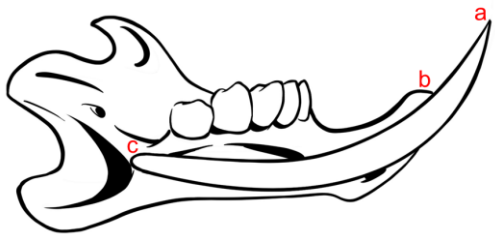
At the beginning of the study (on zero-day), the mice had their two lower incisors measured with digital pachymeter (MTX®) from the incisal edge to gingival margin (Figure 1). Then, a marking groove in the gingival margin was done in all teeth with a diamond disc (KG Sorensen®, Cotia, SP, Brazil) using a low speed handpiece (Beltec®, Araraquara, SP, Brazil). The measurement of the length between the gingival margin and the groove was repeated four times in an interval of ten days (day 2, day 4, day 7 and day 10).

On the 10th day, the animals were sacrificed through isoflurane inhalation. The lower incisors of each animal were carefully dissected out of the bone and measured with pachymeter from the incisal edge to the apical loop (Figure 1).

Statistical analysis

Data were analyzed with Statistical Package for Social Sciences program version 23.0 (SPSS Inc., IBM, Chicago, Ill., USA). The descriptive analysis included the calculation of mean and standard deviation of the incisor length from the incisal edge to gingival margin as well as from the incisal edge to apical loop. The mean and standard deviation of the eruption rate considered the measurement (from the gingival margin to the mark) on the 10th day divided per 10.

Figure 1 – Diagram of a mouse lower incisor



Legend: point ‘a’ to point ‘b’ is the length from the incisal edge to gingival margin. Point ‘a’ to point ‘c’ is the curved length from the incisal edge to apical loop.

Results

The mean eruption rate of the lower incisors per day was 0.18 ± 0.05 mm and ranged from a minimum of 0.07 mm and a maximum of 0.25 mm. So, the mean eruption rate expected per week is 1.26 mm. On the day 2, no noticeable tooth eruption was seen in any of the animals. On the day 4, a new erupted portion of the teeth was measureable in 4 out of 5 animals. On the day 7, a new portion of the teeth was present in both incisors of all animals.

The mean length from the incisal edge to the gingival margin (erupted tooth) was 2.64 ± 0.8 mm and ranged from a minimum of 1.90 mm and a maximum of 4.39 mm. Then, a mouse takes on average 14.6 days for a complete renew of the erupted portion of the lower incisors.

The mean curved length of a whole incisor, from the incisal edge to apical loop, measured after dissection, was 10 mm. Thus, a mouse takes on average 55.5 days for a complete renew of the whole lower incisors.

Table 1: Total length of the erupted portion and eruption rate of right and left lower incisors of five C57Bl/6 strain mice measured in millimeters.

Animal	Incisor	Total length of the erupted portion [a]	New erupted portion [b]				Eruption rate per day
			Day 2	Day 4	Day 7	Day 10	
1	Right	4.39	0	0.42	0.84	1.07	0.11
	Left	3.78	0	0.42	1.05	1.21	0.12
2	Right	1.90	0	0	0.68	0.70	0.07

	Left	1.90	0	0	0.38	1.67	0.17
3	Right	2.62	0	1.00	1.32	[c]	0.22[d]
	Left	2.62	0	1.00	1.50	[c]	0.25[d]
4	Right	2.28	0	0.36	1.48	2.13	0.21
	Left	2.28	0	0.36	1.48	2.14	0.21
5	Right	2.32	0	1.02	1.46	2.15	0.22
	Left	2.40	0	0.55	1.28	2.15	0.22

[a] Measured from the gingival margin to the incisal edge.

[b] Measured from the gingival margin to the marking groove.

[c] It was not possible to do the measurement because the lower incisors broke down.

[d] The eruption rate per day was based on the growth up to the day 7.

Discussion

The tooth eruption rate of rats has been reported as ranging from 0.4 to 0.6 mm per day (5-8). Only one study evaluating the eruption rate of lower incisors of mice was found in the literature (4). This is a study from the 60's that used the MRCTO strain and reported an eruption rate of 0.15 ± 0.05 mm per day. So far, no studies have been found reporting the tooth eruption rate of the C57BL6 mice or other available mice strains commonly used in the experiments nowadays. In the present study, the eruption rate of C57Bl/6 strain mice was 0.18 ± 0.05 mm per day. It corresponded to less than half of the eruption rate reported for rats and was similar to the eruption rate reported for the MRCTO mice.

The animals showed relatively similar eruption rates for both incisors, except for one (animal 2) that presented a slower eruption particularly of the right incisor. The tooth eruption in rodents is mediated by several cytokines and growth factors delivered by the dental follicle(9). The activity of osteoclasts and bone resorption is also required as demonstrated by the delay in tooth eruption after the administration of bisphosphonates in rats(10). The animals in the present study were isogenic and exposed to same environmental conditions and access to food and water. Hence, we do not suppose that genetic or environmental factors could have caused a delay on tooth eruption in one of the animals.

It was observed that the incisors of rats erupted more rapidly when they were shortened to stop them bitten upon (unimpeded) in comparison with incisors that were not shortened (impeded)(11). However, during the present study all the measurements were done in incisors that were occluding with the opposite teeth. In one of the animals, because the lower incisors broke down in the line of the marking groove, between the days 7 and 10, the measurement of the day 7 was the last one used to calculate the eruption rate per day.

A study reported that upper and lower incisors of mice have on average a total length of 7 mm and 9 to 10 mm, respectively(12). A similar result was observed in the present study, as the mean total length of the lower incisors was 10 mm. Thus, on average 55.5 days are necessary for a complete renew of the whole lower incisors of mice. A similar period of time was suggested in a study that evaluated the occurrence of enamel defects induced by antitumor drugs in mice of the BALB/c strain. The animals were sacrificed the on days 3, 5, 10, 15 and 60 after a single injection. They observed that enamel malformations were seen until day 15 after the antitumor drug injection. No more malformations were seen in the enamel in the animals sacrificed on day 60 after injection(13). It signalizes that lower incisors take around 60 days to renew on the whole, as we found in the present study.

Nonetheless, a murine model protocol should be planned in the shortest period of time as possible. Reducing cost and diminishing the exposure of the animals to the potential distress of treatments are among reasons. Since not all categories of experiments depend on the complete renew of the tooth, shorter protocols should be considered.

A diagram of a developing lower incisor of a rat (200 g weight) demonstrated the limits of the developmental stages of enamel formation(5). Considering a total length of 26 mm, 7.3 mm (28.1%) represented the pre-secretion and secretion stages, 6.7 mm (25.7%) represented the early maturation stage, 6.0 mm (23,1%) represented the late maturation stage, and 6.0 mm (23.1%) represented enamel (erupted portion). In a lower incisor of C57Bl/6 strain mice with a total length of 10 mm, the pre-secretion and secretion stage would have 2.82 mm, the early and late maturation stages would have 2.58 and 2.3, respectively, and the mature enamel (erupted portion) would have 2.3 mm. As example, if an experiment aimed to evaluate the effect of a certain insult to the late maturation phase of the amelogenesis in a lower incisor of mice, an average of 13 days would be enough to

evaluate the presence of an abnormality in the mature enamel. The late maturation stage would have moved further replacing the erupted portion. A clear understanding of the eruption rate is crucial to plan protocols to study the amelogenesis in rats or mice.

Conclusion

The mean eruption rate of lower incisors of C57Bl/6 strain mice is 0.18 ± 0.05 mm per day.

Acknowledgements

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5.3 **Fever effect on enamel mineralization: a murine model (Manuscrito)**

Running title: Fever and enamel mineralization

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Abstract

Molar-Incisor Hypomineralization aetiology is still unknown, but it has been associated with fever in childhood. This study aimed to evaluate the fever effect on enamel mineralization of lower incisors of C57Bl/ 6 strain mice. Animals were divided into two groups: 6 in the experimental and 6 in the control. A dose of 12 ml/ Kg of 15 % w/v of brewer yeast and 12 ml/ Kg of saline was subcutaneous injected into each experimental and control mouse, respectively, twice a day for three consecutive days. The body temperature was measured, using a digital thermometer, immediately before the injections and 3, 6 and 10 hours after the injections. Considering a tooth

eruption rate of 0.18 ± 0.05 mm per day, the animals were sacrificed 15 days after the first injection. Elemental analysis of the enamel was done using Micro X-ray fluorescence (μXRF). The mapping of the enamel surface analyzed the spatial distribution of Ca, P, Fe and Sr. The quantification of minerals (Ca, P, Sr, Fe, Zn, and Ca:P) was done in two points positioned in the mature enamel and two points in the maturing enamel. Mann Whitney test was used to compare the body temperature before and after the injections as well as to compare the mineral quantity between the experimental and control groups. The mean body temperature of the experimental and control groups before the first injection were 35.9 ± 0.1 ºC and 35.9 ± 0.2 ºC, respectively. The average body temperature of the experimental and control groups after the first injection were 37.4 ± 0.3ºC and 36.3 ± 0.2ºC, respectively. In the experimental group, Fe was concentrated in the incisal third, while in the control group it was distributed along the incisal and medium thirds of the enamel surface. No significant difference was observed in the quantities of Ca, Fe, Zn, and Ca:P ratio between groups. The experimental group showed a higher concentration of P in the maturing enamel ($p = 0.02$) and a lower concentration of Sr in the mature ($p = 0.01$) and maturing enamel ($p = 0.006$). Therefore, this study suggests that fever can disturb ion movement through tight junctions of ameloblasts, modifying the enamel mineralization of lower incisors of C57Bl/ 6 strain mice.

Key words: Dental Enamel. Dental Enamel Hypoplasia. Fever. Models, Animal. Molar Incisor Hypomineralization.

Introduction

Molar-Incisor Hypomineralization (MIH) is a defect of enamel mineralization, which affects at least one first permanent molar with or without incisors involved(1). The hypomineralized enamel is seen clinically as demarcated opacities(2). Structurally the enamel has its mineral density reduced from amelo-dentinal junction to subsurface(3-6). The surface is more mineralized, though it is not so mineralized as the normal enamel(3, 4, 7). The more the enamel is hypomineralized, the less quantity of calcium (Ca) and phosphorous (P) and the more concentration of carbon (C), indicating remains of organic materials in that enamel(8). Fagrell et al (2010)(9) did not find difference of Ca values between normal and hypomineralized enamel,

but just like Bozal et al (2015)(6), observed that C content is greater in the hypomineralized enamel. A study have found lower rate of Ca:P ratio in the defective enamel(8), while other study did not observe difference between normal and hypomineralized enamel(9, 10). Molar-Incisor Hypomineralization aetiology is still unknown, but systematic reviews have shown an association of postnatal factors, such as respiratory diseases, pneumonia, childhood illnesses or fever, with MIH(11, 12). Several congenital defects such as clefts and malformed teeth are caused by fever(13, 14). Tung et al. (2006)(15) induced fever in rats with turpentine and found a radiolucent line at the outermost layer of enamel, suggesting defective mineralization. Ryynänen et al. (2014)(16) cultured mouse molar tooth at 39 °C up to the early maturation stage and also observed disorder in the enamel formation, but the mineralization phase was not evaluated. Observational studies are subject to recall bias, which is inherent in the questionnaires used in retrospective studies. Fever and childhood illnesses are associated with MIH, because they usually occur together in children(11). Indeed, in human beings it is not possible to be sure if MIH aetiology is a combination of several factors, or only high fever or fever combined with childhood illnesses(17). Animal studies can contribute to clarify the MIH aetiology, once it is feasible to evaluate possible causal factors separately. Mice are a good choice of small experimental animals because they are easier to handle, to accommodate due to the minimum space required, and less costly because of low amount of food and drug necessary for tests(18), as examples. In dentistry, mouse incisors, which have the same development stages as human teeth, and are continuously growing have been used as models(19). Thus, this study aimed to evaluate the fever effect on enamel mineralization of lower incisors of C57Bl/ 6 strain mice.

Materials and methods

This study was approved by the Animal Use Ethics Committee of the Arthur Sá Earp Neto University Centre (UNIFASE). All experiments were carried out in accordance with the Brazilian Regulatory Council for the use of animal in research (CONCEA – 11.794 of 08 October 2008).

Animals

Twelve mice of the C57Bl/ 6 strain, raised and kept in the vivarium of UNIFASE, were used in this study. The animals were female, adult, isogenic, aged 3

months and weighed on average 16.66 ± 1.2 g at the beginning of the experiment. They were housed at an average temperature of 21.2 ± 0.7 ºC with free access to lab chow and tap water during the whole period of the experiment. Figure 1 shows a diagram with the experimental procedures. The animals were divided into two groups: 6 mice in the experimental group and 6 in the control group.

Fever induction

The fever induction protocol was defined in a pilot study. A dose of 12 ml/ Kg of 15% w/v of brewer yeast was injected into each experimental mouse. The suspension was prepared with 15 g of commercially available dried brewer yeast (*Saccharomyces cerevisiae*) and 100 ml of saline (0.9 % w/v of Sodium chloride - NaCl). Each experimental animal received 1.8 g of brewer yeast per kilo. Each control mouse received 12 ml/ Kg of saline in the same way. All animals received subcutaneous injections twice a day for three consecutive days. The injections were applied at 7 am and 5 pm after the temperature measurement. To apply the injections, each animal was placed into a test tube with a longitudinal cut. The cut was pointing upwards and a mouse was placed into the tube trough the tail. So, the skin was stretched through the cut and the injection applied as shown the Figure 2.

Figure 1 – Diagram of the experiment

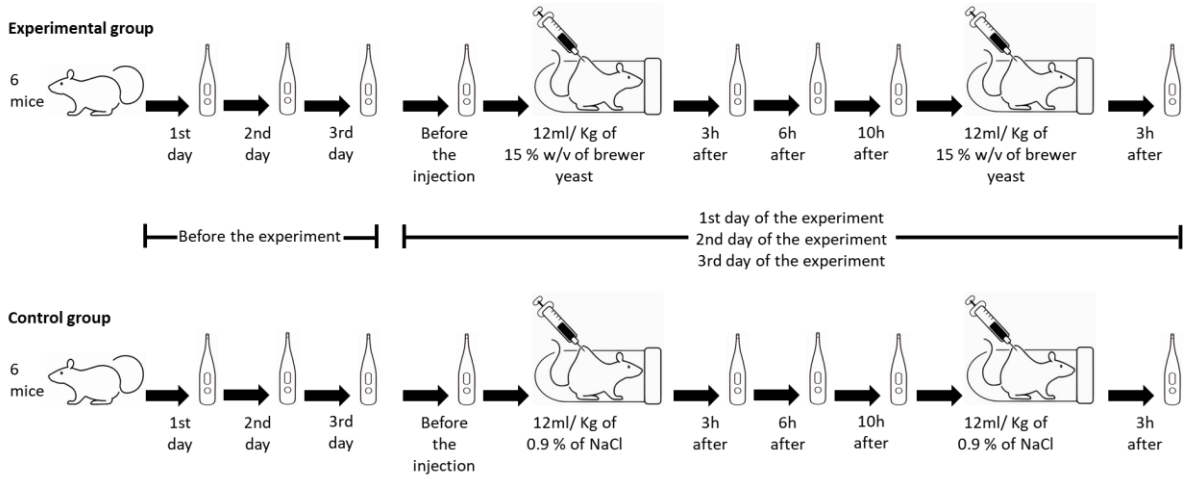
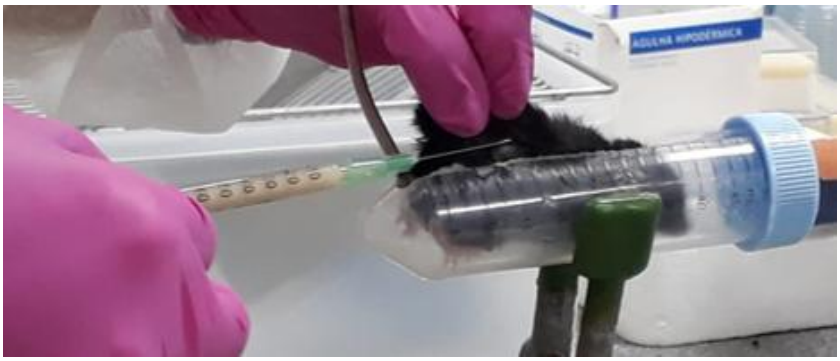


Figure 2 – Application of the subcutaneous injection



Body temperature measurements

The body temperature of the animals was measured using a BAT-7001H digital thermometer with the RET-3 rectal probe appropriate for mice (Physitemp®, Clifton, NJ, USA). The probe was introduced into the colon, 2 cm away from the anus. Before the experiment, the body temperature was measured twice a day for three consecutive days. Then, the body temperature was recorded immediately before the injections and 3, 6 and 10 hours after the injections for three consecutive days. Figure 2 shows a diagram with the experimental procedures.

Sample collection

Considering a tooth eruption rate of 0.18 ± 0.05 mm per day defined in a previous study (unpublished study), the animals were sacrificed through isoflurane inhalation 15 days after the first injection. A lower incisor of each animal was carefully dissected out of the bone and stored in distilled water.

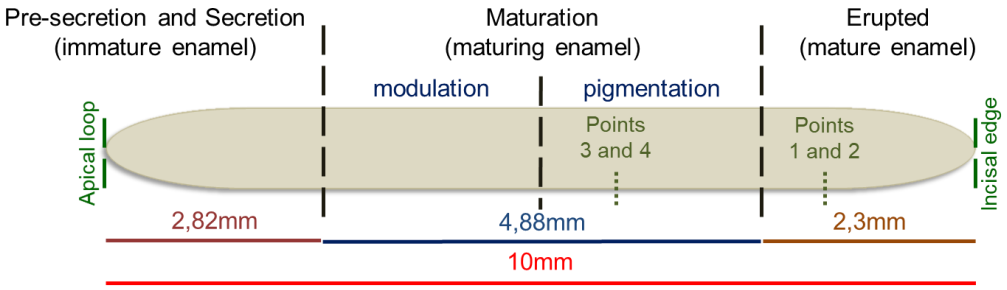
Enamel mineral content measurement

Elemental analysis of all teeth was done using Micro X-ray fluorescence (μXRF). The analysis was performed with the equipment M4 Tornado® (Bruker, Berlin, Germany), which is composed of an X-ray tube with rhodium anode, a polycapillary for the *spot* size of 25 μm, and a detector SDD (Silicon Drift Detector) with an energy resolution of 142 eV to Mn-kα. The mapping of the sample was done with a voltage of 30 kV, current of 600 mA, 12.5 μm of Al filter, step size of 25 μm, during a measurement time of 210 ms per pixel under a vacuum condition of 20

mbar. Spectrum quantification was performed using the in-built software based on a matrix of hydroxyapatite [Ca10 (PO4) 6 (OH) 2].

The mapping of the whole enamel surface was done to analyse the spatial distribution of Ca, P, Fe and Sr. The quantification of minerals was done on the enamel surface in four different points. Two points (1 and 2) were localized 1.3 mm distant from the incisal edge and the other two points (3 and 4), 3.8 mm distant from the incisal edge as shown Figure 3. The determination of the measurement points was based on a diagram of a developing lower incisor of a rat (200 g weight)(20) where the developmental stages of enamel is represented. Considering that a lower incisor of a mouse (20 g weight) has on average 10 mm of length, points 1 and 2 were positioned in the erupted and completely formed enamel while points 3 and 4 were positioned in the unerupted and maturing enamel (Figure 3).

Figure 3 – Diagram representing the position of the four points where the mineral analysis was done in the enamel (based on the diagram of a developing lower incisor of a rat(20))



Statistical analysis

Data were analyzed with Statistical Package for Social Sciences program version 23.0 (SPSS Inc., IBM, Chicago, Ill., USA). The descriptive analysis included the average calculation and standard deviation of environmental temperature where the animals were kept, animal weight, body temperature for experimental and control groups, enamel mineral quantity at points 1 and 2 as well as at points 3 and 4 of all teeth. All evaluated variables presented asymmetrical distribution. The Mann Whitney test was used to compare the body temperature before and after the injections, and the mineral quantity at points 1 and 2 as well as at points 3 and 4 between the experimental and control groups. Statistical significance level was set at 5%.

Results

Body temperature

Figure 4 shows the mean body temperature of the experimental and control groups evaluated during three days before the experiment. The last measurement was done immediately before the first injection. The mean body temperature of the experimental and control groups before the first injection were 35.9 ± 0.1 ºC and 35.9 ± 0.2 ºC, respectively. The body temperature between the groups were not different ($p = 0.6$).

Figure 5 shows the mean body temperature of the experimental and control groups immediately before the first injection and 3, 6 and 10 hours after the first, second and third injections during three consecutive days. The body temperature of the experimental and control groups after the first injection were 37.4 ± 0.3 ºC and 36.3 ± 0.2 ºC, respectively. The mean body temperature of the experimental group was 1.1 ºC higher than the control group, during the fever period. The mean temperature of the experimental group during the fever period was significantly higher than the control group ($p = 0.004$). The body temperature of the experimental group reached the maximum after nine hours of the first injection on the first day and after three hours after the injections on the second and third days. The body temperature ranged from a minimum of 37.2 ºC to a maximum of 38.2 ºC and the average of the maximum temperature of each day was 37.8 ± 0.3 ºC. On average, the body temperature of the experimental group was 1.5 ºC higher than the temperature before the experiment. In the control group, a difference of 0.4 ºC in the mean body temperature was observed before and after the experiment.

Figure 4 – Mean body temperature of the experimental and control groups before the experiment

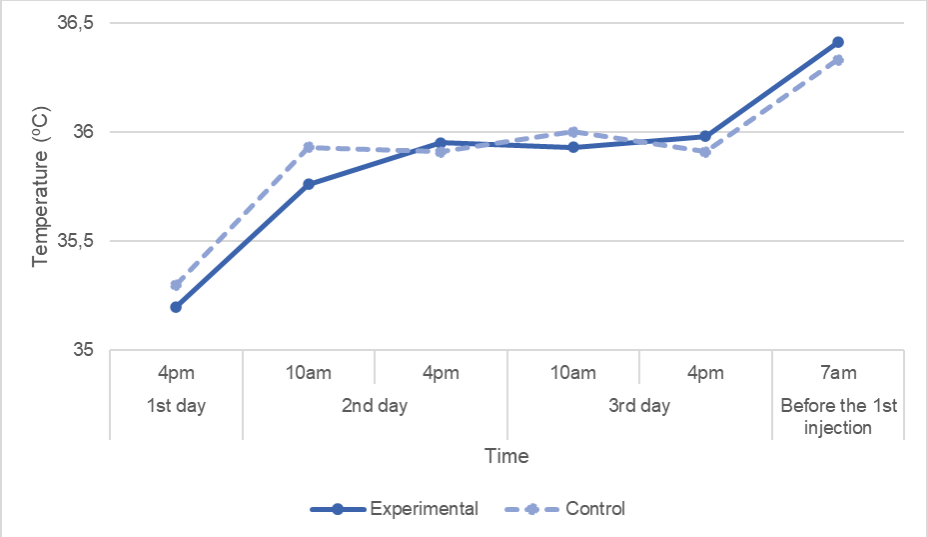
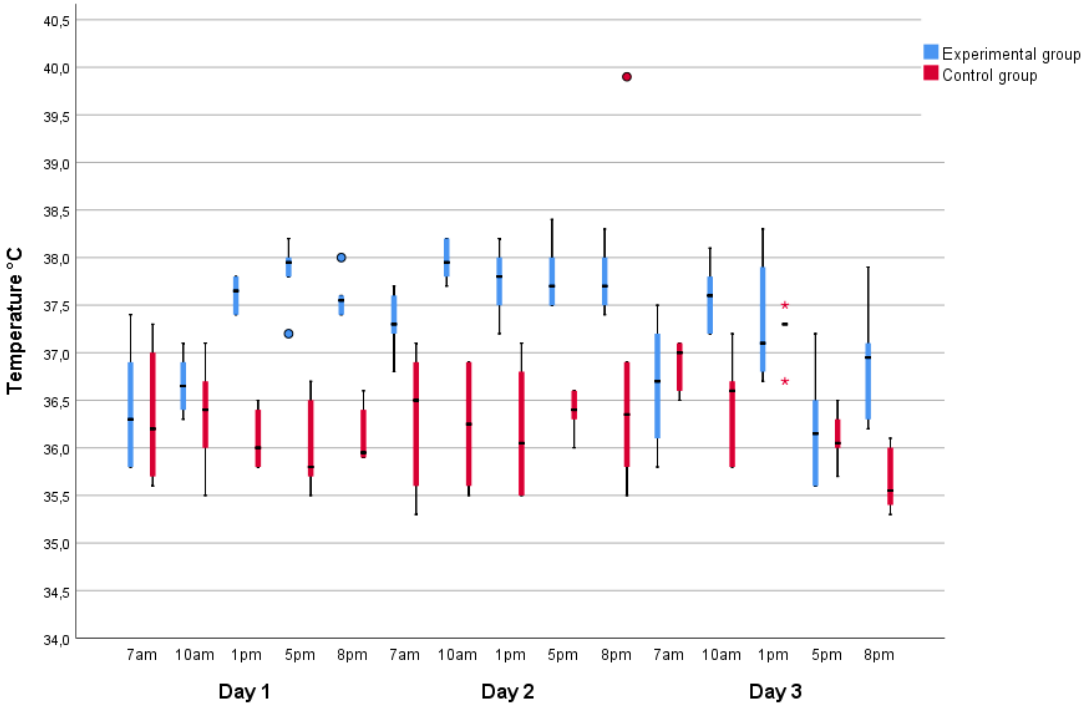


Figure 5 – Mean body temperature of the experimental and control groups immediately before the first injection and 3, 6 and 10 hours after the first, second and third injections during three consecutive days



Enamel mineral content

The elements Calcium (Ca), Phosphorous (P), Strontium (Sr), Iron (Fe), and Zinc (Zn) were identified in μXRF spectrums obtained from the buccal enamel surface of a lower incisor, as shown in Figure 6. Figures 7 and 8 represent the distribution of the identified elements on the enamel surface of the incisors of the experimental and control groups, respectively. Ca, P and Sr were similarly distributed in the experimental and control groups. Ca and P were available in a large amount and distributed over the whole surface in both groups. Although Sr was present in a lower quantity, it was also distributed over the whole surface in both groups. In the experimental group, Fe was concentrated in the incisal third, while in the control group it was distributed along the incisal and medium thirds of the enamel surface.

Table 1 presents the mean quantity and respective medians and standard deviations of Ca, P, Sr, Fe, and Zn in the enamel of the experimental and control groups. Figures are presented for the measurement points 1 and 2 (mature enamel) as well as points 3 and 4 (maturing enamel). Additionally, the Ca:P ratio is also presented. Table 2 shows the result of the comparison between experimental and control groups regarding the mineral quantity at the measurement points 1 and 2 (mature enamel) as well as points 3 and 4 (maturing enamel). No significant difference was observed in the quantities of Ca, Fe, Zn, and Ca:P ratio between groups ($p > 0.05$). The experimental group showed a higher concentration of P in the maturing enamel ($p = 0.02$) and a lower concentration of Sr in the mature ($p = 0.01$) and maturing enamel ($p = 0.006$).

Figure 6 - Micro X-ray fluorescence spectrum obtained from the enamel surface of a mouse lower incisor

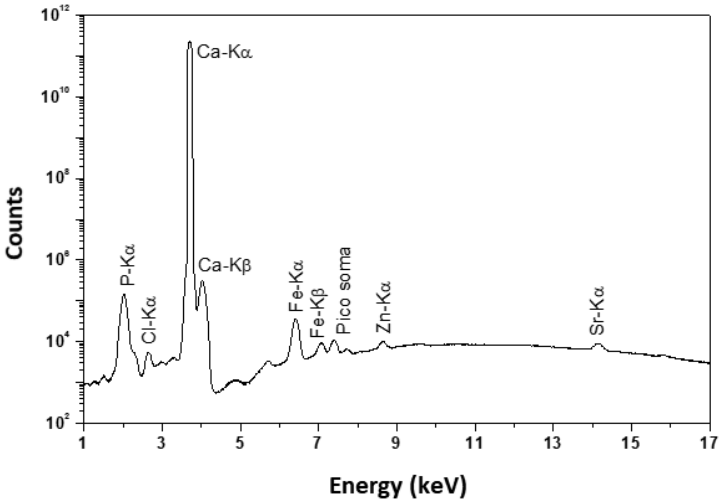
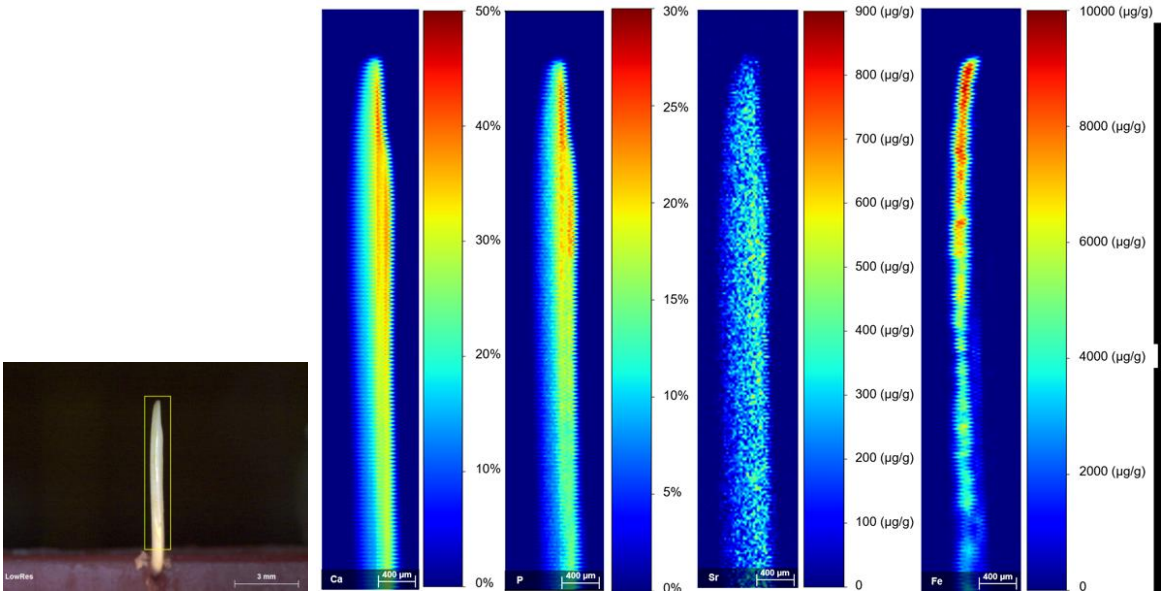


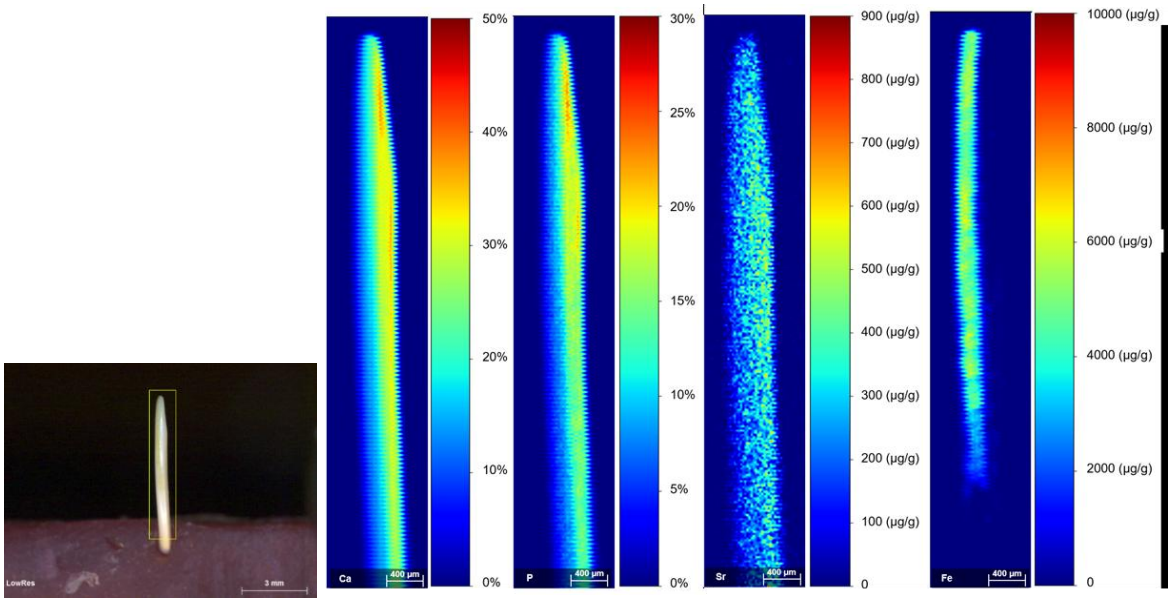
Figure 7 – Front view of the mineral distribution on the enamel surface of a mouse incisor of the experimental group



(a) (b)

Legend: (a) demarcated area for the whole mapping of the enamel surface; (b) Distribution of elements Ca, P, Sr and Fe over the enamel surface.

Figure 8 - Front view of the mineral distribution on the enamel surface of a mouse incisor of the control group



(a) (b)

Legend: (a) demarcated area for the whole mapping of the enamel surface; (b) Distribution of elements Ca, P, Sr and Fe over the enamel surface.

Table 1 – Mean quantity and respective medians and standard deviations (SD) of Ca, P, Sr, Fe, and Zn in the enamel of the experimental and control groups

Minerals	Experimental group						Control group					
	Region of points 1 and 2 Mature enamel			Region of points 3 and 4 Maturing enamel			Region of points 1 and 2 Mature enamel			Region of points 3 and 4 Maturing enamel		
	Mean	Median	SD	Mean	Median	SD	Mean	Median	SD	Mean	Median	SD
Calcium Ca (%)	30.72	29.5	3.24	24.75	25.07	1.27	28.64	28.7	0.76	23.55	23.83	1.08
Phosphorous P (%)	20.98	19.88	2.88	16.07	16.1	1.0	19.33	19.45	0.59	14.7	14.52	0.52
Strontium Sr (ppm*)	277.04	275.97	22.89	276.04	276.84	29.9	341.62	340.09	29.8	339	329.58	20.82
Iron Fe (ppm*)	4874.65	4018.07	1701.62	3393.08	3436.32	666.18	5713.5	5551.02	877.8	3158.17	3183.69	1027.1
Zinc Zn (ppm*)	72.6	70.21	10.95	76.47	75.48	20.5	71.81	71.62	11.13	79.22	79.82	25.27
Ratio Ca:P (%)	1.49	1.5	0.04	1.56	1.55	0.05	1.5	1.5	0.02	1.6	1.61	0.04

*Values in ppm can be transformed in percentage dividing by 10000.

Table 2 – Comparison between experimental and control groups regarding the mineral quantity at the measurement points 1 and 2 (mature enamel) as well as points 3 and 4 (maturing enamel)

Minerals	Region of points 1 and 2 Mature enamel P value*	Region of points 3 and 4 Maturing enamel P value*
Calcium - Ca (%)	0.2	0.1
Phosphorous - P (%)	0.36	0.02**
Strontium - Sr (ppm**)	0.01**	0.006**
Iron - Fe (ppm**)	0.27	0.86
Zinc - Zn (ppm**)	1.0	0.72
Ratio Ca:P (%)	0.85	0.08

*****Mann Whitney test.

**Statistically significant.

Discussion

The present experiment was based on a murine model to evaluate the effect of fever on the mineral composition of the enamel. Previously to the present study, the fever induction protocol was tested in a pilot study to certify that the body temperature of the experimental group would achieve a level high enough to detect a significant different between the experimental and control groups. Once the fever induction protocol was defined, the body temperature of the experimental animals was monitored during three days before the experiment period in order to inure them to manipulation, because hyperthermia can occur also due to stress induction masking fever induction with pyrogen (21).

In the experimental group, an increase of 1.5 ºC in the body temperature was observed. The normal body temperature of mice is 36.5 ºC, and ~1 °C higher is considered fever (22, 23). In the control group, the body temperature showed an increase of 0.4 ºC probably due to stress from manipulation. The slight increase in the body temperature of the control group was not enough to be considered fever and it was significantly lower than the body temperature increase of the experimental group. In the pilot study, it was observed that the body temperature decreases

drastically 12 hours after the injection. Hence, in order to keep fever constant, brewer yeast injection was applied twice a day. Fever was induced for three days, a period similar to the one used by Tung et al. (2006)(15). A study that induced fever in rats using brewer yeast observed that the rectal temperature increased slightly more than 1 ºC (18). Other three studies that also induced fever in rats with yeast observed that the rectal temperature increased 1.6 ºC or ranged from around 38.5 ºC to 40 °C (24-26). Tung et al. (2006)(15) induced fever in rats with turpentine and observed a mean rectal temperature of 38.42 ± 0.79 °C, which was 1.51 ºC higher than the body temperature of control group.

A crucial aspect of studying the effect of any insult to the enamel mineralization is the definition of the correct area of the enamel where the analysis must be done. It has to be done in the portion of the enamel where mineralization occurred during the insult. The tooth eruption rate of the experimental animals was defined in a previous study (unpublished study). So far, no studies have been found in the literature reporting the tooth eruption rate of mice. The upper and lower incisors of C57Bl/ 6 strain mice have on average a length of 7 mm and 9 to 10 mm, respectively(27). Considering that the erupted part has a length of approximately 2.3 mm and that the eruption rate is 0.18 mm per day (unpublished study), the teeth were collected after 15 days in order to guarantee that the erupted part had been completely replaced. An evaluation of lower incisors of the C57Bl/ 6 strain mice reported that early maturation stage, late maturation stage, and erupted enamel are 4, 6 and 9 mm distant from the apical loop, respectively(28). Other study showed that pre-secretion and secretion stage, maturation stage, and erupted enamel occupy the position of 0-3.2, 3.2-7.8, and 7.8-10 mm on the tooth from the apical loop(27). These two studies were used as reference for the definition of the localization of the measurement points where mineral quantity was evaluated in the present study. Hence, two points were localized in the erupted and mature enamel and two points were localized in the unerupted and maturing enamel.

The junctions between ameloblasts allow movement of substances. During normal amelogenesis, they are more permeable during the secretory phase and tighter in the maturation phase(29). Studies have reported that inflammatory mediators, similar to those ones released in the febrile response, can affect the function of tight junctions in salivary gland, and renal epithelial cells(30, 31). This suggests that several local and systemic conditions can allow the passive movement

of great amount of ions and blood proteins between ameloblasts during the maturation stage(32). Besides that, cellular stress may lead to apoptosis of ameloblasts and consequently unregulated ingress of ions and blood proteins into enamel matrix(32).

The quantity of Ca, P, Fe, and Zn in the mature enamel, represented by the measurement points 1 and 2, was not significantly different between experimental and control groups as well as the Ca:P ratio. Similar results were reported by Mihalas et al. (2015)(33) and Souza et al. (2013)(34) regarding the content of Ca and P after chronic treatment of the experimental group with amoxicillin. In the maturing enamel, the higher percentage of P in the experimental group was a conflicting result. However, it has been argued that P detected by mineral analysis may be derived not only from the apatite but also from organic matter in the enamel(9). The lower concentration of Sr in both mature and maturing measurement points in the experimental group suggests a less mineralized enamel. Strontium and Ca have similar chemical behaviour being substitutes for each other in mineralized tissues.(35) So far, no studies based on murine model have been found reporting the concentration of Sr in enamel.

The amount of mineral in general increases from apical loop to incisal edge, and about one-half of the total mineral is in the most mature enamel(27). The spatial distribution of the minerals on the enamel surface showed that Ca, P and Sr were seen over the whole surface in both groups, although more concentrated in the incisal third. Regarding Fe, the higher concentration in the incisal third was more pronounced in the experimental group. The maturing enamel had substantially less Fe than the mature enamel, suggesting that the deposition of Fe was disturbed in the early maturing phase. In a study that tested the effect of amoxicillin in the same mice strain, a decrease in the level of Fe was also observed(33). The function of Fe in enamel is contradictory. A study has concluded that the integrity of enamel does not depend on the presence of Fe(36). On the other hand, iron has been reported as responsible to give resistance to abrasion and cracking(37) and its deficiency has been associated with enamel defects(38). It has been suggested that Fe and Ca are substitutes for each other in hydroxyapatite(39). Iron is deposited into enamel after Ca and P reach the maximum level, and its incorporation may represent the final mineralization(40).

Other studies showed that the elevation of temperature could impair or delay the development of enamel. Tung et al. (2006)(15) have found a radiolucent line at the outermost layer of enamel in 8 out of 14 turpentine-treated rats to induce fever, suggesting defective mineralization. The lower radio density observed along the radiolucent line was interpreted as a consequence of a lower mineral content. However, it is not possible to affirm how the mineral content was affected, since no elemental analysis was done. An irregular orientation of the prisms was observed histologically. The effect of high temperature was also seen by Ryynänen et al. (2014)(16) in cultured mouse molar tooth at 39 °C for 5 days. Probably due to a delayed differentiation of ameloblasts, the enamel formation was postponed. Moreover, the expression of some genes essential for normal enamel development was modified. However, it was not possible to investigate if high temperature interfered in the enamel mineralization because the teeth were cultured up to the early maturation stage.

Comparing normal and hypomineralized enamel in extracted human teeth, Fagrell et al. (2010)(9) did not find difference in the median values for Ca or in the Ca:P ratio. In contrast, Jälevick et al. (2001)(8) saw marked differences between normal and hypomineralized enamel in the composition of Ca, P, and Ca:P ratio and similar content of Sr. Bozal et al. (2015)(6) also saw a similar concentration of Ca and P between control and white opacities, but both were significantly decreased in the yellow-brownish opacities, which are known to be more fragile and prone to fracture(41). The higher concentration of C in the hypomineralized enamel seems to be a consensus between authors (6, 9).

Fever is commonly reported as a potential aetiological factor for enamel hypomineralization. Two systematic reviews on MIH aetiology showed association between fever, respiratory problems, pneumonia, and childhood illnesses and MIH(11, 12). However, authors emphasize the low quality of evidence due to limitations of the observational studies, i.e., a somehow vague description of fever and the fact that retrospective studies are subjected to recall bias. As fever and childhood illnesses occur together in children(11), it is not possible to establish if MIH results from the fever, from the illness, or from a combination of both. In fact, a combination of several factors has been sustained in the aetiology of MIH.

Studies using murine model, designed to isolate fever from other possible aetiological factors supposedly related to enamel hypomineralization, are still scarce.

In the present study, fever affected the spatial distribution of Fe and significantly reduced the concentration of Sr. Therefore, this study suggests that fever can disturb ion movement through tight junctions, modifying the enamel mineralization of lower incisors of C57Bl/ 6 strain mice. However, mechanisms at cellular level need to be better known. Further studies combining a comprehensive mineral investigation with the analysis of C content, and histological aspects are desirable.

Acknowledgements

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CONCLUSÃO

a) O protocolo sugerido de indução de febre com Levedura de cerveja (*Saccharomyces cerevisiae*) em camundongos da linhagem C57Bl/ 6 apresentou-se como um método efetivo e fácil de reproduzir.
b) A taxa média de erupção dos incisivos inferiores de camundongos da linhagem C57Bl/ 6 é 0,18 ± 0,05 mm por dia.
c) O estudo sugere que a febre pode alterar o movimento de íons através das junções entre os ameloblastos, modificando a mineralização do esmalte de incisivos inferiores de camundongos da linhagem C57Bl/ 6. No entanto, mecanismos a nível celular precisam ser melhor compreendidos. Novos estudos combinando uma investigação mineral completa com análise de Carbono e aspectos histológicos são necessários.

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ANEXO A – Comitê de ética no uso de animais

Declaração

Declaramos para os devidos fins que o projeto de pesquisa "Efeito da febre na mineralização do esmalte dentário", orientado pela Professora Dra. Vera Mendes Sovieiro, foi apresentado a este comitê e aprovado pelo mesmo em 30 de maio de 2017.

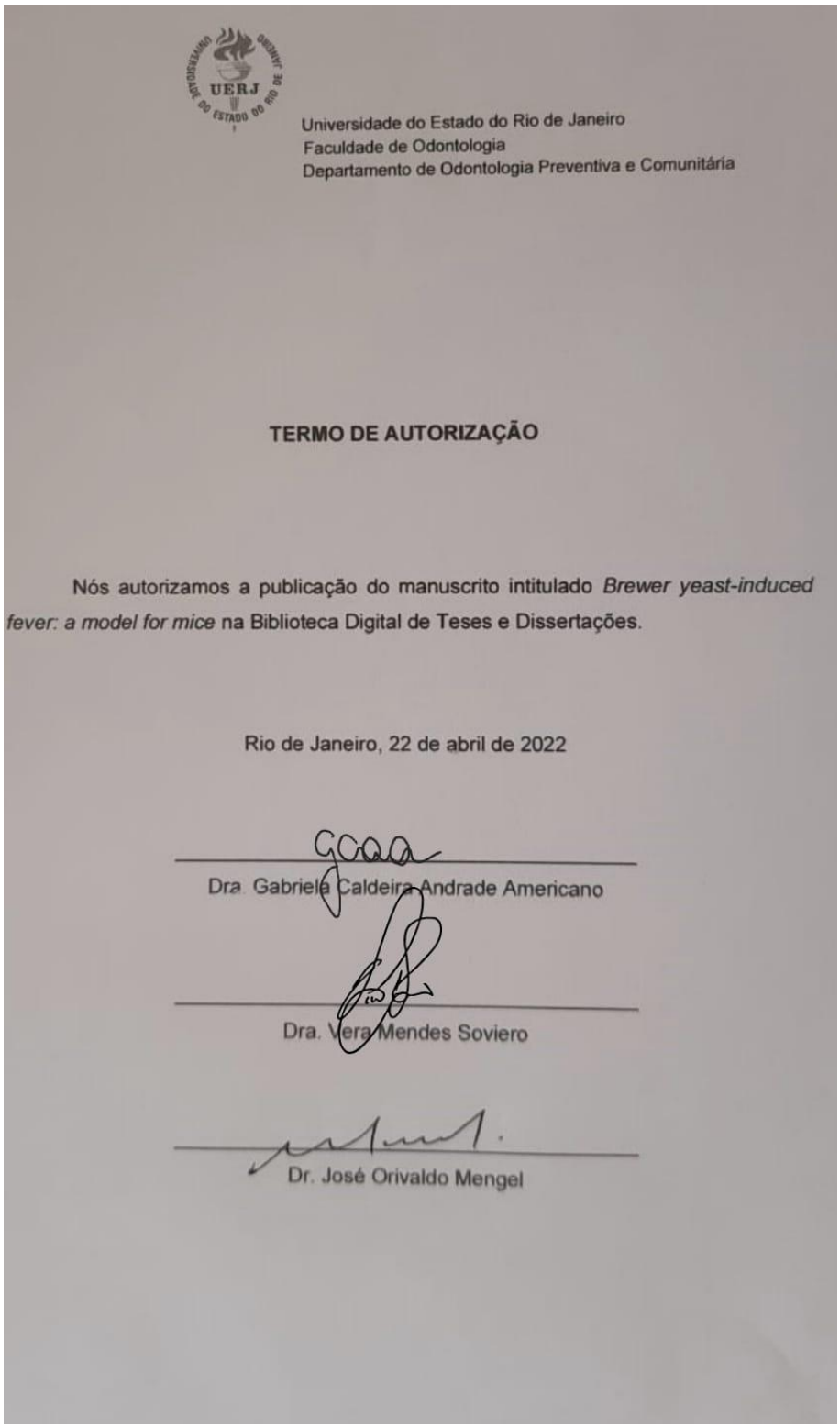
Petrópolis, 08 de fevereiro de 2018.

ceua

fmp

Prof.ª Ana Maria Rodrigues dos Santos
Coordenadora Interina de Pesquisa e Pós-Graduação

ANEXO B – Termo de autorização para publicação do Manuscrito Brewer yeast-induced fever: a model for mice



Universidade do Estado do Rio de Janeiro
Faculdade de Odontologia
Departamento de Odontologia Preventiva e Comunitária

TERMO DE AUTORIZAÇÃO

Nós autorizamos a publicação do manuscrito intitulado *Brewer yeast-induced fever: a model for mice* na Biblioteca Digital de Teses e Dissertações.

Rio de Janeiro, 22 de abril de 2022

Dra. Gabriela Caldeira Andrade Americano

Dra. Vera Mendes Soviero

Dr. José Orivaldo Mengel

ANEXO C – Termo de autorização para publicação do Manuscrito Eruption rate of mouse lower incisors

Universidade do Estado do Rio de Janeiro
Faculdade de Odontologia
Departamento de Odontologia Preventiva e Comunitária

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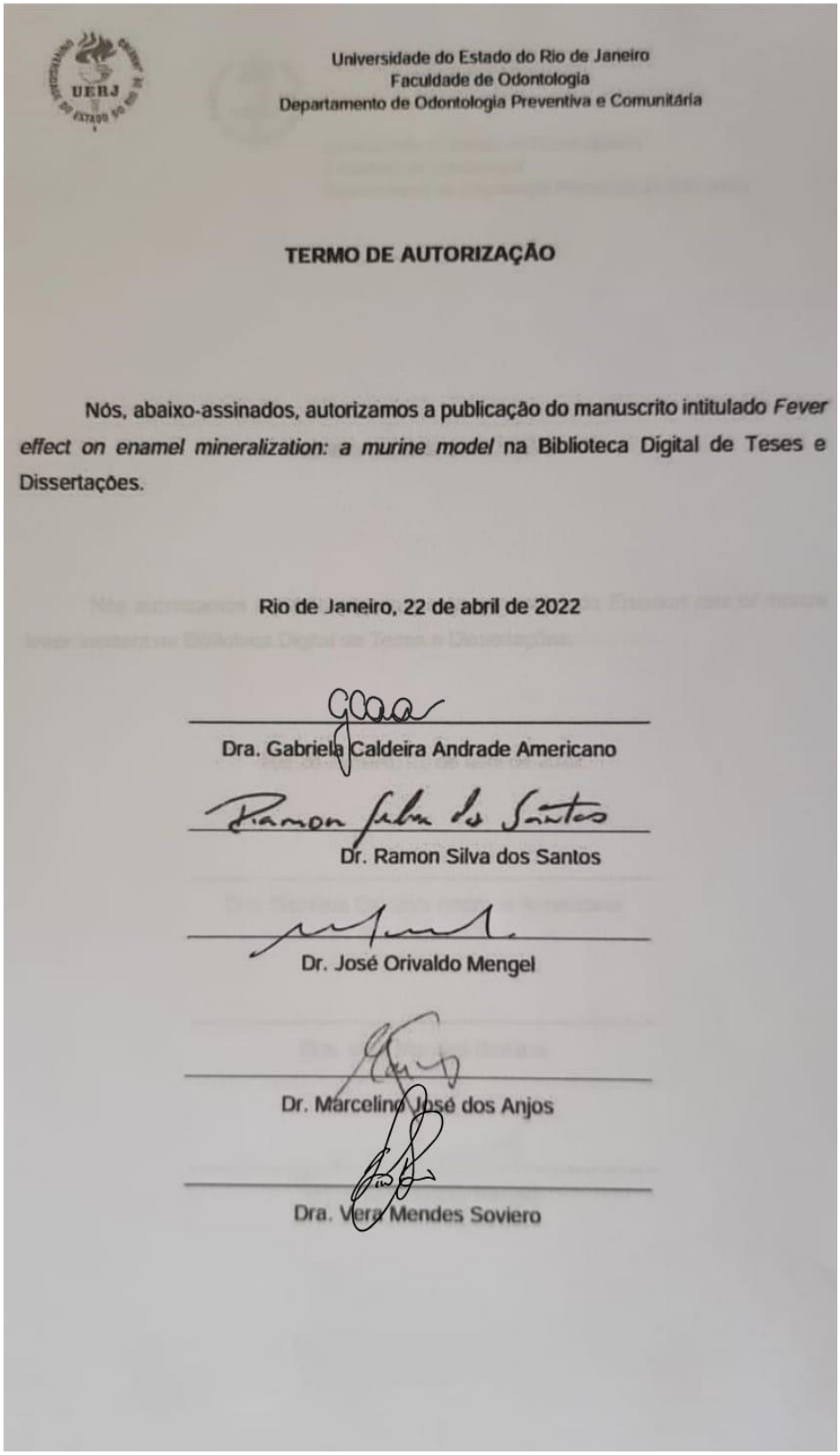
Dra. Gabriela Caldeira Andrade Americano

Dra. Vera Mendes Soviero

Dr. José Orivaldo Mengel

ANEXO D – Termo de autorização para publicação do Manuscrito Fever effect on enamel mineralization: a murine model



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Departamento de Odontologia Preventiva e Comunitária

TERMO DE AUTORIZAÇÃO

Nós, abaixo-assinados, autorizamos a publicação do manuscrito intitulado *Fever effect on enamel mineralization: a murine model* na Biblioteca Digital de Teses e Dissertações.

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Dr. Ramon Silva dos Santos

Dr. José Orivaldo Mengel

Dr. Marcelino José dos Anjos

Dra. Vera Mendes Soviero