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Cirurgiã-Dentista

TRATAMENTO DE DEFEITOS ÓSSEOS COM GEL DE ÁCIDO HIALURÔNICO A 1% EM ANIMAIS NORMAIS E DIABÉTICOS

Tese apresentada à Faculdade de Odontologia de Piracicaba, da Universidade Estadual de Campinas, para obtenção do título de doutor em Clínica Odontológica, Área de Periodontia

Orientador: Antônio Wilson Sallum

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“A percepção do desconhecido é a mais fascinante das experiências. O homem que não tem os olhos abertos para o misterioso passará pela vida sem ver nada.”

Albert Einstein

O ácido hialurônico (HA) é um importante componente da matriz extracelular e desempenha importante papel na cicatrização. Além de sua função durante o processo cicatricial ele também atua na homeostasia dos tecidos e no reparo ósseo. Devido as suas características propomos este estudo com o objetivo de avaliar o efeito do tratamento de defeitos ósseos com ácido hialurônico em ratos normais e diabéticos. Para este trabalho foram utilizados 64 ratos Wistar machos, sendo que 32 destes animais tiveram um quadro de diabetes induzida por meio de injeção única intraperitoneal de estreptozotocina (STZ) (60mg/Kg). Os animais somente foram considerados diabéticos quando o nível de glicose no sangue ultrapassasse 250mg/dL. Dois defeitos de tamanho crítico, de 5mm de diâmetro, foram confeccionados na calota dos animais e os tratamentos 1) gel de HA a 1%; 2) gel de HA a 1% associado a esponja de colágeno absorvível (ACS); 3) ACS; 4) controle (coágulo sanguíneo) foram aleatoreamente distribuídos entre os defeitos. Após 60 dias do procedimento cirúrgico os animais foram sacrificados e os especimes passaram por processamento histológico para posterior avaliação histométrica. Análise histométrica foi realizada tomando duas medidas lineares nos defeitos: tamanho inicial e tamanho final do defeito. A quantidade de preenchimento do defeito foi obtida pela diferença entre os tamanhos inicial e final do defeito e os dados obtidos analisados estatisticamente. Para análise dos dados foram utilizados o teste t de Student para comparação entres os pesos inicial e final dos animais. O teste ANOVA comparou os níveis glicêmicos dos animais diabéticos antes e após a administração de STZ e no sacrifício, e o teste de Tukey foi utilizado para detectar as diferenças significativas. Para comparação dos tratamentos o teste ANOVA one-way foi usado com o teste de Bonferroni *post hoc*. O nível de significânica foi estabelecido a 5%. Os animais apresentaram ganho de peso significativo ($p<0.05$) ao longo do estudo. A indução da diabetes foi bem sucedida, com os níveis glicêmicos ultrapassando 250mg/dL e permanecendo altos até o final dos experimentos ($p<0.05$). A avaliação histométrica demostrou que o HA associado a ACS acelerou o reparo dos defeitos nos animais normais quando comparado aos demais tratamentos ($p<0.05$). Nos animais diabéticos o tratamento com HA favoreceu o reparo ósseo dos defeitos

quando comparado aos defeitos não tratados e aqueles tratados com HA+ACS (p<0.05), sendo no entanto semelhante ao tratamento com ACS (p>0.05). Dentro dos limites deste estudos pode-se concluir que o HA pode ser utilizado como adjunto no tratamento de defeitos ósseos.

Palavras-chave: Diabetes Melitus, Ossos-Regeneração, Biomateriais, Rato

Hyaluronic acid (HA) is an important component of extracellular matrix and has an important role in wound healing. Besides its role in wound healing it also participates in tissue hemostasis e bone repair. Due to these characteristics this study was suggested with the aim of evaluating the effects of HA treatment of bone defects in normal and diabetic animals. Sixty-four male Wistar rats were used in this study, and 32 of these animals underwent diabetes induction by a single intraperitoneal injection of streptozotocin (STZ) (60mg/Kg). The animals were considered diabetic only if their glucose levels were higher that 250mg/dL. Two 5mm round defects were created in the calvaria of the animals and four treatments were randomly distributed: 1) 1% HA gel, 2) 1% HA gel soaked absorbable collagen sponge (ACS), 3) ACS and 4) control (blood clot). Sixty days post-surgery the animals were sacrificed and the specimens processed for histometric analysis. Histometric analyses were performed and 2 measurements were taken: initial and final sizes of the defect. The amount of bone fill was calculated as the difference between the initial and final sizes of the defects and the data statistically analyzed. For statistical analysis Student t test was used to compare initial and final body weights. One-way ANOVA test compared glucose levels between diabetic animals before and after STZ injections and at sacrifice, Tukey test was used to identify significant differences. Comparisons between treatments were performed by one-way ANOVA and Bonferroni *post hoc.* Significance level was set at 5%. The animals had a significant increase in body weight ($p<0.05$) throughout the study. Diabetes induction was successful with glucose levels exceeding 250mg/dL and remaining high until the end of the study ($p<0.05$). Histometric analysis showed that HA associated with ACS improved bone healing in normal animals compared to other treatments ($p<0.05$). In diabetic animals HA treatment showed significantly greater repair than control and HA+ACS treated defects ($p<0.05$), this treatment was similar to ACS alone ($p>0.05$), though. Within the limits of this study it can be concluded that HA can be used as an adjunct in the treatment of bone defects.

Keywords: Diabetes Mellitus, Bone Regeneration, Biomaterials, Rat

Esta tese será apresentada na forma dos seguintes artigos científicos:

1: Association of hyaluronic acid with a collagen scaffold may improve bone healing in critical-size bone defects

(Submitted to *Clinical Oral Implants Research*)

2: Improved healing of critical-size bone defects treated with hyaluronic acid in diabetic animals

(Submited to *Clinical Oral Investigations*)

Sumário

O ácido hialurônico (HA) é um componente essencial da matriz extracelular e exerce muitos papéis importantes na formação e reparo dos tecidos (Laurent e Fraser, 1992; Fraser *et al.,* 1997). Ele é encontrado naturalmente nos tecidos conjuntivos de vertebrados e pode ser extraído do fluido sinovial, pele, tendões e particularmente do corpo vítreo dos olhos, do cordão umbilical e também da crista de galo (Kim *et al.*,1996).

O HA é um dos principais componentes da matriz extracelular e contribui significativamente para a proliferação e migração celular. Também atua na manutenção da homeostase da matriz extracelular e das proteínas plasmáticas (Laurent e Fraser, 1992). Habassed (1997) relata que o HA controla o estado de hidratação e o tráfego de macromoléculas na matriz dos tecidos e atua especialmente na regeneração pós-inflamatória, com atividade especifica na migração dos fibroblastos e da fibrogênese.

Na cicatrização, o HA exerce papel crucial. Durante a fase inicial inflamatória da cicatrização uma grande quantidade de HA acumula-se na ferida e passa a modular a resposta inflamatória e a atividade dos fibroblastos (Chen e Abatangelo, 1999). Além de promover a inflamação o HA também é capaz de reduzir o processo inflamatório graças ao seu efeito protetor contra radicais livres, que danificam as células e perpetuam o processo inflamatório (Presti e Scott, 1994). Após o controle da inflamação a presença do HA na ferida promove a migração e proliferação de células para reparo da ferida.

O papel contraditório exercido pelo HA na inflamação é resultado da interação do HA com os produtos da inflamação, os quais irão atuar em um ciclo de feedback negativo sobre a ativação da inflamação (Chen & Abatangelo, 1999). O TNF-α (fator de necrose tumoral α), importante citocina produzida no processo inflamatório, estimula a expressão de TSG-6 (TNF-stimulated gene 6) em fibroblastos e células inflamatórias. O TSG-6, uma proteína que se liga ao HA, também forma um complexo estável com o

inibidor de proteinase sérica IαI (inibidor inter-α) com efeito sinérgico sobre a atividade inibitória da plasmina (Milner & Day, 2003). A plasmina está envolvida na ativação da cascata proteolítica das metaloproteinases da matriz (MMP) e de outras proteinases, levando ao dano tecidual inflamatório. Desta forma, a ação do complexo TSG-6/IαI, que pode também ser formado pela ligação ao HA da matriz extracelular, pode funcionar como um potente indutor de feedback negativo para moderar a inflamação e estabilizar o tecido de granulação a medida que a cicatrização progride (Milner & Day, 2003; Milner *et al.*, 2006).

As propriedades físicas e bioquímicas do HA são adequadas para o que mesmo desenvolva papel importante no eventos iniciais da osteogênese. É um importante componente da matriz extracelular durante a morfogênese (Toole e Gross, 1971) e grandes quantidades de HA estão presentes durante a transição de célula mesenquimal em cartilagem (Handkey e Lowther, 1976). Hyuang *et al.* (2003) demonstrou, em cultura de células mesenquimais derivadas da calota de ratos, que o HA foi capaz de induzir a proliferação e diferenciação de células osteoprogenitoras em osteoblastos. Sasaki e Watanabe (1995) observaram que o HA foi capaz de acelerar a formação de novo osso por meio da diferenciação e migração de células mesenquimais, em defeitos ósseos criados em ratos. Aslan *et al.* (2006), utilizando o modelo de reparo ósseo em tíbia de coelho, verificou que a associação de enxertos com o gel de HA apresentou maior formação óssea que os defeitos tratados somente com enxerto.

Considerando suas características não-imunogênicas, vem sendo utilizado no tratamento de doenças degenerativas e inflamatórias das articulações dos ossos (Kim *et al.*, 1996), na reposição do fluido sinovial, na liberação de agentes quimioterápicos em implantes cirúrgicos (Jernberg, 1994; Aebischer *et al*., 2001), como meio hidratante e como sistema para encapsulação e liberação controlada de fármacos e cosméticos. Estudos animais mostram que a aplicação de HA exógeno para auxílio na cicatrização é benéfica. O HA levou a uma redução do infiltrado celular no tecido de granulação (Iocono *et al.,* 1998) e acelerou a cicatrização (King *et al.,* 1991; Iocono *et al.,* 1998).

No diabetes melitus, doença metabólica caracterizada por altos níves de glicemia no sangue, a cicatrização, o metabolismo ósseo e a resposta inflamatória encontram-se alteradas. Todas estas alterações são resultado do acúmulo dos produtos avançados da glicosilação (AGEs) (Paul e Bailey, 1996; Friedman, 1999). Sabe-se hoje que várias células apresentam um receptor para AGE (RAGE). A interação deste receptor com os AGEs presentes nos tecidos leva a diversas alterações como por exemplo, alterações nas células endoteliais (Wautier et al., 1996), aumento da quimiotaxia de monócitos (Miyata *et al.,* 1996), aumento da migração e activação de células musculares lisas (Friedman *et al.,* 1994), e síntese alterada de colágeno pelos fibroblastos (Owen *et al.,* 1998).

O acúmulo de AGEs no tecido ósseo tem sido considerado como causa para a formação óssea reduzida (Santana *et al.,* 2003). Os AGEs podem modular o crescimento, diferenciação e atividade dos osteoblastos via os RAGEs presentes nos osteoblastos (Santana *et al.,* 2003; Schwartz*,* 2003). McCarthy *et al.* (2004) sugerem que a modificação do colágeno pelos AGEs altera a adesão dos osteoblastos a matriz extracelular levando a formação alterada do osso.

Neumann *et al.* (1999) observaram *in vitro* que o HA apresenta efeito protetor contra os efeitos dos AGEs nos tecidos. Ao tratar macrófagos com AGE e HA eles observaram que o HA impedia a ligação dos AGEs aos seus receptores na superfíce dos macrófagos e consequentemente a ativação do fator de transcrição NF-κB (factor nuclear kappa B). Sem a ativação de NF-κB não houve a produção e liberação de citocinas por estas células. Entretanto estes resultados só foram observados quando o HA de alto peso molecular (1.2 Mda) foi usado. Os AGEs são encontrados tanto em diabéticos como em pessoas sem esta condição sistêmica, sendo sua produção aumentada nos primeiros devidos aos altos níveis de glicose no sangue. Eles são considerados promotores de inflamação crônica pois estimulam a produção de citocinas e radicais livres.

O HA apresenta várias vantagens sobre a cicatrização bem como tem efeito protetor contra o principal responsável pelas complicações observadas na diabetes, os

AGEs. Apenas dois estudos na literatura relatam o uso tópico de HA para a cicatrização de feridas em diabéticos (Siebenschuh *et al*., 1998; Vasquez *et al*., 2003) e com resultados positivos para a cura desta feridas crônicas. No entanto, não existem estudos que utilizem este polímero para o tratamento de defeitos ósseos em diabéticos. Desta maneira podemos sugerir que o uso de HA exógeno pode ser vantajoso para o tratamento de feridas em diabéticos. As várias evidências sobre os efeitos do HA em osteoblastos nos faz sugerir que o tratamento de defeitos ósseos com este polímero pode ser vantajoso no tratramento destes defeitos em diabéticos bem como em não-dabéticos.

Avaliar os efeitos do tratamento de defeitos ósseos críticos com gel de ácido hialurônico sobre o reparo ósseo em ratos normais e diabéticos.

Association of hyaluronic acid with a collagen scaffold may improve bone healing in critical-size bone defects

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Running title: Hyaluronic acid effects on bone healing

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Keywords: wound healing, critical size defect, hyaluronan

Abstract

Objectives: To evaluate the effects of 1% hyaluronic acid gel in combination with absorbable collagen sponge in the healing of critical-size calvaria defects in rats.

Material and methods: Thirty-two adult Wistar rats were used. Two 5-mm-diameter critical-size defects were created and the treatments were randomly distributed as follows: **1)** 1% hyaluronan gel (HA); **2)** 1% HA gel soaked absorbable collagen sponge (ACS); **3)** control (blood clot); and **4)** ACS. The animals were sacrificed 60 days post-surgery when biopsies were collected and processed for histology and histometric analysis. Bone fill was measured as the difference between initial and final defect sizes. Parametric tests were used to analyze differences between treatments and body weight gain in each treatment group. Significance level was set at 5%.

Results: Histological analysis showed bone formation on the edges of the defects, although very limited, and a thin layer of connective tissue occupying the midportion of the defects in control and ACS groups. Defects filled with HA and HA+ACS had a thicker layer of connective tissue and more new bone formed in the margins of the defects. Linear histometric measures showed that association HA+ACS had significantly greater bone fill than the control and ACS treated groups ($p<0.05$).

Conclusion: Hyaluronic acid association with a collagen scaffold can improve new bone formation in critical size defects.

Introduction

Bone maintenance and bone regeneration have become essential concepts in the treatment of periodontal disease; in the healing of tooth extraction sockets and in the utilization of dental implants (Nyman et al., 1982; Cardaropoli et al., 2005; Araújo & Lindhe, 2009). Currently, great efforts are being made not only to maintain and prevent bone loss, but also to augment and regenerate bone around teeth and implants.

Hyaluronic Acid (HA) is a high molecular weight polysaccharide ubiquitously distributed in the extracellular space and its highest concentrations are found in soft connective tissues. In recent years, HA has been reported to play critical roles in a wide variety of biological events, such as wound healing, chondrogenesis, osteogenesis, the immune response, and migration of rat transformed cells (Kubler and Urist, 1990; Boskey *et al.,* 1991; Lesley and Hyman, 1992; Noble *et al.,* 1993; Turley *et al.,* 1993). Sasaki and Watanabe (1995) showed that HA is capable of accelerating new bone formation through mesenchymal cell differentiation, in bone created wounds in the animal model. They were able to demonstrate that bone formation had already been induced at day 4 after the application of HA. Hyaluronic acid possesses biochemical and physical properties suitable to perform an important role in the early events of osteogenesis. It is a prominent extracellular matrix component during bone morphogenesis (Toole and Gross, 1971) and large amounts of HA are present during the transition from mesenchymal cell to cartilage (Handkey and Lowther, 1976). In terms of its correlations with wound healing mechanisms and hard tissue development (Hay, 1980), HA can be thought as a "primer" in cell regeneration.

Looking at these findings we can suggest that the association of HA with a sustained release device, such as collagen sponges, could help improve bone regeneration. This study focuses on evaluating, histometricaly, the potencial of 1% HA gel in the local treatment of critical-size cortical bone defects.

Material and methods

Animals

Thirty-two 12-week-old male Wistar rats were used in this study. Their mean body weight was 250g. The animals were kept in plastic cages with access to food and water *ad libitum*. Prior to the surgical procedures all animals were allowed to acclimate to the laboratory environment for a 5 day period. The protocol was approved by the University of Campinas Institutional Animal Care and Use Committee.

Experimental Surgery

General anesthesia was obtained by intramuscular administration of ketamine (50mg/kg) (Dopalen®, Agribrands Brasil Ltda., Paulínia, SP, Brazil) and xylazine chloridrate (15mg/Kg) (Virbaxil®; Virbac do Brasil Indústria e Comércio LTDA, Roseira, SP, Brazil). The surgical site was shaved and washed with iodine. An L-shaped incision was made and a full-thickness flap including periosteum was reflected, exposing the calvaria bone. In each animal two 5mm-diameter round defects were created, one on each side of the mid-sagittal suture, with a trephine bur in a dental handpiece under constant irrigation of sterile saline. The trephined bone was removed from the surgical site. Extreme care was taken to avoid injury to the midsagittal blood sinus and dura mater. The periosteal and skin flaps were returned and sutured with 5-0 silk suture. Each animal was included in two different treatments. Four treatments were evaluated: **1)** 1% HA gel; **2)** 1% HA gel soaked onto a pre-cut absorbable collagen sponge (ACS) (Hemospon, Montevideo, Uruguay); **3)** control (blood clot); and **4)** ACS. Sixty days post-surgery animals were euthanized by intracardiac perfusion with 10% neutral buffered formalin solution.

Histometric evaluation

Calvaria specimens were fixed in 10% buffered formalin for 48 hours, and decalcified in 10% EDTA for 30 days. The specimens were washed with tap water, dehydrated with ascending concentrations of ethyl alcohol, xylene, and infiltrated with paraffin. Serial

sections (6 µm) parallel to the mid-sagittal suture were cut from the center of each osteotomy and stained with Mallory´s Trichrome.

Two histologic sections, representing the center of the original surgical defect, were selected for the histologic and histometric analyses. All analyses were performed by an examiner blinded with respect to the treatment rendered. The images of the histologic sections were captured by a digital camera connected to a light microscope with an original magnification of x2.5. The distances between the margins of the residual bone defect and the margins of the originally created defect were linearly measured by means of an image analysis system (Image-Pro Plus, Media Cybernetic, Silver Springs, MD, USA). The two distances were subtracted and the amount of bone fill calculated. The reproducibility of the measurement method was evaluated with double measurements on 20 randomly selected sections within a 2-week interval.

Statistical analysis

Person's correlation test was used to evaluate intra-examiner agreement for the histometric evaluation ($R2=0.99$, $p<0.001$). The histometric results were analyzed using the one-way ANOVA test with Bonferroni test *post-hoc* test for comparisons between treatments. The Student t test was used to compare initial and final body weights. Significance level was set at 5%.

Results

Healing was uneventful in all animals included in the study. No signs of post-operative infection were observed. Animals showed a significant increase in body weight throughout the study as shown in Table 1.

Defects left untreated (control) and with ACS were mainly occupied by a thin layer of connective tissue and a limited amount of new bone. (Fig.1a and b). In the defects filled with HA and HA+ACS a thicker layer of connective tissue was observed and greater amount of new bone formation was found in the defect margins (Fig. 1c and d), as compared to control and ACS groups.

Linear histometric measurements showed that the association of HA with collagen sponge accounted for a greater bone fill compared to control and ACS alone ($p<0.05$). Differences between the other treatments, however, were not statistically significant ($p>0.05$) (Table 2). There were no significant difference between HA and HA+ACS groups ($p>0.05$) (Table 2).

Discussion

The aim of this study was to evaluate the effects of HA on bone healing in a critical size calvaria defect model. The calvaria defect, a standardized full-thickness trephine defect that will not heal during specific extended observation periods, has been used successfully to evaluate bone regeneration in connection with different biomaterials. Compared to other experimental bone defects, is a convenient model for studying bone regenerative materials because of its effective accessibility and the lack of fixation requirements (Schmitz & Hollinger, 1986; Mardas *et al.,* 2002; Donos *et al.,* 2004). In the present study, complete closure of the control defect did not occur 60 days after surgery which indicates that the 5 mm defect used fulfilled the requirements for a critical size defect as reported in other studies (Mardas *et al*., 2008; Yu *et al.,* 2010; Nagata *et al.*, 2010).

The present study demonstrated that 1% HA gel was not able to improve bone formation when used alone. The same results were observed by Brazão *et al*. (2010) in a radiographic evaluation, where the same treatments were evaluated. However, a study by Mendes *et al.* (2008), using the same concentration of HA gel, observed increased bone formation in tooth sockets. This difference may be a result of the type of defect used to evaluate the effectiveness of this treatment. It is known that tooth sockets cannot be considered critical size defects since they spontaneously heal within a few weeks (Devlin, 2000).

Treatment of defects with absorbable collagen scaffold soaked in HA showed better results than the defects treated solely with the scaffold. These results differ from the one of Wiedmann-Al-Ahmad *et al*. (2005) where higher bone proliferation rates were observed for HA in comparison to other biomaterials studied. However, this study used

in vitro conditions testing which biomaterial would favor proliferation of osteoblast-like cells and not an *in vivo* model. Aslan *et al*. (2006) documented that HA needs an osteoconductive scaffold to be effective, as their findings showed that associating HA with bone grafts improved the rate of bone formation in each evaluation period. Similar results were observed in the present study. Collagen sponges are well characterized carrier systems that provide a sustained release of biomolecules with a putative role in bone regeneration (Friess *et al*., 1999; Geiger *et al*., 2003). The present study showed that the use of the sponge as a carrier system was beneficial to the healing of the HA+ACS treated defects.

Hyaluronic acid is found in different molecular weights and it has been proposed that a specific molecular weight is responsible for specific behaviors in the organism. Different authors examined the effects of various hyaluronic acid molecular weights on osteogenesis. Pilloni and Bernard (1998) showed that low molecular weight HA accelerated osteogenesis *in vitro* in calvaria-derived mesenchymal cells. In contrast to this study, Sasaki and Watanabe (1995) described that high molecular HA increases bone formation after bone marrow ablation in comparison to untreated controls. According to Mendes *et al.* (2008) the application of a high molecular weight HA (1% HA gel) in wounds would benefit both the inflammatory and proliferative phases of wound healing. Hyaluronidases available in the wound would degrade the high molecular weight HA into low molecular weight HA allowing for the benefits of this molecule in the angiogenesis process (Slevin *et al.*, 2002; Takahashi *et al.,* 2005).

It would be interesting to evaluate the rate of angiogenesis and proliferation of fibroblasts and osteoblasts into the wounds to verify the various effects of HA during wound healing in this type of defect. Further analyses are under way to identify the real effects of this molecule in the healing of critical size defects.

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and a beta-tricalcium phosphate biomaterial on local bone formation in a critical-size rat calvarial defect model. *Journal of Clinical Periodontology* **37**: 419-426.

Tables

Table 1. Initial and final body weight (g) of animals (Mean±SD)

Initial body weight	Final body weight
249.84±83.8	442.74±29*

*indicates statistical difference between initial and final body weights (Student *t* test; $p<0.0001$)

Table 2. Amount of bone fill (mean ± SEM) (mm) of treatment groups

Treatments	Bone Fill
Control (n=8)	0.50±0.02*
ACS (n=8)	0.56±0.05#
HA (n=8)	0.70±0.14
HA+ACS (n=8)	0.96±0.14

*indicates statistical differences between control and HA+ACS groups (ANOVA; $p<0.05$)

#indicates statistical differences between ACS and HA+ACS groups (ANOVA, $p<0.05$).

Figures

Figure 1a-d. Panoramic view of surgical defects showing new bone formation. Arrows indicate margins of original defect (Mallory's Trichrome, bar=0.1mm)

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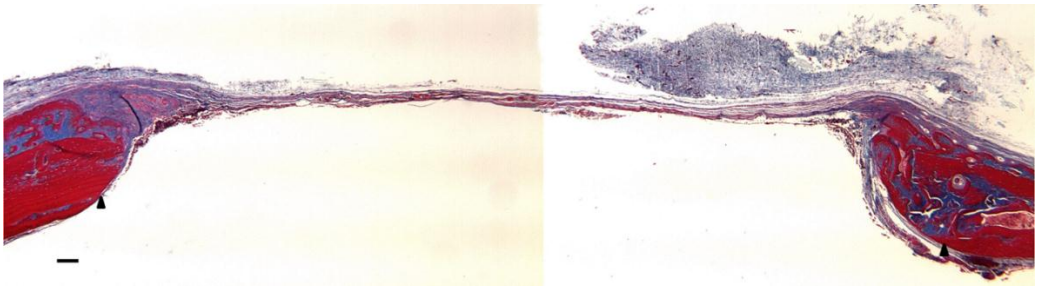
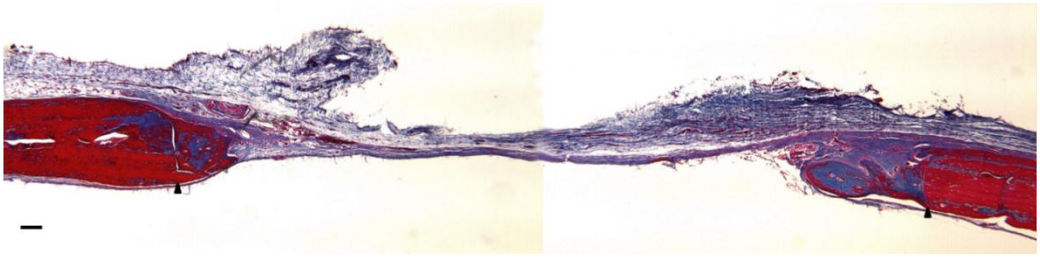
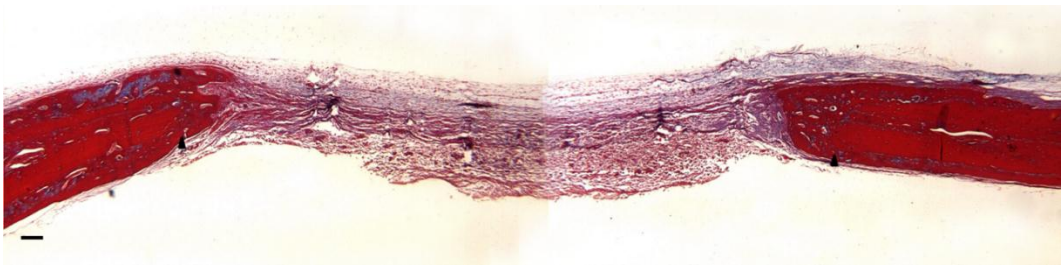


a. Control treated defect



b. ACS treated defect



c. HA treated defect

d. HA+ACS treated defect

Improved healing of critical-size bone defects treated with hyaluronic acid in diabetic animals

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Running title: Hyaluronic acid effects on diabetic bone healing

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Keywords: Diabetes; Hyaluronic Acid, Wound Healing, Critical Size Defect

Abstract

Objectives: To evaluate the effects of hyaluronic acid (HA) in the healing of critical-size bone defects in an animal model of diabetes.

Material and methods: Type 1 diabetes was induced by streptozotocin injections (60mg/kg) in Wistar rats and diabetic state confirmed by glucose levels above 250mg/dl. Thirty-two animals were included in the study and 8 weeks after diabetic condition was confirmed two 5 mm critical-size defects were created in the calvaria. Four treatments were tested: **1)** control (blood clot); **2)** absorbable collagen sponge (ACS); **3)** 1% HA gel and **4)** 1% HA gel soaked ACS. The animals were sacrificed at 60 days when biopsies were collected and processed for histometric analysis. Bone fill was measured as the difference between initial and final defect sizes. Student t test was applied to compare initial and final body weights. One-way ANOVA test compared glucose levels before and after STZ injection and at sacrifice and Tukey test used to identify the differences. One-way ANOVA test was used for comparisons between treatments and Bonferroni test was used as a *post hoc* test. Significance level was set at 5%.

Results: High glucose levels were obtained by STZ injection and these levels remained high until the end of the study. HA-filled defects (1.40±0.17) had more newly formed bone than control (0.88±0.06) and HA+ACS (0.89±0.07) defects ($p<0.05$). Defects treated with ACS (1.84±0.24) were not statistically different from HA, but were from control and HA+ACS defects.

Conclusion: The results of this study showed that bone healing was delayed in diabetic animals and treatment of bony defects with HA was able to improve healing in this model.

Introduction

Diabetes, a metabolic disease characterized by hyperglycemia, causes acute and chronic complications in the body because of the absence, deficiency or inefficiency of insulin. Bone metabolism is impaired in diabetes either through the direct effects of hyperglycemia or via the more long-term effects of vascular disease. Many studies have demonstrated that treatment of diabetic animals with insulin, to improve glycemic control, have reversed the effects of diabetes on bone repair (Shyng *et al.,* 2001; Mishima *et al.,* 2002; Follak *et al.,* 2003; McCracken *et al.,* 2006).

Disturbed formation of collagen, capillary basement membrane thickening, impairment of the host's defense mechanisms and bacterial invasion (Mattson *et al.,* 1998) have all been proposed as causes of disorganized wound healing in severely diabetic rats. Alterations in collagen metabolism are thought to be responsible for impaired wound healing observed in diabetes. These alterations are due to the accumulation of advanced glycosylated end products (AGEs) (Paul & Bailey, 1996). High glucose concentrations over time result in the formation and accumulation of AGEs. The role of AGEs in various chronic diabetic complications is well documented, and that includes retinal, kidney, nervous, and circulatory dysfunction (Brownlee, 1992; Friedman, 1999). The mechanisms by which AGEs induce these complications are complex and unclear. However, it has been postulated that the formation of AGE-derived collagen cross-links results in alterations in collagen solubility, causing alteration in structural and functional properties of connective tissues.

Hyaluronic acid (HA), also known as hyaluronan, is an important component of the extracellular matrix in which most tissues differentiate and also an essential component of many extracellular matrices in mature tissue (Laurent & Fraser, 1992). Among the biologic functions of HA cell attachment, mitosis, migration, wound healing and inflammation are the most studied ones (Knudson & Knudson, 1993; Toole, 2001). It influences and enhances tissue regeneration through its ability to retain large amounts of water (Nakamura *et al.,* 1993). Hyaluronic acid has also been implicated in increased osteoblastic bone formation *in vitro* through increased mesenchymal cell differentiation

and migration (Sasaki & Watanabe, 1995; Huang *et al.,* 2003). Locally applied HA does not result in any phagocytosis (Forrester & Balazs, 1980) or immunological reactions (Larsen *et al.,* 1993) and it has been used in humans for eye surgery (Balazs, 2008), regeneration of tympanic membrane (Goa & Benfield, 1994), and local treatment of osteoarthritis (Altman, 2010). Due to its many benefits in wound healing it is appropriate to suggest that treatment of diabetic wounds with exogenous HA could be advantageous to the impaired wound healing observed in this systemic condition, as it was observed by Vasquez *et al.* (2003). In their study treatment of diabetic foot ulcers with HA improvement healing compared to control treatment. However, studies evaluating HA treatment in bone healing of diabetics are not available. Therefore the present study aims at evaluating the effects of hyaluronic acid on wound healing of critical size calvaria defects in an animal model of type 1 diabetes.

Material and methods

Animals

Thirty-two 4-week old male Wistar rats were used in this study. Their mean body weight was 150g. The animals were kept in plastic cages with access to food and water *ad libitum*. Prior to the surgical procedures all animals were allowed to acclimate to the laboratory environment for a 5 day period. The protocol was approved by the University of Campinas Institutional Animal Care and Use Committee.

Blood glucose levels were measured with a glucometer (Accu-check Active, Roche, Brazil) before diabetes induction. Diabetes was induced in 4-week old animals by a single intraperitoneal injection of streptozotocin (STZ) (Sigma, St Louis, MO), 60mg/Kg, dissolved in citrate buffer (pH 4.5). Two days after STZ injection, blood glucose was measured in all animals. Any animal with blood glucose level higher than 250mg/dL was considered diabetic and included in the study.

Experimental Surgery

Eight weeks after induction of diabetes, blood glucose and body weight were again measured. General anesthesia was obtained by intramuscular administration of

ketamine (50mg/kg) (Dopalen®, Agribrands Brasil Ltda., Paulínia, SP, Brazil) and xylazine chloridrate (15mg/Kg) (Virbaxil®; Virbac do Brasil Indústria e Comércio LTDA, Roseira, SP, Brazil). The surgical site was shaved and aseptically cleaned with iodine. An L-shaped incision was made and a full-thickness flap including periosteum was reflected, exposing the calvaria bone. In each animal, two 5mm round defects were created, one on each side of the mid-sagittal suture, with a trephine in a low-speed handpiece under constant irrigation of sterile saline. Each animal was included in two different treatments. Four treatments were evaluated: **1)** control (blood clot); **2)** absorbable collagen sponge (ACS) (Hemospon, Montevideo, Uruguay); **3)** 1% HA gel and **4)** 1% HA gel soaked ACS. Soft tissues were repositioned and sutured with 5-0 silk suture (Ethicon, São Paulo, SP, Brazil). After the surgical procedure the animals received an intramuscular injection of 24,000 IU penicillin G-benzathine (Pentabiótico Veterinário pequeno porte, Fort Dodge Saúde Animal Ltda, Brazil). Sixty days post-surgery, blood glucose and body weight were measured again and animals were then euthanized by intracardiac perfusion with 10% neutral buffered formalin solution.

Tissue processing and histometric evaluation

The area of the original defect and surrounding tissues were removed *en bloc*. The specimens were fixed in 10% buffered formalin for 48 hours, and decalcified in 10% EDTA for 30 days. The specimens were washed with tap water, dehydrated with ascending concentrations of ethyl alcohol, xylene, and infiltrated with paraffin. Serial sections (6 µm) parallel to the mid-sagittal suture were cut from the center of each osteotomy defect and stained with Mallory´s Trichrome.

Two histologic sections, representing the center of the original surgical defect, were selected for histometric analyses. All analyses were performed by a blind examiner. The images of the histologic sections were captured by a digital camera (Digital Kocam CCD câmera; DMI, São Paulo, Brasil) connected to a light microscope (Axioskop 2 plus®; Zeiss, Jena, Germany) with an original magnification of x2.5. The distances between the margins of the residual bone defect and the margins of the originally created defect were linearly measured by means of an image analysis system (Image-Pro Plus, Media

Cybernetic, Silver Springs, MD, USA). The two distances were subtracted and the amount of bone fill calculated in millimeter.

Statistical analysis

One-way ANOVA test was used to compare blood glucose values before and after STZ injection and at sacrifice and the Tukey test was used to identify the differences. The histometric results were analyzed using the one-way ANOVA test with Bonferroni test *post hoc*. Significance level was set at 5%.

Results

After STZ injection, animals that presented elevated blood glucose levels started to show decreased growth and weight gain compared to the animals that did not become diabetic after injection (data not shown). The animals gained weight throughout the study showing that, even though diabetes leads to weight loss or reduced weight gain, the animals remained healthy even with high glucose levels (Table1).

Injection of STZ was able to induce a diabetic state in the animals which was reflected by the high blood glucose levels compared to the values before injection ($p<0.01$). These levels were kept high until the end of the experiment (Table 2).

Defects treated with HA (1.68 ± 0.13mm) showed significant improvement in bone healing compared to control (0.88 ± 0.07mm; $p<0.05$) and HA+ACS treatments (0.89 ± 0.08mm; $p<0.05$). Associating HA and ACS (0.89 ± 0.07mm; $p<0.05$) did not improve healing, in fact this treatment showed much less bone fill than ACS alone (1.84 ± 0.22mm) (Table 3) ($p<0.05$). Treatment with ACS was also significantly better than control ($p<0.05$). Bone healing patterns are illustrated by figures 1a through 1d.

Discussion

Animal models using either streptozotocin or alloxan to induce diabetes have been extensively used to evaluate wound healing (Mishima *et al.,* 2002; McCracken *et al.,* 2006; Mariano *et al.,* 2010). Diabetes has a deleterious effect on osseous turnover due to decreased osteoblast and osteoclast activities and numbers, and a lower percentage

of osteoid surface and osteocalcin synthesis, as well as increased time for mineralization of osteoid (Piepkorn *et al.,* 1997; Herrero *et al.,* 1998; Kemink *et al.,* 2000)

This study is the first to evaluate the effects of the treatment of critical size bone defects with hyaluronic acid in diabetic animals. HA-filled defects showed a 2-fold (almost a 1.5-fold) increase in newly formed bone compared to control and HA+ACS defects. Higher rates of infection and impaired inflammatory response are seen in diabetes (Seifter *et al.,* 1981). The anti-inflammatory (Chen & Abatangelo, 1999) and anti-infectious properties of HA (Muto *et al.,* 2009), as well as prevention of damage to granulation tissue by oxygen free radicals (Foschi *et al*.,1990), with consequent reduction of AGE accumulation in diabetic tissue, are possible mechanisms that can be suggested to explain the improved healing in HA treated defects compared to control.

Defects treated with ACS showed a 2-fold increase in bone formation compared to control defects. Collagen sponges function as a biological scaffold for permeation of fibroblasts and capillaries (Burton *et al.,* 1978), as well as a topical hemostatic, promoting activation of platelets and coagulation factors (Palm & Altman, 2008). The use of ACS in the defect may have favored greater accumulation of platelets compared to control defect, and maybe a greater concentration of growth factors were present in the defect. Platelet-derived growth factor (PDGF) has been shown to enhance osteoblast migration and proliferation and can be considered a key factor in bone formation (Ai-Aql *et al.,* 2008). Therefore it can be speculated that ACS provided a niche for accumulation of platelets and concentration of growth factors leading to an enhanced bone formation in ACS-treated defects.

Association of HA+ACS showed worse results than ACS alone and HA. Association of HA with a carrier system, which has been shown to provide sustained release of biomolecules (Geiger *et al.,* 2003), appeared to be ineffective and even detrimental to wound healing. These results are somewhat unexpected since improved bone healing was observed in ACS and HA defects. There are no studies in the literature to support this finding. One can suggest that when associating ACS with HA there was no synergy

in the effects of both treatments. We can hypothesize that HA did not allow platelet accumulation into the sponge, since the sponge structure was soaked in HA and therefore reduced the collagen effects on blood clotting. Another explanation could be that application of ACS with HA may have reduced the hygroscopic capacity of HA (Nakamura *et al.,* 1993) and in consequence the regenerating effects of this molecule.

This study is the first to evaluate the effects of HA treatment on bone healing in diabetic animals. The present results showed that treatment of bone defects with HA was beneficial to diabetic animals compared to the control group. However, it showed similar results to ACS suggesting that it could be used as an alternative treatment. Further studies are necessary to evaluate the mechanisms in which HA affects bone healing in diabetes.

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Tables

Table 1. Mean and SD of initial and final body weight (g) of diabetic animals

Initial body weight	Final body weight	*p* value
181.20±12.5	281.41±45.4	0.0001

Table 2. Means and SEM of initial, after STZ injection and final blood glucose (mg/dL) values

Initial blood glucose	STZ blood glucose	Final blood glucose
135.37 ± 5.05	389.87 ± 15.56*	510.75 ± 13.3*,#

* $p<0.01$, compared to initial blood glucose by one-way ANOVA and Tukey's tests

#$p<0.01$, compared to STZ blood glucose by one-way ANOVA and Tukey's tests

Table 3. Means (mm) and SEM of Bone Fill

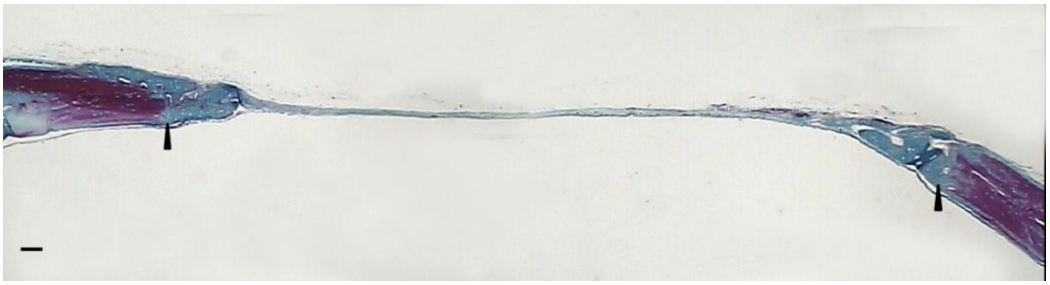
TREATMENT	BONE FILL
CONTROL (n=8)	0.88 ± 0.07*,**
ACS (n=8)	1.84 ± 0.22
HA (n=8)	1.68 ± 0.13
HA+ACS (n=8)	0.89 ± 0.08*,**

*p<0.05, compared to ACS treatment by one-way ANOVA and Bonferroni's tests

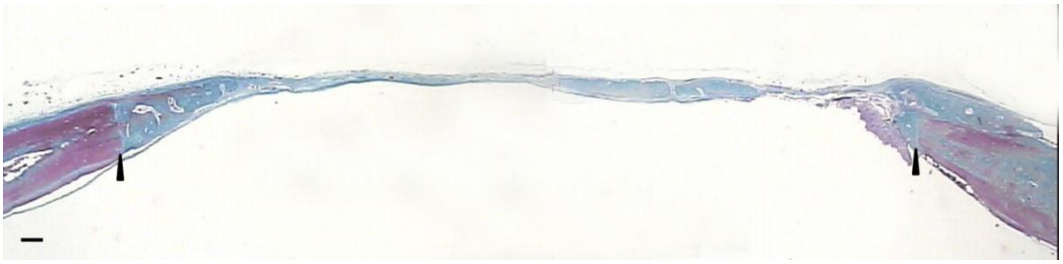
**p<0.05, compared to HA treatment by one-way ANOVA and Bonferroni's tests

Figure 1. Panoramic view of surgical defects showing new bone formation. Arrows indicate margins of original defect (Mallory's Trichrome, bar=0.1mm)

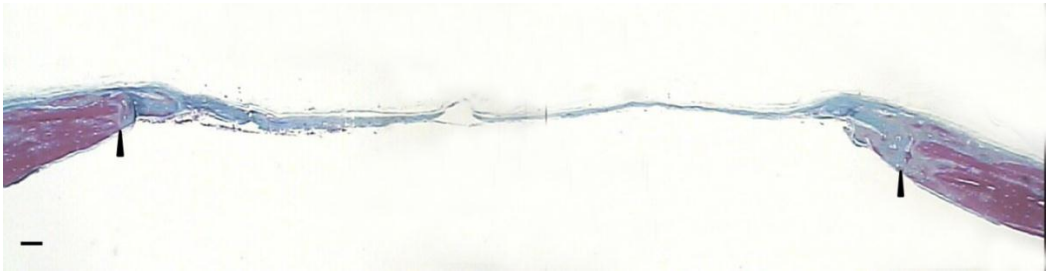
a. Control treated defect



b. ACS treated defect



c. HA treated defect



d. HA+ACS treated defect

Dentro dos limites dos estudos aqui apresentados pode-se concluir que:

1. O tratamento de defeitos ósseos com gel de ácido hialurônico a 1% associado à esponja de colágeno pode trazer benefícios para o reparo ósseo de animais normais;
2. A aplicação tópica do gel de ácido hialurônico a 1% pode acelerar o reparo de defeitos ósseos em animais diabéticos.

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* De acordo com a norma da UNICAMP/FOP, baseadas na norma do International Commitee of Medical Journal Editors – Grupo de Vancouver. Abreviatura dos periódicos em conformidade com o Medline.

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C E R T I F I C A D O

Certificamos que o Protocolo nº **1493-1**, sobre **"Avaliação do uso do gel de ácido hialurônico a 1% sobre o reparo de defeitos ósseos em calvária de ratos diabéticos - avaliação radiológica e histométrica"**, sob a responsabilidade de **Prof. Dr. Antônio Wilson Sallum / Beatriz de Brito Bezerra**, 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/Unicamp em **31 de março de 2008**.

C E R T I F I C A T E

We certify that the protocol nº **1493-1**, entitled **"Evaluation of 1% hyaluronic acid gel effect on bone repair in critical-size calvarial defects in diabetic rats - radiographic and histometric analysis"**, 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 **March 31, 2008**.

Campinas, 31 de março de 2008.

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