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FACULDADE DE ODONTOLOGIA DE PIRACICABA

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CIRURGIÃO-DENTISTA

"INFLUÊNCIA DE FATORES AMBIENTAIS E GENÉTICOS RELACIONADOS COM A AGENESIA DENTAL E MICRODONTIA"

Tese apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas para obtenção do Título de Doutor em Biologia Buco Dental, Área de concentração em Histologia e Embriologia.

Orientador: Prof. Dr. Sérgio Roberto Peres Line

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Vejo em teus olhos, mãe, toda a doçura
Que espelha tua vida terna e calma,
No teu sorriso há a simples candura
Que existe no mais fundo de tua alma.

Tu és um anjo bom em nosso lar,

Nosso refúgio e toda a esperança
E quando tu nos vem aconselhar,
Surge sobre nós a paz e a bonança.

São felizes os dias a teu lado,
Tu és um presente idolatrado,
Representas, enfim, fé e guarida.

E digo-te, feliz com teu carinho:
- **Se sou de tua vida um pedacinho,**
Tu és, por certo, toda a minha vida.

Adaptado de Maria Amélia Porto Alegre Amaral, 1968.

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Eu costumava ter medo do amor
Eu costumava ficar sozinho
Nunca pensei que encontraria
Alguém para me apoiar

Mas como uma benção
Você entrou na minha vida
Quando minha fé se foi
De algum modo você me encontrou

Você tocou minha mão quando eu me perdi
Abraçou-me com seu amor
Em seus braços é onde pertenço

Você é meu abrigo
Minhas lágrimas e sorrisos

A luz do Sol e o vento
Você é meu anjo
Você é a alegria que o amor pode trazer

O amor crescerá e nos elevará
O amor apenas começou
E eu acredito
Nada pode nos derrubar

Eu posso ver nos seus olhos
A flama que nunca desvanece
Porque meu coração é onde você pertence

Tradução de **You are**
Laura Pausini, 2003.

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Faz de mim um instrumento da Tua Paz;
Onde há ódio, faz que eu leve o Amor;
Onde há ofensa, que eu leve o Perdão;
Onde há discórdia, que eu leve a União;
Onde há dúvidas, que eu leve a Fé;
Onde há erros, faz que eu leve a Verdade;
Onde há desespero, que eu leve a Esperança;
Onde há tristeza, que eu leve a Alegria;
Onde há trevas, que eu leve a Luz;
Ó Mestre! Faz que eu procure menos
Ser consolado, do que consolar;
Ser compreendido, do que compreender;
Ser amado, do que amar.....
Porquanto;
É dando que se recebe;
É perdoando que se é perdoado;
É morrendo que se vive para a vida eterna.

Muitas vezes as pessoas são egocêntricas,
ilógicas e insensatas.
Perdoe-as assim mesmo.

Se você é gentil, as pessoas podem acusá-lo
de dissimulado, interesseiro.
Seja gentil assim mesmo.

Se você é um vencedor, terá alguns falsos
amigos e alguns inimigos verdadeiros.
Vença assim mesmo.

Se você é honesto e franco, as pessoas
podem enganá-lo.
Seja honesto e franco assim mesmo.

O que você levou anos para construir, alguém
pode destruir de uma hora para outra.
Construa assim mesmo.

Se você tem paz e é feliz, as pessoas podem
sentir inveja. Seja feliz assim mesmo.

O bem que você faz hoje pode ser esquecido
amanhã. Faça o bem assim mesmo.

Dê ao mundo o melhor de você, mas isso
pode nunca ser o bastante. Dê o melhor de
você assim mesmo.

Veja você que, no final das contas, é entre
você e Deus. Nunca foi entre você e as outras
pessoas.
Madre Teresa de Calcutá

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Um amigo é assim

É fácil afastar-se
Assim como você também ele tem os seus problemas
Mas quando necessitar ele estará aqui
Um amigo é assim

Não perguntará nem como, nem o porquê...
Te escutará e lutará por você
E depois tranqüilo lhe sorrirá
Um amigo é assim

E recorda-se que até quando você viver
Se um amigo estiver com você, não se perderá
Em estradas erradas, percursos de quem
não tem na vida um amigo assim

Não há jamais necessidade de palavras
Só com um olhar entenderá
Que depois de um não ele lhe dirá sim
Um amigo é assim

E se lembre que até quando você desejar
Para sempre ao seu lado o encontrará
Perto de você, nunca cansado porque:
Um amigo é a coisa mais bela que há

Mas se lembre que até quando você viver
Se um amigo estiver com você, jamais o trairá
Só assim descobrirá que
Um amigo é a coisa mais bela que há

E lembre-se que até quando você viver
Um amigo é a coisa mais verdadeira que há
É a companhia da maior viagem que faz
Um amigo é qualquer coisa que não morre jamais

Tradução de Un amico é cosi
Cheope, Laura Pausini 1995.

RESUMO

O termo hipodontia pode ser definido como ausência congênita de um ou mais dentes e microdontia como dente com alterações dimensionais. Os dentes mais afetados são os terceiros molares, segundos pré-molares e incisivos laterais. A odontogênese pode ser afetada diretamente por fatores genéticos e ambienatis podendo ser destacado os genes PAX9 e MSX1, e a desnutrição e álcool. O objetivo deste estudo foi avaliar a influência de regiões dos genes supracitados e da ingestão crônica de álcool durante a gravidez na odontogênese, através de análises de polimorfismos genéticos e morfologia de molares de ratos. No primeiro estudo foi analisado o DNA genômico de 130 indivíduos afetados com agenesia e 110 indivíduos controles. Após a obtenção e extração do DNA, a região G/C-915 do gene *PAX9* (NCBI ref SNP ID: rs 2073247) foi amplificadas por reação em cadeia da polimerase (PCR), os polimorfismos foram analisados com duas seqüências diferentes de primers. Os géis foram corados pelo nitrato de prata. Os dados foram analisados através das Simulações de Monte Carlos (programa Clump) e teste Qui-quadrado ao nível de significância de 5%. As análises mostraram que o polimorfismo da região promotora do PAX9 G/C-915 teve alta freqüência em indíviduos com hipodontia de terceiros molares. No segundo estudo foram estudadas região dos genes PAX9 e MSX1, embora, não houve relação da região estudada dos introns do MSX1 com agenesia de terceiros molares, na região do gene C-160T do gene *PAX*9 (www.ncbi.nlm.nih.gov/SNP ref 2073247) houve relação entre polimorfismo e agenesia de terceiros molares. Com relação a fatores ambientais, o objetivo deste trabalho foi avaliar os efeitos do álcool administrado durante a gravidez e na lactação em molares inferiores de ratos. Observou-se uma redução nas dimensões e microdureza Knoop desses elementos dentais.

Palavras chaves: agenesia, microdontia, PAX9, MSX1, microdureza, odontogênese, polimorfismo e álcool.

ABSTRACT

The term hypodontia can be defined as a congenital absence of one or more teeth and microdontia teeth with alterations dimensional. The most affected teeth are the third molars, seconds premolars and lateral incisors. Genetic and environmental factors such as PAX9 and MSX1 genes malnutrition and alcohol can directly affect the odontogenesis. The objective of this study was to analyze the influence of gene regions and chronic alcohol intake during pregnancy in odontogenesis, through analysis of genetic polymorphism and morphology of rats molars. In the first study was analyzed the genomic DNA of 130 affected individuals with agenesis and 110 controls. After obtaining and extraction of DNA, the region. After DNA extraction, the region G/C-915 *PAX9* gene (NCBI ref SNP ID: rs 2073247) was amplyfied by polymerase chain reaction (PCR). The polymorphic sites were analyzed by two different primers sequences. The gel bands were stained by silver nitrate. Data were analyzed by Monte Carlo simulations (Clump software) and Chi-squared test (x^2)with the significance level 5%. The analysis showed that the PAX9 promoter region polymorphism of the G/C-915 had high frequency in individuals with third molars hypodontia. In the second study were studied region of genes PAX9 and, though, there was no relationship in the region of MSX1 introns with third molars agenesis, in the region of C-160T the gene PAX9 (www.ncbi.nlm.nih.gov / SNP ref 2073247) was relationship between polymorphism and third molars agenesis. About to environmental factors, the objective of this thesis was assessing the alcohol effects administered during pregnancy and lactation in lowers molars rats. There was a reduction in size and Knoop microhardness in these dental elements.
Key words: agenesis, microdontia, PAX9, MSX1, microhardness, odontogenesis, polymorphism and alcohol.

SUMÁRIO

1. INTRODUÇÃO

Agenesia dental e hipodontia são termos apropriados para significar a ausência congênita de um ou mais (até seis) dentes permanentes e/ou decíduos (Stewart & Poole, 1982). Oligodontia é definida como a ausência congênita de seis ou mais elementos, excluindo-se os terceiros molares (Schalk-Van Der Weide et al.,1994). Na maioria das vezes em que os indivíduos possuem oligodontia, os dentes apresentam-se com dimensões reduzidas podendo ter morfologia anômala, além de possuírem erupção dental tardia (Schalk-Van Der Weide et al., 1994). Já microdontia pode ser definida como diminuição das dimensões de um ou mais elementos dentais com ou sem alterações morfológicas (Tekin et al., 2007).

Altug-Atac & Erdem (2007) relataram em seu estudo que as anomalias mais encontradas em pacientes que recebem intervenção ortodôntica são a hipodontia e microdontia.

Os dentes mais comumente atingidos são os terceiros molares (20%), segundo molares (3,4%) e os incisivos laterais superiores (2,2%) (Simons et al.,1993; Peres & Line, 2005). Esta ausência resulta de um distúrbio durante os estágios iniciais da formação do dente (Proffit e Fields, 1995). Existem muitos fatores que podem estar associado a não formação do elemento dental, dentre eles podemos citar a genética, expressão de mudanças evolutivas na dentição, condições sistêmicas, como raquitismo, sífilis, severos distúrbios intra-uterinos, inflamações localizadas ou infecções, displasia congênita e fatores ambientais, como irradiações, uso abusivo de álcool e drogas (Moyers, 1991).

A odontogênese é o processo de formação do elemento dental e pode ser didaticamente dividida em três fases principais: Iniciação, Brotamento e Morfodiferenciação. Ela é mediada por inúmeros fatores difundíveis no tecido que determinam o local no arco em que o dente irá se formar (Neubuser et al., 1997; Peters & Bailing 1999, Jernvall & Thesleff 2000; Jernvall et al., 2000).

A agenesia dental pode ser causada por uma falha no processo de odontogênese, porém, a ausência de terceiros molares, segundos pré-molares

superiores, e incisivos laterais superiores, pode sersuperiores, e incisivos laterais superiores, pode ser entendida como uma variação da normalidade (quando aparece como uma forma isolada), devido a sua presença freqüente na população (Peres & Line, 2005).

A atual Biologia Molecular permite a identificação de cada um dos genes e suas proteínas em muitos processos no organismo, permitindo a identificação e o entendimento dos mecanismos genéticos responsáveis pela diversidade fenotípica existente. Essa pode ser influenciada por polimorfismos genéticos que atuam durante o desenvolvimento embrionário e crescimento.

Polimorfismos genéticos podem ser entendidos como variações genéticas que ocorrem naturalmente em uma população, numa freqüência superior a 1%. Estas variações podem ter um efeito direto sobre a expressão genética e afetar a função protéica (Peres & Line, 2005). Estudos recentes têm mostrado que polimorfismos em regiões codificadoras e regiões reguladoras da transcrição e até mesmo nos introns parecem ser freqüentes, e que estas variações podem ocasionar alterações nas características fenotípicas (Borges-Saito, 2006). Esses polimorfismos devem estar atuantes durante o desenvolvimento embrionário e crescimento.

Diversos genes têm sido implicados no desenvolvimento do dente e mutações genéticas associadas com agenesia dental foram identificadas. Mutação no gene homeobox PAX9 foi associada com a agenesia dental afetando principalmente segundos pré-molares e todos os molares em uma família (Stockton et al., 2000). Estudos recentes mostram a associação entre mutação no gene PAX9 com agenesia dental em humanos (Wise et al., 2002, Jumlongras et al., 2004, Peres & Line, 2005).

O gene PAX9 assim como o MSX1 são altamente expressos no mesênquima dos germes dentais em desenvolvimento, especialmente nos estágios de botão e capuz (Peters & Bailing, 1999). Durante a embriogênese vários brotos epiteliais se formam na região do diastema entre incisivos e molares, entretanto evidências mostram que o controle do número de dentes parece acontecer principalmente

durante a fase de broto. A atrofia destes brotos epiteliais parece estar associada com a expressão diminuída do gene PAX9 (Keranen et al 1999).

Thesleff & Aberg (1997) afirmaram que o desenvolvimento dentário dos ratos ocorre de maneira semelhante ao do ser humano quanto aos tipos celulares e mecanismos de formação, desta forma é um ótimo modelo experimental.

A odontogênese pode além de ter interferência de fatores genéticos, ter interferentes ambientais, como por exemplo, o consumo abusivo de álcool.

O consumo de álcool em todos os países do mundo torna-se um problema de saúde pública crescente a cada dia, pois há grande número de pessoas que o consomem compulsivamente, e requer tratamento específico para isto. Segundo a Organização das Nações Unidas cerca de 20% da população dos países em desenvolvimento são alcoólicos, sendo estimado que no Brasil, esta taxa seja de 10% (Reginato et al, 1986; Fortes & Cardo, 1991).

Willians-Hemby et al. (1996) salientaram a importância da utilização de modelos animais, auxiliando o estudo das alterações provocadas pelo consumo crônico de álcool em seres humanos, uma vez que este acarreta inúmeros problemas sociais, econômicos, físicos e psíquicos.

Estudos demonstraram que o uso crônico de álcool pode promover amplas variações neurofisiológicas (Allan& Harris, 1987; Sanna et al., 1993), morfológicas (Willians-Hemby et al., 1996), eletrofisiológicas (Siggins & Bloom 1980; Glenn & Parson., 1992) e metabólicas (Eckardt et al., 1988; Grünwald et al., 1993), que consequentemente causam alterações em outros órgãos.

Quando consumido em doses elevadas durante a gestação, consiste em um fator de risco grave para o feto, podendo promover prejuízos mentais, além de físicos, tais como malformações congênitas no feto, defeitos craniofaciais e orodentais, decorrentes das interferências no desenvolvimento embrionário normal (Reginato et al., 1986; Wekselman et al., 1995).

Segundo Chaudhuri (2000), Armant & Saunders (1996) e Sadler (2001) a ingestão de níveis elevados de álcool durante a gravidez causa sérios defeitos de nascimento, como a síndrome alcoólica fetal (SFA) que é responsável pelo retardo

do crescimento, deficiências no sistema nervoso central (SNC) e por anomalias faciais.

A Síndrome Fetal Alcoólica (SFA) foi primeiramente descrita no início da década de setenta, para descrever um padrão observado em filhos de mães dependentes do álcool. Esta droga pode promover alterações nas concentrações de prostaglandinas fetais, resultando em vasoconstrição generalizada e um quadro de hipóxia para o feto, causando decréscimo no número de células que deveriam estar sendo formadas (Ribeiro & Gonzalez,1995; Koren et al., 1996; Correia, 2000).

Os efeitos do álcool no desenvolvimento craniofacial e orodental incluem a formação de dentes pequenos, defeitos na maxila (Church et al., 1997) e no esmalte. Também podem ser vistas alterações nas células da epiderme do germe do dente no epitélio interno do esmalte durante o odontogênese.

Rawat (1976) demonstrou que o álcool atua inibindo a síntese protéica e também verificou os efeitos do álcool ingerido durante a gestação na atividade e nos níveis de ATP no coração, cérebro e fígado. Em seus resultados observou uma redução da atividade da enzima ATP sintetase, quando comparado com os animais controle. Fadel & Persaud (1992) observaram que havia redução no desenvolvimento do embrião ocorria na região craniofacial, proncipalmente no prosencéfalo, ocasionado pelo no número de somitos e nos arcos branquiais.

O álcool pode influenciar o desenvolvimento das estruturas orais, como por exemplo, alterações ultraestruturais nos ameloblastos secretores do germe dentário, independente da quantidade de álcool administrada. (Matthiessen & Rómert, 1988). Guerrero et al. (1996), analisando o efeito do álcool sobre o fator de crescimento epidermal (EGF) na odontogênese de camundongos, descreveram que a imunoexpressão desse fator, observada na cúspide média do 1° molar inferior era forte e homogênea no grupo controle, porém fraca e heterogênea no tratado. Também verificaram que os germes dentários dos animais tratados eram morfométricamente menores.

Diante do exposto, o objetivo deste trabalho foi verificar a influência de fatores genéticos, através dos genes PAX9 e MSX1, e ambientais, através da indução do alcoolismo no processo de odontogênese.

2. CAPÍTULO 1

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ASSOCIATION BETWEEN POLYMORPHISM IN THE PROMOTER REGION (G/C-915) OF *PAX9* GENE AND THIRD MOLAR AGENESIS

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Purpose: Hypodontia is the congenital absence of one or more (up to six) permanent and/or deciduous teeth, being one of the most common alterations of the human dentition. Genetic polymorphisms are variations of DNA sequences occurring in a population. This study investigated whether G-915C single nucleotide polymorphism (SNPs) in the *PAX9* gene promoter is associated with hypodontia in humans. Material and Methods: The polymorphism in region G/C-915 of *PAX9* gene (NCBI ref SNP ID: rs 2073247) of 240 patients was analyzed, being 110 controls and 130 individuals with third molar agenesis. After DNA extraction, the region of interest was amplified by PCR technique using two different primers. The significance of the differences in observed frequencies of polymorphisms in both groups was assessed by odds-ratio and chi-squared test with 95% confidence interval.

Results: Genotype CC was more frequent in patients with agenesis (11.5%) compared to the control (1.8%), while GG was more prevalent in the control group (39.1%) compared to the individuals with agenesis (26.2%). Conclusion: These data showed that the allele C could be associated with the third molar agenesis.

Uniterms: Hypodontia; *PAX9* transcription factor; Tooth abnormalities; Genetic polymorphism.

INTRODUCTION

Hypodontia, the congenital absence of one or a few teeth, is one of the most common developmental alterations of human dentition. Hypodontia is not a serious public health problem, but it may cause masticatory (temporomandibular joint disorders) and speech dysfunctions and create esthetic problems with orthodontic and prosthetic impairments[29]. In the past few years, several growth and transcription factors have been shown to be expressed in developing teeth[26]. The direct participation of several genes in tooth development was demonstrated by the lack of teeth in mutant knockout mice models[7]. Autosomal dominant forms of hypodontia have been shown to be caused by mutations in the *MSX1* and *PAX9* genes in human families[25]. However, the origin of the isolated sporadic agenesis, the most common form of hypodontia in humans, is still unknown. To date only a limited number of mutations of *MSX1* and *PAX9* have been proven to be associated with severe hypodontia (oligodontia) in humans[11]. *PAX9* is a transcription factor that is expressed in dental mesenchyme at initiation, bud, cap and bell stages of odontogenesis[15]. Protein products of this gene serve as transcription factors that are responsible for the crosstalk between epithelial and mesenchymal tissues and are essential for the establishment of the odontogenic potential of the mesenchyme[23,27]. It has been found that *PAX9*-deficient mice lack teeth and pharyngeal pouches derivates and have severe craniofacial anomalies[16].

The expression of *PAX9* in the mesenchyme appears to be a marker for the sites of tooth formation as it occurs before any morphological manifestation of this process12. Mutations in this gene have been shown to be associated with autosomal dominant forms of oligodontia (agenesis of more than 6 teeth, MIM 604625) in humans[10,25]. In most families, mutations occur in exon 2 affecting the function of *PAX9* paired domain that is responsible for the binding of this protein to DNA target sequences. Affected individuals have severe tooth agenesis with absence of most molars, second premolars and some incisors. *PAX9* is critical for the regulation of BMP*4* expression through its paired domain rather than *MSX1*[13].

Polymorphisms are a mechanism by which individuals may exhibit variations within the range of what is considered biologically normal. Gene polymorphisms have also been associated with susceptibility to diseases. Most polymorphisms are single nucleotide exchanges that occur at a high frequency in the human genome and may affect the function of genes. Although it has been demonstrated that mutations in the coding sequences of *PAX9* are a causative factor in familiar forms of severe tooth agenesis, polymorphisms in this gene have not been associated with sporadic tooth agenesis. Previous studies have shown that two polymorphisms in the *PAX9* promoter region seem to be associated to the dental agenesis in human beings (G-1031A, T-912C T-160C).

The purpose of the present study was to investigate whether the G-915C single nucleotide polymorphism (SNPs) in the *PAX9* gene promoter is associated with hypodontia in humans[15,22].

MATERIAL AND METHODS

Subject Selection and Sampling

One hundred and thirty unrelated Caucasian individuals with hypodontia, without signs of other disorders and 110 healthy control individuals (without hypodontia) were interviewed and documented. Congenital absence of teeth was confirmed by X-ray examination. No other dental anomalies were observed in the subjects. The sampling of epithelial buccal cells was performed as previously described15. Briefly, individuals undertook a mouthwash of 5 mL 3% glucose. Following mouthwash, a sterile wooden spatula was used to scrape oral mucosa. The tip of the spatula was then shacked into the retained mouthwash solution. Buccal epithelial cells were pelleted by centrifugation at 2000 rpm for 10 min. The supernatant was discarded and the cell pellet resuspended in 500 µL extraction buffer [10 mM Tris-HCl (pH 7.8), 5 mM EDTA, 0.5% SDS]. The samples were then frozen at -20oC until used for DNA extraction. After defrosted, samples were incubated overnight with 100 ng/mL proteinase K (Sigma Chemical Co., St. Louis, MO, USA) at 37℃ with agitation. DNA was then purified by sequential

phenol/chloroform extraction and salt/ethanol precipitation. DNA was dissolved in 70 µL TE buffer [10 mM Tris (pH 7.8), 1 mM EDTA]. The concentration was estimated by measurements of OD 26015.

Polymerase Chain Reaction (PCR)

Amplification of the *PAX9* gene fragment was performed with the oligonucleotides 5'AGC CTG AAT CCT GTG TGC AC-3´ (forward) and two reverses to amplify the region with alleles G or C. To amplify the alelles G the oligonucleotide 5'GAA ATA TTT TCG TGA ATT TGG GAG'-3´ was used and the C allele was amplified with the oligonucleotide 5'ATT TTC GTG AAT TTG GGAC'-3´ (NCBI ref SNP ID: rs 2073245). Reactions were conducted in a total volume of 50 µL, containing: 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1 mM of each primer, 200 mM each dATP, dCTP, dGTP and dTTP, 25 mM MgCl2 and 2.5 units Taq DNA polymerase (Amersham Pharmacia Biotech, Uppsala, Sweden). The thermal cycling conditions consisted of an initial denaturation at 95℃ for 5 min followed by 35 cycles of 95℃ for 60 sec, annealing at 56℃ for 70 sec, and a final extension at 72℃ for 70 sec. Reactions were performed on a *GeneAmp® PCR System 2400* thermocycler (Perkin Elmer).

PCR products of *PAX9* were submitted to 10% polyacrylamide gel electrophoresis and the DNA bands were labeled by silver staining[15].

Statistical Analysis

The significance of the differences in observed frequencies of polymorphisms in both groups was assessed by odds-ratio and chi-squared test with 95% confidence interval. All statistical analysis was conducted using BioEstat program version 2.0. Differences were considered significant at $p<0.05$[15].

RESULTS

The statistical analysis showed significant differences in the alleles and genotypes frequencies in the promoter polymorphisms of *PAX9* gene (G-915C). The C allele was found in 31.4% of the control group and in 42.5% of the test group (individuals with hypodontia) (p=0.0014). The G/G and C/C genotypes frequencies were 39.1% and 1.8% in the control group and 26% and 11.4% in the test group respectively (p=0.0045). The frequencies of different alleles and genotypes of the *PAX9* gene are shown in Table 1. The association was confirmed when the CC genotype is confronted with CG/GG. Individuals with CC genotype seemed to be seven times more susceptible to develop hypodontia (OR= 7.04; 95% CI= 1.1-15.2; p=0.0034).

DISCUSSION

The G-915C polymorphism in the 5' flanking region of *PAX9* gene could be associated with hypodontia in humans. CC genotype was found at a significant higher frequency in individuals with hypodontia. Analysis of human families shows a strong effect of *PAX9* mutations on third molar agenesis, whereas mutations in this gene have a weaker but fairly evident effect on the development of incisors and premolars[4-6,18-20,24,28]. Three other polymorphic alleles (T-160C, G–1031A and T–912C) located close to the polymorphic region evaluated here were also associated with hypodontia in humans[22]. Families bearing *PAX9* mutation had tooth agenesis as the only clinical sign. The relative lack of pleiotropy of mutations in *PAX9* and on other genes affecting the dental field would account for the common variation in the form and number of teeth observed in humans and other mammals[2]. This would allow genetic changes to affect the earlier phases of tooth development, without interfering significantly with the development of other organs[16].

Experimental and theoretical approaches have stressed the importance of cis-regulatory DNA sequences that control the transcription of genes as the basic modular unit that determine the positional information in developing structures[3].

The *cis*-regulatory sequences of many genes are organized into independent modules that regulate the transcription of specific tissues at specific times during development[7]. The regulatory modules are usually constituted of multiple binding sites for transcription factors that can be located near the transcription start site, or thousand of base pairs away[1]. Mutations within individual modules can enhance or repress gene transcription in a tissue specific manner, allowing a mutation to exert its effect on a few or even a single morphogenetic field[7]. A relevant fact is the relative lack of pleiotropic effects of *PAX9* gene mutations in humans. Families bearing *PAX9* mutation had tooth agenesis as the only clinical sign. The relative lack of pleiotropy of mutations in *PAX9* gene and on other genes affecting the dental area would account for the common variation in the form and number of teeth observed in humans and other mammals[2]. This would allow genetic changes to affect the earlier phases of tooth development, without interfering significantly with the development of other organs.

For example, the down-regulation of *Msx1* expression in *PAX9*-deficient mice and decreased levels of Bmp4 expression in *PAX9* and *MSX1* homozygous null mice indicate that the three genes act within the same signaling pathway. Although recent studies of human *PAX9* mutations have enabled structure-function correlations, the precise molecular mechanisms that contribute to tooth agenesis are poorly understood. The functional studies of a previously described mutation (L21P) in the amino-terminal subdomain of the *PAX9* paired domain demonstrate impaired transcriptional activation of the *MSX1* and Bmp4 promoters. It is likely that the proline cyclic side chain blocks the main chain nitrogen atom and chemically prevents it from forming a hydrogen bond[13].

In humans, the changes in the number of teeth tend to occur in the reverse of the sequence that teeth are formed during development, which also characterizes the general pattern of tooth loss observed during the evolution of placental mammals[17]. Variations in tooth number may have represented an important factor in the diversification of mammalian species. Like hypodontia, most evolutionary changes in tooth number resulted from the loss of one or two

elements, in most cases the last member of a tooth family. In this regard, the study of genetic polymorphisms

in tooth agenesis is a specially suited model to understand the association of gene polymorphisms and changes in morphology. Teeth are serially homologous structures and the effects of gene variations on the development of these structures can be easily quantified[8]. Individuals with distinct polymorphic alleles may exhibit subtle and specific phenotypic variations in dental patterning. In this sense, association studies between gene polymorphisms and hypodontia as well as other mild malformations that reflect qualitative defects of embryogenesis[14], may help understanding the molecular mechanisms responsible for the phenotypic variations that may occur in distinct human populations and ethnic groups.

TABLE 1- Genotype and allele distribution of the G-915C polymorphism of *PAX9* gene in the control and hypodontia (third molar) groups

G-915C	Control		Third molar agenesis	
Genotype	n	%	n	%
GG	43	39.1	34	26.2
GC	65	59.1	81	62.4
CC	2	1.8	15	11.5
p				0.0045
CC	2	1.8	15	11.5
GG/GC	108	98.2	115	88.5
p*				0.0034
OR (95%CI)				7.04(1.1-15.2)
Allele	n	%	n	%
G	151	68.6	150	57.5
C	112	31.4	110	42.5
p				0.0014

*p**: chi-squared test.

The first mutation described in human *PAX9* gene was an insertion of an additional G within the paired box sequence at nucleotide 219 (219InsG) of exon 2 in a family with oligodontia. Since then eight additional mutations in the coding region of *PAX9* gene were reported being associated with oligodontia in humans[8]. Seven out of the nine reported mutations were located in the paired domain at exon 2. Recently, it has been shown that the 219InsG frameshift mutation dramatically reduces DNA binding of the *PAX9* paired domain and supports the hypothesis that loss of DNA binding is the pathogenic mechanism by which the

mutation causes oligodontia[9]. Although mutations in the coding sequences of *PAX9* and *MSX1* genes are a causative factor in familiar forms of oligodontia6, the genetic origin of the isolated sporadic agenesis, the most common form of hypodontia in humans, is still unknown. We report here that three other polymorphisms (T-160C, G-1031A and T-912C) in the 5' flanking region of *PAX9* gene are associated with hypodontia in humans. Other alleles, such as C (-160), A (-1031) and C (-912) were found at a significant higher frequency in individuals with hypodontia. A comparative analysis of mouse, rat and human *PAX9* 5' flanking sequences shows that the T-160C is located in a poorly conserved region when human and rodent (mice and rat) sequences are compared. Although the present study showed a positive association on genotype and odds ratio, the results must be confirmed by studies with other populations and functional experiments, such as homologous recombination in mice, gel shift analysis and reporter gene systems.

CONCLUSION

The findings of this study showed that the allele C could be associated with third molar agenesis.

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3. CAPÍTULO 2

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Suggestive Associations Between Polymorphisms in *PAX*9, *MSX*1 Genes and Third Molar Agenesis in Humans

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Abstract: Hypodontia, the congenital agenesis of one or few permanent teeth is among the most common alterations in human dentition. PAX9 and MSX1 genes have critical roles in craniofacial development. Mutations in these genes cause severe tooth agenesis in humans and mice. The aim of the present work was to study the association of the CA repeat in the first intron of *MSX*1 gene and the C-160T polymorphism in the promoter region of *PAX*9 gene and hypodontia in humans, with emphasis on third molar agenesis. DNA extracted from buccal epithelial cells was amplified by the Polymerase Chain Reaction. Denaturing Gel Electrophoresis, DNA sequencing and PCR-RLFP were employed on the investigation of the polymorphisms. The 169 bp allele of *MSX*1-CA repeat was the most prevalent in both groups. Borderline associations were found for *MSX*1 gene. The 169-bp allele was more frequent in individuals with hypodontia (OR=1.9; 95% CI= 1.0 – 3.6; p=0.05) and 169/175 genotype was less prevalent in individuals with hypodontia (OR=0.4; 95% CI=0.2 – 0.9; p=0.05). The CC genotype of the *PAX*9 C-160T polymorphism was found at a significant higher frequency in individuals with hypodontia (p=0.0009). A separated analysis of individuals with third molar agenesis also revealed a positive association with the CC genotype (p=0.0007).

Key Words: Hypodontia, tooth agenesis, gene polymorphisms, *PAX*9, *MSX*1, CA repeat, C-160T polymorphism, Human dentition, Restrict Fragment Length Polymorphism.

INTRODUCTION

Hypodontia (MIM 106600), the congenital absence of one or a few teeth, is one of the most frequent alterations in the human dentition [1]. It can occur as a familial (autosomal dominant, recessive or X-linked) or isolated sporadic trait [2]. The most common permanent teeth missing are the third molars. Third molar agenesis has been associated with morphological parameters such as maxillary jaw dimensions [3] and late formation of tooth germs [4]. Several homeobox genes are known to be expressed during odontogenesis [5,6]. The expression of these genes controls the growth and formation of developing teeth. The homeobox gene *MSX*1 (Muscle Segment homeobox 1) and the *PAX*9 gene, a member of a transcription factor family characterized by a common motif the DNA-binding paired domain, are expressed in dental mesenchyme at initiation, bud, cap and bell stages of odontogenesis [7,8]. Protein products of these genes serve as transcription factors that are responsible for the cross talk between epithelial and mesenchymal tissues and are essential for the establishment of the odontogenic potential of the mesenchyme [5,6,9,10]. Msx1- and Pax9- deficient mice lack teeth and have severe craniofacial anomalies [8,11]. Mutations in these genes were shown to be associated with autosomal dominant forms of oligodontia (agenesis of more than 6 teeth, MIM 604625) in humans. Individuals with mutations in the *PAX*9 gene have severe tooth agenesis with absence of most molars, second premolars and some incisors [12-20].

*MSX*1 mutations also cause severe tooth agenesis with absence of second premolars [21-25]. The first and third molars and first premolars are also frequently affected. Also, the CA repeat region in *MSX*1 gene has been associated with limb deficiency in humans [26]. Recently, polymorphisms in putative transcription regulatory regions of these genes have been associated with hypodontia in humans [27]. The analysis of *MSX*1 gene polymorphisms (CA repeat in the first intron) of *MSX*1 gene, however, did not include any cases with third molar agenesis and did not allow any conclusion regarding the effect of that polymorphic site on third molar agenesis. The analysis of polymorphisms located at

approximately 1100 bp from transcription start site of *PAX*9 gene revealed a moderate association with third molar agenesis [28]. The aim of the present work was to study the association of the CA repeat of the first intron of *MSX*1 gene (www.ncbi.nlm.nih.gov/SNP ref 3836612) and the C-160T polymorphism in the promoter region of *PAX*9 gene (www.ncbi.nlm.nih.gov/SNP ref 2073247) with third molar agenesis.

MATERIAL AND METHODS

Subject Selection and Sampling

One hundred twenty two (122) unrelated Caucasian individuals with tooth agenesis, without signs of other disorders, and one hundred and one (101) healthy control individuals (without hypodontia) were interviewed and documented (Table **1**).

Congenital absence of teeth was confirmed by X-ray analysis. Subjects of both gender were collected, and all of them were of Caucasian origin. There was an overrepresentation of females in the cases as well as in controls (Table **2**).

The sampling of epithelial buccal cells was performed as previously described [29]. Briefly, individuals undertook a 5ml 3% glucose mouthwash for 1 min. Following mouthwash, a sterile wood spatula was used to scrape oral mucosa. The tip of the spatula was then shacked into the retained mouthwash solution. Buccal epithelial cells were pelleted by centrifugation at 2000 rpm for 10 min. The supernatant was discarded and the cell pellet resuspended in 500 ul of extraction buffer [10 mM Tris-HCl (pH 7.8), 5 mM EDTA, 0.5% SDS].

The samples were incubated overnight with 100 ng/ml proteinase K (Sigma Chemical Co., St. Louis, MO, USA) at 37°C with agit ation. DNA was then purified by sequential phenol/chloroform extraction and salt/ethanol precipitation. DNA was resuspended in 70 ul extraction buffer. The concentration was estimated by measurements of OD 260. Ethical approval was granted by the Ethics Committee of the Piracicaba Dental School.

Polymerase Chain Reactions (PCR)

Amplification of the *MSX*1 gene fragment was performed with the oligonucleotides 5'-GGG CAT GTT GAT GTC TGC TGAC-3' (forward) and 5'-TTAGAT TGTCAT CAG TCCTC-3' (reverse) [26], whereas amplification of *PAX*9 promoter fragment was performed with the oligonucleotides 5'-CCCACCTATAGC CTT AACTT-3' (forward) 5'-CTCTTTCAGGCTAGCTCCCC-3' (reverse) (NCBI ref SNP ID: rs 2073247). Reactions were conducted in a total volume of 50 µl, containing: 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1µM of each primer, 200 µM each dATP, dCTP, dGTP and dTTP, 25 mM MgCl2 and 2.5 units Taq (Thermus aquaticus bacteria) DNA polymerase (Amersham Pharmacia Biotech, Uppsala, Sweden). The thermal cycling conditions consisted of an initial denaturation at 95℃ for 5 min followed by 35 cycles of 95℃ for 1 min, annealing at 57℃ (*MSX1*) or 56℃ (*PAX9*) for 1 min, and a final extension at 72℃ for 1 min. Reactions were performed on a *GeneAmp® PCR System 2400* thermocycler (Perkin Elmer).

Denaturing Gel Electrophoresis and Sequencing

PCR products of *MSX*1 gene were mixed with denaturing buffer (50% formamide, 0.001% bromophenol blue and 0.001% xylene cyanol) and heated at 94ºC for 3 min. Denatured samples were snap-chilled on ice and submitted to denaturing poliacrylamide gel electrophoresis [30]. The DNA bands were evidenced by silver staining [31]. *MSX*1-CA repeat base-pair-alleles sizes were confirmed automatic DNA sequencing. Briefly, DNA bands were eluted from the gels and PCR reamplified. PCR products were purified by Wizard® SV Gel and PCR Clean-Up System (Promega Corporations, Madison, Wi, USA) and submitted to automatic DNA sequencing in a total volume of 20 µl, containing 3,2 µM forward primer and 8 µL *Big Dye Terminator Ready v.2.0* (Applied Biosystems).

Restriction Endonuclease Digestion

For the analysis of the C-160T promoter polymorphism of *PAX*9 promoter the PCR products were digested with 2.0 units of the restriction enzyme BspEI

(New England *Biolabs*, Beverly, MA, USA). The enzyme cut the allele T generating two fragments of 45 bp and 185 bp, while allele C remains uncut (230 bp). The digest was mixed 5 µl of loading buffer and electrophoresed on a 10% vertical non-denaturing polyacrylamide gel. The DNA bands were evidenced by silver staining.

Statistical Analysis

The significance of the differences in observed frequencies of polymorphisms in both groups was assessed by Odds-Ratio and chi-squared test (*x2*) test with a 95% confidence interval. All statistical analysis was conducted using BioEstat program version 2.0. Differences were considered significant when $p<0.05$.

RESULTS

The statistical analysis of the associations of allele and genotype frequencies of *MSX*1 CA repeats with hypodontia is shown on Table **3**. There was no significant association between the CA repeat alleles, genotype frequencies and hypodontia. A more specific analysis of the cases with third molar agenesis (n=92, this group includes individuals that besides third molar agenesis presented agenesis of other teeth), or exclusively with individuals presenting third molar agenesis (n=79) did not reveal any positive associations. An analysis excluding individuals third molar agenesis (n=29) revealed a borderline association between *MSX*1-CA 169-base-pair allele and hypodontia (p=0.05) (OR=1.9; 95% IC=1.0-3.6). The 175-base-pair was significantly less frequent in this group than in control group (p=0.02) (OR =0.1; 95% IC=0.01 – 0.9). The frequencies of the 175-bp allele in individuals with hypodontia excluding third molar agenesis (n=29) and control group was 1.7% and 12.9%, respectively.

The frequencies of the 169-bp allele in the group hypodontia excluding third molar agenesis and control group was 70.7% and 55.4%, respectively. The 171/175 genotype was the less prevalent, whereas the 169/169 genotype was the most prevalent. The distribution of cases by type of tooth missing and by selected

characteristics of the probands was calculated (Table **1**). The genotype 175/169 was more frequent in the control group than in the hypodontia group (p=0.05).

This result, however, may be spurious due to the small number of individuals bearing this genotype. The statistical analysis did show significant differences in the genotypes frequencies of the C-160T promoter polymorphism of *PAX*9 gene between the two sample groups (Table **4**). The CC genotype was found in 4.5% of the Control group and in 17.7% of Test group (individuals with tooth agenesis)(p= 0.0009). The TT and CT genotypes frequencies were 34.2% and 61.3% in the Control group and 40.7% and 41.6% in the Test group, respectively. The association was confirmed when the CC genotype is confronted with CT/TT. Individuals with CC genotype seemed to be about four times more susceptible to develop hypodontia (OR=4,6; 95% CI=1.6-12.6; p(chisquared test)=0.002). The frequencies of different alleles and genotypes of the *PAX*9 gene are shown in Table **4**. The genotype distribution for the studied polymorphism was consistent with the assumption of Hardy-Weinberg equilibrium in both groups (p>0.09). A separated analysis of the cases with third molar agenesis (n=84) showed a positive association between the genotype frequencies and hypodontia (Table **4**). The frequencies of CC genotypes in Control and in the group with third molar agenesis were 4.5% and 17.9%, respectively (p=0.0007). The frequencies of TT and CT genotypes in the group with third molar agenesis were 44% and 38.1%, respectively. The association was confirmed when the CC genotype was confronted with TT/CT. Individuals with CC genotype seemed to be about four times more susceptible to develop third molar agenesis (OR= 4.6; 95% CI= 1.6-13.2; p(chi-squared test)= 0.002).

DISCUSSION

The first mutation described in the human *MSX*1 gene was an Arg31Pro missense insertion. This mutation was likely to interfere with the binding of the protein to the target DNA sequences, since the arginine is a highly conserved residue located in the homeodomain site [21]. Since then several mutations in the

coding region of *MSX*1 gene were reported being associated with oligodontia in humans [21-25]. Recently, the CA repeat of the first intron of *MSX1* gene was associated with hypodontia in humans [27]. This study, however, did not include any cases with third molar agenesis, not allowing any conclusion regarding the effect of this polymorphic site on third molar agenesis. The results presented here show that the CA repeat site is not associated with third molar agenesis. In fact, analysis of human families showed a stronger effect of *MSX*1 mutations on second premolar agenesis, whereas these mutations have a weaker, but fairly evident, effect on the development of incisors and third molars [21-25]. It is also possible, however, that the susceptibility for third molar agenesis is influenced by other polymorphic sites present in the coding sequences of the *MSX*1 gene. The results presented here showed that the *MSX*1-CA 169-base-pair allele was found at a significant higher frequency in individuals with developmentally missing teeth, excluding third molars. Our data confirm the results of Vieira *et al*. [27] using an independent sample and a distinct approach (case control study). Although this polymorphic site has been associated with limb deficiency and hypodontia [26,27] it is not possible to affirm that the variations of CA repeats can modulate the transcriptional activity of the *MSX*1 gene. DNA sequencing of the homeodomain bearing the second exon of *MSX*1 gene in 13 individuals with premolar agenesis failed to show a positive association [32]. A comparative analysis of mouse, rat, dog, chimpanzee and human sequences shows that the CA repeat is conserved among human and chimpanzee and is not present in the first intron of dog, rat and mouse *MSX*1 gene (http://genome.ucsc.edu/cgi-binchr4:4981696-4981720). The analysis of the CA repeat flanking regions on MatInspector software (http://www.genomatix.de/cgibin/./eldorado/main.pl) revealed the presence of core sequences for three putative transcription factor binding sites: Myc associated zinc finger protein MAZ (GAGG), Myeloid zinc finger protein MZF1 (GGGG), and the Zinc finger transcription factor ZBP-98 (CCCC). These three binding sites are located downstream from the CA repeat.

Although there is no specific study on the expression of these factors on tooth development is possible that the variations in the length of the *MSX*1-CA repeat may affect the binding affinity of these transcription factors. In fact, length polymorphisms in CA repeats were shown to modulate the transcriptional activity of several genes [33]. The C-160T polymorphism in the 5' flanking region of *PAX*9 gene was associated with hypodontia in humans. Genotype CC was found at a significant higher frequency in individuals with hypodontia. A separated analysis of the cases with only third molar agenesis (n=84) presented similar results. In fact, analysis of human families shows a strong effect of *PAX*9 mutations on third molar agenesis, whereas *PAX*9 mutations have a weaker but fairly evident effect on the development of incisors and premolars [12-20]. Two other polymorphic alleles (G–1031A and T–912C) located over 800 base-pairs upstream from the polymorphic site analyzed here were also associated with hypodontia in humans [28]. The statistical analysis, however, showed that the p values for the C-160T polymorphism were over 10-fold smaller than the values reported by the two other upstream sites. Likewise the Odds-Ratio values were about two-fold higher. Although the results presented in this paper are suggestive, is not possible to affirm the C-160T polymorphism can affect the transcriptional regulation of *PAX*9 gene. A comparative analysis of mouse, rat, dog, chimpanzee and human shows that the C–160T polymorphism is located in a highly conserved TTC**C**CC box, where the C allele (bold) is conserved among these species. The analysis of the polymorphic sites on MatInspector software (http://www.genomatix.de/cgi-bin/./eldorado/main.pl) revealed that the C allele is located within a Zinc Finger Transcription Factor ZBP-98(CCCC) and an Olfactory Neuron-Specific Factor (TCCC) core binding sites. Furthermore, the C allele (bold) is located on a GC rich region (C**C**CCGGAGG). These regions, known as GC boxes are binding sites for a family of transcription factors known as SP1-like transcription factors [34]. These proteins control the transcription of many genes that regulate the embryonic development in vertebrates [35]. The association of the C–160T polymorphism with tooth agenesis in humans, allied with the evolutionary conservation suggest that

this region may play a role on the transcriptional regulation of *PAX*9 gene. It is important to mention, however, that the results obtained using this approach have to be further validated by other independent studies and by studies involving *in vitro* or *in vivo* laboratory investigation [36]. Although the present study showed a positive association on genotype frequencies and Odds Ratio the results must be confirmed by studies with other populations and functional experiments such as homologous recombination of conserved consensus sequences in mice, gel shift analysis, and reporter gene systems.

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ABREVIATIONS

Bp = Base pair

BspEI = A *E. coli* strain that carries the BspI gene
from Bacillus species

*MSX*1 = Muscle Segment homeobox 1

*PAX*9 **=** DNA-binding paired domain 9

PCR-RLFP = Polymerase Chain Reaction- Restriction
Length Fragment Polymorphisms

SNP = Single Nucleotide Polymorphisms

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Table 1. Aspects of the Population Studied

Individuals Characteristic	N(%)
Number of teeth missing	
1	38(31.93)
2	43(36.13)
3 or more	38(31.93)
Type of teeth more often missing	
(Total teeth missing = 272)	
Third molar	199(73.16)
Lateral incisor	43(15.8)
Second premolar	15(5.51)
Canines	5(1.83)
Central incisor	4(1.47)
First premolar	3(1.1)
Second molar	3(1.1)
Number of cases missing molars	92 (76)
Number of cases missing incisors	23 (19)
Number of cases missing premolars	14 (11.5)
Number of cases missing canines	4 (3.3)

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Table 2. Proportion Between Males and Females in Controls and Cases of Hypodontia

Individual Characteristics	Control	Cases	Total
Gender distribution			
Male	38 (37.6%)	33 (27.2%)	71
Female	63 (62.4%)	88 (72.8%)	151
Total	101	121	222

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Table 3. Distribution of Alleles and Genotypes *MSX1* CA Repeat, Case Versus Control Individuals

	Control (n=101)		Hypodontia (n=121)		Third molar hypodontia only (n=79)		Hypodontia, excluding third molar (n=29)		Third molar hypodontia (may present agenesis of other teeth) (n=92)	
	n	%	n	%	n	%	n	%	n	%
Alleles										
[a]169	112	55.4	146	60.3	90	56.2	41	70.7	105	57.1
OR(95%CI)							**1.9(1.0 – 3.6)**			
p		0.3				1.0		**0.05**		0.82
171	17	8.4	18	7.4	15	9.4	0	0	18	9.8
p		0.8				0.9				0.8
173	47	23.3	55	22.7	35	21.9	16	27.6	39	21.2
p		1.0				0.8		0.6		0.7
175	26	12.9	23	9.5	20	12.5	1	1.7	22	11.9
OR(95%CI)							**0.1(0.01 – 0.9)**			
p		0.3				1.0		**0.02**		0.9
Genotypes										
169/169	30	29.7	49	40.5	30	37.5	14	48.3	35	38
p		0.1				0.3		0.1		0.3
169/173	28	27.7	33	27.3	16	20	13	44.8	20	21.7
p		0.9				0.3		0.1		0.4
169/175	17	16.8	9	7.4	9	11.2	0	0	9	9.8
OR(95% CI)		**0.4 (0.2 – 0.9)**								
p		**0.05**				0.4				0.2
169/171	7	6.9	6	4.9	5	6.2	0	0	6	6.5
169/171	7	6.9	6	4.9	5	6.2	0	0	6	6.5
173/173	6	5.9	8	6.6	7	8.7	1	3.4	7	7.6
173/175	5	4.9	5	4.1	4	5.0	1	3.4	4	4.3
171/171	4	4	4	3.3	3	3.7	0	0	4	4.3
175/175	2	2	3	2.5	2	2.5	0	0	3	3.3
171/173	2	2	1	0.8	1	1.2	0	0	1	1.1
171/175	0	0	3	2.5	3	3.7	0	0	3	3.3

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Table 4. Genotype Distribution of the C-160T Polymorphism of *PAX9* Gene in Control and Hypodontia Groups

C-160T	Control		Tooth agenesis		Third molar agenesis	
Genotype	n	%	n	%	n	%
TT	38	34.2	46	40.7	37	44
CT	68	61.3	47	41.6	32	38.1
CC	5	4.5	20	17.7	15	17.9
p			0.0009		0.0007	
CC	5	4.5	20	17.7	15	17.8
CT/TT	106	95.5	93	82.3	69	82.2
*p**			0.002		0.002	
OR (95%CI)			4.6 (1.6-2.6)		4.6(1.6-13.2)	

*p**: chi-squared test.

4. CAPÍTULO 3

O presente artigo foi enviando a European Journal of Oral Science no dia 04/11/2007, conforme carta de confirmação do Editor. (Anexo 01)

Effect of maternal ethanol intake on the morphology and mineralization of the rat mandibular molars.

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Effect of maternal ethanol intake on the morphology and mineralization of the rat mandibular molars.

Eur J Oral Sci

ABSTRACT

Alcohol consumption is a public health problem. When consumed during pregnancy may cause severe malformations in fetus, known as fetal alcohol syndrome (FAS). The caracteristics of this alterations occur principally in the craniofacial region. This work have the goal the study the molar alterations in pups rats submitted to chronic alcoholism during pregnancy and lactation. To induce the alcoholism, weekly doses of alcohol were administered in increasing dilution scale of: 5%, 10%, 15%, 20% e 25% before fertilization. The molar mandibles were removed and analyzed by scanning for eletronic microscopy and microhardness. The results showed that the alcohol group had lower body weight, mandibular dimensions and molar cusps sizes when compared to control animals. Alcohol consumption also decreased significantly enamel and dentin microhardness It could be concluded that alcohol affects the morphologic and biochemical nature of the teeth when animals are chronically exposed to alcohol from the period of fecundation.

Key words: alcohol; microhardness; morphology; odontogenesis, molar**.**

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INTRODUCTION

Alcohol consumption during pregnancy is a significant public health problem and is an established cause of serious birth defects and developmental delay, collectively described as fetal alcohol syndrome (FAS) (1,2). The first article that reported this pathology was published in 1968 by Lemoine and collaborators (3). FAS is characterized by retardation of craniofacial growth, central nervous system (CNS) deficiencies and a characteristic set of facial anomalies (1,2,4). FAS is estimated to occur in 0.5–2 per 1000 live births (5).

To help define the risks associated with maternal alcohol use during pregnancy, researchers have sought to understand the mechanisms of the teratogenic effects of alcohol on various tissues (2). It has been demonstrated that the effects of FAS in animals models, are comparable with those in humans and this fact permits to study the teratogenic effects of alcohol on tissue development during embriologic period (6).

Alcohol is capable of crossing the placentary barrier and reaching the embryo. The adverse effects of alcohol are seen in practically all the systems, but the exact mechanism involved in this process still is unknown. Alcohol can retard fetal development, modify cell differentiation and growth, inhibit cellular migration, and interfere with the metabolism of carbohydrates, proteins and lipids (7).

Experimental works showed that ethanol consumption during pregnancy can also affect the development of dentition (2). The aim of the present study is to evaluate the morphology, size and hardness of the mandibular molars, in the offspring of rats that had chronically ingested alcohol.

MATERIAL AND METHODS

1. Animals and treatment

Wistar (Ratus novicergus) SPF (specific pathogen free) were used. The animals were supplied by the Multidisciplinary Biological Investigation Center ("Centro Multidisciplinar de Investigação Biológica CEMIB – UNICAMP"), housed in collective cages with a maximum of 5 animals, in a climatized room (24 ± 4℃) and with a light/dark cycle of 12/12 h. The animals received the same solid diet *ad libitum*.

To study the effects of ethanol on dental development, the animals were divided into two groups of 15 animals, denominated: control and chronic alcohol, because of alcohol exposure during fecundation.

To induce the alcoholism, weekly doses of ethanol were administered in the increasing dilution scale of: 5%, 10%, 15%, 20% e 25%. The gradual administration of alcohol was intended to adapt the animal to this study model. The pre-fertilization treatment period was 42 days.

After this period the female rats were placed together with non alcoholic male rats for mating. During the pregnancy period and lactation the rats received the ethanol solution with a concentration of 25%. All the procedures performed in this project were in accordance with the Guidelines of the Ethics Commission on Animal Experimentation of the State University of Campinas.

2. Anesthesia and Sample Collection

Three months after birth, the animals were weighed and received an intramuscular solution of xilazine chloride (25 mg/Kg - Rompum®; Bayer S.A, São

Paulo, SP, Brazil) and ketamine chloride (50 mg/Kg - Dopalen®; Agribrands do Brasil LTDA, Paulínia, S.P, Brazil) (8). The mandibles were removed and immersed in formalin.

3. Mandibles measures

The linear dimensions were evaluated on the lateral jaw radiographic and the parameters utilized are indicated on the Fig 1.

4. Analyses and Samples Preparation for Scanning Electronic Microscopy (SEM)

The mandibles were mounted on stubs and sputter coated (Sputter CoaterMED 010. Balzers, Liechtenstein) for 120 seconds, to obtain gold covering of approximately 10 nanometers on the occlusal faces of the teeth.

The samples were observed by scanning electronic microscope JSM-5600 LV of JOEL (Tokyo, Japan), operating at an acceleration of 11 kV to enable high resolution images of the molars, in which the following measurement of the crowns were made: linear vestibular-lingual measurements of the cuspids of these molars, according to Chowdhury and Bromage in 2000 (9).

In order to take the mesio-distal measurements, the distances between the most external points of the first (mesial) and second cuspids crown were measured (Fig 2).

5. Analyses and Sample Preparation for Microhardness Testing

In the microhardness test, the molars were cut in the longitudinal direction, to expose the inner enamel and dentin. After this procedure, the samples were embedded in an acrylic polymer, and submitted to sequential polishing in an electric polishing machine (Maxgrind, Solotest, São Paulo-SP, Brazil), with 400, 600 and 1200β grain abrasive aluminum oxide disks, water cooled, and with

diamond pastes (Top, Gold and Ram, Arotec Ind e Com Ltda, Cotia-SP, Brazil) of 6, 3, 1 and 1/2 µm on felt cooled with mineral oil. Microhardness measurements were made in a microhardness meter (Future Tech FM-1e, Tokyo, Japan) with a Knoop type probe and static load of 25 g and 5 g during 5 s to analyze the enamel and dentin, respectively. Five indentations with spacing of 20µm were made in 3 distinct areas in inner enamel and dentin of first inferior molar. Data are expressed in Knoop Hardness Number (KHn) (10).

6. Statistical Analysis

Statistical analysis was done by the use of the nonpaired Student's-*t* test. Values of *p* lower than 0.05 were indicative of statistical significance.

RESULTS

The body weight was lower in the alcohol group (298±22g) when compared to control group (327±13g, $p<0.05$). The mandibular measures were lower in alcohol group than control group. The mean A measure in the control group was 28.8±0.37mm and the alcohol group 27.8±0.34mm ($p<0.05$). The mean B measure in the control group was 12.2±0.25, while the alcohol group showed 11.9±0.22mm ($p>0.05$), and the C measure ($p<0.05$), control group showed 29.2±0.48mm compared to 28.6±0.36mm ($p<0.05$). These results show the alcohol group was significantly smaller in A and C measures.

Table 1 shows the results regarding the mandibular molars. The first molar the mesio-distal length (MD), mesial cuspid length, middle cuspid and distal cuspid length of this element were analyzed. Statistical analysis showed no difference between the control and alcohol groups in the MD length of the tooth, but in the chronic alcoholism group, it was observed that the other three measurements were significantly smaller in this group when compared with the control ($p < 0.0143$ for the mesial cusp, $p < 0.0003$ for the middle cusp and $p < 0.0001$ for the distal cusp).

In the second molar, all the values of the chronic alcoholism group were statistically lower when compared with the control ($p<0.05$). The analysis of the third molars showed that all measures of the alcohol treated animals were significantly smaller than controls.

The microhardnes analysis showed that enamel and dentin microhardness were reduced in ethanol treated animals, with a reduction of 16.4% and 33,9% respectively ($p<0.05$) (Table 2).

DISCUSSION

Our results indicate a significant decrease of about 10% in tooth size in rats from alcoholic mothers. It is interesting to note that the tooth size decrease was about the same magnitude of whole body and mandibular size decrease (8%). It was, however, significantly less than the size decrease observed by Jimenez-Farfan, 2004 (7) in the tooth germs during morphogenesis. It is plausible to infer that ethanol will retard the growth of tooth germ. The delay in development observed in the early phases may be compensated by an extended developmental period. In fact, ethanol consumption during pregnancy has been associated with delayed eruption of molar teeth in mice and monkeys (11). Possible molecular mechanisms responding for this effect are the alterations in the expression of EGF, EGF-receptor and erb-2 that are mediators of cell proliferation (7,12)

The effects of ethanol have been studied mainly in bone. Ethanol intake can decrease trabecular bone density, cortical area mature bone strength, inhibit bone formation (13), and repair (14). The effects of ethanol on the mineralization of dental tissues, however, have not been studied. We showed that maternal ethanol intake caused a significant decrease in mineral content in both dentin (33,9%) and enamel (16,5%) of rats, as seen by microhardness analysis. The observation of ameloblasts from fetuses of mini-pigs from alcoholic mothers revealed severe mitochondrial alterations that could interfere with mineral secretion (16). A similar decrease in humans would have important clinical implications as less calcified

teeth would be more prone be affected by extrinsic factors leading to abrasion, pigmentation and possibly caries (15,16). The results presented here suggest a link between maternal alcohol consumption and dental hypomineralization, further research, however, is needed in order to evaluate the implications and clinical relevance in the oral health of FAS children.

It could be concluded that alcohol affects the morphologic and biochemical nature of the teeth when animals are chronically exposed to alcohol from the period of fecundation.

ACKNOWLEDGMENTS

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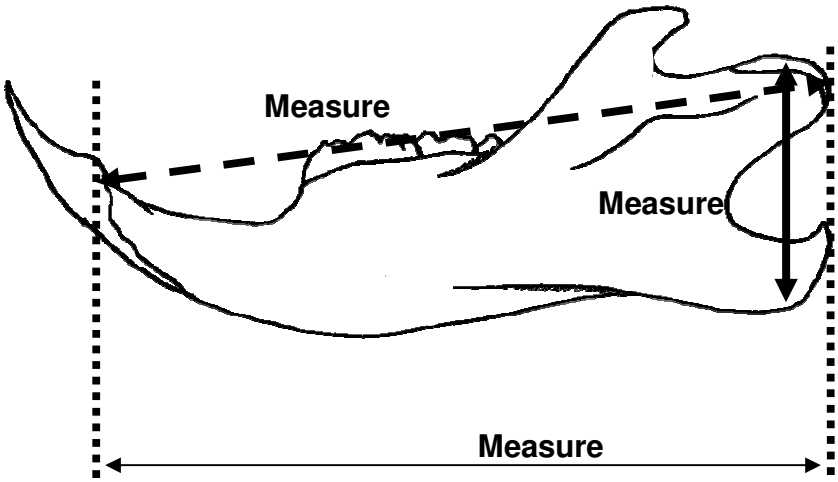

Figure 1. Lateral aspect of left mandible showing the lineal measures utilized.

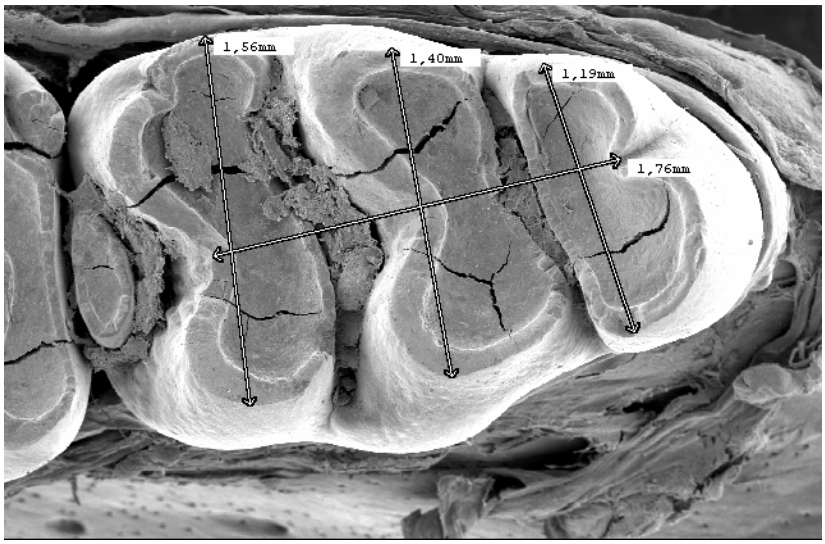

Figure 2. Molar measure schemes utilized.

Mandibular Molars Sizes		
First Molar	**Control (mean ± sem)**	**Alcohol (mean ± sem)**
Lenght	1.806 ± 0.03	1.816 ± 0.03
Mesial Cuspids	1.237 ± 0.02	1.136 ± 0.03 *
Middle Cuspids	1.500 ± 0.03	1.328 ± 0.03 *
Distal Cuspids	1.673 ± 0.02	1.470 ± 0.03 *
Second Molar		
Length	1.145 ± 0.01	1.100 ± 0.01 *
Mesial Cuspids	1.672 ± 0.03	1.591 ± 0.01 *
Distal Cuspids	1.673 ± 0.03	1.560 ± 0.01 *
Third Molar		
Length	1.312 ± 0.03	1.222 ± 0.01 *
Mesial Cuspids	1.356 ± 0.03	1.254 ± 0.02 *
Distal Cuspids	1.114 ± 0.05	1.013 ± 0.02 *

Table 1. Results of dimensions of the mandibular molars of control and Fetal Alcohol Syndrome rats. * $p<0.05$.

Molars Microhardness		
	Control (mean ± sem)	**Alcohol (mean ± sem)**
Enamel	352 ± 28.6	294 ± 14.9*
Dentin	52.8 ± 9.6	34.9 ± 4.6 *

Table 2. Microhardness results of rat molars. * $p<0.05$.

5. CONCLUSÕES

- O polimorfismo na região promotora do gene PAX9 G/C-915 (NCBI ref SNP ID: rs 2073247) e C-160T (NCBI ref SNP ID: rs 2073247) está relacionado com a agenesia dental de terceiros molares, porém o intron do gene MSX1 não apresentou relação com agenesia.
- Em ratos, o álcool administrado na gravidez interferiu na odoNtogênese de molares inferiores ocasionando dentes menores e tamanho reduzido da mandíbula, além de ocasionar hipoomineralização do esmalte e da dentina.
- Fatores genéticos e ambientais podem estar associados com a agenesia ou microdontia.

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ANEXOS

Data:	Sun, 4 Nov 2007 09:22:00 -0500 (EST)
De:	oral.sciences@odontologi.gu.se
Para:	fabiojbianchi@yahoo.com.br
Assunto:	European Journal of Oral Sciences - Manuscript ID EOS-3195-MAN-07

04-Nov-2007

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Thank you for submitting your manuscript entitled "Effect of maternal ethanol intake on the morphology and mineralization of the rat mandibular molars." to the European Journal of Oral Sciences. It has been successfully submitted online and is presently being given full consideration.

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CERTIFICADO

Certificamos que o Protocolo nº **1025-1**, sobre "**EFEITOS DO ÁLCOOL ETÍLICO NO PERÍODO GESTACIONAL NA MORFOLOGIA E MICRODUREZA DE MOLARES INFERIORES DE RATOS**" sob a responsabilidade de **Prof. Dr. Sérgio Roberto Paes Line / Tiago Franco de Oliveira** está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética na Experimentação Animal (CEEA)-IB-UNICAMP em reunião de 22 de maio de 2006.

CERTIFICATE

We certify that the protocol nº **1025-1**, entitled "**EFFECT OF THE ALCOHOL IN THE GESTACIONAL PERIOD IN THE MORPHOLOGY AND MICROHARDNESS OF INFERIORS MOLAR RATS**", is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). This project was approved by the institutional Committee for Ethics in Animal Research (State University of Campinas - UNICAMP) on May 22, 2006.

Campinas, 22 de maio de 2006.

Profa. Dra. Ana Maria A. Guaraldo
Presidente

Fátima Alonso
Secretária Executiva

UNICAMP

COMITÊ DE ÉTICA EM PESQUISA

UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

Certificamos que o Projeto de pesquisa "Associação entre agenesia dental em humanos e polimorfismos genéticos na região promotora do gene pax9 e análise funcional dos polimorfismos", protocolo CEP nº 044/2004, dos Pesquisadores *Fábio José Bianchi* e *Sérgio Roberto Peres Line*, está de acordo com a Resolução 196/96 do Conselho Nacional de Saúde - MS e foi aprovado pelo Comitê de Ética em Pesquisa da Faculdade de Odontologia - UNICAMP.

We certify that the research project "Association between human dental agenesy and genetics poliformifisms on the promoter region of pax9 gene and functional analyze of these polimorfisms", register number 044/2004, of *Fábio José Bianchi* and *Sérgio Roberto Peres Line*, is in agreement with the recommendations of 196/96 Resolution of the National Health Committee - Brazilian Health Department and was approved by the Research Ethics Committee of the School of Dentistry of Piracicaba - State University of Campinas - UNICAMP.

Piracicaba - SP, Brazil, June 16 2004

Profa. Dra. Cinthia Pereira Machado Tabchoury
Secretaria
CEP/FOP/UNICAMP

Prof. Dr. Jacks Jorge Júnior
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