UNIVERSIDADE ESTADUAL DE CAMPINAS

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Expressão de INS-R e IGF-IR em glândulas salivares de camundongos espontaneamente diabéticos após tratamento glicêmico prolongado

Este exemplar corresponde à redação final da tese defendida pelo(a) candidato (a) Eduardo José Caldeira

e aprovada pela Comissão Julgadora.

Tese apresentada ao Instituto de Biologia para obtenção do Título de Doutor em Biologia Celular e Estrutural, na área de Anatomia.

Orientadora: Profa. Dra. Valéria Helena Alves Cagnon Quitete

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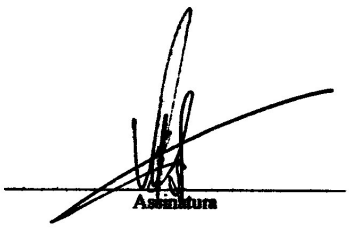

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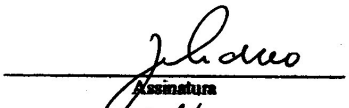

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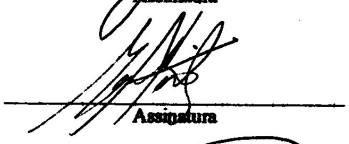

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DEDICATÓRIA
À DEUS...

"Porque tu és a minha rocha e a minha fortaleza, pelo que, por amor do teu nome, guia-me e encaminha-me."(Salmo 31:3)

AOS AMORES DE MINHA VIDA

MEUS PAIS, JOSÉ JULIO E MERCEDES,

"Pelo amor único e infinito; fraterno, amigo e confidente que sempre zela e se oferece por inteiro."

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"Não sei o que o mundo vai pensar dos meus trabalhos, mas o que me parece é que tenho sido apenas uma criança que brinca à beira do mar, encontrando uma concha mais agradavelmente colorida do que outra, enquanto o imenso oceano da verdade se estende inexplorado diante de mim."

(Isaac Newton)

SUMÁRIO

RESUMO

O Diabetes Mellitus compromete as glândulas salivares, alterando a morfologia e os mecanismos de salivação, os quais são fundamentais para a saúde bucal. Assim, o objetivo do presente estudo foi analisar os efeitos do tratamento insulínico prolongado sobre as células responsáveis pelo processo secretor das glândulas salivares parótidas e submandibulares de camundongos diabéticos autoimunes, além da expressão dos receptores de insulina (INS-R) e dos receptores dos fatores de crescimento homólogos a insulina (IGF-IR). Um total de 45 camundongos fêmeas foram divididos em três grupos: 18 Nod diabéticos positivos (grupo I), 18 Nod diabéticos tratados com insulina (grupo II) e 9 controles BALB/C (grupo III). O grupo II foi dividido em dois subgrupos com 9 animais que receberam insulina após 10 dias e 20 dias da expressão do diabetes. Da mesma forma os camundongos dos grupos I e III receberam diariamente solução fisiológica. Amostras das glândulas salivares foram analisadas em microscopias de luz, eletrônicas de transmissão e varredura e imunohistoquímica. Os resultados demonstraram que após tratamento insulínico prolongado os níveis glicêmicos dos animais retornaram a padrões de normalidade, contudo ainda observou-se alterações celulares como atipia celular, desorganização das biomembranas, aumento dos componentes fibrilares e alterações na expressão dos receptores de INS e de IGF-IR. Assim, a partir dos resultados pôde-se concluir que o diabetes provocou importantes mudanças tanto nas glândulas parótidas como nas submandibulares mesmo quando controlado com tratamento insulínico, o que pode levar a deficiências nos processos de manutenção e renovação tecidual, além de comprometer os mecanismos funcionais.

I-INTRODUÇÃO

DIABETES – GENERALIDADES

O Diabetes Mellitus é uma doença crônica que afeta o metabolismo de proteínas, carboidratos e gordura resultando em severas desordens metabólicas. A principal característica dessa patologia é a hiperglicemia refletindo uma deficiência na utilização dos carboidratos, resultado de uma anormalidade na secreção de insulina ou do efeito desta sobre os tecidos além de exacerbado acúmulo celular de lipídios e lipoproteínas (Robbins et al., 1989; Quissel et al., 1994; Conget, 2002).

O pâncreas é uma glândula tanto de secreção exócrina como endócrina, sendo a porção endócrina representada por aproximadamente um milhão de unidades celulares microscópicas agrupadas denominadas “Ilhotas de Langherans”. Essas ilhotas são agregados de diferentes tipos celulares dos quais 70% estão representados por células beta. As células beta secretam a insulina estimuladas pela concentração de glicose no sangue (Nattras & Halles, 1988). Em seres humanos, pode-se classificar o Diabetes Mellitus em tipo I e II. O diabetes tipo II ou não dependente de insulina é caracterizado por estabilidade e relativa insensibilidade à insulina bem como ausência de cetoses (Ritz, 2002). A obesidade é uma das características marcantes desse tipo de diabetes, a qual leva a diminuição do número de receptores de insulina nas células alvo tornando a quantidade de insulina ineficiente. Já o diabetes tipo I ou insulino dependente apresenta severa ou absoluta falta de insulina e grande tendência à cetose (Elenberg & Rifkin, 1962; Robbins, 1989).

No Brasil, o Diabetes Mellitus é um dos principais problemas de saúde sendo a quarta causa mortis. Contudo 50% das pessoas diabéticas desconhecem que estão com a doença e 8% da população na faixa etária de 30 a 70 anos de idade expressam diabetes representando cerca de 10 milhões de pessoas (Ministério da Saúde, 2002; Pace et al., 2003). Nos Estados Unidos, o diabetes é responsável por 2% dos óbitos estando entre as dez doenças que mais matam naquele país. Além disso, estudos experimentais e clínicos vêm demonstrando que essa patologia debilita os portadores levando-os a várias complicações como: ateriosclerose, nefropatias, retinopatias, neuropatias, perda de peso, alterações nos processos de cicatrização tecidual, atrofias celulares em epitélios de revestimento, aumento da incidência de infecções e alterações celulares em glândulas sexuais acessórias (Gomore & Goldner; 1943, Fushini et al., 1980; Ho, 1990; Stefan, 1996; Cagnon et al., 2000; Carvalho et al., 2003; Caldeira et al., 2004).

Apesar do conhecimento das várias complicações que o indivíduo diabético apresenta, dúvidas existem sobre a etiologia dessa doença. Esse fato deve-se a vários fatores tais como: os diversos perfis clínicos e subclínicos, a idade com que a doença manifesta-se e a incidência nos diferentes grupos étnicos e geográficos avaliados (Creutzfeld, 1976).

Assim sendo, a utilização de diferentes espécies animais que apresentam o Diabetes, tanto por indução química ou por espontaneidade é freqüente. Para obtenção de um animal diabético por indução química são utilizadas drogas como a estreptozotocina e a aloxana (Seethalakshmi et al., 1987; Saprykina, 1998; Perez et al., 1998; Kovacs et al., 1998; Palomar-Morales et al., 1998; Lee et al., 1999; Yildirim et al., 1999; Orie & Anyaegbu, 1999; Ravikumar & Anuradha, 1999; Avedano et al., 1999). Os animais utilizados nesses experimentos são tanto cães como roedores da linhagem C57BL/Ksj homozigotos diabéticos

(db/db), o Wistar Chinês e o Nod (diabético não obeso), apresentando os dois últimos síndrome espontânea semelhante ao Diabetes humano tipo II e I, respectivamente (Herberg & Coleman, 1977; Makino et al., 1980). Tanto nos animais tratados quimicamente como nos espontaneamente diabéticos caracterizam-se sintomas como: polidipsia, poliúria, polifagia e emaciação o que é próximo à sintomatologia humana, simulando condições patológicas para observações experimentais.

DIABETES E GLÂNDULAS SALIVARES MAIORES

A relação entre o Diabetes Mellitus e os efeitos desse sobre as glândulas salivares em especial, alterações celulares, processos inflamatórios, diminuição dos componentes bioquímicos e fluxo salivar têm sido estudado tanto experimentalmente como na clínica (Hunger et al., 1996; Szczepanski et al., 1998; Chavez et al., 2000; Watanabe et al., 2001; Takahashi, 2002). Em pacientes pediátricos diabéticos, observaram-se aumento dos níveis protéicos e da enzima amilase na saliva (Lopez et al., 2003). Já em estudos com pacientes diabéticos adultos e experimentos com camundongos espontaneamente diabéticos, os constituintes salivares como eletrólitos e a concentração total de proteína da saliva mostraram-se significantemente diminuídos. Também, observou-se além da diminuição do fluxo salivar a ocorrência de severa lesão inflamatória com células mononucleares localizadas inicialmente ao redor dos vasos sangüineos e posteriormente ao redor dos ácinos e ductos (Bloch, 1965; Goillot et al., 1991; Hu et al., 1992; Pozzilli et al., 1993; Humphreys-Beher 1998; Yamano et al., 1999). Contudo, não há consenso entre os diferentes estudos clínicos quanto à efetiva alteração dos constituintes bioquímicos da saliva, nem tão pouco sobre a diminuição do fluxo salivar (Chavez et al., 2000).

Os diferentes estudos experimentais vêm correlacionando positivamente freqüentes infiltrados de células inflamatórias ao fator pró-inflamatório, o qual leva a inflamação da glândula submandibular em camundongos Nod diabéticos. Contudo, alguns resultados imunohistoquímicos relacionam este fator pró-inflamatório ora inibindo e ora acelerando estes processos inflamatórios (Jacob et al., 1990; Yang et al., 1994). Ainda, em roedores comprovadamente diabéticos observou-se aumento da atividade da enzima cisteína, a qual esta envolvida no mecanismo de morte celular programada nas glândulas parótida e submandibular (Robinson, 1996; Kong et al., 1998; Yamachika et al., 1998). Outros experimentos, utilizando ratos diabéticos induzidos quimicamente e camundongos espontâneamente diabéticos, evidenciaram diminuição do volume dos ácinos, diminuição do peso da glândula submandibular, decréscimo dos ductos granulares e da densidade dos grânulos secretores, acúmulo de gotas lipídicas tanto nas células acinares como nos ductos intercalares, além do retardo no crescimento da glândula parótida e submandibular (Anderson, 1983; High et al., 1985; Hu et al., 1992; Anderson et al., 1994; Leigh et al., 1994; Szczepanski et al., 1998).

Também, é descrito que os níveis dos fatores de crescimento homólogos à insulina (IGF), polipeptídios responsáveis pela homeostase funcional das glândulas salivares, são diminuídos em glândulas submandibulares de camundongos jovens espontâneamante diabéticos. Porém, em camundongos diabéticos adultos os níveis de IGF aumentam progressivamente. Apesar de controvertido, a presença desse fator de crescimento em glândulas salivares de camundongos diabéticos adultos pode estar relacionada à ocorrência de linfócitos T o que provoca o aumento da expressão do IGF (Hayashi, 1994; Kerr et al., 1995; Mustafa et al., 2001).

A literatura também descreve a ação da insulina sobre os tecidos glândulas salivares. Em experimento com camundongos Nod espontaneamente diabéticos, mesmo com controle glicêmico por 78 horas, demonstrou diminuição dos níveis de insulina na saliva bem como no plasma sangüíneo (Kerr et al., 1995). Em estudos com ratos e camundongos quimicamente diabéticos, tratados com única dose insulínica, não se observaram diferenças significantes no peso da glândula parótida, demonstrando a recuperação no peso deste órgão após o tratamento insulínico (Anderson, 1986). Também, após tratamento com dose única de insulina, ratos induzidos quimicamente ao diabetes mostraram aumento da síntese protéica na saliva produzida por glândulas submandibulares. Ainda, caracterizou-se em ratos induzidos quimicamente ao diabetes com tratamento insulínico diário por 7 dias a recuperação dos níveis de secreção salivar (Anderson, 1983).

Já Chan et al. 1993, após administrarem insulina por 7 dias em ratos quimicamente diabéticos não evidenciaram recuperação dos níveis protéicos na secreção salivar de glândulas submandibulares. Assim, os autores também relataram que esses achados não refletem a recuperação total da função nem tão pouco da morfologia glandular (Anderson & Shapiro, 1980; Watanabe et al., 2001). Como, demonstrado em outro experimento com reposição insulínica por períodos de 3 horas, 1, 3 e 7 dias em ratos diabéticos, onde não observou-se a reestruturação morfológica da glândula parótida sugerindo que o mecanismo pelo qual a insulina atua nas glândulas salivares é complexo e ainda não esta totalmente esclarecido (Anderson, 1983).

GLÂNDULAS SALIVARES MAIORES

A saliva é produzida pelas glândulas salivares as quais drenam suas secreções na cavidade bucal (Mjör & Fejerskov, 1990). Estas secreções apresentam íons, proteínas, imunoglobulinas e insulina além de fatores de crescimento homólogos a insulina (IGF). Estes fatores de crescimento são sintetizados principalmente nas células dos ductos granulares e estriados das glândulas submandibulares e parótidas (Patel, 1986; Ryan et al., 1992; Kerr et al., 1995; Katz, 2003). As ações tanto do IGF quanto da insulina são mediadas por receptores específicos. Em camundongos, durante a morfogênese glandular, pôde-se observar que a expressão dos receptores de IGF (IGF-R) e de insulina (INS-R) são mais evidentes no epitélio do que no mesênquima (Jaskoll & Melnick, 1999). A principal função do IGF-R é mediar às ações do IGF (Mustafa et al., 2001).

O IGF contribui para a homeostase funcional de diferentes tecidos, além da atividade mitótica de várias células incluindo fibroblastos, condroblastos, osteoblastos e células musculares lisas, sendo este fator controlado por hormônios pituitários (Zumstein & Stiles, 1987). Os níveis dos fatores de crescimento homólogos à insulina são similares em camundongos machos e fêmeas, indicando não haver diferenças sexuais (Kerr et al., 1995). As glândulas salivares são consideradas reservatórios de vários fatores de crescimento, incluindo da insulina e do IGF-I, mas a função fisiológica desta reserva ainda não está totalmente esclarecida (Kagami et al., 2000).

Anatomicamente, as glândulas salivares tanto em seres humanos como em roedores são divididas em maiores e menores, sendo as maiores as glândulas parótidas, submandibulares e sublinguais e as menores as labiais, da bochecha, linguais e palatinas. Também, é descrito que as glândulas salivares são divididas funcionalmente em serosas, mucosas e mistas.

As glândulas salivares menores espalham-se por toda a extensão da mucosa bucal e estão localizadas tanto na lâmina própria como na submucosa (Pinkstaff, 1980; Mjör & Fejerskov, 1990). A secreção serosa das glândulas salivares menores diminui progressivamente da região labial para a orofaringe, onde apresenta apenas secreção mucosa (Tomasi, 1977).

As glândulas salivares maiores, tanto em seres humanos como em roedores, possuem padrão comum sendo usualmente descritas como tubuloacinares. Nas glândulas submandibulares ocorrem túbulos serosos, ductos granulares e os ácinos seromucosos os quais são formados por células colunares mucosas associadas às células semilunares serosas (Tomasi, 1977; Pinkstaff, 1980). As células serosas apresentam extenso retículo endoplasmático granular perinuclear, pequenos grânulos eletron-densos na região apical e complexo de golgi conspícuo. As células mucosas possuem núcleo localizado na região basal, o retículo endoplasmático granular localizado basolateralmente, o complexo de golgi proeminente contendo inúmeras vesículas e as mitocondrias apresentando-se na região perinuclear. Os grânulos de secreção mucosos ocupam a maior parte do citoplasma supranuclear (Taga & Sesso, 1979; Pinkstaff, 1980; Mjör & Fejerskov, 1990). Os ácinos serosos, das glândulas parótidas de roedores, produzem secreção rica em mucoproteína sendo também chamados de ácinos seromucosos apresentando esses uma única camada celular com formato piramidal distribuídas ao redor de lúmem central. As células colunares possuem núcleo esférico localizado na região basal. O citoplasma basal é intensamente basófilo, enquanto o apical é eosinófilo e granular. A basofilia é devido a sua função secretora protéica (Pinkstaff,1980).

II-JUSTIFICATIVA E OBJETIVO

A saliva produzida pelas glândulas salivares contribui na defesa biológica e nos processos digestivos mantendo a homeostase fisiológica do organismo. Estudos clínicos e experimentais vêm demonstrando que o diabetes mellitus compromete a morfologia e os mecanismos de salivação os quais são relevantes para a patogênese de doenças relacionadas às glândulas salivares (Edgar & O'Mullane, 1996; Chavez et al., 2000; Takahashi et al., 2002). Assim, o objetivo do presente estudo foi analisar os efeitos do tratamento insulínico prolongado sobre as células responsáveis pelo processo secretor das glândulas salivares parótidas e submandibulares de camundongos diabéticos autoimunes e também sobre a expressão dos receptores de insulina (INS-R) e sobre os receptores para fatores de crescimento homólogos a insulina (IGF-IR). Além disso, pretendeu-se correlacionar o comportamento celular frente aos processos de manutenção e renovação tecidual das glândulas salivares.

III-MATERIAIS E MÉTODOS

Um total de 45 camundongos fêmeas com 18 semanas de idade, pesando em média 20 gramas provenientes do Centro de Bioterismo da Unicamp (CEMIB, Campinas, São Paulo), foram divididos em 5 grupos: 9 camundongos Nod diabéticos por 10 dias (grupo 1), 9 camundongos Nod diabéticos por 20 dias (grupo 2), 9 camundongos Nod diabéticos por 10 dias (grupo 3), 9 camundongos Nod diabéticos por 20 dias (grupo 4) e 9 camundongos BALB/c não diabéticos (grupo 5). Os animais dos grupos 3 e 4 receberam diariamente insulina via subcutânea na região dorsal (Insulina NPH Altamente Purificada, Biobrás,

Montes Claros, MG, Brasil) na dosagem de 0.20 mg/100g (4 a 5 U) de peso corpóreo durante 20 dias (Anderson, 1983). Os animais dos grupos 1, 2 e 5 receberam diariamente injeções via subcutânea de solução fisiológica pelo mesmo período simulando as condições experimentais do tratamento insulínico. Os animais dos grupos 3 e 4 foram controles negativos do grupo 1 e 2 comprovadamente diabéticos. Nos animais pertencentes aos três grupos experimentais se fez monitoramento diário do sangue, onde foram avaliados os níveis de glicemia (mg/dl), sendo considerado diabético valores superiores a 400 mg/dl (Hu et al., 1992; Shirai et al., 1998). O sangue desses animais foi coletado e analisado no aparelho MediSense Optium (MediSense Optium, Abbott, Bedford, MA, USA). No momento do sacrifício, os camundongos fêmeas de todos os grupos experimentais foram submetidos à coleta de esfregaços vaginais para determinação do ciclo estral. Para não ocorrer interferências de determinando ciclo, não foi priorizado fases específicas (Todos os procedimentos de acordo com o Comitê de Ética Animal-COBEA). A seguir, os animais foram anestesiados com Francotar/Virbaxil (1:1), na dosagem de 0,25 ml/100g de peso corpóreo e procedeu-se à coleta das amostras para análises em microscopias de luz, eletrônica de transmissão e varredura e imunohistoquímica para expressão do IGF-IR e do INS-R. Todos os animais estudados receberam como dieta sólida ração Purina® (Nuvilab CR 1, SP, Brasil) na forma de grãos e água ad-libitum. Ao longo do período experimental foram realizadas mensurações diárias dos consumos de líquido e sólido dos animais estudados. Os pesos corpóreos (g) também foram verificados no início e no final do experimento.

III.1- Macroscopia

As glândulas salivares parótida e submandibular foram retiradas através da dissecação com lâmina de bisturi cirúrgico n.º 15, tomando como referência à orelha externa do camundongo, a base da mandíbula, o ápice da mandíbula e o osso esterno. A incisão realizada foi em forma de "Y" com aproximadamente 4,0 cm. Os músculos esternomastóideo e masseter não foram removidos (Figura 1).

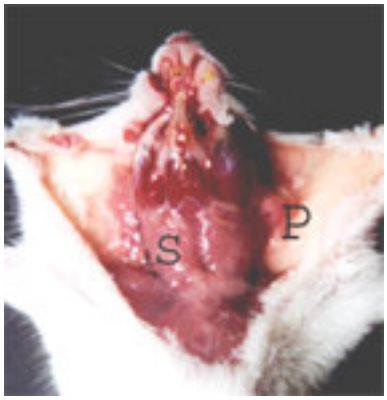

Figura 1: Coleta das Glândulas Salivares. Observa-se as Glândulas Submandibulares (S) e as Glândulas Parótidas. x 5.

III.2- Microscopia de Luz

Análise Estereológica

As amostras das glândulas parótidas e submandibulares foram coletadas, de cinco animais de cada grupo experimental, fixadas em Bouin (Solução aquosa saturada de ácido pícrico- 75 ml, formol- 25 ml, ácido acético glacial- 5 ml) por 12 horas para posterior processamento e inclusão em parafina.

Para a inclusão em parafina, os tecidos foram lavados em álcool 70% e, a seguir, sofreram desidratação em uma série crescente de álcoois (álcool 80 % - 2 vezes, álcool

absoluto – 3 vezes; 1 a 2 horas cada). Posteriormente, os fragmentos foram diafanizados em xilol durante 1 a 2 horas até que se tornassem translúcidos. Os fragmentos foram incluídos em parafina e polímeros plásticos (Paraplast Plus, Polysciences, Niles, IL) a 56^0 C durante aproximadamente 1 hora; passando, em seguida, para nova parafina na mesma temperatura.

Os tecidos foram orientados e posicionados cuidadosamente no fundo das formas plásticas, visando à obtenção de cortes histológicos transversais. Os blocos foram trimados para a obtenção de superfícies planas e seccionados com cinco micrômetros de espessura. A seguir, os fragmentos foram colocados sobre as lâminas albuminizadas e levadas para a estufa a 60^0 C. Após a preparação dos cortes, estes foram corados com hematoxilina/eosina para estudo morfológico geral e tricrômico de masson (Behmer et al., 1976), sendo posteriormente fotografados no fotomicroscópio Axioskop Zeiss, em objetivas planacromáticas de 40 x e 100 x, usando-se filmes KodaK Tmax de 135 mm, ASA 100.

Estes cortes foram utilizados para a quantificação dos volumes citoplasmático e nuclear, onde foram medidos os diâmetros de 40 núcleos de cada animal, totalizando 200 núcleos por grupo experimental. Escolheram-se núcleos com limites definidos e cortes planos em toda extensão. As medidas foram realizadas com auxílio de ocular de 10X graduada com régua e acoplada ao microscópio de luz Olympus CBB, fixando-se as observações com a objetiva da 100X. Após as medições dessas estruturas, se procedeu a calibração da ocular com lâmina especial, provida de divisões de 0,01 mm (10 μm), visando transformar as unidades da ocular em micrometros. A partir destes valores foram calculadas as médias dos volumes dos núcleos através:

$$V=4/3\ \pi\ r^3$$

Para núcleos esféricos, sendo “r” o raio do núcleo e

$$V=4/3\ \pi\ (d/2)^2\ D/2$$

Para núcleos elípticos, sendo “d” diâmetro menor e “D” diâmetro maior.

Além disso, foram medidas para determinação quantitativa as frações de volume (Vv) ocupadas pelo núcleo e pelo citoplasma. Essas medidas foram realizadas através de uma ocular 10X contendo um retículo de integração quadrilátero com 400 pontos acoplado ao microscópio de luz Olympus CBB e objetiva de 40X. Foi realizada a contagem de pontos localizados sobre o núcleo e citoplasma de células de quatro campos previamente definidos. A fração de volume ocupada pelo núcleo em relação ao citoplasma foi calculada utilizando a seguinte formula:

$$Vv=p/P$$

Onde Vv equivalem à densidade de volume ou fração de volume (%)

p equivale ao número de pontos sobre o núcleo

P equivale ao número total de pontos ou soma dos pontos sobre o núcleo e citoplasma nos diferentes campos.

O volume citoplasmático foi calculado a partir da relação entre Vv do citoplasma, Vv do núcleo e do volume do núcleo. A determinação do volume celular foi feita a partir da soma dos valores obtidos para os volumes nuclear e citoplasmático.

Todas as mensurações foram realizadas através de ocular graduada (10 mm/100, Zeiss) acoplada ao microscópio Olympus CBB equipado com lente de 100X (Weibel, 1979; Mandarim de Lacerda, 1995). O softwear The Image-pro® Express Version 4 foi utilizado com objetiva de 10X para determinar através da delimitação do diâmetro total a área relativa ocupada pelo estroma e pelas células acinares.

III.3- Microscopia Eletrônica

Para análise de microscopia eletrônica de transmissão às amostras foram coletadas de quatro animais de cada grupo experimental, fixadas em Karnovsky (solução de Paraformaldeído 4% e glutaraldeído 2,5% em tampão fosfato Sörensen) por 24 horas (Karnovsky, 1965). Em seguida, os fragmentos foram submetidos ao complemento da fixação por imersão no mesmo fixador por mais 12 horas e pós-fixados em tetróxido de ósmio a 1 % em tampão fosfato, 0.1 M, pH 7.4 por duas horas. A seguir, os fragmentos foram lavados em água destilada e contrastados com acetato de uranila a 0.5 % em solução aquosa de sacarose por 12 horas posteriormente, os fragmentos foram desidratados em série crescentes de acetona e incluídos em resina (Araldite Polysciences, Niles, IL). Em seguida, os blocos foram cortados em um ultramicrótomo LKB 8800 ultratome III com 0.5 a 0.8 micrometros de espessura. Os cortes semifinos foram realizados com navalha de vidro, montados em lâminas e corados com azul de toluidina a 1 %, para escolha da região desejada. A seguir fizeram-se cortes ultrafinos os quais foram montados em telas de cobre de 200 mesh e pós–contrastados com acetato de uranila e citrato de chumbo. Posteriormente, foram examinados e fotografados em um microscópio eletrônico de transmissão LEO – 906.

Para análise em microscopia eletrônica de varredura os mesmos animais foram utilizados. As amostras foram pós-fixadas em glutaraldeído 2,5% por 12 horas, após este período foi realizado a criofratura e preparo para análise (Wahlqvist et al., 1996). O material foi examinado e fotografado no microscópio eletrônico de transmissão LEO-906 e no microscópio eletrônico de varredura JEOL JSM 5800LV do Laboratório de Microscopia Eletrônica do Instituto de Biologia (Unicamp, Campinas, São Paulo).

III.4- Imunohistoquímica

Parte das amostras retiradas para microscopia de luz foi congelada em nitrogênio líquido, a seguir cortes de 12 µm de espessura foram realizados utilizando o Criostato (Microm HM 505 E, CA, USA). Os cortes foram fixados utilizando metanol e acetona (1:1) a 4^0C por períodos de 3 minutos e pós-fixados em paraformoldeido a 4% durante 10 minutos, a seguir os cortes foram lavados em solução salina tamponada (PBS). Posteriormente, as amostras foram incubadas em solução bloqueadora durante 1 hora em temperatura de 20^0C para bloquear marcações inespecíficas. A seguir os anticorpos primários para receptores de INS-R (Santa Cruz, CA, USA) e para receptores de IGF-IR (Santa Cruz, CA, USA) foram diluídos em solução bloqueadora (1:50) e aplicados sobre os cortes durante 12 horas à 4^0C. Posteriormente os cortes foram lavados em tampão PBS e incubados em anticorpo secundário fluoresceina-conjugada (anti-rabbit IgG-FITC, Santa Cruz, CA, USA) diluído em solução bloqueadora (1:100). Após, os cortes foram novamente lavados com PBS e montados em 1.4-diazabicyclo [2.2.2] octane (DABCO, para microscopia fluorescente, Sigma, St. Louis, USA) e observados ao Microscópio Confocal (MRC 1024UV, Hercules, CA, USA). Para estas observações foi utilizado o comprimento de onda de 488nm para excitação da fluoresceina e os ajustes de contraste, brilho e diâmetro da íris foram constantes para todos os grupos experimentais. Para obtenção das imagens utilizou-se objetiva de 40X (1.4 NA, imersão em água). Para controle negativo da imunomarcação parte das amostras não foi incubada em anticorpo primário. A intensidade da imunomarcação foi padronizada em intensa (+++), moderada (++) e fraca (+) de acordo com a concentração e distribuição dos receptores nos cortes teciduais (Markopoulos et al., 2000).

III.5- Análise Estatística

O estudo estatístico foi realizado para as seguintes variáveis: peso corpóreo inicial (g), peso corpóreo final (g), volume nuclear (μm^3), volume citoplasmático (μm^3), área relativa ocupada pelo estroma e pelas células acinares (%), consumo de ração (g) e consumo de líquido (ml), através da análise dos perfis médios entre os grupos e a variância paramétrica e não paramétrica complementada com os intervalos de confiança simultânea pelo teste de Tukey & Scheffé (Norman & Streiner, 1994). Todo o estudo foi realizado com 1% de significância (Montgomery, 1991).

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Stereology and Ultrastructure of the Salivary Glands of Diabetic Nod Mice Submitted to Long-Term Insulin Treatment

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ABSTRACT

Insulin-dependent diabetes mellitus compromises the salivary glands, altering their morphology and the mechanisms of salivation, which are fundamental for oral health. Thus, the aim of the present study was to determine the effects of prolonged insulin treatment on the morphology of the salivary glands in Nod mice. Forty-five female mice were divided into five groups: nine positive diabetic Nod mice for 10 days (group 1), nine positive diabetic Nod mice for 20 days (group 2), nine diabetic Nod mice for 10 days (group 3), nine diabetic Nod mice for 20 days (group 4), and nine nondiabetic BALB/c mice (group 5). Animals of groups 3 and 4 received 4–5 U of insulin daily, whereas animals of groups 1, 2, and 5 received the same dose of physiological saline simulating the experimental conditions. Samples of the salivary glands were analyzed by light, transmission, and scanning electron microscopies. The results showed intense alterations in diabetic animals characterized by nuclear and cytoplasmic atrophy, biomembrane disorganization, an increase in fibrillar components of the extracellular matrix, and the presence of inflammatory cells. Insulin treatment exerted positive effects on the recovery of the changes resulting from the diabetic state in both parotid and submandibular glands but the pattern continued to be altered. It can be concluded that, in addition to compromising the processes of tissue maintenance and renewal, tissue destructuring leads to alterations in functional mechanisms in both diabetic animals and animals submitted to glycemic control.

Key words: salivary gland; autoimmune diabetes; insulin treatment

Insulin-dependent diabetes mellitus affects approximately 10% of Western diabetic patients. This type of diabetes results from absolute insulin deficiency caused by the autoimmune destruction of β-cells (Hamilton and Blackwood, 1977). The relationship between diabetes mellitus and both cellular alterations and changes in salivary components has been studied in experimental and clinical investigations (Chavez et al., 2000; Watanabe et al., 2001; Takahashi et al., 2002). Generally, a decrease in salivary flow and in salivary components is observed in experimental animals and diabetic patients (Anderson and Shapiro, 1980; Chavez et al., 2000). Regarding the morphology, experiments conducted on chemically induced diabetic rats and autoimmune diabetic mice have demonstrated a reduction in acinar volume, growth retardation, a weight reduction of the parotid and submandibular glands, a

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TABLE 1. Body weight variation (final weight − initial weight) and fluid and solid intake in the experimental groups

Group	Weight variation (g)	Fluid intake (ml)	Solid intake (g)
I	-2.5 ± 1.2^a	171.9 ± 11.7^a	57.2 ± 3.0^a
II	-2.3 ± 1.4^a	173.6 ± 11.2^a	56.9 ± 2.7^a
III	1.4 ± 1.4^b	25.7 ± 3.8^b	46.8 ± 5.6^b
IV	1.1 ± 1.2^b	23.3 ± 3.0^b	48.9 ± 5.7^b
V	1.6 ± 1.1^b	31.9 ± 4.1^b	37.6 ± 4.2^b
Statistical result	12.9 ($P < 0.01$)	1139.3 ($P < 0.001$)	38.8 ($P < 0.001$)

Data are reported as mean ± S.D.
[a,b,c]Different letters indicate statistical differences at the 1% level of significance.

decline in the number of granular ducts and in the density of secretory granules, as well as accumulation of lipid droplets in acinar cells and intercalated ducts (Anderson, 1983; High et al., 1985; Hu et al., 1992; Anderson et al., 1994; Leigh et al., 1994). In addition, the occurrence of an inflammatory process consisting of mononuclear cells located around blood vessels, acini, and ducts has been reported (Goillot et al., 1991; Hu et al., 1992; Pozzilli et al., 1993; Humphreys-Beher, 1998; Yamano et al., 1999). On the other hand, an increase in protein synthesis in the submandibular glands has been observed in chemically induced diabetic rats after they had received a single dose of insulin. However, the authors did not characterize both functional and morphological recovery of the glands (Anderson and Shapiro, 1980; Watanabe et al., 2001). Also, no significant recovery of parotid gland weight was observed in other studies administering a single dose of insulin to chemically induced diabetic rats (Anderson, 1987; Pinkstaff, 1996). Similarly, another experiment conducted on diabetic rats submitted to insulin replacement for 7 days failed to characterize the morphological and functional restructuring of the parotid and submandibular glands (Anderson, 1983; Chan et al., 1993). Thus, considering the functional involvement of the salivary glands in the organism's biological defense and in digestive processes, in addition to the relevance of therapeutic effects of insulin on the recovery of these glands, the aim of the present study was to determine the effects of long-term insulin treatment on the morphology of the salivary glands in NOD mice.

MATERIALS AND METHODS

Animals and Tissue Preparation

Forty-five female mice aged 18 weeks, provided by Animal Care Center (CEMIB/State University of Campinas), were divided into five groups: nine positive diabetic Nod mice for 10 days (group 1), nine positive diabetic Nod mice for 20 days (group 2), nine diabetic Nod mice for 10 days (group 3), nine diabetic Nod mice for 20 days (group 4), and nine nondiabetic BALB/c mice (group 5). Animals of groups 3 and 4 were administered subcutaneously into the dorsal region with insulin (highly purified mixed NPH insulin; Biobrás, Montes Claros, Minas Gerais, Brazil) at the dose of 0.20 mg/100 g (4–5 U) for 20 days (Anderson, 1983). Animals of groups 1, 2, and 5 received daily injections of physiological saline for the same period of time to simulate the experimental conditions of insulin treatment. Solid (Nuvilab CR 1, São Paulo, Brazil) and fluid intake was quantified on a daily basis. Blood glucose levels were measured twice a day in each animal (MediSense Optium; Abbott, Bedford, MA), with animals presenting values higher than 400 mg/dl being considered diabetic (Hu et al., 1992; Shirai et al., 1998). All animals were anesthetized with Francotar/Virbaxil (1:1/0.25 ml/100 g body weight) and sacrificed (according to Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation, COBEA). Samples of the salivary glands were then removed for light, transmission, and scanning electron microscopic analyses. For light microscopy, salivary gland samples obtained from five animals of each group were fixed in Bouin's solution (picric acid solution), embedded in plastic resin (Araldite; Polysciences, Niles, IL), and stained with hematoxylin and eosin (H&E) and Masson's trichrome (Behmer et al., 1976). Photomicrographs were obtained with a Nikon photomicroscope. For scanning and transmission electron microscopies, four animals were ultilized (Karnovsky, 1965; Wahlquist et al., 1996). Specimens were then dehydrated, embedded in plastic resin (Polysciences), cut into ultrathin sections with an LKB ultramicrotome, and stained with uranyl acetate and lead citrate (Watson, 1958; Reynolds, 1963). The material was examined and photographed under a LEO-906 transmission electron microscope and JEOL JSM 5800LV scanning electron microscope at the Laboratory of Electron Microscopy, Institute of Biology, State University of Campinas.

Stereological Procedures

Cytoplasmic and nuclear volumes as well as relative area were measured. Nuclear and cytoplasmic volumes were recorded as the average of 200 measurements per experimental group. Long and short axes were measured, and the mean nuclear volume was calculated considering the nuclei to be ellipsoid (Weibel, 1979). The Image-Pro Express version 4 software with a 10× objective was used to determine the % relative area (stroma × acinar cells).

Statistical Analysis

The nonparametric Tukey and Scheffé tests were used to analyze body weight variations (final weight minus initial weight), relative area, and mean nuclear and cytoplasmic volume (μm^3), followed by a multiple-comparison test involving all group pairs (Norman and Streiner, 1994). The level of significance between groups was set at 1% (Montgomery, 1991).

RESULTS

Blood Glucose Levels

Mean blood glucose level was 140 mg/dl in groups 3–5 and 850 mg/dl in groups 1 and 2.

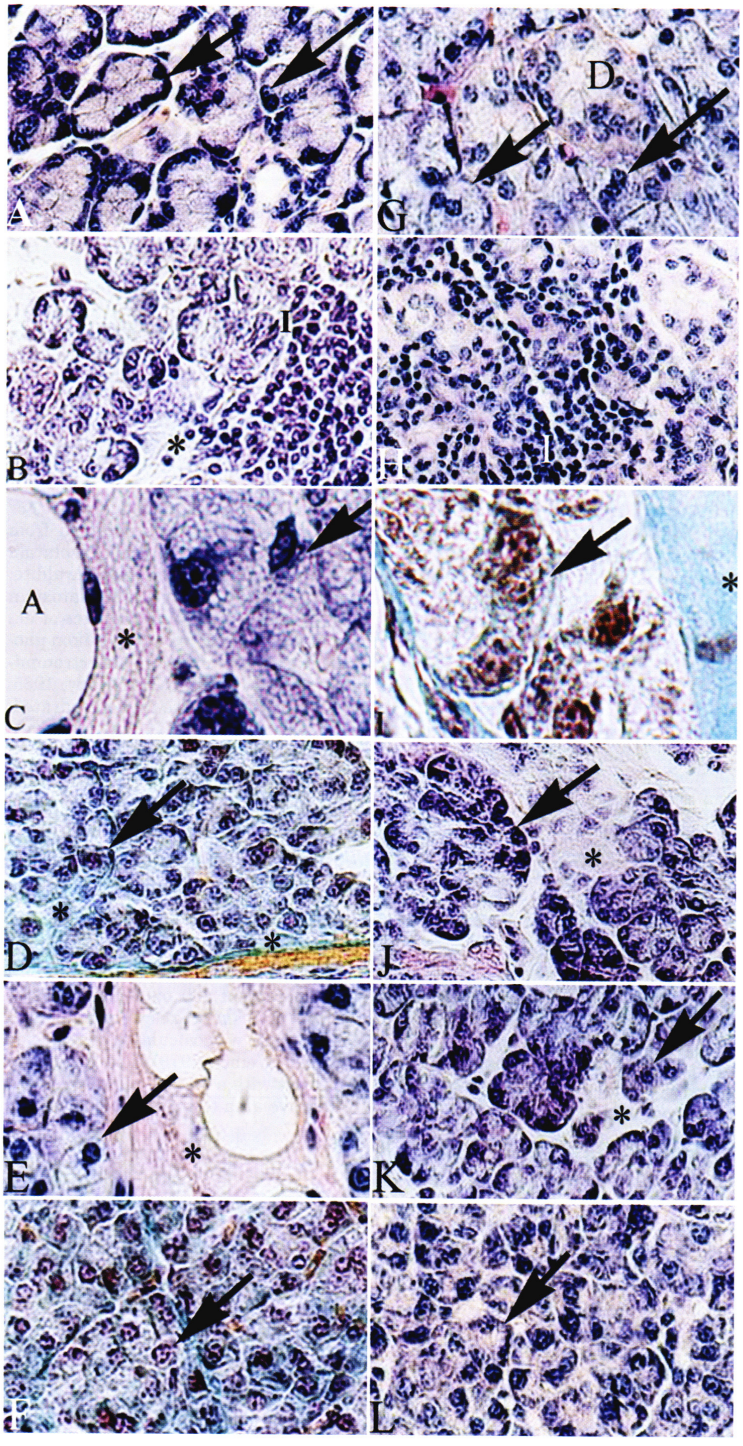

Fig. 1. Photomicrograph of the parotid gland. **A:** Group 5 serous acini (short arrow) and nuclei (long arrow). Magnification: 250×. H&E. **B** and **C:** Group 1 inflammatory processes (I) and enlarged stroma (asterisk). Magnification: 250×. H&E. Cellular atrophy (short arrow), enlarged stroma (asterisk), and adipocytes (A). Magnification: 750×. H&E. **D:** Group 2 reduced cells (short arrow) and enlarged stroma (asterisk). Magnification: 250×. Masson. **E:** Group 3 atrophied cells (short arrow) and enlargement of the stromal space (asterisk). Magnification: 250×. H&E. **F:** Group 4 reduced cellular volume (short arrow). Magnification: 250×. Masson. Photomicrograph of the submandibular gland. **G:** Group 5 seromucous acini (short arrow), nuclei (long arrow), and striated duct (D). Magnification: 250×. H&E. **H:** Group 1 inflammatory cells (I). Magnification: 250×. H&E. **I:** Group 1 enlarged stroma (asterisk) and atrophied cells (short arrow). Magnification: 750×. Masson. **J:** Group 2 reduced cells (short arrow) and enlarged stromal space (asterisk). Magnification: 250×. H&E. **K:** Group 3 cellular atypia (short arrow) and enlargement of the stromal compartment (asterisk). Magnification: 250×. H&E. **L:** Group 4 cellular atypia (short arrow). Magnification: 250×. H&E.

TABLE 2. Nuclear (n) and cytoplasmic (c) volume in the different experimental groups

Group	Treatment	Parotid gland (n)	Parotid gland (c)	Submandibular gland (n)	Submandibular gland (c)
I	Saline	38.2 ± 1.7[a]	91.4 ± 10.5[a]	31.9 ± 2.9[a]	87.2 ± 9.5[a]
II	Saline	15.3 ± 4.6[b]	52.7 ± 11.7[b]	12.2 ± 1.8[b]	41.8 ± 6.9[b]
III	Insulin	85.7 ± 9.7[c]	189.7 ± 36.5[c]	79.4 ± 11.4[c]	170.6 ± 48.6[c]
IV	Insulin	74.6 ± 19.5[d]	138.6 ± 36.7[d]	67.9 ± 13.7[d]	126.0 ± 30.4[d]
V	Saline	304.0 ± 96.2[e]	465.2 ± 134.6[e]	223.2 ± 10.6[e]	380.7 ± 19.6[e]

Data are reported as mean ± S.D.
[a,b,c,d,e]Different letters indicate statistical differences at the 1% level of significance.

Body Weight and Fluid and Solid Intake

Groups 1 and 2 showed a significant body weight loss compared to group 5. In contrast, in groups 3 and 4, body weight gain was lower than in group 5, but clearly higher than in groups 1 and 2 (Table 1).

Light Microscopy

Parotid gland. In control mice (group 5), adjacent serous acini consisting of columnar cells were noted (Fig. 1A, Tables 2 and 3). In animals of group 1, at 10 days of effective diabetic state, atrophic cells and inflammatory cells were observed, as well as enlargement of the interacinar space and the presence of adipocytes scattered throughout the stroma (Fig. 1B and C, Tables 2 and 3). In diabetic animals of group 2, pleomorphic serous acini and enlarged interacinar space (Fig. 1D, Tables 2 and 3) were observed. After insulin treatment, atrophied serous acini were noted in animals of group 3 but at a lower intensity than in group 1 and 2 animals. Interacinar spacing was demonstrated by an increase in glandular stroma (Fig. 1E, Tables 2 and 3). Animals of group 4 were characterized by a marked volumetric reduction of serous acini after insulin treatment compared to group 3 animals (Fig. 1F, Tables 2 and 3).

Submandibular gland. In control mice (group 5), adjacent seromucous acini formed by cuboidal cells were observed (Fig. 1G, Tables 2 and 3). In animals of group 1, atrophic cells and enlarged stroma were observed, as well as the presence of inflammatory cells (Fig. 1H and I, Tables 2 and 3). In group 2 animals, pleomorphic acini with significant reduction in cytoplasm and nucleus were observed, as well as a clearly visible interacinar space (Fig. 1J, Tables 2 and 3). After insulin treatment, animals of group 3 demonstrated acinar atrophy, which, however, was accompanied by relative recovery when compared to the other diabetic groups without insulin treatment. The interacinar space was clearly visible, with enlargement of stromal elements (Fig. 1K, Tables 2 and 3). Atypical acini were observed in diabetic animals of group 4 after insulin treatment (Fig. 1L, Tables 2 and 3).

Electron Microscopy

Parotid gland. Control animals showed a glandular epithelium consisting of columnar serous cells. In group 5 animals, a nucleus located in the basal region was observed, which was delimited by a regular nuclear envelope and homogeneous chromatin distribution. The cisternae of the parallel granular endoplasmic reticulum were located in the perinuclear region. Mitochondria and secretory granules were distributed in the apical and perinuclear region. The plasma membrane was regular. Collagen fibers were observed in the glandular stroma (Fig. 2A–C). In group 1 animals, atrophic cells were noted. Spherical mitochondria were present in the perinuclear region. The enlargement of the interacinar space was characterized by the accumulation of collagen fibers (Fig. 2D). In group 2, the alterations were characterized by reduced acini. The endoplasmic reticulum cisternae were dilated and digestive vacuoles were present, as well as an irregular plasma membrane (Fig. 2E and F). In group 3 animals, atrophic acini, mitochondria, and granular endoplasmic reticulum were present in the perinuclear region. The interacinar space was enlarged (Fig. 2G). A decrease in cell volume was also observed in group 4 animals. Mitochondria, granular endoplasmic reticulum, as well as apical secretory granules were noted. A folded plasma membrane delimited the enlarged interacinar space, which was characterized by intense accumulation of collagen fibers (Fig. 2H and I).

Submandibular gland. The glandular acini were seromucous and cuboidal. In group 5 mice, the nucleus was delimited by a regular nuclear envelope and showed homogeneous chromatin distribution. Seromucous secretory granules were located in the apical region. Mitochon-

TABLE 3. Relative area (%) of the acinar cells and stroma of salivary glands

Group	Treatment	Parotid gland (n)		Submandibular gland (c)	
		Acinar cells	Stroma	Acinar cells	Stroma
I	Saline	58[a]	42[a]	54[a]	46[a]
II	Saline	37[b]	63[b]	36[b]	64[b]
III	Insulin	61[a]	39[a]	60[a]	40[a]
IV	Insulin	42[b]	58[b]	39[b]	61[b]
V	Saline	80[c]	20[c]	80[c]	20[c]

Data are reported as mean ± S.D.
[a,b,c]Different letters indicate statistical differences at the 1% level of significance.

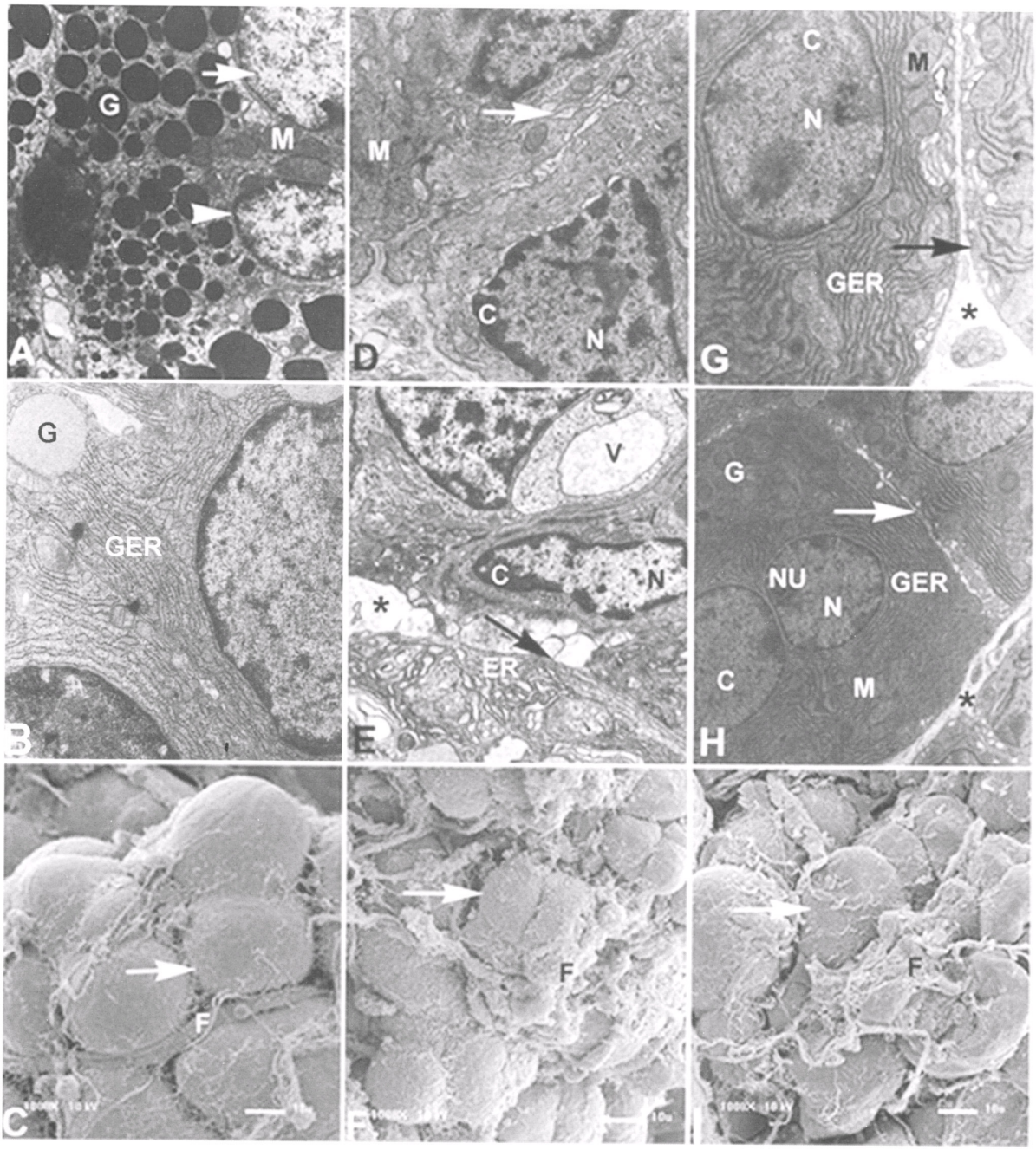

Fig. 2. Electron micrographs of the parotid gland. **A:** Group 5 serous acini containing a clearly visible nucleus (arrowhead) with homogeneously distributed chromatin (arrow), mitochondria (M), and secretory granules (G). Magnification: 5.397×. **B:** Group 5 granular endoplasmic reticulum (GER) and secretory granules (G). Magnification: 11.625×. **C:** Group 5 serous acini (arrow) and collagen fibers (F). Magnification: 1,000×. **D:** Group 1 atrophied serous acini characterized by an irregular plasma membrane (arrow) nucleus (N) and chromatin close to the nuclear envelope (C). Mitochondria (M) are present. Magnification: 11.625×. **E:** Group 2 atrophied cells and enlarged interacinar space (asterisk), irregular plasma membrane (arrow), as well as atypical nucleus (N) with condensed chromatin close to the nuclear envelope (C). Digestive vacuoles (V) and dilated endoplasmic reticulum (ER). Magnification: 11.625×. **F:** Group 2 reduced acini (arrow) and increased fibrillar components (F). Magnification: 1,000×. **G:** Group 3 atrophied cells and enlarged stroma (asterisk), a regular plasma membrane (arrow), as well as the nucleus (N) with chromatin (C). Mitochondria (M) and granular endoplasmic reticulum (GER). Magnification: 11.625×. **H:** Group 4 reduced cells, enlarged stroma (asterisk), and a regular plasma membrane (arrow). Nucleus (N) shows homogeneously chromatin (C) and a nucleolus (NU). Granular endoplasmic reticulum (GER), secretory granules (G), and mitochondria (M). Magnification: 11.625×. **I:** Group 4 atrophied acinus (arrow) and the increased fibrillar components (F). Magnification: 1,000×.

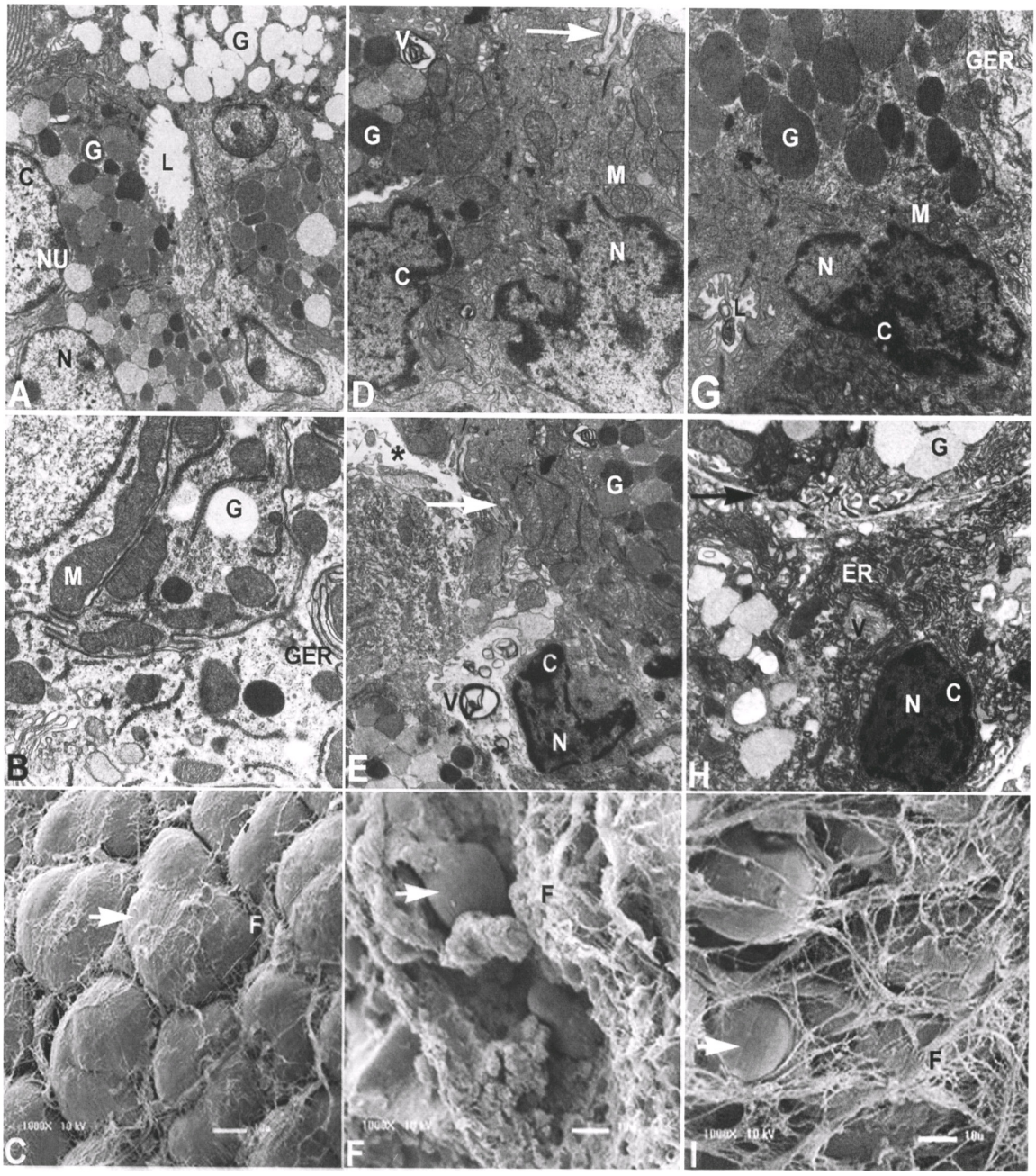

Fig. 3. Electron micrographs of the submandibular gland. **A:** Group 5 seromucous acini with nucleus (N) and chromatin (C). Cytoplasm containing seromucous secretory granules (G) and a lumen of the acini (L). Magnification: 4.176×. **B:** Group 5 granular endoplasmic reticulum (GER), secretory granules (G), and mitochondria (M). Magnification: 11.625×. **C:** Group 5 seromucous acini (arrow) and collagen fibers (F) are also observed. Magnification: 1,000×. **D:** Group 1 atrophied acini, an irregular plasma membrane (arrow), and nucleus (N) containing condensed chromatin close to the nuclear envelope (C). Cytoplasm with mitochondria (M), secretory granules (G), and digestive vacuoles (V). Magnification: 11.625×. **E:** Group 2 reduced cells, enlarged stroma space (asterisk), an irregular plasma membrane (arrow), and atypical nucleus (N) with chromatin close to the nuclear envelope (C). Cytoplasm with digestive vacuoles (V) and secretory granules (G). Magnification: 11.625×. **F:** Group 2 atrophied acinus (short arrow) and fibrillar components (F). Magnification: 1,000×. **G:** Group 3 reduced cells and nucleus (N) with chromatin (C). Mitochondria (M), granular endoplasmic reticulum (GER), secretory granules (G), and a lumen of the acini (L). Magnification: 11.625×. **H:** Group 4 cellular atrophy delimited by a regular plasma membrane (arrow) and nucleus (N) with chromatin close to the nuclear envelope (C). Endoplasmic reticulum (ER), secretory granules (G), and digestive vacuoles (V). Magnification: 11.625×. **I:** Group 4 reduced acinar volume (arrow) and increase in fibrillar components (F). Magnification: 1,000×.

dria and granular endoplasmic reticulum with parallel and flattened cisternae were noted in the perinuclear region. The plasma membrane was regular and collagen fibers were observed in the interacinar space (Fig. 3A–C). Group 1 animals showed cellular reduction. Mitochondria located in the perinuclear region, secretory granules, as well as digestive vacuoles were observed. The plasma membrane was irregular and the basal lamina was folded, delimiting an enlarged interacinar space (Fig. 3D). In group 2, marked volumetric reduction was noted. The atrophic cytoplasm contained mitochondria, as well as digestive vacuoles. Seromucous secretory granules were observed in the apical region. The plasma membrane was also irregular (Fig. 3E and F). In group 3, atrophic acini were observed. Perinuclear mitochondria, granular endoplasmic reticulum, and secretory granules were noted. The plasma membrane was regular (Fig. 3G). In group 4, the animals showed reduced cells. Digestive vacuoles, secretory granules, and dilated endoplasmic reticulum cisternae could be identified. The plasma membrane was regular (Fig. 3H and I).

DISCUSSION

The present study showed that, although the diabetic animals consumed a larger amount of ration and fluid, they had an important fall in their body weight. Whereas in animals submitted to prolonged glycemic control, recovery of the body weight was observed. The diabetes mellitus provokes metabolic disorders in various organ systems, including a reduction in body weight and destructuring of different tissues as demonstrated by atherosclerosis, retinopathies, nephropathies, sexual disorders, and alterations in healing processes, in addition to compromising the lining mucosae (Daubresse et al., 1978; Fushini et al., 1980; Makino et al., 1980; Ho, 1990; Cagnon et al., 2000; Conget, 2002; Caldeira et al., 2004). However, an increase in body weight has been observed in diabetic rats submitted to glycemic control for 7 days (Anderson, 1983; He et al., 2004). These findings indicate that diabetes compromises general body metabolism, leading to weight loss, and that glycemic control is an effective treatment for the recovery of body weight in diabetic animals. With respect to glycemia, diabetic animals not submitted to insulin treatment showed elevated levels of glucose, whereas these levels returned to normal in animals submitted to glycemic control, similar to control group. According to Hu et al. (1992), normal glucose levels are close to 180 mg/dl in control animals, with levels higher than 400 mg/dl being considered an effective diabetic state. Thus, the present findings confirm the diabetic state of the animals and demonstrate the efficacy of insulin treatment in the control of glycemic levels.

Morphological alterations in the salivary glands were detected not only in the uncontrolled diabetic groups but also in those on glycemic control. Some of the structural changes observed in the present experiment have also been reported in the literature. Several investigators found that, on average, after 20 days of effective diabetic state both chemically induced diabetic rats and autoimmune diabetic mice showed a reduction in submandibular gland weight, a decrease in granular ducts and in the density of secretory granules, and accumulation of lipid droplets in acini and intercalated ducts, in addition to growth retardation of the parotid and submandibular glands (Anderson, 1983; High et al., 1985; Hu et al., 1992; Anderson et al., 1994; Leigh et al., 1994). Furthermore, clinical and experimental studies have demonstrated a reduction in secretory and salivary components, as well as a severe inflammatory reaction accompanied by the presence of mononuclear cells first located around blood vessels and then around acini and ducts (Goillot et al., 1991; Hu et al., 1992; Pozzilli et al., 1993; Humphreys-Beher et al., 1998; Yamano et al., 1999; López et al., 2003). Differences in the tissue responses between parotid and submandibular glands have already been reported for chemically induced diabetic rats, mainly in terms of parotid gland weight, which did not show significant alterations (Anderson and Johnson, 1981). Also, chemically induced diabetic rats submitted to glycemic control for a period of 1 hr to 7 days demonstrated recovery of salivary secretion levels and a decline in the accumulation of lipid droplets in the cytoplasm of parotid and submandibular salivary glands (Anderson, 1983; Morris et al., 1992; Watanabe et al., 2001). However, the authors emphasized that this treatment does not reflect total recovery of glandular function or morphology. These studies underline the fact that the mechanism whereby diabetes and insulin affect salivary glands continues to be unknown (Anderson and Shapiro, 1980). Based on the present results, it can be concluded that diabetes provokes structural alterations in both the parotid and submandibular glands, leading to deficiencies in the processes of tissue maintenance and renewal, in addition to compromising functional mechanisms. Moreover, it can be observed that despite glycemic control, important cellular disorganizations continued to be present, especially in the submandibular glands, demonstrating that even prolonged insulin treatment was not sufficient to cause structural reorganization of the salivary glands.

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The Anatomical Record

"IGF-I and Insulin receptor expression in the salivary glands of diabetic NOD mice submitted to long-term insulin treatment"

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ABSTRACT

Studies have demonstrated that metabolic disorders such as diabetes change the functional mechanism not only of insulin but also of the insulin-like growth factor in different organs, including the salivary glands. However, glandular features related to cellular and molecular mechanisms involved in the actions of these elements in the salivary glands remain unclear, as well as the contribution of these polypeptides in the glandular pathogenesis. Then, the aim of this work was to characterize the IGF-I and insulin receptor expression in the submandibular and parotid glands of autoimmune diabetic mice, correlating to therapeutic effects of long-term insulin treatment on these receptors. A total of 45 female mice were divided into 5 groups with 9 mice each. Group 1 (diabetic Nod mice for 10 days), Group 2 (diabetic Nod mice for 20 days), Group 3 (diabetic Nod mice for 10 days with insulin treatment for 20 days), Group 4 (diabetic Nod mice for 20 days with insulin treatment for 20 days) and Group 5 (non diabetic mice). At the end of the experimental period, the animals were anesthetized and sacrificed. Next, fragments from the salivary glands were collected and processed for immunohistochemical analysis. The results showed that the prolonged diabetic state led to a steadily increased IGF-I receptor expression. Nevertheless, the insulin receptor expression was gradually decreased. Thus, it can be concluded that not only was the IGF-I receptor expression affected by the diabetic state but also the insulin receptor expression. Moreover, the period of the diabetic state was directly related to compromising the expression of these receptors. In spite of the insulin treatment having recovered the glycemic levels, the expression of insulin and the IGF-I receptors did not reach the standard level, which certainly hampered glandular function.

INTRODUCTION

Various biological processes such as the control of metabolic activities, cellular differentiation, cellular proliferation, apoptotic processes and tumorigenesis are influenced by biologically active peptides including several hormones. Among them are fibroblastic growth factors (FGF), vascular endothelium growth factors (VEGF), transforming growth factors (TGF), insulin-like growth factor (IGF) and insulin (INS) (Zumstein and Stiles, 1987; Ryan et al., 1992; Plum et al., 2005; Curigliano et al., 2005; Yakar et al., 2005). All these processes take place in different tissues including salivary glands, the central nervous system and accessory sex glands among others. (Yee et al., 1989; Ryan et al., 1992; Kerr et al., 1995; Schillaci et al., 1998; Djavan et al., 2001; Wetterau et al., 2003; Zhao et al., 2004; Bahr and Groner, 2005; Marszalek et al., 2005).

Insulin is found in blood plasma after being secreted by beta cells from the pancreas, stimulated by the blood glucose level. (Nattras and Halles, 1988). Also, insulin-like growth factors, including IGF-I and IGF-II, are found in blood plasma, besides different cellular types from mammals can synthesize and export them. The IGF regulatory system in each organ is tissue-specific (Djavan et al., 2001).

In the salivary glands, insulin-like growth factors are synthesized in the acinar cells, especially, in the region of the secretary ducts not only in the human species but also in rodents (Smith and Patel, 1984; Hansson and Tunhall, 1988; Ryan et al., 1992; Humphreys-Beher et al., 1994). IGF and insulin effects are mediated by specific membrane receptors. There are two membrane IGF receptors, which resemble the insulin receptor. Thus, IGF-I, IGF-II and insulin can all blind to the IGF-I receptor with a ratio affinity of 100:10:1, respectively (Jones and Clemmons, 1995).

Diabetes mellitus is a metabolic disorder in which the functional mechanisms of both insulin and IGF are changed (Kerr et al., 1995). Studies showed that chemically induced diabetes in both rats and mice led to diminished insulin level in the acinar cells of the parotid and submandibular glands after periods, which varied from 7 to 27 days. (Smith and Patel, 1984; Patel et al., 1986). Also, Kerr et al. (1995) observed diminished insulin level, besides increased IGF levels from the saliva of diabetic mice. The authors concluded that these changes impaired the secretory processes in the salivary glands. In addition, another study verified a steady increase in the expression of the IGF receptors in the salivary glands from autoimmune diabetic mice as a result of their diabetic state (Mustafa et al., 2001). According to Zaka et al. (2005), analyzing in-vitro nervous cells, high concentrations of IGF are related to cellular phosphorylation. This phosphorylation is responsible for changes in the cellular processes of apoptosis, due to enzymatic alterations, besides activating the insulin receptors. Also, clinical studies demonstrated that overexpressed IGF could be considered a stimulatory factor for cancer cell proliferation of different glands, as well as for the occurrence of metastasis (Smith and Patel, 1984; Patel et al., 1986; Yee et al., 1989; Denley et al., 2005; Neuvians et al., 2005).

Therefore, specialized literature has shown the fundamental role of insulin and IGF in the homeostasis maintenance of the salivary glands. However, the cellular and molecular events involved in the actions of these elements not only in the normal glandular processes but also in the pathological conditions, remain unclear.

Taking into consideration the above, the main objective of this study was to characterize the expression of the IGF and insulin receptors in autoimmune diabetic mice, associated to therapeutic effects of long-term insulin treatment on these receptors.

MATERIALS AND METHODS

Animals and Tissue Preparation

Forty-five female mice aged 18 weeks, provided by the Animal Care Center (CEMIB/UNICAMP), were divided into five groups: 9 positive diabetic Nod mice for 10 days (1), 9 positive diabetic Nod mice for 20 days (2), 9 diabetic Nod mice for 10 days (3), 9 diabetic Nod mice for 20 days (4), and 9 non-diabetic BALB/c mice (group 5). Animals from groups 3 and 4 were subcutaneously administered a 0.20mg/100 g dose (4 to 5 U) of insulin (highly purified mixed NPH insulin, Biobrás, Montes Claros, Minas Gerais, Brazil) in the dorsal region for 20 days (Anderson, 1983). Animals from groups 1, 2 and 5 received daily injections of physiological saline for the same period of time to simulate experimental conditions of insulin treatment. Solid (Nuvilab CR 1, SP, Brazil) and fluid intake was ad libitum. Blood glucose levels were measured twice a day in each animal (MediSense Optium, Abbott, Bedford, MA, USA), with animals presenting values higher than 400 mg/dl being considered diabetic (Hu et al., 1992; Shirai et al., 1998). All animals were anesthetized with Francotar/Virbaxil (1:1/0.25 ml/100 g body weight) and sacrificed (according to the Ethical Principles for Animal Research established by The Brazilian College for Animal Experimentation-COBEA). Samples of the salivary glands were removed for immunohistochemical analyses. The material was examined and photographed under a Confocal microscope (MRC 1024UV, Hercules, CA, USA) at the State University of Campinas (UNICAMP).

Confocal Microscopy

The samples of the salivary glands were removed and frozen in liquid nitrogen, and cut into 12 µm thick sections by means of Microm cryostat (Microm HM 505 E, CA, USA).

The sections were fixed with icecold methanol (3 min) and acetone (3 min) (1:1), and air-dried for 30 min, followed by fixation in 4% paraformaldehyde for 10 min and washed with phosphate buffered saline (PBS). Next, the specimens were incubated with blocking solution for 1 h at room temperature to block nonspecific binding. Primary rabbit Insulin R (Santa Cruz, CA, USA) and rabbit IGF-IR (Santa Cruz, CA, USA), antibodies were diluted in blocking solution (1:50) and applied to the sections overnight at 4^0 C. The slides were washed with PBS and the material was incubated with a secondary fluorescein-conjugated antibody (anti-rabbit IgG-FITC, Santa Cruz, CA, USA) diluted 1:100 in blocking solution. After washing with PBS, the sections of the glands were mounted in 1.4-diazabicyclo [2.2.2] octane (DABCO, mounting medium for fluorescence microscopy, Sigma, St. Louis, USA) and then observed with a confocal microscope. A wavelength of 488 nm was used to excite the fluorescein. The settings for contrast, brightness, and iris diameter were adjusted and kept constant during all observations of control and treated groups. A 40X1.4 water immersion objective was used for the confocal image. Sections from salivary glands that were not stained with primary antibody for insulin Rα and IGF-IRα, and were used for negative controls. Staining intensity was graded either strong (+++), moderate (++), or weak (+) according to concentration and distribution of the receptor in the sectioned tissues (Markopoulos et al., 2000).

RESULTS

Blood Glucose Levels

The average blood glucose level was 140 mg/dl in groups 3, 4 and 5. The animals from groups 1 and 2 had an average blood glucose level of 850 mg/dl.

Confocal Microscopy

Parotid Gland

The IGF-I receptors showed moderate and uniform expression in the mice from the control group (Group 5), which were identified, specially, in the secretory ducts. In contrast, Insulin receptors (INS-R) presented strong expression, placed in the acinar cells and especially in the glandular ducts. (Figures 1A,1B and Table1). IGF-I receptors placed near ductal cells were strongly expressed in the animals of group 1 at 10 days of effective diabetic state. Also, the expression of the INS-R was verified in the glandular duct regions, however they showed moderate expression (Figures 1C, 1D and Table 1). Strong expression of the IGF-I receptors, placed in the glandular duct cells can be seen in the diabetic animals of Group 2. Nevertheless, INS-R presented weak expression (Figures 1E, 1F and Table 1). After insulin treatment, the IGF-I receptors showed strong expression in the diabetic animals for 10 days (Group 3). On the other hand, the expression of the INS-R was considered moderate in relation to those in Group 5. In addition, both of these receptors were placed near the ductal cells (Figures 1G, 1H and Table 1). Group 4, which is formed by diabetic animals for 20 days with insulin treatment, showed strong expression of the IGF-I receptors. Although, the expression of INS-R was weak in the glands from these animals (Figures 1I, 1J and Table 1).

Submandibular Gland

In the animals of the control group (group 5), the IGF-I receptors showed moderate expression, placed in the cells of the secretory ducts. However, the expression of the INS-R was considered strong, situated in the cells of the secretory ducts (Figures 2A, 2B and Table 1). Strong expression of the IGF-I receptors, placed in the glandular ducts can be observed in the diabetic mice for 10 days (group 1). Nevertheless, the INS-R showed moderate

expression, situated near ductal cells (Figures, 2C, 2D and Table 1). Strong expression of the IGF-I receptors, shown especially close to the secretory ducts, was verified in the diabetic mice for 20 days (group 2). Although, the occurrence of the INS-R showed weak expression (Figures, 2E, 2F and Table 1). After insulin treatment, diabetic animals for 10 days (group 3), showed strong expression of the IGF-I receptors, in relation to immunological expression verified in the animals of the control group (group 5). In contrast, the expression of the INS-R was characterized as moderate, in particular when it was related to the control group (group 5). Both of these receptors were placed in the ductal cell regions (Figures 2G, 2H and Table1). In the diabetic mice for 20 days (group 4), despite receiving insulin treatment, the cells from the glands of these animals showed strong expression of the IGF-I receptors, which was homogeneously distributed not only in the glandular ducts but also in the acinar membranes. The INS-R presented weak expression (Figures 2I, 2J and Table 1).

DISCUSSION

Animals from the diabetic group without insulin treatment presented high glucose level, however in the animals submitted to glycemic control these levels presented normal rates, similar to those characterized in the control group. According to Hu et al. (1992) normal glucose levels in rodents are close to 180mg/dl, while effective diabetic state levels are over 400 mg/dl. Therefore, it can be concluded that all animals studied showed effective diabetic state, as well as the insulin treatment per se being efficient, leading to balanced glycemic levels.

The immunostaining of IGF receptors demonstrated that the effective and long diabetic state causes intense and steady expression of these receptors. On the other hand, the

expression of the INS receptors was gradually diminished, which had directly proportional effect on the duration of the diabetic occurrence.

The expression of the IGF levels and their receptors have been related to various autoimmune diseases such as diabetes and Sjögren's syndrome. Mustafa et al. (2001) verified a steady increase in the levels of the IGF receptors in the submandibular glands from adult Nod diabetic mice, in comparison to young diabetic mice. The authors concluded that these changes altered glandular homeostasis. Also, Kerr et al. (1995) observed that diabetic animals showed increased IGF levels, after analyzing the relative volume of saliva produced by the parotid and submandibular glands from Nod mice, aged 21 weeks. The authors associated this increase to lymphocytic infiltrated occurrence, as well as the possibility of affinity between IGF-I and INS receptors. The interaction between IGF and insulin receptors could be considered a compensatory factor to maintain tissue homeostasis due to decreased insulin levels in the saliva. In addition, Kobayashi et al. (2003) characterized increased IGF receptor expression in the wall of the aorta artery of chemically induced diabetic rats. The authors attributed the rise of the receptors levels as a consequence of the long-term hyperinsulinemia.

In addition, IGF and IGF receptor levels have been related to Sjögren's syndrome, in autoimmune disease that leads to harmful structural and physiological alterations in various organs, resembling those found in diabetic patients. According to Katz et al. (2003), patients with Sjögren's syndrome had decreased IGF receptor expression in their salivary glands in comparison to healthy people. In contrast, Markopoulos et al. (2000) verified increased IGF levels in the salivary glands from patients who presented Sjögren's syndrome. The authors suggested that this rise works as a compensatory mechanism in relation to the decrease of the IGF receptors.

Still, some authors associated the rise of the growth factors, including IGF-I, to cancerigenic cell proliferation and stimulation of metastasis occurrence. Regarding mammals' gland neoplasia and submandibular gland adenocarcinoma in humans, there is a rise of the IGF level in the neoplastic cells, which is possibly related to intense cellular proliferation and mitotic activity in the tissues (Yee et al., 1989; Figueroa and Yee, 1992; Kyakumoto et al., 1990). In another study, a patient with prostate cancer showed increased IGF levels, which were linked to cellular proliferation with consequent neoplasia evolution (Wolk et al., 1998; Oliver et al., 2004). On the other hand, Janssen et al. (2004) did not observe increased IGF levels in blood plasma, as well as not establishing a positive association between IGF level and prostatic specific antigen in men with prostate cancer. These authors inferred the connection between IGF level and neoplasic development, however, the stimulation of mitogenic activity remains unclear (Katz et al., 1995; Janssen et al., 2004; Marszalek et al., 2005).

Furthermore, based on the present results, it can be concluded that the steady long term of the diabetic state leads to increased IGF receptor expression, which could suggest impairment of the cellular mechanisms in the salivary glands with possible changes in the apoptotic and mitotic processes. In addition, it can be inferred that the raise of the IGF receptor expression could be related to an attempt to keep the glandular homeostasis during diabetes. It can even suggest the possibility of there being an association between IGF and INS receptors, which would be trying to establish a compensatory mechanism towards hyperinsulinemia.

Insulin has an important function in the modulation of the cellular processes in the different tissues and salivary glands. According to Schillaci et al. (1998) the decrease in the modulation between insulin and its receptors in the human species, with infiltration of

lymphofocytic cells, could lead to tissue dysfunction. Other studies observed a diminished insulin level in the salivary glands from diabetic mice and rats for periods varying from 7 to 27 days. These insulin levels are indicated as harmful to secretory function not only in the parotid gland but also in the submandibular gland (Smith and Patel, 1984; Patel et al., 1986).

Other works, focusing on diabetic animals, verified that the insulin replacement per se was not efficient to recover morphophisiological aspects of the salivary glands, after different periods of diabetic occurrence (Anderson, 1983; High et al., 1985; Hu et al., 1992; Anderson et al., 1994; Leigh et al., 1994; Szcsepanski et al., 1998; Caldeira et al., 2005). These facts are indicated as being responsible for the action of the insulin on the tissues, which remain unclear (Anderson and Shapiro, 1980; Caldeira et al., 2005).

Finally, according to present results it can be concluded that the injurious effects caused by the diabetic state on the insulin receptor expression in the salivary glands are proportional to duration of the diabetic state. Moreover, it can be concluded that the glycemic control was not crucial to have the normal expression of these receptors in the glandular tissues. Still, it can suggest that the alterations in the IGF and insulin receptor expression, when analyzed as a group, can operate in synergism in the misbalance of the glandular homeostasis. Then, it can be concluded that the insulin treatment per se despite restoring the glycemic level did not stabilize all IGF and insulin receptor levels, showing that molecular processes, which control the tissues, are extremely complex and deserve new investment.

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FIGURE LEGENDS

Figure 1: Immunolocalization of the insulin and IGF-I receptors on the parotid glands. A: Non-diabetic mice (Group 5); secretory ducts cells showing moderate IGF-I receptor expression (arrow). B: Non-diabetic mice (Group 5); intense insulin receptor expression, placed, specially, in the glandular duct cells (arrow). C: diabetic mice for 10 days (Group 1); secretory duct cells presenting intense IGF-I receptor expression (arrow). D: diabetic mice for 10 days (Group 1); glandular duct cells with moderate insulin receptor expression (arrow). E: diabetic mice for 20 days (Group 2); glandular duct cells showing intense IGF-I receptor expression (arrow). F: diabetic mice for 20 days (Group 2); secretory duct cells showing weak insulin receptor expression (arrow). G: diabetic mice for 10 days with insulin treatment (Group 3); intense IGF-I receptors expression (arrow). H: diabetic mice for 10 days with insulin treatment (Group 3); moderate insulin receptor expression (arrow). I: diabetic mice for 20 days with insulin treatment (Group 4); secretory duct cells presenting intense IGF-I receptor expression (arrow). J: diabetic mice for 20 days with insulin treatment (Group 4); weak insulin receptor expression (arrow). Magnification: 400X.

Figure 2: Immunolocalization of the insulin and IGF-I receptors on the submandibular gland. A: Non-diabetic mice (Group 5); secretory duct cells showing moderate IGF-I receptor expression (arrow). B: Non-diabetic mice (Group 5); glandular duct cells with intense insulin receptor expression (arrow). C: diabetic mice for 10 days (Group 1); secretory duct cells showing intense IGF-I receptor expression (arrow). D: diabetic mice for 10 days (group 1); moderate insulin receptor expression near glandular ducts (arrow). E: diabetic mice for 20 days (Group 2); intense IGF-I receptor expression in the secretory duct

cells (arrow). F: diabetic mice for 20 days (Group 2); weak insulin receptor expression in the glandular duct cells (arrow). G: diabetic mice for 10 days with insulinic treatment (Group 3); intense IGF-I receptor expression (arrow). H: diabetic mice for 10 days with insulinic treatment (Group 3); moderate insulin receptors expression. I: diabetic mice for 20 days with insulinic treatment (Group 4); intense immunostaining of the IGF-I receptors, specially, in the glandular ducts (arrow). J: diabetic mice for 20 days with insulinic treatment (Group 4); weak insulin receptor expression (arrow). Magnification: 400X.

Figure 1

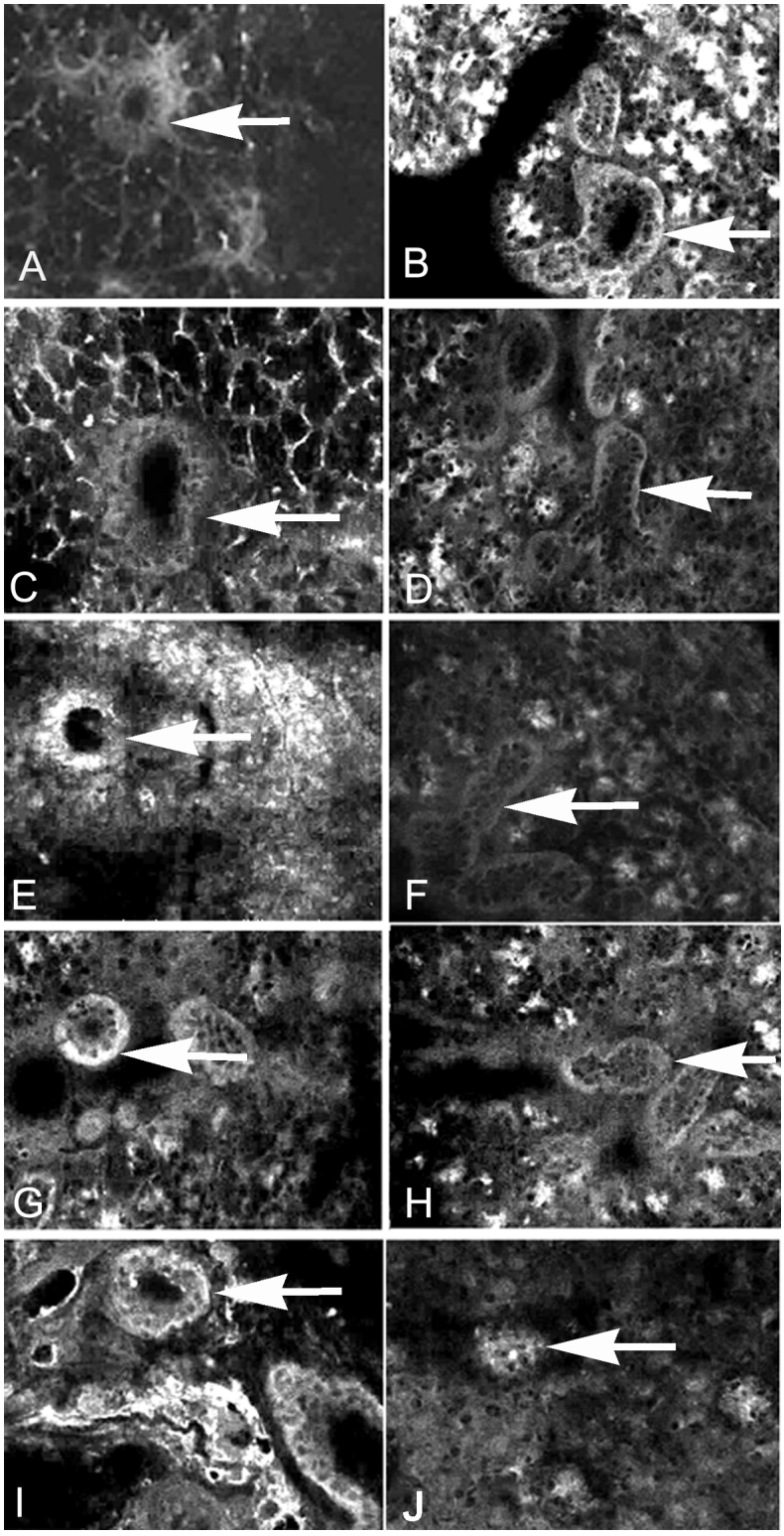
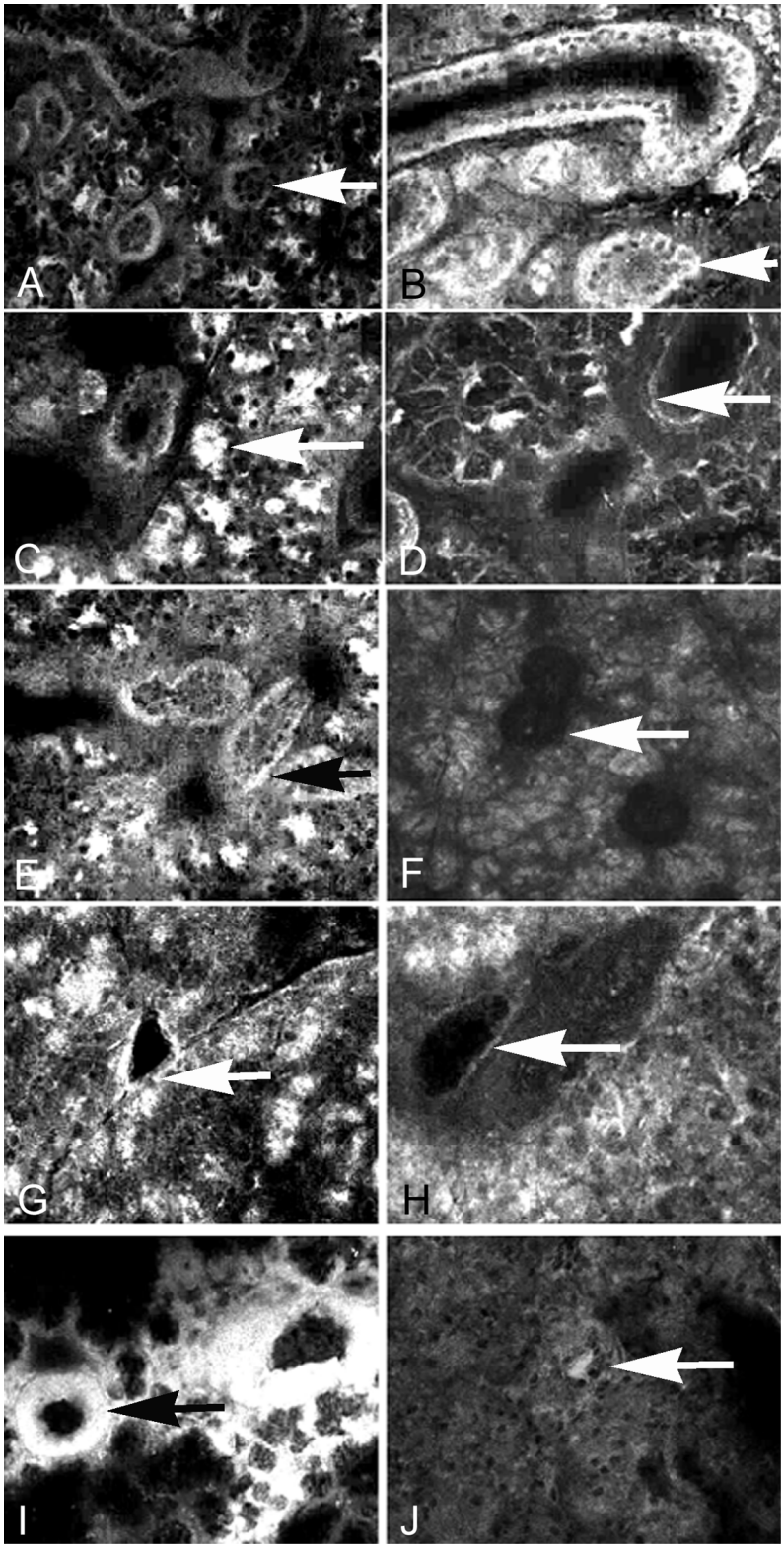


Figure 2



TABLE

Table 1. Distribution of the immunostaining of the IGF-I and insulin receptors according to their intensity of expression on the salivary glands.

Groups	Parotid Gland		Submandibular Gland	
	IGF-IRα	INS-Rα	IGF-IRα	INS-Rα
1	+++	++	+++	++
2	+++	+	+++	+
3	+++	++	+++	++
4	+++	+	+++	+
5	++	+++	++	+++

Intense (+++), moderate (++) and weak (+)

IV-DISCUSSÕES E CONCLUSÕES FINAIS

1) Os níveis glicêmicos nos animais dos grupos 1 e 2 caracterizaram o efetivo estado diabético, bem como os níveis glicemicos nos animais dos grupos 3, 4 e 5 confirmaram a eficácia do tratamento insulínico no controle da hiperglicemia.

2) O diabetes autoimune comprometeu o metabolismo corporal geral levando a perda de peso nos animais. Já o controle glicêmico demonstrou ser tratamento eficaz na recuperação do peso corpóreo nos animais diabéticos.

3) O diabetes autoimune levou a alterações estruturais tanto às glândulas parótidas como às submandibulares.

4) O controle glicêmico prolongado não reorganizou a morfologia dos tecidos glandulares.

5) O estado diabético prolongado levou ao aumento da expressão dos receptores de IGF-I, o que certamente sugere o comprometimento dos mecanismos funcionais celulares com possíveis alterações nos processos mitóticos e apoptóticos. O aumento da expressão dos receptores de IGF-I pode estar relacionado à tentativa de manter a homeostase glandular frente aos efeitos do diabetes ou ainda uma possível interação

entre o IGF-I e os receptores de insulina, na tentativa de compensar a hiperinsulinemia.

6) Os efeitos nocivos causados pela hiperglicemia sobre a expressão dos receptores de insulina nas glândulas salivares foram proporcionais ao tempo de efetivo estado diabético.

7) O controle glicêmico prolongado não foi determinante para a normalização da expressão dos receptores de IGF-I e de insulina nos tecidos glandulares.

8) Ainda, os presentes resultados sugeriram que as alterações na expressão dos receptores de IGF-I e de insulina, em conjunto, podem atuar sinergicamente no desequilíbrio da homeostase glandular.

9) O tratamento insulínico per se apesar de restabelecer os níveis glicêmicos, não estabilizou totalmente os níveis de expressão dos receptores de IGF-I e de insulina, demonstrando que os processos moleculares que controlam os tecidos glandulares são complexos e necessitam de novos estudos.

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