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"DETECÇÃO DE METALOPROTEINASES DA FAMILIA DAS GELATINASES E DE SEUS INIBIDORES NO LIGAMENTO INTERPÚBICO DO CAMUNDONGO, DURANTE A PRENHEZ"

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SUMÁRIO

RESUMO

Durante a prenhez de alguns roedores ocorre um acentuado processo de remodelação da sínfise púbica (SP). No camundongo, esta articulação fibrocartilaginosa é gradativamente formada por um tecido conjuntivo fibroso, resultando no ligamento interpúbico (LI) entre os coxins de cartlagem na etapa final da prenhez. Logo após o parto, este ligamento é rapidamente remodelado e o espaço entre os ossos púbicos se fecha por volta do quinto dia pós-parto.

Sabe-se que o hormônio relaxina facilita o crescimento e remodelação dos órgãos do trato reprodutor feminino, alterando a regulação dos processos bioquímicos envolvidos na remodelação da matriz extracelular (MEC) do útero gravídico e da cérvice. Porém pouco se conhece sobre a sínfise em relação ao envolvimento de metaloproteinases (MMPs) nessa remodelação. Assim como, da participação de células inflamatórias, que poderiam estar envolvidas no processo de remodelação da sínfise púbica do camundongo durante a prenhez.

Neste estudo, foram utilizadas sínfises de camundongos virgens e ligamento interpúbico de animais prenhes para a detecção de MMPs *in vivo* e *in vitro* (explante de 24 horas). As MMPs da família das gelatinases (MMP-2 e MMP-9) foram caracterizadas pela zimografia e por meio de ensaios imunohistoquímicos, assim como a MMP-8 (colagenase 2) associada, quase que exclusivamente, com o processo inflamatório. Também foram utilizadas microscopia eletrônica de transmissão e microspia de luz associada às colorações seletivas para evidenciar os tipos celulares que poderiam estar participando do processo de remodelação da sínfise púbica.

A zimografia demonstrou a presença das gelatinases MMP-2 e MMP-9, ambas como pré e pró-enzimas, na SP e no LI. Imunohistoquímica detectou a reação positiva de gelatinases e também de MMP-8 em células semelhantes aos fibroblastos. A caracterização morfológica evidenciou que as células semelhantes a fibroblastos na sínfise de animais virgens estão próximas a matriz pericelular, ao passo que durante a prenhez, há um aumento deste sub-compartimento, entre célula e matriz. As análises qualitativas e quantitativas mostram um reduzido número de granulócitos na sínfise de animais virgens e no ligamento intepúbico durante a prenhez.

Dados obtidos neste trabalho suportam a hipótese de que as gelatinases estão envolvidas na remodelação da sínfise por processos intrínsecos, evidenciando que tanto as modificações do fenótipo de células semelhantes a fibroblastos, como as modificações químicas da MEC, são fundamentais para o rearranjo desta articulação durante a prenhez, parto e pós-parto no camundongo. A extensão da remodelação da MEC sugere o papel dos hormônios da prenhez na modulação da interação célula-matriz independentemente de uma reação do tipo inflamatória descrita em outros órgãos durante a prenhez.

ABSTRACT

During pregnancy of some rodents a deep remodeling occurs in the pubic symphysis (PS). In the mouse, these fibrocartilagenous articulation are gradually replaced by a dense connective tissue, forming the interpubic ligament (IL) at the end of pregnancy. Right after birth, this ligament is rapidly remodeled and the space between the two bones is quickly closed, five days after birth.

It is well known that hormones such as relaxin, facilitates the growth and remodeling of organs in female reproductive tract, changing the regulation of biochemical processes that are involved in the remodeling of the extracellular matrix (ECM) of the uterus and cervix during pregnancy. However, little are known about pubic symphysis in when the subject is the involvement of metalloproteinases (MMPs) in this remodeling, as well as the participation of inflammatory cells that could be involved in the remodeling process of the mouse pubic symphysis during pregnancy.

In this study were used pubic symphysis of virgin mice and interpubic ligament in pregnant animals for the detection of MMPs *in vivo* and at in vitro (24h explants). MMPs from the gelatinase family (MMP-2 and MMP-9) were characterized by zymography and by immunohistochemistry essays, also MMP-8 (collagenase 2) that is believed to be associated almost exclusively with an inflammatory process. Transmission electron microscopy and light microscopy was also used associated with selectively staining, to evidence type of cells that could be involved in the pubic symphysis remodeling.

Zymography demonstrated the presence of gelatinases MMP-2 and MMP-9 both in inactive and active forms, at the PS and IL. Immunohistochemistry, at the pubic symphysis and interpubic ligament, detected the expression of gelatinases and also MMP-8 in cells that were similar to fibroblasts. Morphological characterization evidenciated that cells that are similar to fibroblasts in the pubic symphysis of virgin animals are closely related to the pericellular region, being that during pregnancy, these spaces suffers an increase in this sub-compartment between cell and matrix. Qualitative and quantitative analysis demonstrated little granulocytes in the pubic symphysis of virgin animals and in the interpubic ligament during pregnancy.

Results that were obtained from this work support the hypothesis that the gelatinases could be involved in pubic symphysis remodeling through intrinsic processes, being evident that, both cellular phenotype modifications and also chemical modifications of the ECM, are fundamental to the rearrange of this articulation during mouse pregnancy, birth and pos-partum. The extensive remodeling of the ECM suggests hormonal role of the pregnancy in the modulation of the interaction between cell-matrix independently of an inflammatory reaction in other organs during pregnancy.

1. INTRODUCÃO

1.1 A sínfise púbica e o canal de parto durante a prenhez.

O conjunto de elementos que existem para unir dois ou mais ossos entre si em seu ponto de encontro é denominado articulação ou junta e classificado morfologicamente com base na sua estrutura. O termo sínfise representa uma modalidade de articulação que significa literalmente "crescendo junto" (Ham, 1972; Gamble *et al.*, 1983).

A sínfise púbica é uma articulação pouco extensível presente na confluência dos ossos púbicos, onde são recobertos por coxins de cartilagem hialina. Esses coxins estão unidos por um tecido fibroso denso que se funde com a cartilagem, resultando em uma estreita zona de transição fibrocartilaginosa. Toda essa estrutura é revestida dorsal e ventralmente por um tecido conjuntivo denso, contínuo ao periósteo dos ossos, que na sínfise corresponde aos ligamentos superior e arqueado (Talmage, 1947 a, b; Hall, 1947; Ham, 1972).

Quanto ao canal de parto sabe-se que o amplo amolecimento da cérvice uterina bem como contrações coordenadas do útero são reconhecidamente pré-requisitos para a formação do canal em algumas espécies de mamíferos (Sherwood,1994; Garfield *et al.*, 1998). Além do dimorfismo sexual dos ossos pélvicos, o aumento da flexibilidade das sínfises sacro-ilíacas e/ou pélvica no final da prenhez contribui para reduzir os traumas do parto. Especificamente no que diz respeito à sínfise púbica durante a prenhez, verifica-se em algumas espécies, que sua expansão proporciona condições para que essa flexibilidade ocorra de modo satisfatório. A expansão ocorre de modo mais evidente em espécies de mamíferos nas quais o feto é proporcionalmente maior que nos adultos. Dentre estas se destacam os modelos animais: camundongo, (Crelin, 1969; Sherwood, 1994), cobaia (Talmage, 1947a-b; Wahl *et al.*, 1977), coelho (Hashem *et al.*, 2006) e morcego (Crelin & Newton, 1969).

Durante a prenhez, ossos púbicos se separam e a sínfise dá lugar a um ligamento. No camundongo, a expansão gradual do tecido conjuntivo fibroso induzida por hormônios esteróides tem início por volta do décimo segundo dia da prenhez. Posteriormente verifica-se o amolecimento desse tecido conjuntivo, ou "relaxamento", gradual do ligamento interpúbico, atribuído à ação da relaxina (Contribuições originais estão descritas em Sherwood, 1994-2005). Nesses animais os ossos começam a se separar no décimo segundo dia da prenhez e progridem à razão de aproximadamente 1mm por dia até o parto que ocorre no décimo nono dia do período gestacional. No momento do parto, o ligamento pode medir de 5 a 6mm (Horn, 1960). Esta abertura é muito importante para garantir o parto seguro, facilitando a passagem dos fetos pelo canal de parto. Logo após o parto, este ligamento é rapidamente remodelado e o espaço entre os ossos púbicos se fecha rapidamente. Por volta do quinto dia pós-parto ele quase que fecha completamente.

Estudos realizados desde a década de 20 têm demonstrado que existe marcante diversidade entre espécies no que diz respeito à estrutura, fonte

tecidual, regulações de síntese e secreção da relaxina. Conseqüentemente, a extrapolação dos achados de uma espécie animal para outra é desaconselhada (Sherwood, 2005). No camundongo, tais modificações resultam em elevado grau de plasticidade dessa articulação, onde em um primeiro momento ocorre proliferação da cartilagem hialina, com pouca ou nenhuma absorção óssea (Hall, 1947). No final da prenhez, as células do ligamento são fibroblastos que se dividem e se rearranjam ao longo de fibras colágenas para constituírem o ligamento interpúbico no final da prenhez (Hall, 1947; Crelin, 1969).

As modificações observadas na sínfise durante a prenhez oferecem fortes indícios de que a liberação controlada de enzimas proteolíticas contribua para que a matriz extracelular do disco interpúbico fortemente coloidal pobre em água da sínfise de animais virgens, se altere para dar lugar a um tecido conjuntivo fracamente coloidal e rico em água que constitui o ligamento interpúbico de animais a termo (Crelin 1969; McDonald & Schwabe, 1982; Sherwood, 1994). Recentemente, Rodríguez *et al.* (2003) relataram à infiltração de eosinófilos no ligamento interpúbico da cobaia nas etapas finais da prenhez, de modo semelhante ao que se observa na reação inflamatória, no entanto não há relatos na literatura de que este fenômeno tenha sido observado em camundongos ou em outros modelos experimentais.

Em animais como a cobaia e o camundongo, as modificações espaço-temporais finamente reguladas da sínfise durante a etapa final da prenhez favorecem o estudo da plasticidade de células e da matriz extracelular do tecido conjuntivo (Wahl *et al.*, 1977; Zhao *et al.*, 1999-2000; Pinheiro *et al.*, 2003 - 2005). Estes autores fizeram tais considerações tendo em mente os seguintes aspectos: 1) a homogeneidade do tecido que constitui o ligamento que se forma no período final da prenhez; 2) o tipo celular predominante no ligamento, com morfologia semelhante aos fibroblastos; 3) que o principal componente da matriz extracelular é representado pelo colágeno que contribui com cerca de 70% do peso seco do ligamento. Estas características não são observadas em outros órgãos do sistema reprodutor, a exemplo do útero que apresenta histo-arquitetura complexa, que tem o músculo como principal componente tecidual, onde a deposição e remodelação de matriz extracelular ocorrem de modo gradual durante a prenhez.

A remodelação da matriz extracelular, deste ligamento, ocorre rapidamente em resposta aos estímulos hormonais favorecendo o estudo de fenômenos envolvendo a síntese, deposição e reabsorção de componentes da matriz (Wahl *et al.*, 1977).

A partir da década de 20, ensaios hormonais para analisar as modificações bioquímicas, fisiológicas e morfológicas do disco interpúbico, cérvice, útero e glândulas mamárias induzidas pela relaxina em diferentes modelos animais evidenciaram que, a relaxina tem ação diversificada em outros órgãos tais como: cérebro, rim, coração, fígado e pulmão, atuando no sistema nervoso central, na homodinâmica e osmorregulação, desempenhando ainda papel antifibrosante. Estas características abriram perspectivas para aplicação terapêutica deste hormônio na cicatrização de feridas, ortodontia, doenças isquêmicas cardíacas, inflamação e câncer (Hayes, 2004; Sherwood, 2005). Os

receptores de relaxina observados em camundongos ooforectomizados foram localizados nos órgãos anteriormente citados, porém estão preponderantemente nas células do disco fibrocartilaginoso e do ligamento interpúbico, estando ausentes nos coxins de cartilagem hialina e nos ossos púbicos (Yang *et al.*, 1992). Posteriormente, se demonstrou que não há separação da sínfise de camundongos fêmeas que sofreram deleção (*knockout*) do gene da relaxina, embora os indivíduos sejam férteis e produzam ninhadas sadias (Zhao *et al.*, 1999-2000).

As modificações químicas, morfológicas da matriz extracelular do disco fibrocartilaginoso interpúbico, devido às alterações hormonais da prenhez, sobretudo dos níveis de relaxina, evidenciou o importante papél de tais na preparação desta articulação para o parto. Tais mudanças se refletem nas alterações da composição da matriz tais como: aumento da proporção proteoglicano-colágeno; composição de fibras do sistema elástico, de hialuronato e água (Chihal & Espey, 1973; Wahl *et al.*, 1977, Viell & Struck, 1987; Zhao *et al.*, 1999-2000; Ortega *et al.*, 2001; Moraes *et al.*, 2003; Pinheiro *et al.*, 2003-2004-2005), paralelamente à ativação de enzimas proteolíticas capazes de induzir o relaxamento da sínfise (Wahl *et al.*, 1977; Weiss *et al.*, 1979; McDonald & Schwabe, 1982). No camundongo o conjunto destas modificações ficou evidenciado nos aspectos ultra-estruturais da ruptura do arranjo helicoidal das fibras que contem colágeno, o que pode contribuir para um aumento da complacência e da extensão do ligamento de modo a facilitar o parto (Pinheiro *et al.*, 2004).

No que diz respeito ao fenótipo de células do tecido conjuntivo da cérvice uterina, resultados obtidos anteriormente em nosso laboratório mostraram que as células semelhantes aos fibroblastos (miofibroblastos) apresentam alterações de seu citoesqueleto que levam o aumento dos microfilamentos de actina, tanto na cérvice uterina humana e do rato (Varayoud *et al.*, 2001; Montes *et al.*, 2002), quanto no ligamento interpúbico de camundongos (Moraes *et al.*, 2003). Tais alterações do citoesqueleto estão relacionadas à percepção, transmissão e transdução de perturbações físicas e químicas da matriz extracelular durante a prenhez e possivelmente contribua para a reaproximação dos ossos pélvicos possibilitando a reaproximação óssea após o parto (Moraes *et al.*, 2003).

1.2 O amolecimento do canal de parto e as metaloproteinases (MMPs).

De modo geral, as modificações que ocorrem no aparelho reprodutor feminino (desde a ovulação até a parturição), são resultantes de extensiva remodelação da matriz extracelular, do arranjo e metabolismo celular. Dentre tais modificações, se destacam as que resultam nos fenômenos da decidualização e implantação embrionária (Abrahamsohn & Zorn, 1993; Paffaro *et al.*, 2003) e aquelas que levam ao amolecimento do canal de parto, importante evento biológico necessário para o parto normal (Junqueira *et al.*, 1980; Leppert, 1995; Ludmir & Shadev, 2000). Este último, descrito freqüentemente como degradação gradual do colágeno, em um processo finamente regulado durante a prenhez e que se acelera nos momentos que antecedem o parto de modo a

proporcionar a dilatação adequada ao canal. O processo de remodelação é associado às células autóctones, epiteliais, fibroblastos e também aos leucócitos circulantes capazes de secretar enzimas proteolíticas responsáveis pela hidrólise de componentes da matriz extracelular e da superfície celular, particularmente as metaloproteinases de matriz MMP-2 e MMP-9, enzimas da família das gelatinases (Bany *et al.*, 2000; Lenhart *et al.*, 2001; Stygar *et al.*, 2002; Becher *et al.*, 2004).

As metaloproteinases de matriz (MMPs) são enzimas que constituem uma família multigênica de mais de 25 enzimas, endopeptidases dependentes de zinco/cálcio e pH fisiológico para sua ativação e responsáveis principalmente pela degradação da matriz extracelular e membrana basal (Hulboy *et al.*, 1997; Nagase & Woessner, 1999; Souza *et al.*, 2001).

As MMPs são produzidas em sua maioria como pro-enzimas (inativas) e secretadas na matriz extracelular e funcionam em condições fisiológicas, são finamente reguladas e são freqüentemente induzidas em áreas com alta remodelação de matriz. As metaloproteinases de matriz podem se auto ativar, como também serem ativadas por outras MMPs ou ainda por outras proteínas (Matrisian, 1990; Hulboy *et al.*, 1997).

Além da remodelação da matriz, as MMPs aparentemente regulam o comportamento celular através da proteólise pericelular, necessária para a quebra de componentes da matriz extracelular, facilitando interações intercelulares (Werb, 1997). Conseqüentemente as MMPs desempenham importantes papéis no processamento de proteínas em vias que levam a sua ativação, desativação e remoção de complexos macromoleculares.

Deste modo, as MMPs tem destacado papel na manutenção da homeostase e influenciam a reorganização da matriz (Vansaun & Matrisian, 2006). A regulação espaço-temporal da degradação da matriz extracelular é essencial para desenvolvimento embrionário, morfogênese, reprodução, reabsorção de tecido e remodelação (Nagase & Woessener, 1999). A remodelação tecidual envolve várias respostas celulares incluindo proliferação, migração, diferenciação e apoptose. As MMPs podem participar desses processos de diferentes maneiras, através de sua ação pode alterar interações célula-célula e célula-matriz, modificar o formato celular e liberar ou ativar fatores de crescimento (Hulboy *et al.*, 1997).

A família das metaloproteinases agrupa colagenases (MMP-1, -8, -13), gelatinases (MMP-2 e -9), estromelisinas (MMP-3, -10 e -11), metaloproteinases de membrana (MT-MMPs) (MMP-14, -15, -16, -17, 24, -25) e outras enzimas que não se encaixam neste grupo, tomando como base o seu substrato específico e especificidade de proteínas da matriz extracelular (Hulboy *et al.*, 1997; Stamenkovic, 2003).

Esta família de proteínas apresenta homologias entre as MMPs, como os domínios fundamentais e também há diferenças de reconhecimento, especificidade para componentes da matriz extracelular entre as enzimas.

MMPs possuem três domínios fundamentais – um identificado como "pré", que contém sinal para sua secreção, outro identificado co "pró" que mantém sua latência e uma região catalítica que contém o sítio ativo onde se liga o zinco

(Hulboy *et al.*, 1997). A maioria das MMPs possui domínios adicionais, somando a estes três domínios, existem outros que definem a que família que uma determinada MMP vai pertencer, devido à associação com diferentes substratos e com células e componentes de matriz (Nagase & Woessner, 1999).

Estudos destinados ao reconhecimento das funções das MMPs e de suas interações e co-localizações têm demonstrado que as colagenases MMP-1, MMP-8, MMP-13 e MMP-14 são as únicas MMPs que podem degradar eficientemente os colágenos dos tipos I, II e III (Somerville *et al.*, 2003). A clivagem dos tipos de colágenos fibrilares faz com que sua estrutura se torne instável dando origem à gelatina. Depois disto, outros membros da família das MMPs, principalmente as MMP-2 e MMP-9 degradam as gelatinas. Metaloproteinases de matriz sempre atuam em conjunto com outras MMPs (Souza *et al.*, 2001; Somerville *et al.*, 2003).

A fibra de colágeno é degradada como um todo gerando fragmentos exatos de ¾ e ¼ do comprimento da molécula intacta, assim como colágenos não fibrilares como IX, XII e XIV (Balbín *et al.*, 1998). A clivagem da tripla hélice do colágeno por estas enzimas modifica propriedades da estabilidade e a solubilidade da molécula que resulta na desnaturação da mesma em gelatina.

A gelatina é suscetível à ação enzimática de algumas proteinases, particularmente às gelatinases (MMP-2 e MMP-9) que contém seqüências de aminoácidos homólogas à de fibronectina, onde se encontra o domínio catalítico, que resulta na capacidade desta enzima em clivar a gelatina. As gelatinases são as metaloproteinases de matriz mais estudadas no trato reprodutor feminino (Stygar *et al.*, 2002).

As enzimas denominadas estromelisinas como MMP-3, MMP-10 e MMP-11 atuam em diversos substratos da matriz extracelular. As duas gelatinases e estromelisinas são capazes de degradar principais componentes da membrana basal, incluindo colágeno do tipo IV, laminina e fibronectina. Existem ainda as MT-MMPs que contém um domínio transmembrana próximo à região carboxi terminal que ancoram estas MMPs a membrana plasmática. Uma das grandes funções da MT-MMPs é a ativação da MMP-2 (Curry *et al.*, 2003).

Se por um lado, a secreção de gelatinases é detectada como hidrolases ativas nos fenômenos fisiológicos de remodelação de órgãos do aparelho reprodutor feminino durante a prenhez e processos patológicos da cérvice uterina (Gaiotto *et al.*, 2004); por outro lado, ainda há controvérsias no que diz respeito à atuação de hidrolases no aumento da extensibilidade, complacência e resistência no estiramento cervical. Recentemente, Buhimschi *et al.*, (2004) sugeriram que as colagenases não influenciam na extensibilidade cervical, e se houver esta influência, ela é pequena se não for nula.

No que diz respeito à remodelação do canal de parto observou-se que as MMPs desempenham importante papel no amolecimento da cérvice a termo, em humanos. O emprego de métodos imunohistoquímicos e de biologia molecular demonstrou que os fibroblastos são a principal fonte de MMP-2 enquanto que a MMP-9 foi detectada de modo esparso nos leucócitos (Stygar *et al.*, 2002).

No que diz respeito à família das metaloproteinases de camundongos caracterizadas como endopeptidases dependentes de zinco e cálcio e com

extensivas seqüências homólogas, verificou-se que ela conta com 23 membros capazes de degradar componentes da matriz extracelular, facilitando a remodelação e migração celular. Destas, as gelatinases GelA e GelB (respectivamente MMP-2 e MMP-9) parecem ser expressas estritamente em tecidos reprodutivos (Nuttall *et al.*, 2004). No que diz respeito à ação destas gelatinases notou-se que elas atuam na remodelação de ligamentos, a exemplo do ligamento periodontal, e que esta atuação pode ser modulada pela elevação da concentração de íons zinco no meio (Souza *et al.*, 2001).

Esses aspectos nos fazem supor que diferentes enzimas possam contribuir para a rápida remodelação de uma estrutura dinâmica com uma grande capacidade de adaptar-se às demandas fisiológicas variadas, como àquelas a que está submetido o ligamento interpúbico de camundongos prenhes. Além das MMPs as enzimas lisossomais como catepsinas B e C e serino proteases identificadas inicialmente por Weiss *et al.*, (1979).

1.3 A regulação das metaloproteinases (MMPs) e seus inibidores.

As atividades das MMPs são reguladas por diferentes inibidores como, por exemplo as *TIMPs – Tissue Inhibitor of Matrix Metalloproteinases,* inibidores endógenos. Estas moléculas protéicas têm a capacidade de inibir específica e reversivelmente às atividades de metaloproteinases da matriz e daquelas, que permanecem ligadas às membranas celulares – *Membrane type* (Noda *et al.*, 2003; Nuttall *et al.*, 2004). Estes antagonistas fisiológicos são altamente conservados e constitutivamente expressos em altos níveis nos órgãos do sistema reprodutor do camundongo (Nuttall *et al.*, 2004). São moléculas secretadas que atuam no meio extracelular (Fassina *et al.*, 2000).

As TIMPs possuem dois domínios com regiões N- e C- terminal, onde cada uma contém seis resíduos de cisteína, formando três pontes dissulfeto. A região N- terminal contém a região de maior homologia entre os quatro tipos de TIMPs que é suficiente para inibição de metaloproteinases. Estes domínios possuem resíduos que interagem com zinco de metaloproteinases ativas. A região C-terminal é importante para interação entre proteínas, e também para interação com pro-MMPs, assim regulando sua atividade (Chirco *et al.*, 2006).

São inibidores endógenos de metaloproteinases. Até recentemente quatro membros da família das MMPs foram identificados e o algarismo anotado na identificação do inibidor indica a ordem seqüencial de suas identificações (Fassina *et al.*, 2000). Os membros da família TIMP-1, TIMP-2, TIMP-3 e TIMP-4 reduzem a atividade de muitas MMPs. A TIMP-2 é expressa constitutivamente e amplamente expressa pelos tecidos do corpo, enquanto que as expressões de TIMP-1, -3 e -4 são mais restritas (Chirco *et al.*, 2006).

As diferenças entre as TIMPs se devem basicamente ao fato de que a TIMP-1 é uma proteína solúvel, glicosilada, com peso molecular por volta de 28KDa. Esta TIMP se liga ao domínio hemopexina da MMP9, diminuindo sua atividade (Fassina *et al.*, 2000); TIMP-2, solúvel, não-glicosilada, com 21KDa de peso molecular, é capaz de se ligar ao domínio de hemopexina de pro-MMP-2 modulando sua atividade. Pequenas concentrações de TIMP-2 associadas às

moléculas de MT1-MMP medeiam à ativação de MMP-2, enquanto em altas concentrações, pode inibir diretamente a MMP-2 e MT1-MMP mediado pela ativação da MMP-2; TIMP-1 se liga preferencialmente à MMP-9 e TIMP-2 à MMP-2 (Hulboy *et al.*, 1997), TIMP-3 proteína insolúvel, associada à matriz, não-glicosilada e com peso molecular de 24KDa tem atuação no controle da invasão do dos tecidos que compões o útero pelo trofoblasto, limitando a degradação da matriz extracelular (Hulboy *et al.*, 1997). TIMP-3 também foi localizada em tecidos como o cartilaginoso, muscular e epitelial (Apte *et al.*, 1994). A TIMP4, proteína solúvel não-glicosilada, de peso molecular de 23KDa, a última proteína a ser caracterizada, tem participação em processos de remodelação matriz de órgãos como coração durante a embriogênese e do ovário durante a maturação, ruptura do folicular e luteólise (Fassina *et al.*, 2000; Rahkonen *et al.*, 2002).

Mesmo com seu tamanho muito pequeno, as TIMPs possuem outras funções além de inibir a atividade de MMPs. Podem também estar envolvidas em promover o crescimento celular, se ligar a matriz extracelular, indução e redução da apoptose (Fassina *et al.*, 2000).

As contribuições anteriormente citadas evidenciaram que o equilíbrio entre a expressão e ativação de metaloproteinases e seus inibidores constituem um dos principais mecanismos para manutenção da funcionalidade da matriz, e regulação de eventos de remodelação que contribuam para processos fisiológicos, a exemplo daquele que permite a remodelação da sínfise púbica do camundongo durante a prenhez. Estes aspectos nos fizeram supor que, diferentes MMPs e seus inibidores TIMPs, poderiam contribuir para uma rápida remodelação de uma estrutura dinâmica com uma grande capacidade de ajuste sobre diferentes demandas fisiológicas, como o ligamento interpúbico em camundongos durante a prenhez.

1.4 Principais fontes celulares de enzimas envolvidas nos processos de remodelação do canal de parto.

Além das similaridades da composição bioquímica da matriz extracelular da cérvice e do ligamento interpúbico no final da prenhez foi demonstrado na cobaia que as modificações que levam ao relaxamento ligamento têm semelhanças com o processo inflamatório (Rodríguez *et al.*, 2003). Tal adaptação tem como base o influxo de células inflamatórias como principal evento regulatório da ruptura da integridade, da matriz o que resulta no amolecimento da cérvice em humanos e modelos animais (Junqueira *et al.*, 1980; Osmers *et al.*, 1995; Bokstrom *et al.*; 1997; El Maradny *et al.*, 1997; Knudsen *et al.*, 1997; Luque *et al.*, 1997; Mackler *et al.*, 1999; Hertelendy *et al.*, 2004).

Na sínfise púbica da cobaia, durante a etapa final da prenhez, foi verificado um aumento significativo de eosinófilos e também imagens histológicas típicas da matriz do tecido conjuntivo em degradação ao redor dos mesmos granulócitos, durante as modificações para a preparação para o parto (Rodríguez *et al.*, 2003). Estes autores consideram a dissolução do tecido conjuntivo interpúbico, como um paralelismo entre o relaxamento da sínfise e o amolecimento da cérvice uterina, sob controle hormonal.

Durante o amolecimento cervical, foi observado que o influxo de células inflamatórias coincide com a elevação da concentração de metaloproteinases de matriz tais como MMP-9 e MMP-8, produzidas por granulócitos presentes no tecido conjuntivo durante a dilatação (Rath *et al.*, 1998). A MMP-8 é também conhecida como colagenase dos neutrofilos e é normalmente armazenada em grânulos citoplasmáticos como pro-MMP-8 e é secretada durante intenso processo inflamatório (Nagase *et al.*, 1991). MMP-8 já foi encontrada em células como os fibroblastos (Van Lint & Libert, 2006).

A investigação de possíveis mecanismos e fontes de agentes responsáveis pelo amolecimento cervical aponta para diferentes possibilidades como: o estiramento cíclico dos tecidos durante o trabalho de parto, iniciando a quimiotaxia de neutrófilos que por sua vez promove a degradação de fibras de colágeno da cérvice uterina, finalmente contribuindo para o início e aceleramento do amolecimento cervical (Takemura *et al.*, 2004); também, a proximidade entre óstio interno da cérvice e âmnio, que atuaria como fonte quimiotática para granulócitos (Kelly, 2002). Porém de acordo com Timmons & Mahendroo (2006), o papel das células inflamatórias no início do amolecimento cervical na prenhez normal é controverso.

De acordo com Timmons & Mahendroo (2006), por volta do décimo oitavo dia da prenhez do camundongo verifica-se a migração de células inflamatórias, mas não ativação das mesmas na cérvice uterina, antes do amolecimento cervical do camundongo. Grande parte de células inflamatórias na cérvice no décimo oitavo dia da prenhez é representada por monócitos que se mantêm inativos até o parto. Verificou-se também aumento significativo de eosinófilos após o parto, além disto, foi observado que na ausência de neutrófilos, o parto acontece normalmente. Tal ocorrência poderia ser uma evidência que estes granulócitos não possuem uma participação crucial na preparação para o amolecimeto cervical (Timmons & Mahendroo, 2006).

A manutenção da viabilidade celular no explante de tecidos do aparelho reprodutor feminino semelhante àquela observada *in vivo,* possibilita a observação de células com potencial para expressar sua atividade enzimática e migrando assim da matriz para o suporte empregado na cultura, sem que haja alterações severas do fenótipo das células, uma vez que colagenases exógenas não foram utilizadas para possibilitar a liberação das mesmas (Sharma *et al.*, 2002).

Nestas condições, as células estruturais residentes expressam citocinas regulatórias chaves (CD40, IL-8 e PGE2) que podem recrutar e ativar neutrófilos capazes de induzir o amolecimento cervical imediatamente antes do parto (King *et al.*, 2001). Deste modo, julgamos oportuno investigar a expressão imunohistoquímica da MMP-8, também conhecida como colagenase dos neutrófilos, colagenase 2. Sendo uma colagenase capaz de degradar eficientemente os colágenos dos tipos I, II e III (Somerville *et al.*, 2003) que são encontrados na sínfise púbica do camundongo e que vão dar início ao processo de degradação.

Também empregaremos neste estudo as técnicas histológicas semelhantes àquelas empregadas por Rodríquez *et al.,* (2003), para a

caracterização de granulócitos presentes no ligamento interpúbico da cobaia, bem como o estudo ultra-estrutral para a exploração do comportamento das células do ligamento que poderiam estar envolvidas no processo de relaxamento desta estrutura *in vivo* e no explante, durante a etapa final da prenhez.

2. OBJETIVOS

Dada a importância da secreção e ativação de enzimas proteolíticas, induzidas pela relaxina, durante as alterações que levam à formação do canal de parto de camundongo, nós nos interessamos em examinar o envolvimento de metaloproteinases e de seus inibidores no processo de remodelação do ligamento interpúbico que se forma durante a última semana da prenhez. Os objetivos deste estudo foram:

1. Identificar a presença de granulócitos no ligamento interpúbico de camundongo prenhez no final da gestação que poderiam fazer parte do processo de remodelação da sínfise púbica durante a prenhez.

2. Identificar a expressão imunohistoquímica de metaloproteinases de matriz: colagenases-MMP8 e gelatinases-MMP2 e MMP9 em populações celulares do ligamento interpúbico do camundongo na etapa final da prenhez.

3. Identificar a expressão imunohistoquímica dos inibidores de metaloproteinases: TIMP1 (inibidor de MMP9) e TIMP2 (inibidor de MMP2) em populações celulares do ligamento interpúbico do camundongo na etapa final da prenhez.

3. ARTIGOS

Os resultados do presente trabalho permitiram a elaboração de dois artigos científicos que serão submetidos para publicação:

1. Renata Giardini Rosa[1], Christiane Aparecida Badin Tarsitano [2], Stephen Hyslop.[2], Áureo Tatsumi Yamada[1], Olga Maria S. Toledo[3] and Paulo Pinto Joazeiro.[1]

RELAXATION OF THE MOUSE PUBIC SYMPHYSIS DURING LATE PREGNANCY IS NOT ACCOMPANIED BY THE INFLUX OF GRANULOCYTES

2. Renata Giardini Rosa[1], Christiane Aparecida Badin Tarsitano [2], Stephen Hyslop[2] , Áureo Tatsumi Yamada[1], Olga Maria S. Toledo[3] and Paulo Pinto Joazeiro.[1]

INVOLVEMENT OF METALLOPROTEINASES (MMP-2 AND MMP-9) AND THEIR INHIBITORS (TIMP-1 AND TIMP-2) IN THE REMODELING OF THE MOUSE PUBIC SYMPHYSIS DURING LATE PREGNANCY.

RELAXATION OF THE MOUSE PUBIC SYMPHYSIS DURING LATE PREGNANCY IS NOT ACCOMPANIED BY THE INFLUX OF GRANULOCYTES

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ABSTRACT

In some animals, such as mice and guinea pigs, a hormonally controlled mechanism increases the flexibility of the pubic symphysis and enhances the cervical remodeling necessary for safe delivery. Cervical ripening during pregnancy is associated with a paradoxical influx of leukocytes. However, the changes in cell metabolism during relaxation of the mouse pubic symphysis for delivery have not been extensively studied. In this work, we used light microscopy and transmission and scanning electron microcopy, as well as immunohistochemistry for MMP-8, to investigate the involvement of granulocytes or resident stromal cells in the relaxation of the virgin pubic symphysis during late pregnancy (days 18 and 19, before delivery) *in vivo* and in explanted joints. MMP-8 was studied because this collagenase is a hallmark for cervical ripening associated with the influx of granulocytes during late pregnancy. Extensive dissolution and disorganization of the extracellular matrix was seen around fibroblastic-like cells in late pregnancy. However, in contrast to the cervix (positive control), morphological and immunohistochemical analyses revealed that there was no characteristic cellular inflammatory response in the interpubic tissue. Staining for MMP-8 was observed in chondroid and fibroblastic-like cells of virgin and relaxed interpubic ligament, respectively. Similarly, no inflammatory response was seen in the extensive remodeling of the pubic joint during mouse delivery. These results indicate that constitutive stromal cells may do not have an important role in tissue degradation during remodeling of the pubic symphysis in pregnancy.

Keywords: Metalloproteinases, Remodeling, Extracellular Matrix, Pubic Symphysis.

INTRODUCTION

During pregnancy, structural modifications of the pelvic girdle that allow walking and ensure safe passage of the fetus at birth occur in many mammalian species, including mice (Crelin, 1969; Hall, 1947; Sherwood, 1994), guinea pigs (Rodriguez *et al.*, 2003; Talmage, 1947a, b; Wahl *et al.*, 1977), rabbits (Hashem *et al.*, 2006) and bats (Crelin and Newton, 1969).

The main modifications in the pubic symphysis involve finely tuned tissue remodeling of the pubic joint during late pregnancy. At this stage, the mouse pubic joint becomes separated by a flexible, elastic interpubic ligament that is 5-6 mm long at parturition. Following labor, the ligament undergoes rapid involution and returns to its original size by the third or fourth day postpartum (Crelin, 1969; Hall, 1947; Sherwood, 1994). Steroid hormones and relaxin are involved in reproductive tract physiology and help to regulate the processes involved in remodeling of the extracellular matrix (ECM) (for original contributions, see Sherwood, 1994-2005). Mature condrocytes released from their lacunae de-differentiate and become fibroblast-like cells that arrange themselves transversely along the collagen fibers to form a new structure that resembles a true ligament (Crelin, 1969; Moraes *et al.*, 2003). This cell behavior may increase the flexibility of the pubic symphysis in pregnant mice.

The progressive modifications that occur in the mouse pubic joint during pregnancy involve alterations in the composition and abundance of connective tissue, as well as the rearrangement of collagen and elastic fibers. At term, the rupture and rearrangement of helicoidal collagen fibers increase the pliancy and relaxation of the ligament, thereby facilitating parturition in mice (Crelin, 1969; Chihal and Espey, 1973; Hall, 1947; Moraes *et al.*, 2003; Ortega *et al.*, 2001; Pinheiro *et al.*, 2003, 2004, 2005; Zhao *et al.*, 1999, 2000). In addition, the activation of proteolytic enzymes capable of relaxing the symphysis relaxation has also been described (McDonald and Schwabe, 1982; Wahl *et al.*, 1977; Weiss *et al.*, 1979). Biochemical and morphological changes similar to those seen in the ECM of the mouse pubic symphysis have also been reported for nipples, vagina and cervix (Zhao *et al.*, 1999, 2000).

In addition to morphological and biochemical changes in the ECM, the adaptation of the reproductive tract for delivery also involves an inflammatory-like response. In a currently accepted model of connective tissue remodeling, the influx of inflammatory cells is a major regulatory event for initiating connective tissue ripening in the uterine cervix of humans and animals (Bokstrom *et al.*, 1997; El Maradny *et al.*, 1997; Hertelendy *et al.*, 2004; Junqueira *et al.*, 1980; Knudsen *et al.*, 1997; Luque *et al.*, 1997; Mackler *et al.*, 1999; Osmers *et al.*, 1995).

While studying relaxation of the pubic symphysis in pregnant guinea-pigs, Rodriguez *et al.* (2003) noted a significant increase in the number of granulocytes, especially eosinophils, that was closely related to dissolution of the interpubic connective tissue during the preparatory changes for delivery. These authors concluded that the granulocytic infiltration of guinea-pig interpubic

connective tissue resembled an inflammatory response similar to that described for cervical ripening at parturition.

Different cellular sources and mechanisms have been proposed for the actions of ripening agents, including: 1) cyclic mechanical stretch of tissue by labor that initiates neutrophil chemotaxis, resulting in the degradation of collagen that contributes to the initiation and/or acceleration of cervical ripening (Takemura *et al.*, 2004), 2) changes in the biological response to glucocorticoids that enhance pro-inflammatory events to cause cervical ripening at parturition (Stygar *et al.*, 2002) and 3) the proximity of the internal *os* of the cervix and the fetal membranes that could be sources of ripening agents such as chemotactic factors (Kelly, 2002).

The influx of inflammatory cells coincides with a rise in the levels of matrix metalloproteinase (MMP) -1, -13 and -8 that degrade fibrillar collagen, and MMP-9 that degrades basement membranes (Aronsson *et al.*, 2005; Rath *et al.*, 1998; Sennström *et al.*, 2003). MMP-8, also known as neutrophil collagenase-2, is normally stored in neutrophil granules as pro-MMP-8 and is secreted during acute inflammatory processes (Nagase *et al.*, 1991). Sennström *et al.* (2003) reported an 80-fold increase in MMP-8 levels during cervical ripening and suggested that this MMP may be important for initiating remodeling in female reproductive tissue.

Despite the "traditional paradigm that the influx of inflammatory cells is a major regulatory event in the initiation of cervical ripening" (Hassan *et al.*, 2006), Timmons and Mahendroo (2006) recently demonstrated that normal mouse cervical ripening does not require a typical inflammatory response. According to these authors, there is evidence for the migration of inflammatory cells into the cervix before the onset of cervical ripening, but no evidence for their activation.

Considering the limited morphological evidence for a time-dependent accumulation of leukocytes during pubic separation in preparation for normal delivery (we are aware of only one such study in the literature, done in guinea-pigs), as well as the controversy surrounding the occurrence of a typical inflammatory response during cervical ripening in mice, we investigated whether relaxation of the interpubic joint in pregnant mice resembled a typical inflammatory process. We also investigated the expression and distribution of MMP-8 in the interpubic joint (*in vivo*) using immunohistochemistry and examined the cell behavior in explanted joints of virgin and late pregnant mice. The use of 24 h explants allowed us to assess time-dependent tissue remodeling in the absence of a major inflammatory input (Flick *et al.*, 2006).

MATERIAL AND METHODS

Animals

Outbred female Swiss mice (~90 days old) were obtained from the Multidisciplinary Center for Biological Investigation (CEMIB) at UNICAMP and mated with males of the same strain as described by Moraes *et al.* (2003). The presence of a vaginal plug the next morning was considered as day one of pregnancy. Virgin mice in estrus (Shorr, 1941) and pregnant mice on day 18

(D18, one day before parturition) and day 19 (D19, day of parturition but before delivery) were sacrificed between 11:00 and 12:00h a.m. after anesthesia with a mixture of ketamine and xylazine chloride (100-200 mg/kg and 5-16 mg/kg, i.p., respectively; Agribrands do Brasil, Jacareí, São Paulo, Brazil). Following laparotomy, the medial portions of the pubic bones, symphysis and ligaments were removed and processed. The cervix was excised and used as a control when necessary. All of the surgical procedures were done using aseptic technique, and the experimental protocols were approved by the institutional Committee for Ethics in Animal Experimentation (CEEA/IB/UNICAMP, protocol no. 1143-1).

Explant cultures

Three animals per group (virgin, D18, and D19) were sacrificed, and the interpubic tissue was immediately cleaned from any fat, muscle, bone and fascia. The tissue fragments, smaller than 0.5 mm (in size), that constituted the explant were taken from triplicates of virgin pubic symphysis and from pregnant mice D18 and D19 interpubic ligament. The explants were cultured in a round glass cover slip in 24 well plates with Dulbecco's Modified Eagle Medium (DMEM) – Gibco BRL, with gentamicin, (40 mg/ml), and with 10 % fetal inactivated calf serum, according to Souza *et al.* (2001). The cultures were incubated in a 5% CO_2 atmosphere at 37ºC for 24 h after which the explants were removed and processed for transmission electron microscopy (TEM) and the cells that had migrated from the explant onto the coverslips were processed for scanning electron microscopy (SEM).

Light microscopy

Five mice from each group (virgin, pregnant D18 and pregnant D19) were used for light microscopy. The interpubic tissue (symphysis and ligaments) and cervix were dissected and fixed *in situ* in 4% paraformaldehyde (Merck, Darmstadt, Germany) in 0.1 M phosphate buffer, pH 7.2, and then removed and immediately immersed in the same fixative solution for 24 h. The samples were then dehydrated in a graded ethanol series and embedded in paraffin at 58ºC. Sections 5 µm thick were mounted on glass slides coated with 0.1% poly-L-lysine (Sigma Chemical Co., St. Louis, MO, USA), deparaffinized and stained with a variety of selective stains. Collagen was detected by staining with Sirius red - F3B (Pinheiro *et al.*, 2004), eosinophils were detected with a modified Sirius red method (Wehrend *et al.*, 2004 a,b) and neutrophils were stained with Giemsa (Rodríguez *et al.*, 2003).

For immunohistochemistry in interpubic tissues, the sections were deparaffinized and heated in a Panasonic (model NN 7809-BH) microwave oven (1380W, and two cycles of 180s). Endogenous peroxidase activity was blocked using 3% H_2O_2 followed by incubation in 5% skimmed milk.

The sections were then incubated with anti-MMP-8 polyclonal rabbit antibody (RP1MMP8 - Triple Point Biologics, Forest Grove, OR, USA) diluted 1:400 in 0.01 M phosphate buffer and 0.2% BSA. After incubation overnight at 4°C, a secondary anti-rabbit antibody (DAKO immuno/biotinylated GxR E04321)

was applied for 1 h. The reactions were developed using a streptavidin-peroxidase conjugate (Vector, USA) and a mixture of 3.3-diaminobenzidine (0.5 mg/ml; Sigma) and 0.3% hydrogen peroxide as substrate. The sections were counterstained with Harris´ hematoxylin and mounted in permanent mounting medium under a coverslip (Moraes *et al.*, 2003). Sections of cervix were used as positive controls and, for negative controls, the primary antibody was replaced by non-immune rabbit serum. The sections were examined and photographed with a Nikon Eclipse E800 light microscope.

Electron microscopy

After sectioning for light microscopy, samples of interpubic tissue were processed for SEM. Deparaffinized sections were hydrated and incubated for 30 min in 0.02 M phosphate buffer, pH 7.4, containing 0.005 M sodium bisulphite and 0.0005 M EDTA followed by incubation in 0.05% papain (Merck) in this same buffer for 90 min at 37°C (Montes and Junqueira, 1988). The sections were then washed twice in distilled water for 10 min and fixed in 2% glutaraldeyde-1% tannic acid for 1 h at room temperature. After a further wash, the samples were post-fixed in 1% osmium tetroxide for 1 h at 4°C, dehydrated in a graded ethanol series and critical point-dried with CO_2 in a Balzers CPD 030 critical point dryer. The specimens were coated with gold in a Balzers SCD 050 sputterer and then examined in a JEOL 5800 LV scanning electron microscope operated at 5-10 kV. Coverslips used as explant supports were also fixed, dehydrated and processed for SEM as described above.

For TEM, fragments of fresh interpubic tissue and 24 h cultured explants were fixed in a solution containing 1.25% formaldehyde, 2.5% glutaraldeyde and 0.03% picric acid in 0.1 M sodium cacodylate buffer, pH 7.2, for 2 h at room temperature. After post-fixation in 1% osmium tetroxide for 1 h at 4°C, the samples were dehydrated in a graded ethanol series and embedded in epoxy resin Epon 812 (Electron Microscope Science). Ultrathin sections were collected on cooper grids, stained with uranyl acetate and lead citrate and examined with a LEO 906 transmission electron microscope.

RESULTS

Light microscopy and SEM of transverse sections showed the general histoarchitecture of the pubic joint in adult virgin female mice (Fig 1). The central fibrocartilaginous disk was the major component that provided mechanical support and neutralized the forces of traction and shearing by limiting movement of the joint. The histological and ultrastructural organization of the pubic girdle in D18 and D19 pregnant mice (Fig. 2) agreed with the classic description of joint relaxation as "transformation of a colloidal-rich, water-poor phase (seen here as hyaline and fibrocartilaginous tissues) to a colloidal-poor, water-rich phase (seen here as a loosely arranged interpubic ligament)" (Crelin, 1969).

Giemsa staining (Fig. 3A-C) and modified Sirius red staining (Fig. 3D-F) (Rodríguez *et al.*, 2003) were used to screen for the presence of cells characteristic of an inflammatory response. No migrating granulocytes were seen

in virgin fibrocartilaginous disks (Fig. 3A, D) or in the relaxed interpubic ligament of D18 (Fig. 3B, E) or D19 (Fig. 3C, F) mice. In contrast to the symphysis and relaxed interpubic ligament, neutrophils were detected in the cervical stroma of virgin females (Fig. 4A) and in D18 and D19 (during ripening) mice (Fig. 4B). Eosinophils were detected in pubic bone marrow (Fig. 4C).

MMP-8 was detected in virgin fibrocartilaginous disks and in the stroma of the relaxed interpubic ligament of D18 and D19 females (Fig. 5). In virgin mice, MMP-8 was present in the cartilaginous caps (not shown) and chondroid cells of the fibrocartilaginous disk (Fig. 5A). In pregnant mice, MMP-8 was detected in the cytoplasm of chondrocytes of the cartilaginous caps and in fibroblast-like cells on the day D18 (Fig. 5B, C), although some of the latter cells on the D19 showed no MMP-8 immunoreactivity (Fig. 5C). Cervical tissue (positive control) showed strong staining for MMP-8 (not shown).

SEM and TEM were used to examine fresh (*in vivo*) and 24 h explanted (*ex vivo*) interpubic tissue from virgin and late pregnant mice (D18 and D19). Figure 6 shows the typical aspects of "de-differentiation of mature cartilaginous tissue, in which chondroid cells are released from their lacuna and became fibroblast-like when enmeshed in the web of collagen fibers" (Crelin, 1969). However, ultrastructural analysis revealed no signs of an influx of inflammatory cells during interpubic tissue relaxation.

DISCUSSION

The histological and ultrastructural characteristics of interpubic relaxation seen here in female Swiss mice agreed with previous descriptions of the reorganization of the pubic joint histo-architecture during late pregnancy in mice (Crelin, 1969; Moraes *et al.*, 2003; Pinheiro *et al.*, 2004, 2005) and guinea-pigs (Chihal and Espey, 1973; Rodríguez *et al.*, 2003; Wahl *et al.*, 1977). Although the enlargement of the pubic gap can be partly explained by the development and growth of the interpubic ligament, it is the final fiber remodeling (disassembly, untwisting and rupture) and invasion of the tissue by water molecules attracted to this site by proteoglycans and glycosaminoglycans that allow the length of the pubic articulation to increase (Pinheiro *et al.*, 2004, 2005; Viell and Struck, 1987; Zhao *et al.*, 2000). These changes result from well-defined biochemical processes stimulated particularly by relaxin. This phenomenon is not restricted to the reproductive system but also occurs in the degeneration of fibrocartilaginous tissue and of the female temporomandibular joint disk through the modulation of matrix metalloproteinase expression (Hashem *et al.*, 2006; Naqvi *et al.*, 2005).

Light and electron transmission microscopy of *in vivo* and explanted (*ex vivo*) tissues revealed no neutrophils or eosinophils in the pubic symphysis of virgin mice or in the relaxed interpubic ligament at term. In contrast, inflammatory cells were detected in the cervix, used as a positive control. These findings differ from those of Rodríguez *et al.* (2003) who identified neutrophil and eosinophil, leukocytes in the interpubic ligament of pregnant guinea-pigs before delivery. These authors concluded that relaxation of the guinea pig pubic symphysis at parturition resembled an inflammatory process. In contrast to these findings,

Timmons and Mahendroo (2006) reported an influx of inflammatory cells into the cervix of mice before the onset of ripening, but no subsequent activation of these cells, which suggested that in this species there is no need for an inflammatory response such as that seen in guinea-pigs. This conclusion is contrary to the general opinion that an influx of leukocytes is intimately associated with ECM degradation during cervical ripening at term (Balbín *et al.*, 1998; Junqueira *et al.*, 1980; Kelly, 2002; Luque *et al.*, 1997; Luque and Montes, 1989; Takemura *et al.*, 2004).

MMPs are involved in the rapid remodeling of organs and structures such as ovaries, uterus, cervix, pubic symphysis and nipples. The dramatic ripening of the cervix involves a cascade of MMPs that includes MMP-8 (collagenase-2), MMP-3 (stromelysin) and gelatinases (MMP-2 and -9). Indeed, high levels of MMP-8 (Sennström *et al.*, 2003); MMP-3 (Chien *et al.*, 2005) and MMP-2 and -9 (Stygar *et al.*, 2002) have been observed at parturition. MMP-8 was originally thought to be expressed solely in neutrophil precursors during late myeloid maturation, but it is now recognized that this enzyme can be expressed in a wide range of cells, including chondrocytes and fibroblasts (Van Lint and Libert, 2006). MMP-8 is the only collagenase to be stored in cells rather than being synthesized and is released on demand. This enzyme is also the only MMP known to cleave aggrecan, a major proteoglycan, in addition to type I collagen (Maymon *et al.*, 2000).

The immunohistochemical detection of MMP-8 in chondrocytes and chondroid cells of virgin pubic symphysis agreed with the expression of this enzyme reported for growth plate cartilage and mature chondrocytes during growth and aging (Bae *et al.*, 2003; Macotela *et al.*, 2006). In addition, the focal or punctate staining for MMP-8 seen in the cytoplasm of fibroblast-like cells of the interpubic ligament on D18 and D19 (relaxed) also agreed with the intracellular storage of this protein. The hormonal control of MMP-8 expression in fibroblasts of the relaxed mouse interpubic ligament may be similar to that seen in these cells during cervical ripening (Sennström *et al.*, 2003). While these assays do not determine the activity of the enzyme, the location of the protein may indicate a potencial function for this gene.

MMP-8 is the first MMP involved in the degradation of fibrillar collagens during remodeling of the pubic symphysis in pregnancy, followed by the gelatinases MMP-2 and MMP-9, as occurs in the cervix (Stygar *et al.*, 2002). MMP-8 produced by human chondrocytes (and possibly other vertebrate species) can also cleave prolactin (PRL) to generate biologically active peptides that prevent angiogenesis under normal conditions and protect cartilage (Macotela *et al.*, 2006). Hence, the production of MMP-8 in the mouse pubic joint may contribute to the restoration of this articulation after delivery.

Changes in the interaction between the ECM and fibroblastic-like cells in the interpubic ligament at the end of pregnancy are an important aspect of remodeling. Although MMPs greatly facilitate the streaming migration of cells through surrounding connective tissue, these enzymes may be sequestered by extracellular matrix proteins until needed (Vansaun and Matrisian, 2006), and the complete inhibition of all proteases does not stop cellular migration in this region

(Wolf *et al.*, 2003). These characteristics show that, like the cervix, the pubic symphysis is a dynamic structure, with a high capacity to adapt to different physiological situations (Moraes *et al.*, 2003).

Twenty-four hour cultures of tissue explants from virgin joints and the interpubic ligament on D18 and D19 (relaxed) provide a means of studying the behavior of condroid cells in the mouse pubic symphysis, as originally described by Crelin (1969) where also the mature cartilaginous chondroidal cells released from the lacunae of such explants de-differentiate into fibroblastic-like cells in the interpubic ligament during pregnancy.

The explant method used in this study enable us to observe cells that have the potential to express their enzymatic activity liberating themselves from the matrix and migrating out from the explant to the coverslips, without any damage caused by enzymatic digestion as collagenase is usually used for releasing cells from extracellular matrix.

Thus, organ or explant cultures in which heterogeneous cellular viability and differentiated state are preserved as a result of auto- and paracrine interactions can be relied upon, in general, for molecular or cytoarchitectural research in short-term (24–96 h) as it was observe in the reproductive tissue culture studies (Sharma *et al.*, 2002). The presence of ruptured collagen network among the fibroblasts mainly in the explanted pregnant interpubic ligament suggests that the fibroblastic cell continues to survive and maintain its morphological and biochemical integrity despite incubation. More over the use of explants has the advantage that tissue responses can be observed independently of any influence by systemic inflammation or pain, although there may be an initial, transitory inflammatory response following isolation of the tissue (Flick *et al.*, 2006).

In the present study, examination of the cells and cell-ECM interactions in fresh and explanted tissue from virgin joints and from the interpubic ligament on D18 and D19 showed that chondroidal cells retained their round profile whereas fibroblasts were loosely associated with the underlying fibrillar ECM and migrated spontaneously on D19. Fibroblast migration is an integral part of biological processes such as wound healing and embryogenesis (Dunlevy and Couchman, 1993), and intrinsic factors, including MMP-8, secreted by pubic cells may enhance cellular locomotion in relaxed ligament. The detection of migrating fibroblasts confirmed that interpubic cells are phenotypically similar to myofibroblasts and are involved in sealing the pubic gap after parturition (Moraes *et al.*, 2003). This could explain why relaxation of the mouse pubic symphysis does not involve a typical inflammatory response.

In conclusion, our data indicate that relaxation of the pubic symphysis in mice does not require a typical inflammatory response. This conclusion is supported by the absence of polymorphonucelar neutrophils and eosinophils in the symphysis *in vivo* and in 24 h cultures of tissue explants from virgin or pregnant mice.

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FIGURE LEGENDS

Fig. 1. Light and scanning electron micrographs of the pubic symphysis of an adult virgin female mouse. A - Sirius red stained section showing all components of the joint. B - Details of the densely packed extracellular matrix and small elliptical chondroid cells of the fibrocartilaginous disk. C - Low power electron micrograph of the same specimen as in panel A showing the smooth transitions among the components of the stromal joint. D - Electron micrograph of the fibrocartilaginous disk. Note the well-defined lacunae of chondroid cells in the densely packed collagen fibers of the extracellular matrix. FD - central interpubic fibrocartlaginous disk with a stromal region rich in collagen fibers, HC - hyaline cartilage caps, PB - pubic bones, with skeletal muscle (sm) insertions. Bars: A, C - 100 μm, B - 70 μm, D - 10 μm.

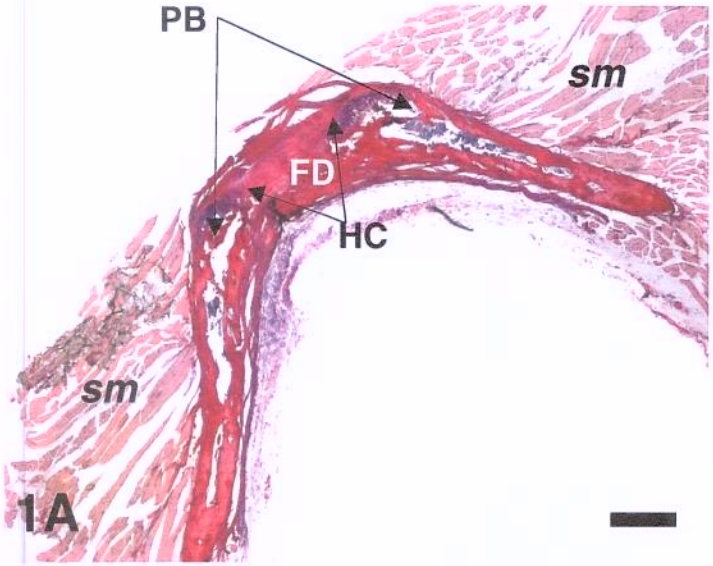
Fig. 2. Light and scanning electron micrographs of transverse sections of the interpubic ligaments of mice during late pregnancy. A - Sirius red stained section showing the major components of the symphysis on the 18th day of pregnancy. Note that the gap between the pubic bones is greatly enlarged (compare with Fig. 1A, same magnification) and that long, thick, densely packed collagen fibers are arranged parallel to each other and to the major axis of the symphysis. B - Detail showing the elliptical nuclei of fibroblast-like cells that form the interpubic ligament. Wavy collagen fibers show a zig-zag arrangement that corresponds to crimp (dotted lines). C - Scanning electron micrograph of the same specimen as shown in A. Note the fiber organization and crimp (low power view). D - Scanning electron micrograph of the interpubic ligament on the day of parturition (D19). Note the irregular and disrupted collagen fibers; separation of the bundles revealed thin, isolated fibers (★ , compare with Fig. 2C). HC - hyaline cartilage caps, IL - interpubic ligament, PB - pubic bones. Bars: A – 100 μm, B-D – 10 μm.

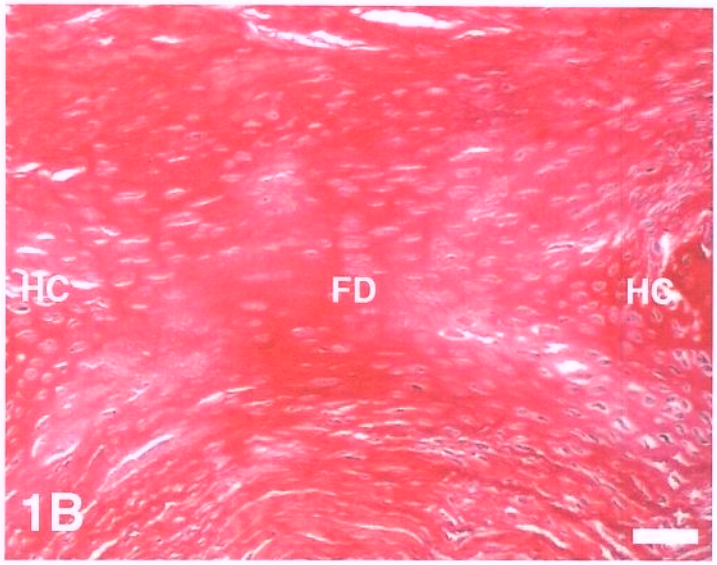
Fig. 3: Transverse sections of the pubic symphysis of adult virgin female mice (A, D) and of the interpubic ligament on D18 (B, E) and D19 (C, F). Staining was done with Giemsa (A-C) or modified sirius red counterstained with hematoxylin (D-F). There was no leukocyte infiltration in virgin fibrocartilaginous tissue or in the interpubic relaxed ligament on D18 and D19. Bars: 40 µm.

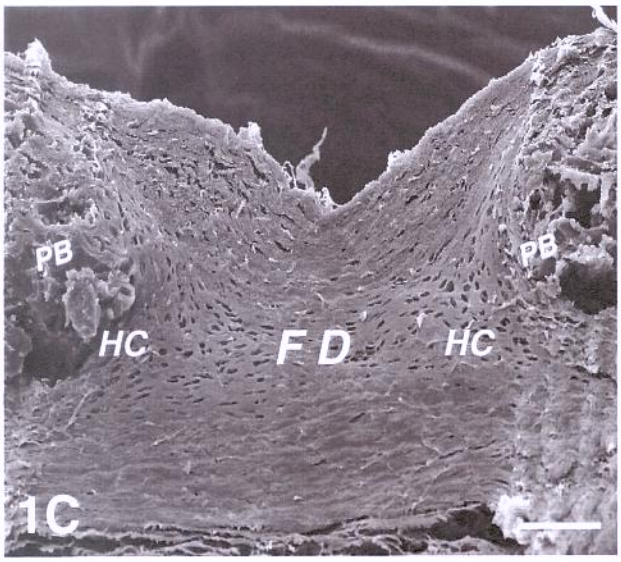
Fig. 4: Histological sections (controls) stained with Giemsa or sirius red were used to identify inflammatory cells. The cervix was used as a positive control for Giemsa stained neutrophils (A, B). In this tissue, inflammatory cells (arrow) were involved in remodeling of the cervix during pregnancy. Pubic symphysis bone marrow was used as a positive control for eosinophilic granulocytes stained with a modified sirius red method. These inflammatory cells were detected only in this area (C, arrow/inset). Bars: 40 μm.

Fig. 5: Immunohistochemical staining for matrix metalloproteinase MMP-8. A - Interpubic fibrocartlaginous disk from a virgin mouse. The arrow indicates MMP-8 in the cytoplasm of an elliptical condroid cell. B-C – MMP-8 in the cytoplasm (arrows) of fibroblastic-like cells of the interpubic ligament on D18 (B) and D19 (C) (relaxed). Note the cells with focal or punctate staining throughout the cytoplasm for MMP-8 also agreed with the intracellular storage of this protein (insets) while other cells were negative for MMP-8 (dotted line). Bars: 10 μm.

Fig. 6: Electron micrographs of cells from fresh (*in vivo*; A, D and G) and explanted (*ex vivo*; B, C, E, F and H-J) tissue from a virgin fibrocartilaginous disk and from the interpubic ligament on D18 and D19 (relaxed) of pregnancy. Panels A, B, D, E, G and H were obtained by transmission electron microscopy, and panels C, F, I and J were obtained by scanning electron microscopy. Cells from the virgin fibrocartilaginous disk (A) were round, had a smooth nuclear outline and were closely associated with the extracellular matrix. In 24 h explants (B), the cells were more elongated, the nucleus had a more condensed chromatin and interaction with the extracellular matrix was less evident. D and G - The interpubic ligament on D18 and D19, respectively, contained fusiform fibroblast-like cells loosely associated with the extracellular matrix (particularly in G) whereas explants of this ligament on D18 (E) and D19 (H) contained star-shaped cells rich in cytoplasmic projections (particularly on D19). C – A typical round chondroid cell with short microvillosities from a virgin fibricartilaginous disk. On D18 (F) and D19 (I), the cells showed spreading with the presence of phyllopodia, suggestive of movement, particularly on D19. J - Disrupted mesh of the fibrillar matrix on D19. Bars: A-D, F, I – 1 μm, E – 0,7 μm, G – 0,4 μm, H –3 μm, J – 10 μm.







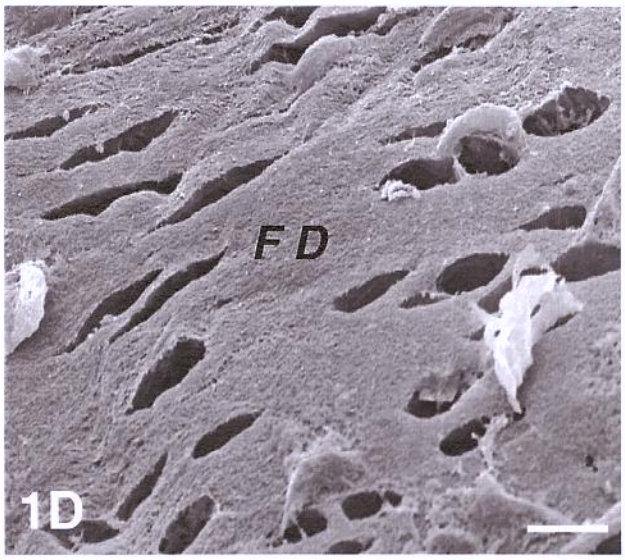
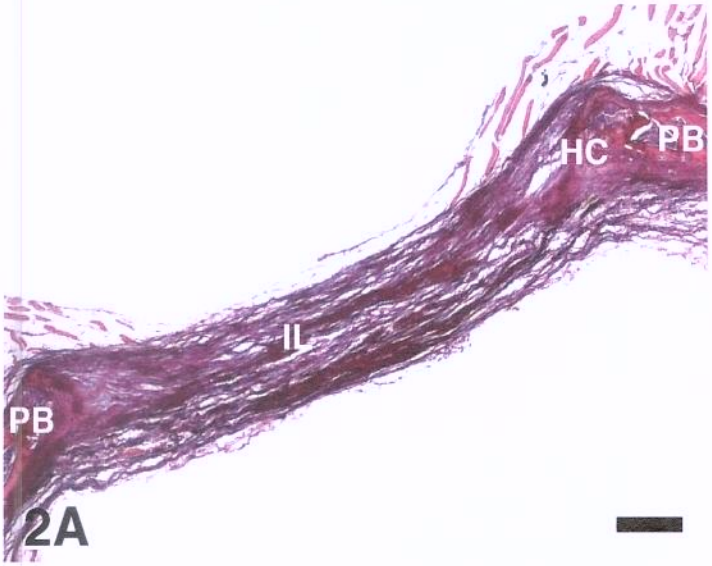
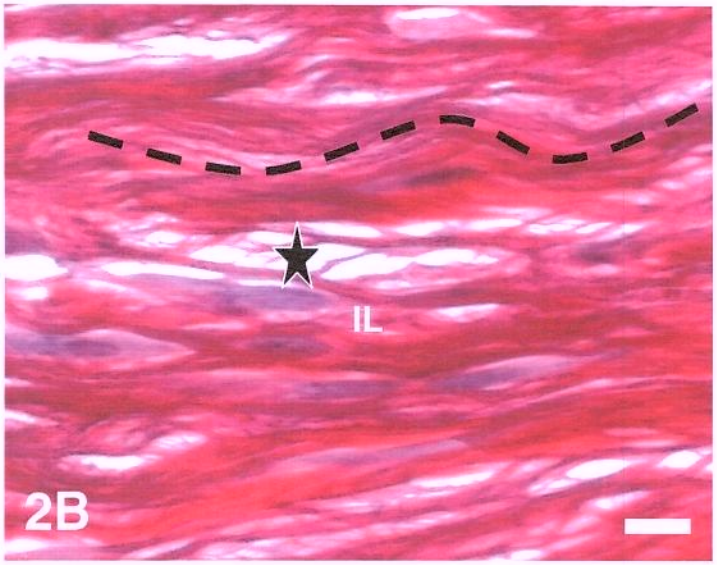
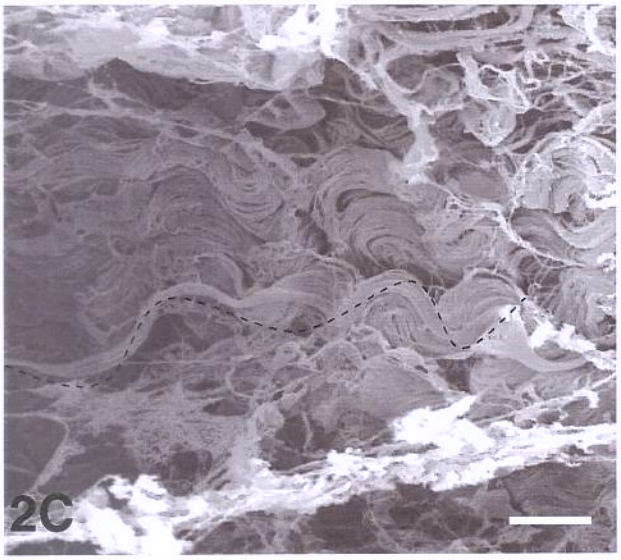
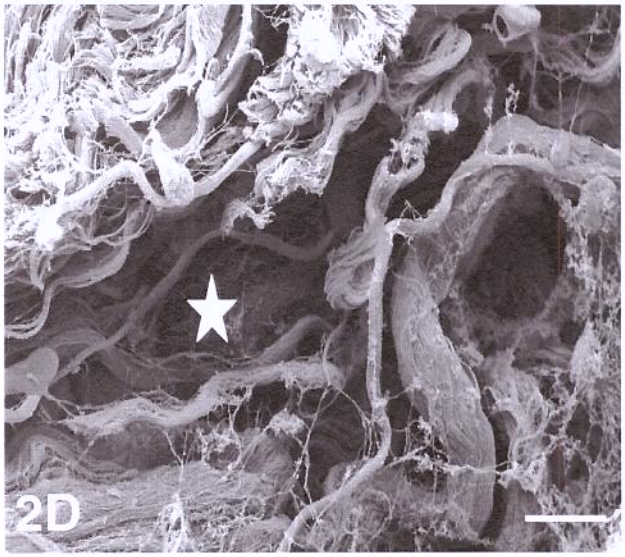


Figure 1









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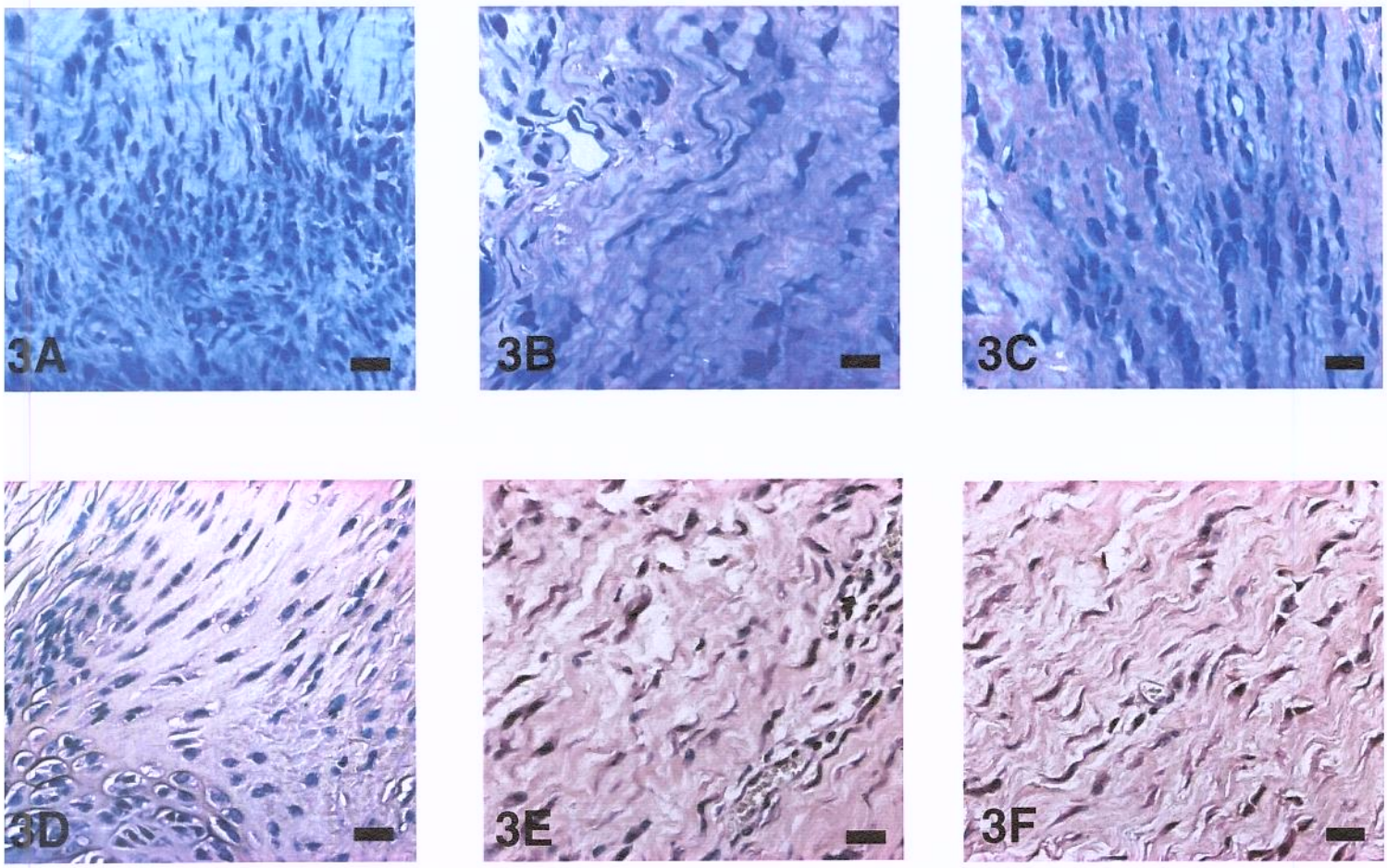

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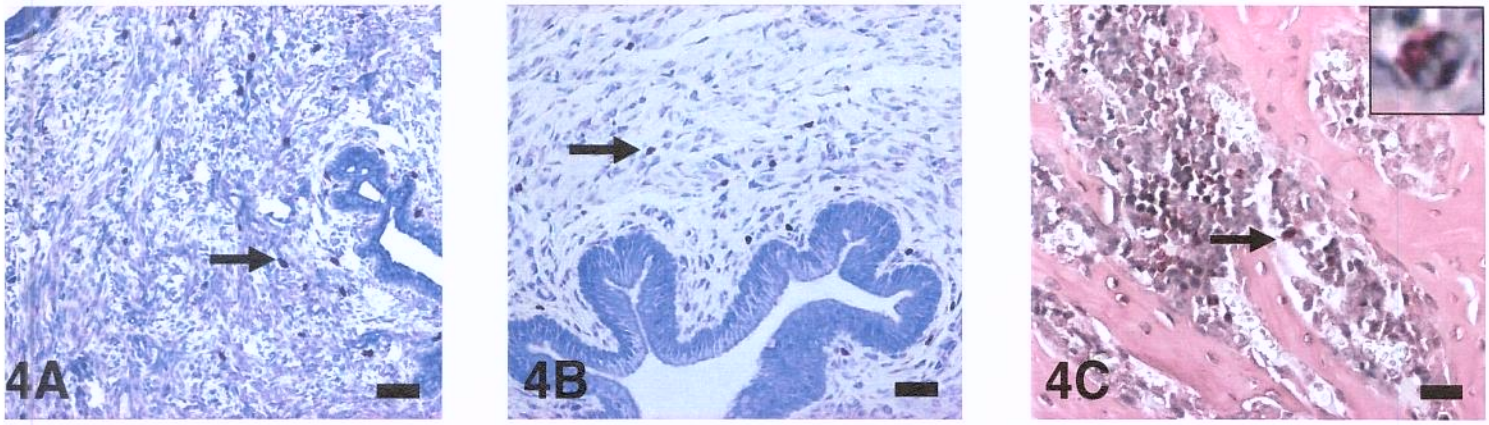

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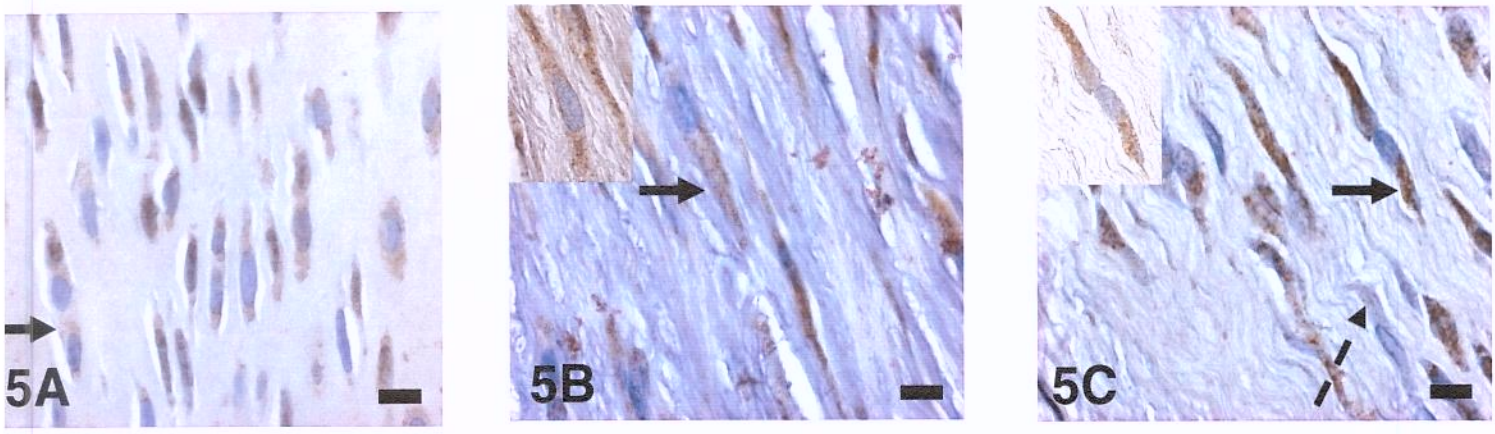

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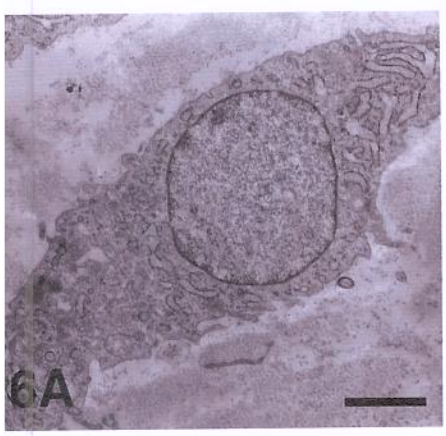

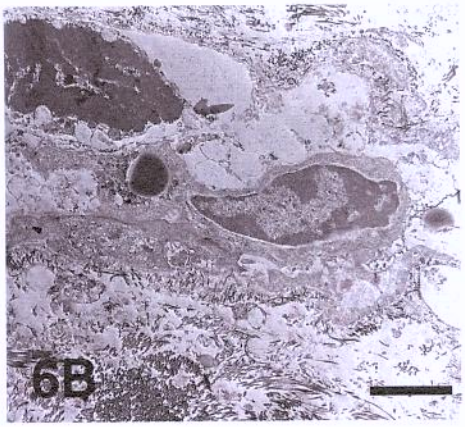

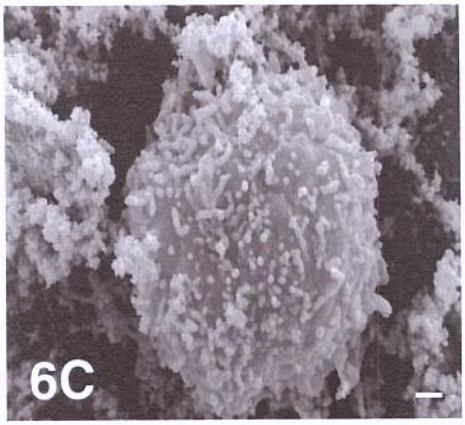

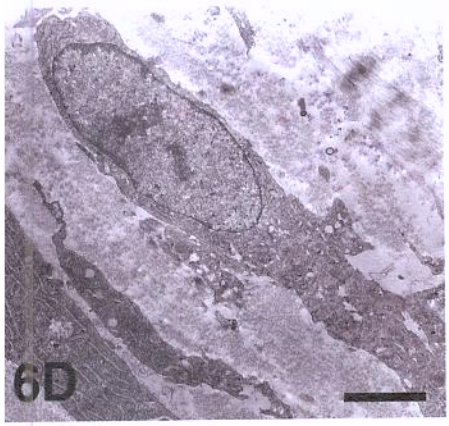

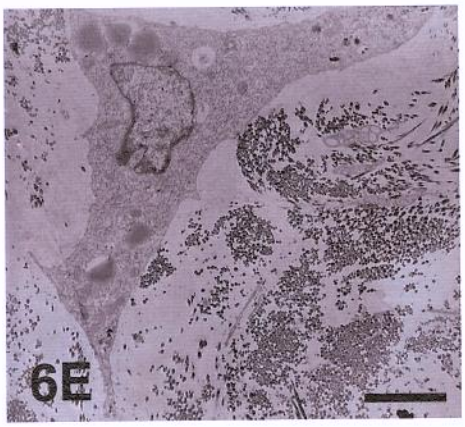

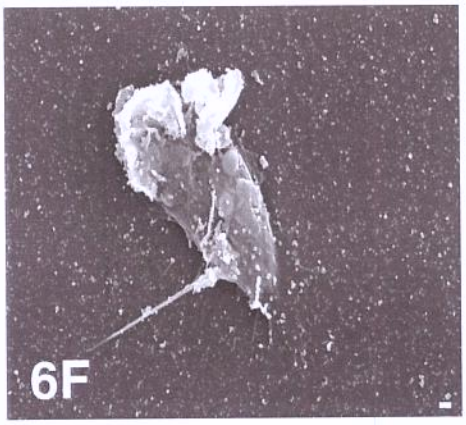

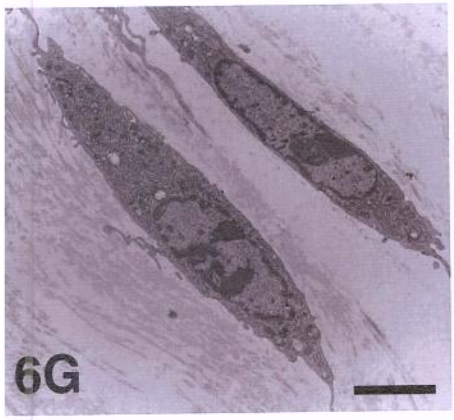

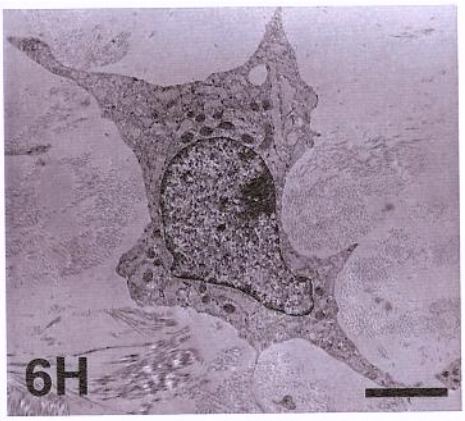

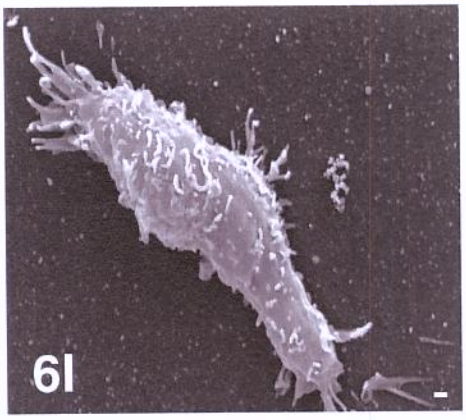

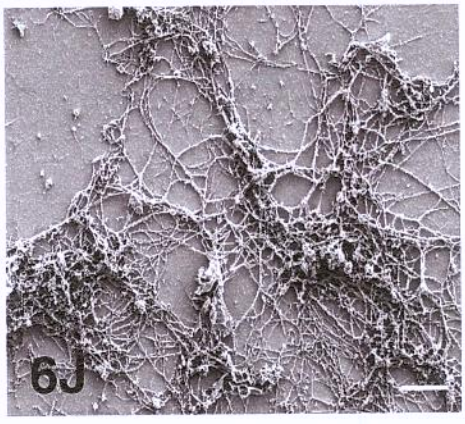

Figure 6

INVOLVEMENT OF METALLOPROTEINASES (MMP-2 AND MMP-9) AND THEIR INHIBITORS (TIMP-1 AND TIMP-2) IN THE REMODELING OF THE MOUSE PUBIC SYMPHYSIS DURING LATE PREGNANCY.

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ABSTRACT

Hystological and morphological studies of the pubic symphysis in mice, showed deep modifications of this articulation during pregnancy. The medial ends of pubic bones and the cartilage caps and its extremity of the pubic bones, partially, in early stages of pregnancy, are absorbed, and the scarce connective tissue in the interpubic ligament, that was observed in virgin animals, undergoes to radical modifications that allowed the separation of the pelvic bones for the passage of the fetuses at birth and at this time, the pubic joint becomes separated by a flexible and elastic interpubic ligament. Each new cell and extracellular matrix (ECM) arrangements in the interpubic tissue are dynamic adaptations to provide mechanisms for smooth transfer of forces and protect efficiently the birth canal. Immediately after the parturition, following labor, the ligament undergoes rapid involution on the 3 rd or 4 th day, and around the second week it got back together almost completely. Proteolytics enzymes during the differentiations of the pubic bones, during pregnancy, may be involved in this process. It is known that they are represented by collagenases, catepsins and dipeptidases. Nevertheless, there are almost no reports that recognize the specific role of metalloproteinases (MMP) and their inhibitors, tissue inhibitors of metalloproteinases (TIMPs) in the remodelation of the mouse pubic symphysis and ligament. Studies that are destined to the recognition of the functions and co-localizations of MMPs and their inhibitors have showed that, they act selectively in the degradation of the supramolecular arrangement of the different types of collagen. These alterations provide elevate ECM and cellular plasticity that results in liberation of condrocytes from its lacunae. After that they acquire volumed fibroblasts profile and they rearrange along collagen fibers that constitute the interpubic ligament in the end of pregnancy. Studies that focalize morphological and functional characteristics of the components of the pubic symphysis and interpubic ligament, can contribute to the knowledge of biomechanical behavior of the connective tissue during extensive differentiations that the articulations suffer. This behavior was involved in the formation of the birth canal and the impact in the remodeling process on the physiology of the organs.

Keywords: interpubic ligament, matrix metalloproteinases, mouse, pregnancy, tissue inhibitors of metalloproteinases

INTRODUCTION

In a variety of animals, including mice (Crelin, 1969; Sherwood, 1994), guinea pigs (Talmage, 1947a,b; Wahl *et al.*, 1977), rabbits (Hashem *et al.*, 2006) and bats (Crelin & Newton, 1969), pregnancy is accompanied by structural modifications in the pelvic girdle to facilitate the safe passage of the fetus at birth.

The principal modifications seen in the mouse and guinea-pig pubic symphysis include an estrogen-mediated "separation" of the pubic bones to allow the gradual expansion of the fibrous connective tissue in the ligament, and a relaxin-mediated "relaxation" that helps to soften the connective tissue. These processes involve the action of proteolytic enzymes released by chondrogenic cells and fibroblasts along the collagen fibers during the formation of the interpubic ligament. The resulting alterations in the symphyseal tissue produce a new structure that resembles a true ligament (Crelin, 1969; Gamble, 1983, Moraes *et al.*, 2003).

During the last week of pregnancy, the main physiological changes include: (a) growth of a ligament, characterized by the deposition of coarse, densely packed collagenous fibers that are very well organized along the major axis of the symphysis, (b) tissue remodeling and collagen degradation through the action of proteases (mainly metalloproteinases), (c) an increase in water-retaining extracellular matrix glycosaminoglycans, such as hyaluronate, that provide additional hydration to increase the pliability of the tissue, and (d) partial resorption of the medial ends of the pubic bones and cartilaginous caps.

Hormones such as estrogen, progesterone and relaxin that are required for relaxation of the cervix and symphysis (Hall, 1947) also have a central role in the chemical, physiological and morphological changes of the interpubic disk, cervix, uterus and mammalian glands (Sherwood, 1994; Hayes, 2004). In virgin, ovariectomized mice, relaxin receptors occur primarily in cells of the interpubic ligament and are absent from pubic bones and the cartilaginous pad (Yang *et al.*, 1992). In knockout mice without the relaxin gene there is no expansion of the symphysis although the mice are fertile and produce healthy young (Zhao *et al.*, 1999-2000).

The hormone-mediated changes that prepare the interpubic fibrocartilaginous disc for parturition include alterations in the composition of the connective tissue, with increases in the content of proteoglycans, collagen, elastic fibers and water (Chihal & Espey, 1973; Moraes *et al.*, 2003; Ortega *et al.*, 2001; Pinheiro *et al.*; 2003; Wahl *et al.*, 1977; Zhao *et al.*, 1999-2000), and a concomitant activation of proteolytic enzymes capable of inducing symphysis relaxation (Wahl *et al.*, 1977; Weiss *et al*, 1979; McDonald & Schwabe, 1982). These changes result in the rupture of the helicoidal arrangement of collagen fibers in order to increase the compliance and extendibility of the ligament prior to parturition (Pinheiro *et al.*, 2004).

Many of the modifications seen in the female reproductive system from ovulation up to parturition result from extensive extracellular matrix (ECM) deposition, remodeling and cellular metabolism, and include events such as embryo implantation and decidualization (Abrahamsohn & Zorn, 1993) and

softening of the birth canal (Junqueira *et al*, 1980; Leppert, 1995). Softening of the birth canal involves a gradual, regulated degradation of collagen during pregnancy, and this process is accelerated moments before parturition to provide biomechanical dilation at the moment of birth.

The remodeling of the interpubic symphysis is mediated by epithelial cells, fibroblasts and immune cells that secrete matrix metalloproteinases (MMPs) capable of hydrolyzing extracellular and cell surface components (Nagase *et al.*, 1999; Somerville *et al.*, 2003). The ability of MMPs to degrade components of the ECM means that they are involved in a variety of processes, including cell migration, tissue remodeling and wound healing (Nagase, 1999). MMPs are zinc-dependent proteinases that are secreted in a latent form by cells such as fibroblasts, endothelial cells, polymorphonuclear cells, keratinocytes and macrophages (Lanchou *et al.*, 2003; Somerville *et al.*, 2003; Sennstrom *et al.*, 2003) and can be activated by other MMPs. Matrix metalloproteinases 2 and 9 (MMP-2 and MMP-9) are members of the gelatinase family of proteases that can degrade collagen type IV (Bany *et al.*, 2000; Becher *et al.*, 2004; Lenhart *et al.*, 2001, 2002; Stygar *et al.*, 2002).

The activities of MMPs are regulated by TIMPs (tissue inhibitor of matrix metalloproteinases) and RECK (reversion-inducing cysteine-rich protein with Kazal motifs) that can specifically and reversibly inhibit MMPs (Noda *et al.*, 2003; Nuttall *et al.*, 2004). These inhibitors, particularly the TIMPs, are spatially and tightly regulated at several levels such that an imbalance between MMPs and TIMPs provides a means of modulating wound healing (Vaalamo, 1999; Kleiner *et al.*, 1999).

Increased expression of TIMP-1 (28 kDa) and TIMP-2 (21 kDa) (inhibitors of MMP-9 and MMP-2, respectively) has been observed following relaxin-induced remodeling of the birth canal, particularly in the uterine cervix (Lenhart *et al.*, 2001,2002). TIMP-1 is enriched in reproductive organs whereas TIMP-2 expression is constitutive and widely expressed throughout the body (Chirco *et al.*, 2006). TIMP-1 and TIMP-2 can stimulate growth in a wide variety of cell types, including keratinocytes, fibroblasts and breast cancer cells (Chirco *et al.*, 2006).

In this study, we examined the expression of MMPs and their inhibitors during the extensive tissue remodeling required for safe delivery in mice. In particular, we studied this occurrence of these proteins in the interpubic ligament of pregnant mice.

MATERIALS AND METHODS

Animals

Male and virgin female Swiss mice (*Mus musculus*) (~90 days old, 25-30 g) were obtained from the Multidisciplinary Center for Biological Investigation (CEMIB) at UNICAMP. The mice were house at 25±2°C on a 12 h light/dark cycle with free access to water and standard pelleted rodent chow. The animal protocols were approved by the institutional Committee for Ethics in Animal Experimentation (CEEA/IB/UNICAMP, protocol 1143-1) and the experiments

were done in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Academy of Science, 1996).

Pregnant females were obtained by housing virgin females overnight with males (2:1 ratio). The presence of vaginal "plugs" the following morning indicated successful mating and this day was designated as the first day of pregnancy (D1). Delivery was expected on day 19. Virgin mice in estrus were used as controls. Estrus was determined by vaginal smears according to Shorr (1941).

Samples of pubic symphysis were collected during pregnancy (D18 and D19, before parturation). When required, the mice were anesthetized with a mixture of ketamine (Dopalen, Agribrands of Brazil, Brazil) 100-200 mg/Kg and xylazine chloride (Anazedan, Agribrands of Brazil, Brazil), 5-16 mg/kg, i.p.) and were killed between 11:00 a.m. and 12:00 a.m. After laparotomy, the medial portions of the pubic bones on the symphysis or ligaments were removed and processed according to the subsequent analysis to be done. All surgical procedures were done using aseptic technique.

Tissue collection

For light microscopy, five mice were used for each experimental group, and three mice per group were studied for electron microscopy. The pubic symphysis were dissected and fixed *in situ* with the appropriate fixative for 10 min after which the tissue was removed and immediately immersed in the same fixative. Twenty-four hour cultures of pubic symphysis were used for transmission (TEM) and scanning (SEM) electron microscopy. Tissues for zymography were removed and immediately frozen in liquid nitrogen prior to storage at -70°C. For zymography, tissue homogenates were obtained from pools of 10 virgin, 8 D18 and 8 D19 mice.

Light microscopy

Specimens were fixed in 4% paraformaldehyde (Merck, Darmstadt, Germany) dissolved in 0.1 M phosphate buffer, pH 7.2, for 24 h, dehydrated in a graded ethanol series, and embedded in paraffin at 65ºC. Transversal sections 5 µm thick were mounted on slides coated with poly-L-lysine (0.1% w/v in water; Sigma Chemical Co., St. Louis, MO, USA) and dried for 24 h at 37°C. The sections were stained with Masson trichrome for the morphological analysis.

Cell culture

Explants of the pubic symphysis and interpubic ligament were placed on coverslips in 24-well plates and incubated with Dulbecco's modified Eagle´s medium (DMEM, Gibco BRL) containing gentamicin (40 mg/ml) and supplemented with 10% inactivated fetal calf serum. The cultures were incubated for 24 h at 37ºC in an atmosphere of 5% CO_2 after which the explants were collected and processed for TEM and the coverslips were used for SEM (data not shown).

Electron microscopy

Small fragments of interpubic and symphysis tissue from 24 h cultures and freshly collected tissue were fixed in a solution containing 1.25% formaldehyde, 2.5% glutaraldeyde and 0.03% picric acid in 0.1 M sodium cacodylate buffer, pH 7.2, for 2 h at room temperature, followed by post-fixation in 1% osmium tetroxide for 1 h at 4°C. The material was dehydrated through a graded ethanol series and the tissue fragments and explants were subsequently immersed in propylene oxide/Epon 812 (Electron Microscope Science) (ratios of 1:1 and 3:1, respectively) for 6 h prior to being placed in pure resin overnight and then into molds containing fresh resin to allow polymerization for 72 h at 60°C. Semithin sections (1 μm) were obtained with an LKB model 8800 Ultratome III and stained with 0.5% toluidine blue (Merck) to allow the selection of areas of pubic symphysis for ultrathin sectioning. Ultrathin sections obtained with the same ultratome were double-stained with 2% uranyl acetate and 0.5% lead citrate prior to examination and documentation with a LEO 906 transmission electron microscope.

Detection of MMP activity by zymography

The pubic symphysis and interpubic ligament frozen in liquid nitrogen were ground with a manual mortar, extracted in extraction buffer, homogenized, and briefly sonication with an ultrasonic cell disruptor (Vir Sonic 60 - Virtis) on ice. The homogenate was centrifuged (3.000 rpm, 10 min, 4°C) and the supernatant was stored at -75°C until used. Protein was quantified using a Bradford dye binding assay (BioAgency, São Paulo, SP, Brazil). The activity of MMP gelatinases secreted by the symphysis and ligament was analyzed by zymography after SDS-PAGE (the latter done as described by Laemmli, 1970). Briefly, 50 μg of protein was loaded onto a 7.5% polyacrylamide gels polymerized with 0.1% gelatin (type A, from porcine skin) in an SE260 electrophoresis cell (Amersham-BoRad). After electrophoresis, the gels were washed twice (20 min each) in 2.5% Triton X-100 with gentle shaking and then incubated in 50 mM Tris-HCl, pH 7.8, containing 0.1 M NaCl, 1 M $CaCl_2$, 0.02% sodium azide) at 37°C for 24 h, with gentle shaking. After this incubation, the gels were stained with Coomassie blue G-250 (BioRad). Molecular mass standards (Sigma SDS–PAGE LMW marker proteins) and human standards for MMP-2 and MMP-9 were included in the gels. Was also used as control phenanthroline, a specific inhibitor of metalloproteinase, to make sure that those bands were really from MMPs. A concentration of 0.01M of phenanthroline was used in the 24h incubation.

Immunohistochemical analysis
Antibodies

Antibodies to MMP-2, TIMP-1 and TIMP-2 (anti-rabbit) were purchased from NeoMarkers (LabVision, USA) and antibodies to MMP-9 (anti-mouse) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All antibodies detected active and inactive forms of the respective proteins. The secondary antibodies were obtained from Dako and were Immunoglob/Biotinylated GxR E04321 for MMP-2, TIMP-1 and TIMP-2 and Immunoglob/Biotinylated GxM E04331 for MMP-9. Detection was done with a streptavidinAB-HRP complex (Dako).

Immunohistochemistry

Immunohistochemistry was done as described previously (Varayoud *et al.*, 2001) for the symphysis and interpubic ligament. Briefly, after deparaffinization, the sections were pretreated with microwaves and endogenous peroxidase activity was blocked with 0.3% H_2O_2 and 5% milk in phosphate buffer for 30 min. Sections were incubated with antibodies (MMP-2 1:150, MMP-9 1:100,, TIMP-1 1:400 and TIMP-2 1:150, all diluted in 0.01 M phosphate buffer containing 0.2% bovine serum albumin. After incubation overnight at 4°C overnight, secondary antibody was applied for 1 h and the reactions were developed using streptavidin-biotin peroxidase for 45 min followed by incubation with diaminobenzidine (DAB, chromogenic substrate) (Sigma) for 5 min at room temperature. Samples were counter–stained with Harris hematoxylin and mounted in permanent mounting medium. Each immunohistochemical experiment included negative controls. For negative controls, the primarily antibody was replaced by non-immune rabbit/mouse serum. All experiments were done at least three times and no positive reaction was detected in the negative controls. Sections were observed and photographed with a Nikon Eclipse E800 microscope.

RESULTS

Light microscopy of transversal sections showed that the central region of the pubic symphysis in virgin mice consisted of a fibrocartilaginous disc lying between the hyaline cartilage-covered articular surfaces of the pubic bones. A layer of perichondrial tissue containing numerous fibroblasts covered the entire symphysis (Fig. 1A). The medial fibrocartilaginous disk joined the two caps to fill all of the intervening spaces (Fig. 1B). This structure increased in size throughout pregnancy (Fig. 1C) and the fibrocartilaginous discs arranged as compact dense connective tissue gave rise to the interpubic ligament (Fig. 1D).

For zymography, preliminary experiments with 100 µg, 50 µg and 25 µg (data not shown) suggested that 50 µg of protein was an appropriate amount to use for the tissue pools (Fig. 2A). The assays were done four times to ascertain the pattern for each pool. Active and inactive forms of MMP-2 and MMP-9 were detected in virgin (a) (pubic symphysis) and D18 (b) and D19 (c) (interpubic ligament) samples, although additional bands of activity were also seen. There was a band of activity at ~20-23 kDa that was not seen in virgin mice (Fig. 2A, arrows). Beyond the use of specific patterns of MMP2 and MMP9 of human tissues, to make sure that these bands that were found in the zymography were really from MMPs, there was used an specify inhibitor (Phenanthroline) of MMPs. Zymography with the use of this inhibitor in the incubation solution, showed that all bands that were detected in our experiments were metalloproteinases (Fig.2B). So, the pattern was seen, but the gel was completely blue with no white bands.

In virgin mice, MMP-9 was more abundant than MMP-2 in the pubic symphysis. However, there was a tendency for the amount of active MMP-9 to

decrease with pregnancy. In contrast, both forms of MMP-2 were more abundant in D18 and D19 mice than in virgin mice (Graphic 1A, B).

Electron microscopy was used to examine pubic symphysis obtained from explants cultured for 24 h [Fig. 3A (virgin), B (D18), C (D19)]. In cultured explants, these cells were similar to those seen in tissue sections, but had few projections and the cell-matrix association was not as well-defined. However, the matrix was well organized with few signs of remodeling. At D18 and D19, during this phase, remodeling was more accentuated (Fig. 3B, C) was seen in the 24 h explants. Cells in the explants showed deposition of collagen fibers. Cultured explants showed poorly arranged and loosely packed fibers, with cellular projections that suggested cell motility

Immunohistochemistry detected MMP-2 and MMP-9 and their inhibitors TIMP-1 and TIMP-2. In virgin mice, strong staining for MMP-2 and TIMP-2 was detected in the cartilaginous pad [Fig. 4A (MMP-2), B (TIMP-2)], mainly in the cell cytoplasm. MMP-9 and TIMP-1 were detected in cells of the fibrocartilagenous transition [Fig. 5A (MMP-9), B (TIMP-1), arrow], and TIMP-1 was also detected in the extracellular matrix (Fig. 5B, star).

During pregnancy, MMP-2 [Fig. 4C (D18), E (D19)] and TIMP-2 [Fig. 4D (D18), F (D19)] were co-localized in the cytoplasm of fibroblast-like cells. Qualitative analysis showed that there were no differences between the days D18 and D19 of pregnancy. In contrast, staining for MMP-9 was weak [Fig. 5C (D18), E (D19)], with a slight increase in D18 (Fig. 5C) mice compared to D19 (Fig. 5D, thin arrow). Similarly, there was greater staining for TIMP-1 [Fig. 5D (D18), F (D19)] in the cytoplasm of D18 cells (Fig. 5D, arrow) than in those of D19 (Fig. 5E) and virgin mice. On the other hand, immunostaining of the extracellular matrix was more evident in virgin mice (Fig. 5B) than during pregnancy (Fig. 5D, E).

DISCUSSION

Our histological and ultrastructural analysis shows that the mouse pubic symphysis undergoes marked rearrangement in late pregnancy, as also reported by others (Chihal & Espey, 1972; Pinheiro *et al.*, 2004). Many of these changes are probably mediated by the hormone relaxin which stimulates remodeling, dilatation and parturition in animals (Sherwwod, 1994; Naqvi *et al.*, 2004; Hashem *et al.*, 2006).

The histological alterations seen in tissue fragments and in 24 h cultured explants were similar to those described by Pinheiro *et al.* (2004), namely, the disruption of collagen fibers and a reduction in crimp by D18. These modifications were more intense by D19 (day of birth) and are important for birth canal formation and safe delivery. The ultrastructural analysis revealed the migration of fibroblastic cells in explants from D18 and D19 mice. These cells had a phenotype similar to that of myofibroblasts present in the interpubic ligament of pregnant mice (Moraes *et al.*, 2003) and can migrate in response to stimuli such as TGF-β and PDGF (Larsen *et al.*, 2004). Fibroblastic-myofibroblastic cell

plasticity may have implications for a variety of physiological changes seen in the pubic symphysis (Moraes *et al.*, 2003) and uterine cervix during pregnancy, parturition, and postpartum involution (Varayoud *et al.*, 2001).

The connective tissue of the symphysis and intepubic ligament undergoes extensive remodeling (Sherwood, 1994; Pinheiro *et al.*, 2004) that involves the turnover (synthesis and degradation) of ECM components, mainly via the action of collagenases present in interpubic tissues of non-pregnant and pregnant mice (Wahl *et al.*, 1977; Weiss *et al.*, 1979). Numerous proteases have been implicated in the proteolytic degradation of the ECM (Midwood *et al.*, 2004), with the most prominent being MMPs (Stamenkovic, 2003), which are believed to be the only enzymes capable of degrading fibrillar collagens (Hulboy *et al.*, 1997).

MMPs are involved in a variety of cellular activities, including cell motility, cell-cell interactions, cell-matrix interactions, cell adhesion, matrix degradation, and the release of bioactive signaling molecules (Vansaun & Matrisian, 2006). MMPs may regulate cellular behavior through pericellular proteolysis (Werb, 1997), and changes in pericellular interactions at the end of pregnancy are an important component of tissue remodeling. At this stage, latent MMPs are thought to be sequestered by extracellular matrix proteins until needed (Vansaun & Matrisian., 2006). Nevertheless, complete inhibition of proteolysis does not necessarily stop cellular migration (Wolf *et al.*, 2003).

MMPs can influence the phenotype of the cellular components that were evident in the explant from the 24h culture, altering basic functions of the cell such as proliferation, apoptosis and differentiation (Hulboy *et al.*, 1997). During our 24h culture, in the exlant, was evident that cells did remodeling to try to adept to a new environment and MMPs can be involved in this process. As we detected the presence of both MMP-2 and MMP-9. These can explain the pericellular spaces that were caused in the pubic symphysis during pregnancy, and that is also seen in the 24h culture material. The rupture of the matrix could be done by involvement of MMPs. MMP-9 was also noticed to be involved in cell migration (Imada *et al.*, 1997). MMPs also can modify cellular attachment to the ECM (Mott *et al.*, 2004).

These changing relationships indicate that the pubic symphysis is a dynamic structure with a high capacity to adapt to different physiological situations (Varayoud *et al.*, 2001). The detection of MMP-2 and MMP-9 in ligament fibroblastic cells supports the hypothesis that MMPs facilitate the growth and remodeling of the interpubic symphysis and modifications in the ECM are required for tissue reorganization prior to delivery in mice. Our results agree with the previously reported limited expression of MMP-2 and MMP-9 in reproductive tissues (Nuttall *et al.*, 2004).

The molecular massas of MMP-2 and MMP-9 generally agreed with values for these enzymes in other tissues and species (Souza *et al.*, 2001). The increased activity of MMP-2 and MMP-9 at D18 may have been related to the more intense remodeling during this phase compared to D19. Our findings agree with reports showing that relaxin induces the secretion of MMP-2 and MMP-9 and that these MMPs participate in enlargement of the uterus and cervix by

increasing the level of extracellular proteolysis in the reproductive tract (Arguello-Ramirez *et al.*, 2004; Lenhart *et al.*, 2001).

Zymography also revealed the presence of a low molecular mass gelatinase (20-23 kDa) of a size similar to MMP-7 and MMP-12 (Stamenkovic, 2003; Nénan, 2005). According to Nénan (2005), although MMP-12 (metalloelastase) targets elastin it is also active on gelatin as a substrate. MMP-12 occurs as a proenzyme of 54 kDa that decreases to 22 kDa in the active form. Active MMP7 has a molecular mass in the range reported for the unidentified band seen here (Hulboy *et al.*, 1997) and is present in reproductive tissues (Nuttall *et al.*, 2004). Although we have not investigated the identity of this band any further, elastin (substrate for MMP-12) is known to be crucial in maintaining the structure of the pubic symphysis (Moraes *et al.*, 2004) and MMP-7 is involved in postpartum involution (Stamenkovic, 2003).

The fibrocartillogenuos cells of the pubic symphysis of virgin mice and fibroblasts of the interpubic ligament of pregnant mice expressed MMP-2 and MMP-9. These MMPs have also been detected in human and pig cervix during relaxation of this structure at the end of gestation (Lenhart et al., 2001; Stygar *et al.*, 2002). Stygar *et al.* (2002) pointed out that fibroblasts in the stroma are the principal source of MMP-2 and that leukocytes express primarily MMP-9. In the interpubic ligament, MMP-2 and MMP-9 were detected in fibroblasts, as also reported by Imada *et al.* (1997). Our immunohistochemical findings agreed with the zymographic results which indicated greater expression of MMP-2 than of MMP9 during pregnancy.

TIMP-1 and TIMP-2 were expressed in the same cells in which MMPs were detected. TIMP-1 was also detected in the ECM, often along fibrous structures. In contrast, TIMP-2 expression was concentrated in the cell cytoplasm. These findings are consistent with Naesse *et al.* (2003), who observed similar immunoexpression during remodeling of periodontal tissue.

The pattern of immunoexpression detected in the pubic symphysis was consistent with the rigorous control exerted by TIMPs on MMP activity in the extracellular environment (Curry *et al.*, 2003). Indeed, the tight control of MMP activity is important in order to prevent unregulated tissue destruction (Fassina *et al.*, 2000; Naesse *et al.*, 2003) and maintain normal pubic organ functions (Lenhart *et al.*, 2002). TIMPs, which are produced locally and specifically inhibit MMPs (Brew *et al.*, 2000; Lenhart *et al.*, 2002; Woessner, 1999), are abundant in reproductive tissues, where their levels are hormonally regulated (Curry *et al.*, 2003; Nuttall *et al.*, 2004). TIMP-1 binds preferentially binds to MMP-9, whereas TIMP-2 has a high affinity for MMP-2 (Curry *et al.*, 2003; Naesse *et al.*, 2003).

In conclusion, the results of this study indicate that stromal fibrosblast-like cells of the mouse interpubic ligament were the main source of gelatinases and their inhibitors. These proteins are very probably involved in the tissue remodeling that occurs during pregnancy and immediately prior to parturition. The tightly regulated control of MMP activity during this process provides a means of autoregulation (Maquart *et al.*, 2005) that helps to prevent excessive tissue destruction immediately before and after birth.

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FIGURE LEGENDS

Fig. 1. A-D - Sections of the pubic symphysis of virgin mice showing the main components [pubic bones (PB), hyaline cartilage caps (HC) and a central region with the fibrocartilaginous disk (FD)] of this structure. Panel B shows that the tissue consists basically of one cell type similar to fibrocartilaginous cells. C - Section from D18 of a pregnant mouse showing that the histoarchitecture of the interpubic ligament at this point consists mainly of loosely organized fibroblast-like cells and collagen fibers. D - The spaces (star) in the extracellular matrix indicate early morphological disruption of the ligament. Masson trichrome. Bars: A, C – 70 μm, B,D – 40 μm.

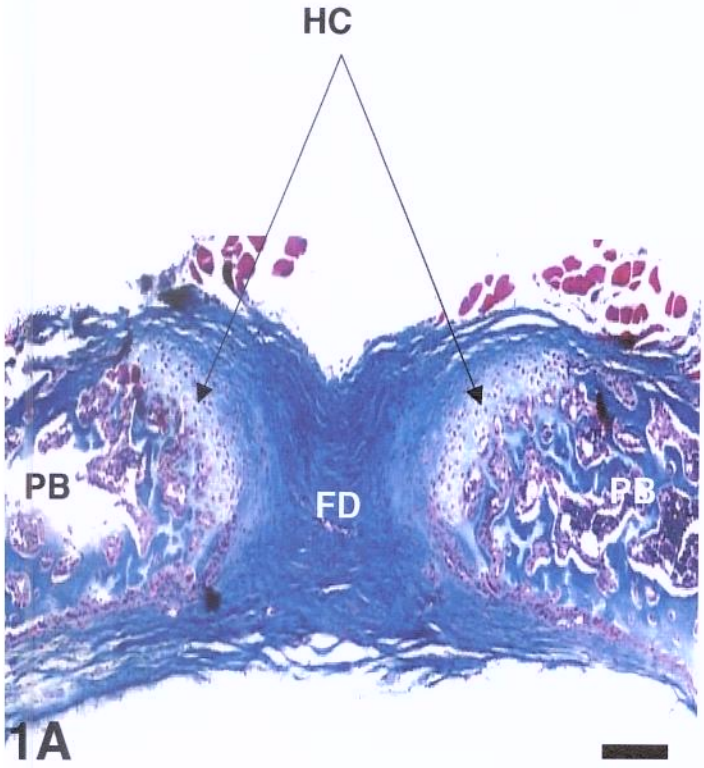
Fig. 2. A-B - Zymography showing gelatinase activity (visualized as light bands) in a homogenatethe from (a) virgin animals, (b) D18, (c) D19. Matrix metalloproteinase (MMP)-9 activity is shown ~ 95 kDa (Inactive form) and 85 kDa (active form) in virgin females and at D18 and D19 of pregnancy. MMP-2 activity is observed in both forms ~ 72kDa (Inactive form) and ~ 66kDa (active form) in all study groups. Fifty micrograms of protein was applied to each lane. Note the low molecular mass band with gelatinolytic activity (2A, thin arrows). 2B – Activities are abolished by incubation with phenanthroline demonstrating this is specifically due to MMPs.

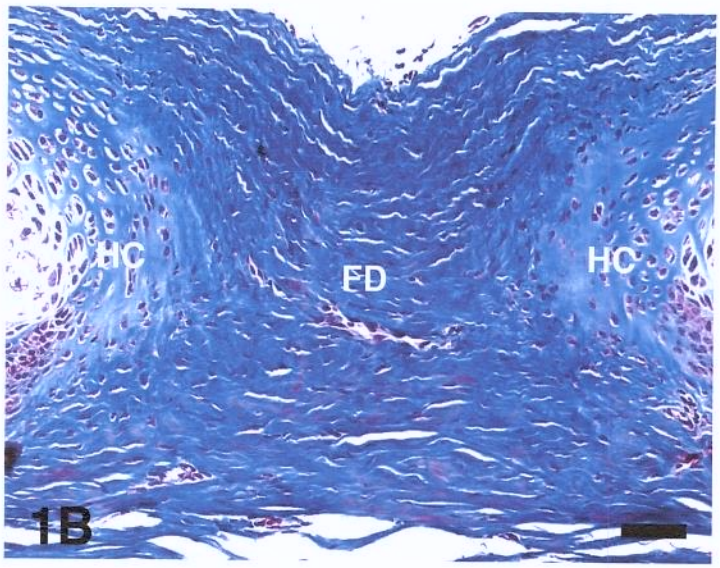
Graphic 1A_B: Metalloproteinase 9 in the pubic symphysis at virgin and during pregnancy D18 and D19 were quantifies as described in methods. The columns represent the mean ± SD of all groups in the inactive and active forms. ***p<0.001, **p<0.01 compared to the virgin and # compared between the pregnancy groups (ANOVA), Fig.1A. Metalloproteinase 2 in the pubic symphysis at virgin and during pregnancy D18 and D19 were quantifies as described in methods. The columns represent the mean ± SD of all groups in the inactive and active forms. ***p<0.001 compared to the virgin (ANOVA), Fig.1B.

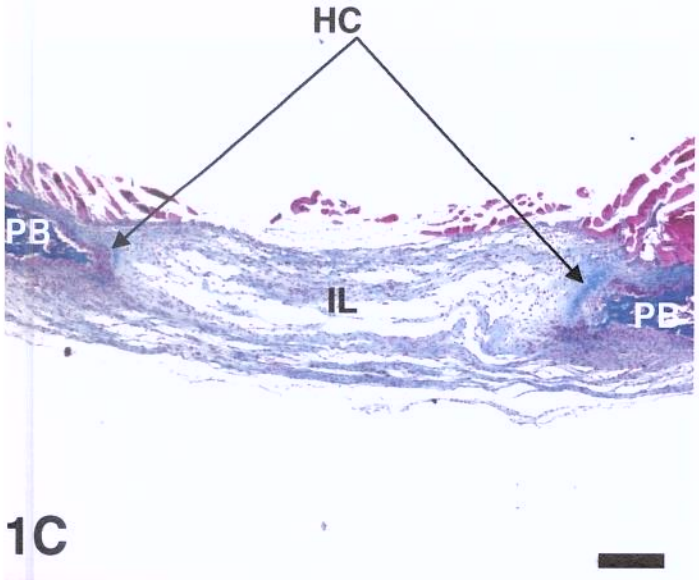
Fig.3A-C: Transmission electron micrograph of pubic symphysis from the 24h culture, showing that cells that were in culture were without tensions rounded cells, similar to those *in vivo* (data not show) in virgin animals. At fig.A, the intimate relation cell/matrix, characteristic of the PS in virgin animals are not as close now, culture cells. At fig.B and C this relation cell/matrix is not that close, probably because this material was in culture. These alterations, suggest that, modifications of matrix components of the cell surface support cell migration in the explant. These aspects are absent in explants from virgin females but are frequent at D18 and D19. Bars - 1μm.

Fig. 4. A-F - Paraffin sections of pubic symphysis from virgin females showing the immunolocalization of MMP-2 (Fig.A) and TIMP-2 (Fig.B) in the cytoplasm of fibrocartilaginous cells (arrow). Figs. C-F - Paraffin sections of the interpubic ligament at D18 (Figs. C, D) and D19 (Figs. E, F) of pregnancy showing the immunolocalization of MMP-2 (Figs. B, D) and TIMP-2 (Figs. D, F). Note the disorganization of the matrix and some crimps. The proteins were detected in the cytoplasm of fibroblast-like cells in the interpubic ligament. Note that MMP-2 and TIMP-2 were colocalized in the same cells (arrows). Bars – 10 μm.

Fig. 5. A-F - Localization of MMP-9 (Fig.A, C and E) TIMP-1 (Fig.B, D and F) in the cytoplasm of fibrocartilaginous cells (arrows) in virgin females (Fig.A-B). Fig. B shows that TIMP-1 was also detected in the matrix (star). Figs. C-F - At D18 (Fig.C, E) and D19 (Fig.D, F) of pregnancy, these proteins were detected in the cytoplasm of fibroblast-like cells that formed the interpubic ligament. MMP-9 and TIMP-1 were colocalized (arrows). Some cells were not marked (E, thin arrow). In late pregnancy, TIMP-1 expression in the extracellular matrix was not as marked as in virgin females. Bar – 10 μm.







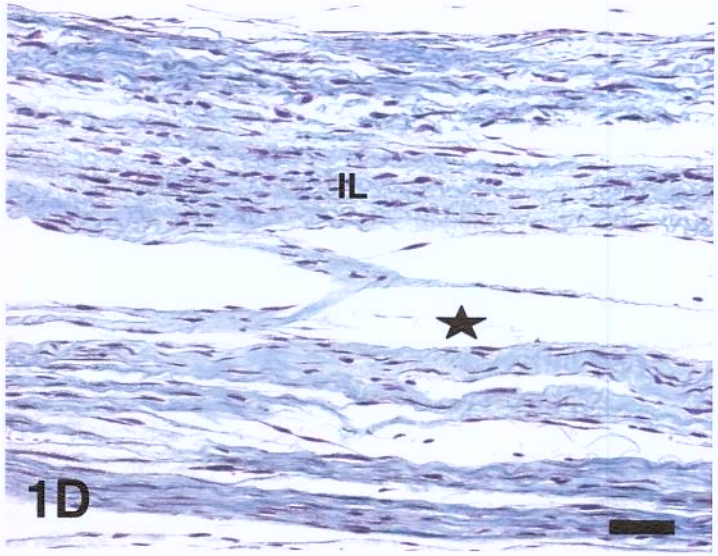


Figure 1

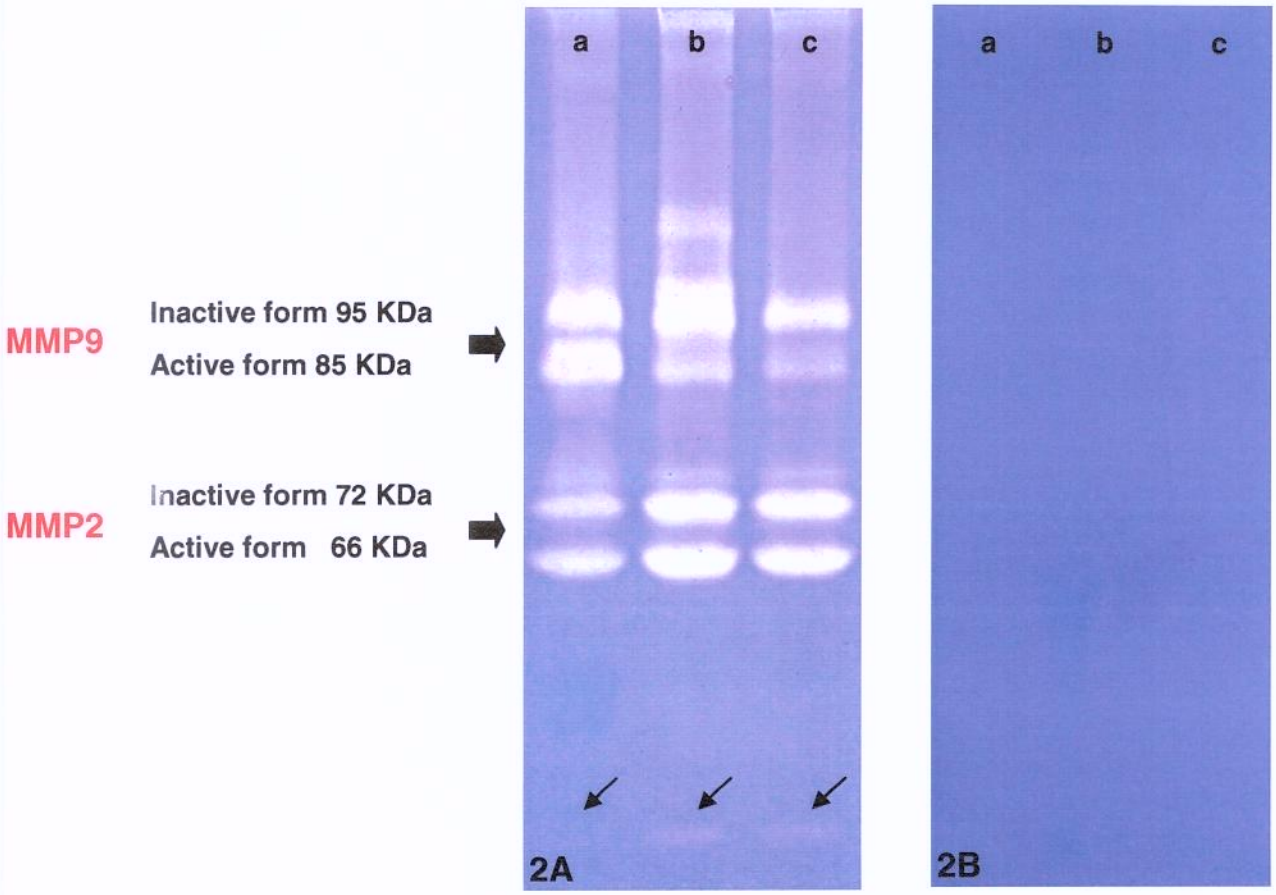
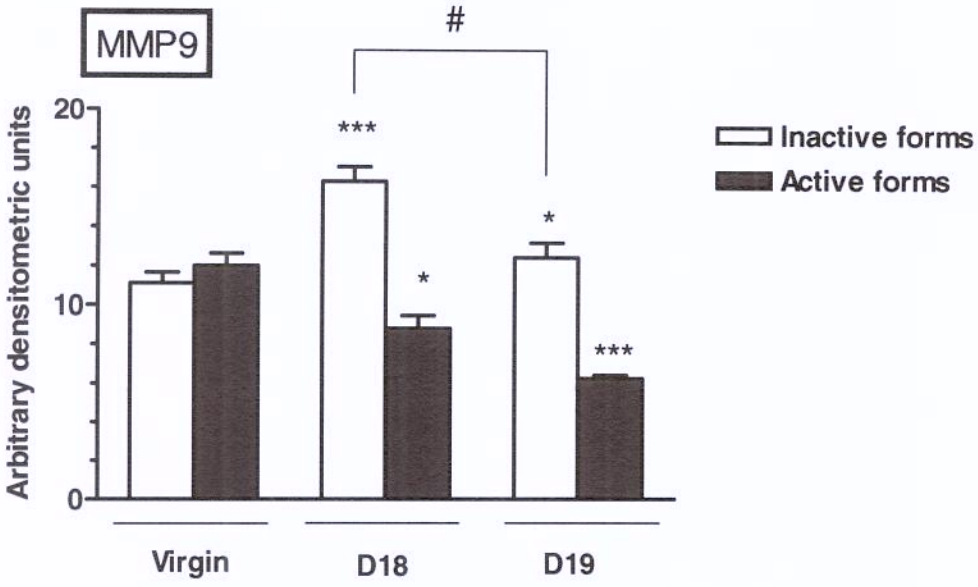
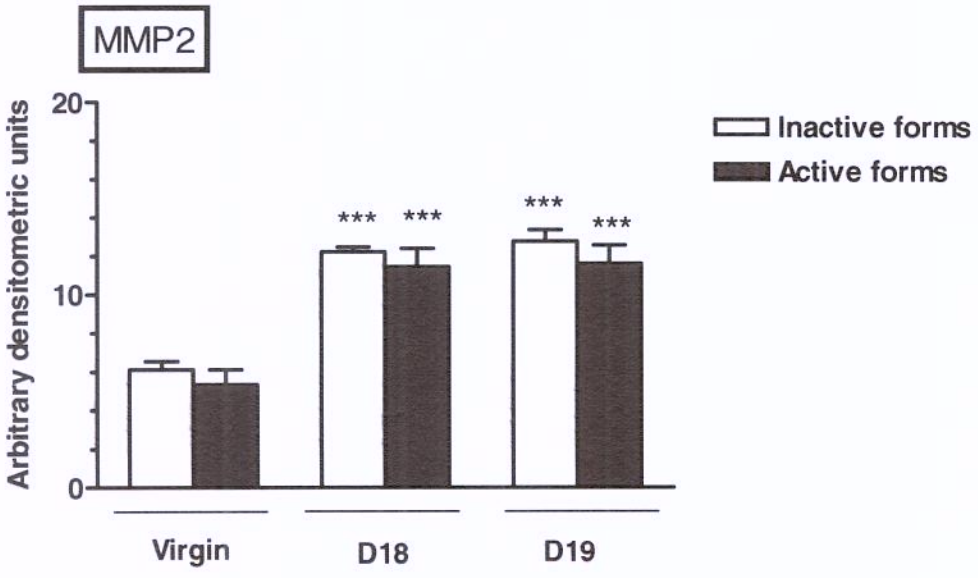

MMP9
Inactive form 95 KDa
Active form 85 KDa
MMP2
Inactive form 72 KDa
Active form 66 KDa
a
b
c
2A
a
b
c
2B

Figure 2

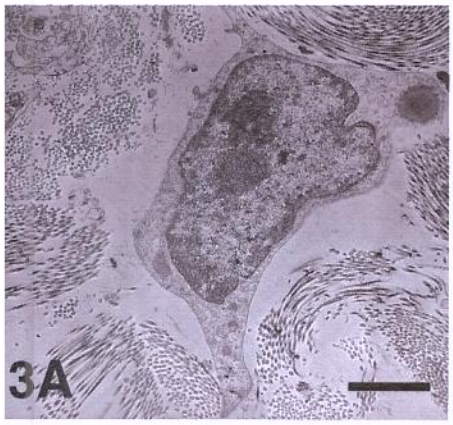


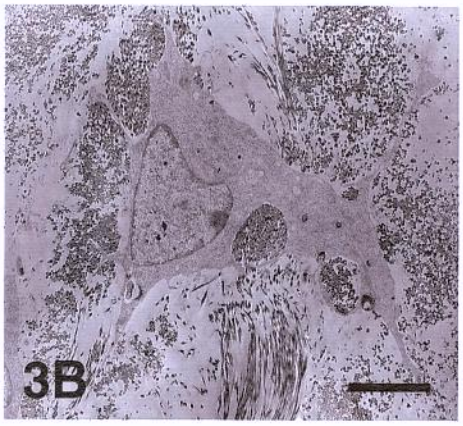
Graphic 1A



Graphic 1B

Graphic 1





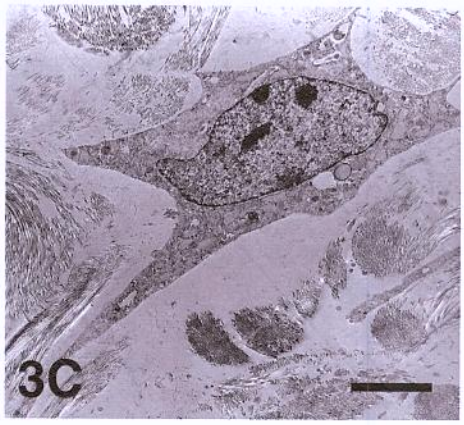


Figure 3

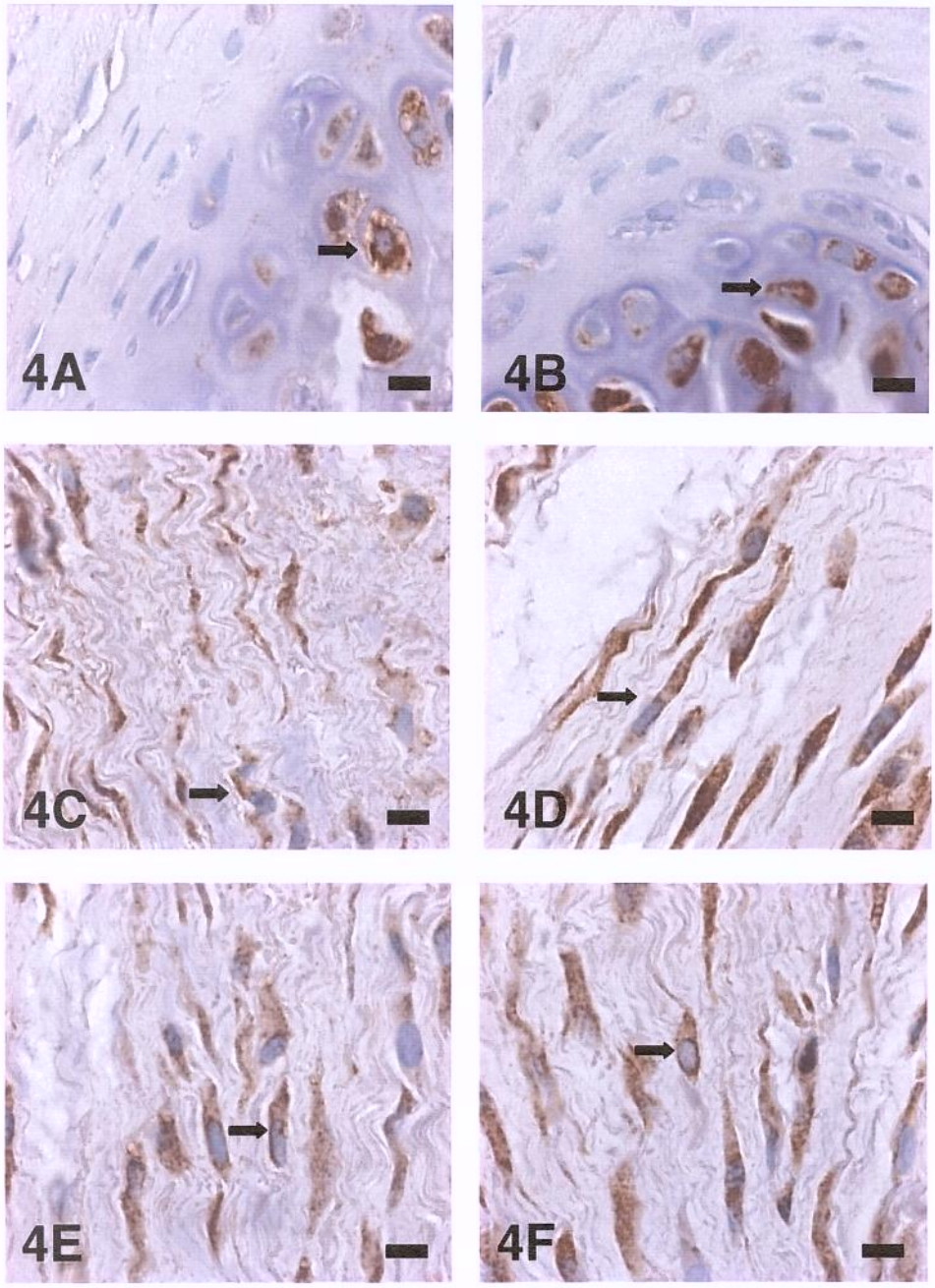


Figure 4

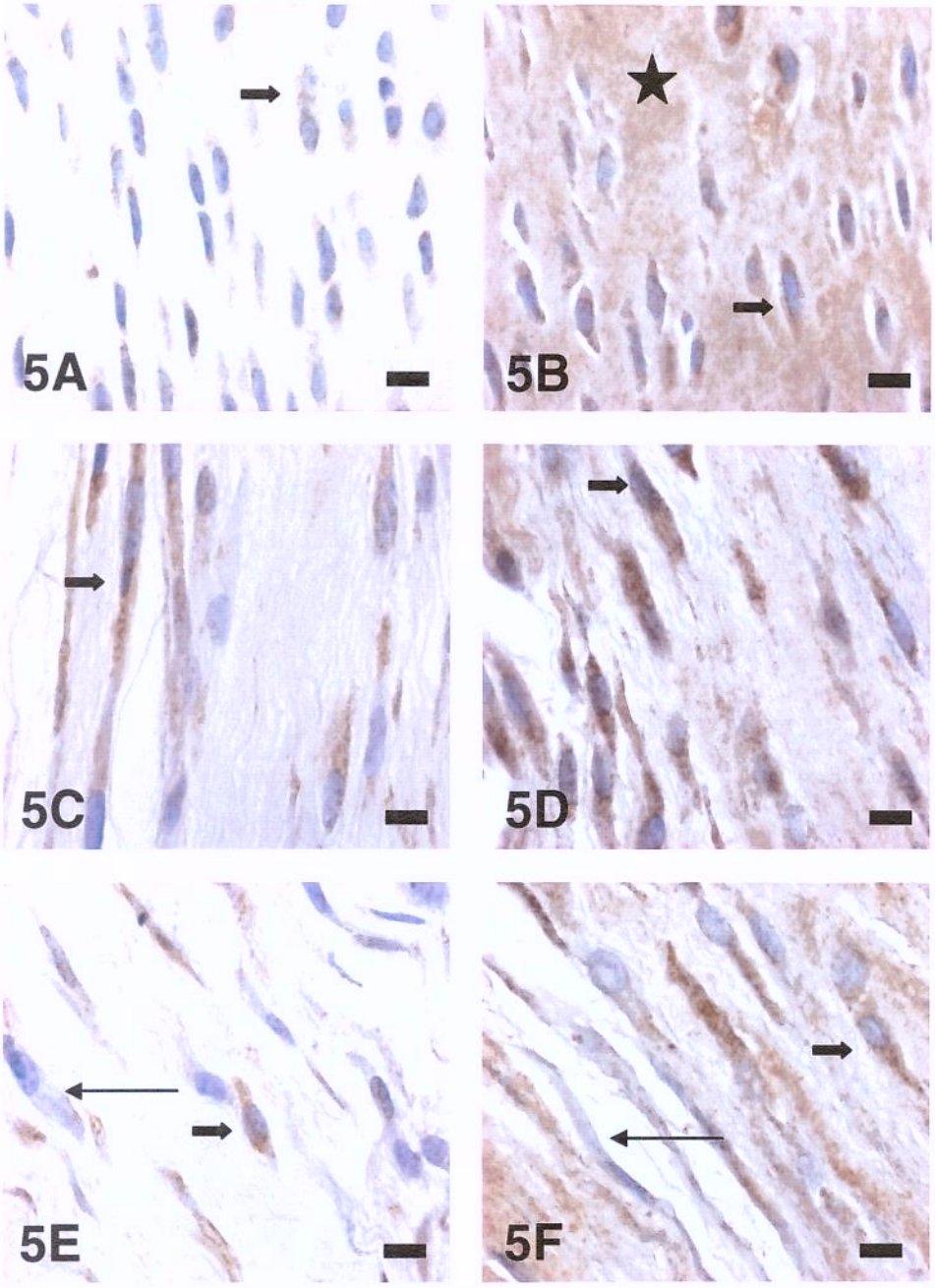


Figure 5

4. CONCLUSÕES

1. Na sínfise púbica de camundongo não foi possível à observação de células inflamatórias no processo de remodelação durante a prenhez, sendo assim, a remodelação da sínfise ocorre não através destas células.
2. A metaloproteinase -8 (Colagenase2) não é exclusiva de processo inflamatório, também participa no processo de remodelação da sínfise púbica do camundongo e é encontrada em células semelhantes a fibroblastos.
3. Na remodelação da sínfise púbica, as metaloproteinases -2 e -9, participam no processo de remodelação ativamente assim como seus inibidores TIMP-1 e -2.
4. O pico da remodelação ocorre no D18 de gestação, onde a taxa de MMP-2 e -9 são mais altas, quando comparadas ao dia do parto.
5. A principal fonte de metaloproteinases -2 e -9 e seus inibidores TIMP-2 e -1 respectivamente são células semelhantes a fibroblastos, no modelo de sínfise púbica do camundongo.

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