PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÂO
DOUTORADO EM FISIOPATOLOGIA E SAÚDE ANIMAL

ELLYN AMANDA FONSECA MARTINS

# AVALIAÇÃO HISTOMOLECULAR DA DIMENSÃO FRACTAL E DA REMODELAÇÃO DA MATRIZ EXTRACELULAR DURANTE O DESENVOLVIMENTO OVARIANO DE FETOS BOVINOS

Presidente Prudente - SP
2020

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Tese apresentada à Pró-Reitoria de Pesquisa e Pós-Graduação, Universidade do Oeste Paulista, como parte dos requisitos para obtenção do título de Doutor – Área de Concentração: Clínica Médica e Reprodução Animal.

Orientador:
Prof. Dr. Anthony César de Souza Castilho

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Presidente Prudente, 06 de Março de 2020.

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## DEDICATÓRIA

In *memoriam* do meu amado filho Miguel Martins Rodrigues, pela brava luta nesta Terra.

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*“Tudo sofre, tudo crê, tudo espera, tudo suporta”. (I Aos Coríntios, 13:7)*

## RESUMO

### Avaliação histomolecular da dimensão fractal e da remodelação da matriz extracelular durante o desenvolvimento ovariano de fetos bovinos

A fisiologia reprodutiva de fêmeas mamíferas é distinta em decorrência de sua fase estral, formação dos gametas femininos e dos folículos ovarianos iniciada no período pré-natal. O aparecimento dos estágios pré-antrais no ovário tem padrão temporal espécie-específico. Em fêmeas de *Bos taurus indicus*, estudos já demonstraram que folículos primordiais, primários e secundários aparecem próximo aos 90, 120 e 150 dias de gestação, respectivamente. Embora os estudos foquem na remodelação da matriz extracelular (MEC) durante a formação e funcionalidade de ovários adultos, há escassez no entendimento sobre os mecanismos que controlam a formação dos folículos pré-antrais durante a gestação. O objetivo do estudo foi caracterizar o fenótipo histomolecular do remodelamento MEC através da dimensão fractal (DF), quantificação de colágeno e perfil de transcritos envolvidos com remodelação de MEC em ovários de fetos bovinos associados à temporalidade de formação dos folículos pré-antrais. Assim, a fim de investigar o remodelamento tecidual ao longo do desenvolvimento ovariano fetal, foi utilizado ovários de fetos para quantificar a DF, o colágeno total e a abundância relativa de mRNA de genes relacionados ao remodelamento da MEC (COL1A1, COL1A2, COL4A1, MMP2, MMP9, MMP14, TIMP1 e TIMP2*).* Para tanto*,* pares de ovários fetais foram obtidos de fêmeas *Bos taurus indicus* com 60, 90, 120 e 150 dias de gestação em matadouro, sendo um deles destinado à extração de RNA total e posterior investigação dos transcritos alvos e o outro para análise de colágeno total e DF. O presente estudo demonstrou que a partir dos 120 dias houve maior área de colágeno total no ovário fetal. Nas análises de coloração em hematoxilina eosina (HE), a DF foi menor aos 150 dias quando comparada aos 60 dias de gestação, todavia, apresentou padrão inverso na coloração de picrosirius. A expressão dos genes alvos da abundância relativa dos transcritos de mRNA para COL1A1, COL4A1, MMP2, MMP14, TIMP1 e TIMP2 foi maior aos 150 dias em comparação ao dia 60. Conclui-se que a dimensão fractal reflete às alterações morfológicas durante a organização estrutural do tecido ovariano fetal e que a expressão de genes relacionados ao remodelamento da MEC é modulada ao longo da gestação em ovários fetais bovinos.



**Palavras-chave:** Bovino. Colágeno. Dimensão fractal. Folículos pré-antrais. Metaloproteinase. Ovário fetal.

## ABSTRACT

### Histomolecular evaluation of the fractal dimension and remodeling of the extracellular matrix during the ovarian development fetal bovine

The reproductive physiology of female mammals is different due to their estrous phase, the formation of female gametes and ovarian follicles that started in the prenatal period. The appearance of preantral stages in the ovary has a species-specific temporal pattern. In females of *Bos taurus indicus*, studies have already shown that primordial, primary and secondary follicles appear close to 90, 120 and 150 days of gestation, respectively. Although studies have focused on remodeling the extracellular matrix (ECM) during the formation and functionality of adult ovaries, there is a lack of understanding about the mechanisms that control the formation of preantral follicles during pregnancy. The aim of the study was to characterize the histomolecular phenotype of ECM remodeling through the fractal dimension (FD), quantification of collagen and profile of transcripts involved with remodeling of ECM in ovaries of bovine fetuses associated with the temporality of formation of pre-antral follicles. Thus, in order to investigate tissue remodeling along fetal ovarian development, fetal ovaries were used to quantify FD, total collagen and relative mRNA abundance of genes related to ECM remodeling (COL1A1, COL1A2, COL4A1, MMP2, MMP9, MMP14, TIMP1 and TIMP2). For this purpose, pairs of fetal ovaries were obtained from *Bos taurus indicus* females at 60, 90, 120 and 150 days of gestation in a slaughterhouse, one of which was destined for the extraction of total RNA and further investigation of the target transcripts and the other for collagen analysis. total and FD. The present study demonstrated that after 120 days there was a greater area of total collagen in the fetal ovary. In the staining analyzes in hematoxylin eosin (HE), the FD was lower at 150 days when compared to 60 days of gestation, however, it presented an inverse pattern in the picrosirius staining. The expression of the target genes for the relative abundance of *mRNA* transcripts for COL1A1, COL4A1, MMP2, MMP14, TIMP1 and TIMP2 was greater at 150 days compared to day 60. It is concluded that the fractal dimension reflects the morphological changes during structural organization of fetal ovarian tissue and that the expression of genes related to ECM remodeling is modulated throughout pregnancy in fetal bovine ovaries.



**Key words:** Bovine. Collagen. Fractal dimension. Preantral follicles. Metalloproteinases. Fetal ovary.

## LISTA DE SIGLAS

CL – Corpo lúteo
*COL1A1* – Colágeno tipo 1 alfa 1
*COL1A2* – Colágeno tipo 1 alfa 2
*COL4A1* – Colágeno tipo 4 alfa 1
CGPs – Células germinativas primordiais
DF – Dimensão fractal
DNA – Ácido desoxirribonucleico
FGFs – Fatores de crescimento fibrobastico
FSH – Hormônio folículo estimulante
GAPDH – Gliceraldeído 3-fosfato desidrogenase
GDF9 – Fator de crescimento de diferenciação
GnRH – Hormônio liberador de gonadotrofina
HE – Hematoxilina eosina
*H2AFZ* – Histona H2A
LH – Hormônio luteinizante
MEC – Matriz extra celualr
MMPs – Metaloproteinases da matrizz extracelular
MMP2 – Metaloproteinase-2 da matriz extracelular
MMP9 – Metaloproteinase-9 da matriz extracelular
MMP14 – Metaloproteinase-14 da matriz extracelular
RNA – Ácido ribonucleico
mRNA – Ácido ribonucleico mensageiro
PCR – Proteina c-reactiva
PGE2 – Prostaglandina E2
PPIA – Peptidilprolil isomerase A
PSR – Picrosirus red
TIMPs – Inibidor tecidual das metaloproteinases
TIMP1 – Inibidor tecidual da metaloproteinase 1
TIMP2 – Inibidor tecidual da metaloproteinase 2
ZP – Zona pelúcida

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# 1 INTRODUÇÃO

O rebanho bovino brasileiro é o maior rebanho comercial do mundo, composto principalmente por raças zebuínas, a raça Nelore (*Bos taurus indicus*) e seus cruzamentos compõe 80% do rebanho de corte e uma das principais características da pecuária é a produção de animais em pastagens (ANUAPEC, 2015). Em 2017 o país possuía um rebanho de 226 milhões de cabeças de bovinos, o que correspondia a 22,64% do total mundial, sendo que o maior rebanho pertence à Índia (IBGE, 2017).

Segundo Lima (1989), a raça Nelore multiplicou-se em grande velocidade, sendo essa grande aceitação devido aos altos índices produtivos e reprodutivo. São apresentadas algumas de suas características funcionais como a rusticidade em adaptar-se em regiões tropicais e subtropicais, alta fertilidade e prolificidade (BLACKBURN; GOLLIN, 2009).

A ocorrência de eventos fisiológicos e morfológicos, que suportam os mecanismos endócrinos, levando à maturidade sexual, é resultante de um adequado desenvolvimento de fêmeas, pois a maturação do eixo reprodutivo ocorre de maneira gradual e associada com as mudanças na composição corporal e no eixo somatotrópico que regula o *status* metabólico do animal para que ocorra o estabelecimento da gestação (DAY; MAQUIVAR, 2014).

A reprodução de fêmeas mamíferas é influenciada por vários fatores, incluindo a formação dos gametas femininos e dos folículos ovarianos é iniciada no período pré-natal, destacando os principais processos morfofisiológicos envolvidos na ovogênese e na foliculogênese.

O ovário das fêmeas de mamíferos é constituído ao nascimento por milhares de folículos primordiais, os quais são considerados o *pool* de reserva dos folículos ovarianos (MARSTROM *et al.*, 2002). Há o interesse crescente em ampliar o conhecimento sobre os mecanismos que modulam o desenvolvimento inicial dos ovários, o que beneficiaria fortemente a produção de alimentos de origem animal, bem como a preservação da biodiversidade, além de viabilizar estratégias para o tratamento da infertilidade na espécie humana (JIANG *et al.,* 2003; KSIAZKEWICZ, 2006).

Na maioria dos animais, a formação de folículos é iniciada durante a vida fetal e ao longo da vida reprodutiva.

Os folículos nos ovários de mamíferos permanecem em repouso por um período variável de tempo, em quiescência. A ativação dos folículos primordiais se dá através da transição da quiescência para a fase de crescimento, é a primeira e essencial etapa caracterizada e o início do crescimento do oócito (McNATTY *et al.,* 1995). Mais de 99,9% destes folículos não atingem a ovulação, visto que a maioria morre por um processo fisiológico designado atresia folicular (MARSTROM *et al.*, 2002; MORAES *et al.,* 2014).

Pode-se, portanto, associar o padrão de expressão de genes candidatos com o surgimento ou o aumento em número de folículos em categorias específicas. Dentre elas os fatores de crescimento fibroblástico (FGFs). A presença desses fatores e de seus receptores tem sido demonstrada em oócitos e/ou células somáticas de folículos pré-antrais (BURATINI *et al.,* 2005; BURATINI et al., 2007; MACHADO *et al.,* 2009). A natureza dinâmica da arquitetura ovariana dos mamíferos requer o remodelamento cíclico da matriz extracelular (MEC), onde a metaloproteinase (MMP) e seus inibidores são componentes decisivos para a quebra do colágeno da membrana basal folicular, teca interna e externa, túnica albugínea e da membrana basal da superfície epitelial (MCINTUSH; SMITH, 1998).

A dimensão fractal (DF) é uma medida não topológica do espaço e define o grau de auto-semelhança que um objeto tem nas suas diferentes escalas de observação (HUYN, 2015). É utilizada para detectar alterações morfológicas sutis em lâminas histológicas, transformando a complexidade da forma em dados quantitativos analíticos, conciliando características estruturais e medidas quantitativas funcionais (PAJEVIC, 2018). Nas ciências médicas, tem sido aplicada como base na caracterização da taxa de proliferação celular em tumores malignos, avaliação da mineralização óssea para o diagnóstico de osteoporose, ou alterações ao padrão molecular das hemácias na drepanocitose (BOSE *et al.*, 2015). Esses conhecimentos também podem fornecer ferramentas fundamentais para maximizar o uso de biotecnologias na reprodução animal.

## 1.1 Hipóteses

O presente estudo foi realizado para verificar as seguintes hipóteses quanto aos mecanismos envolvidos no desenvolvimento ovariano:

A temporalidade gestacional influencia na quantidade de folículos primordiais, primários, secundários e terciários.

A descrição do fenótipo histomolecular da remodelação da MEC com o aumento do colágeno e expressão genica do perfil dos transcritos alvos se difere quanto à temporalidade durante a formação dos folículos pré-antrais.

A importância da dimensão fractal como uma ferramenta para ser agregada como biotecnologia na reprodução animal.

## 1.2 Objetivo

O objetivo do estudo foi caracterizar o fenótipo histomolecular do remodelamento de matriz extracelular (MEC) através dimensão fractal, quantificação de colágeno e perfil de transcritos envolvidos com remodelação de MEC em ovários de fetos bovinos associados à temporalidade de formação dos folículos pré-antrais

# 2 REVISÃO DE LITERATURA

## 2.1 Fisiologia ovariana

### 2.1.1 Ovogênese

Nos mamíferos, o ovário e a gônada feminina são responsáveis pela diferenciação e liberação de um oócito maduro para fertilização e propagação bem-sucedida das espécies. Bem como, na função endócrina para a produção de hormônios que atuam no desenvolvimento das características sexuais secundárias femininas e auxiliam na gestação (DYCE *et al*., 1987).

A ovogênese refere-se à sequência de eventos em que as células germinativas primordiais (CGPs) diferenciam-se totalmente, ou quase totalmente, na fase embrionária, inicialmente em ovogônias e se encerram com a formação do oócito haploide fecundado (MOORE; PERSAUD, 2008).

Durante a fase embrionária, as CGPs, localizadas na parede do saco vitelínico, migram para os ovários em desenvolvimento, onde iniciam o processo de diferenciação (SADEU *et al*., 2006). Perdem suas características de motilidade e sofrem extensiva proliferação celular e redistribuição das organelas citoplasmáticas transformando-se em ovogônias. Estas células apresentam intensa proliferação através da divisão mitótica, mantendo-se diploides (ALBERTS *et al.,* 2010). No fim do ciclo de divisões mitóticas, as ovogônias aumentam de tamanho e entram em primeira prófase meiótica, com a duplicação do ácido desoxirribonucleico (DNA), diferenciando-se, assim, como ovócitos primários (MOORE; PERSAUD, 2008; MONIRUZZAMAN; MIYANO, 2010).

A primeira prófase meiótica é dividida em cinco estádios sequenciais: leptóteno, zigóteno, paquíteno, diplóteno ou dictióteno e diacinese (MOORE; PERSAUD, 1994). No estágio do leptóteno, cada cromossomo se condensa a partir de sua conformação interfásica para produzir um fino fio discreto. O zigóteno começa assim que é iniciada a sinapse e cada gene é trazido em justaposição próxima com seu gene homólogo no cromossomo oposto. As

células entraram no estágio de prófase do paquíteno assim que a sinapse está completa. Quando o ovócito atinge o estágio do paquíteno, ele fica fechado em um folículo (GOSDEN, 1995).

Porém, o processo meiótico no oócito ocorre com a primeira interrupção no estádio na prófase I, antes de completar o estádio de diplóteno ou dictióteno. A formação dos oócitos primários (2n = 60 cromossomos já duplicados) permanece neste estágio da divisão celular até o início da maturação ovocitária no período da puberdade. Em bovinos, a formação dos ovócitos primários ocorre entre 75 e 80 dias após a concepção (McNATTY *et al.,* 1995; McNATTY et al., 2000).

Na puberdade, imediatamente antes da ovulação, com o pico dos hormônios folículo estimulante (FSH) e hormônio luteinizante (LH), os oócito no estágio de vesícula germinativa, que possui o dobro da quantidade normal de DNA desde a interrupção no estádio da primeira prófase meiótica (GOSDEN, 1995). Os oócitos que terminaram seu crescimento retomam a meiose e o núcleo da vesícula germinativa para diacinese (MOORE; PERSAUD, 1994). Segundo Moore; Persaud. (2008), ocorre a quebra da primeira meiose, progressão para metáfase I, anáfase I e telófase I, expulsão do primeiro corpúsculo polar e formação do oócito secundário (n = 30 cromossomos duplicados). Inicia-se a seguir a segunda divisão meiótica, em que o núcleo do oócito evolui até o estádio de metáfase II, quando ocorre a segunda interrupção da meiose (GORDON, 1994). O oócito permanece neste estádio até ser fecundado pelo espermatozoide, quando, então, completa a meiose e expulsa o segundo corpúsculo polar, formando o oócito haploide fecundado (MOORE; PERSAUD, 1994; MOORE; PERSAUD, 2008).

### 2.1.2 Foliculogênese

A foliculogênese inicia na vida fetal, simultaneamente à ovogênese. É o processo contínuo responsável pelo desenvolvimento folicular (transição dos folículos primordiais da fase quiescente para a fase de crescimento), pela liberação de um ou mais oócitos maduros e atresia dos folículos ovarianos em

um intervalo fixo ao longo da vida reprodutiva de uma fêmea até a senilidade (NILSSON; SKINNER, 2001). Todo o processo de crescimento folicular do estágio primordial ao estágio ovulatório leva aproximadamente 180 dias em bovinos (LUSSIER *et al*., 1987).

Na transição da mitose, com a interrupção no dictióteno, na primeira prófase meiótica as ovogônias transformam-se em ovócitos primários e folículos primordiais ao redor do ovócito, com uma camada de 4 a 8 células somáticas, chamadas pré-granulosas, e uma lâmina basal formando a primeira geração de células foliculares (BRAW-TAL; YOSSEFI, 1997; FAIR, 2003). É o primeiro sinal de ativação dos folículos primordiais e a retomada da proliferação das células da granulosa (MARTINS, 2008). As células da granulosa dos folículos primordiais podem ser originadas das células mesoteliais ou das células mesonéfricas, ou ainda de ambas as células (CASTILHO *et al.,* 2013).

Isto sugere que a interação das células teca e granulosa pode desempenhar um papel na regulação do crescimento e diferenciação do folículo em todas as etapas da foliculogênese (PICTON, 2001).

A foliculogênese pode ser dividida em duas fases: 1) fase pré-antral, que é subdividida em ativação dos folículos primordiais e crescimento dos folículos primários e secundários; 2) fase antral, subdividida em crescimento inicial e terminal dos folículos terciários, pré-ovulatório (folículo de *Graaf*) (MARTINS, 2008).

#### 2.1.2.1 Fase pré-antral

Os folículos permanecem quiescentes nos ovários até o recrutamento e o crescimento de um "*pool*" de folículos primordiais da população ovariana. Os fatores e mecanismos responsáveis pela ativação de folículos primordiais, bem como os mecanismos envolvidos na variação do período de início do crescimento folicular são ainda enigmáticos e representam uma das maiores questões relacionadas com a biologia ovariana (FORTUNE *et al.,* 2000).

No processo da fase pré antral, os folículos primordiais, são a unidade fundamental de desenvolvimento do ovário de mamíferos. Estão localizados na

região periférica do córtex ovariano, presença das células de granulosa com formato pavimentoso, sem suprimento sanguíneo e sua nutrição acontece por difusão (MORAES, 2014). Tornam-se folículos de transição, caracterizados pela presença de células da granulosa com formato pavimentoso e cubóide, gradualmente adquirem formato cúbico (VAN DEN HURK, 1997). Os folículos primordiais no bovino foram detectados pela primeira vez no 90º dia de gestação (ERIKSON, 1966).

Folículos primordiais bovinos são ativados para se tornarem folículos primários no 140º dia de gestação (RUSSE, 1983). A mudança de forma é seguida pelo início da síntese de DNA e mitose nas células da granulosa. A ativação é independente da hipófise, e provavelmente é controlado por mecanismos autócrinos / parácrinos (McLAUGHILIN; McLVER, 2009).

Em seguida, originam-se os folículos primários, caracterizado quando o oócito é circundado por uma camada completa de granulosas cubóides (BRAW-TAL; YOSSEFI, 1997; GOURGEON; BUSSO, 2000). Além da mudança da forma das células da granulosa, há um rápido aumento e diferenciação no tamanho do oócito tanto no volume citoplasmático e nuclear, como resultado de um aumento progressivo no nível de síntese de RNA e, terão alcançado um diâmetro de 100 µm. Alguns genes de oócitos são transcritos e traduzidos, incluindo aqueles que codificam as proteínas da zona pelúcida (ZP) (HIRSHFILD, 1991; BRAW-TAL; YOSSEFI, 1997; LIMA-VERDE *et al.,* 2011). Eventos foliculares pré-antrais importantes incluem a expressão do fator de crescimento e diferenciação 9 (GDF9), fator básico de crescimento de fibroblastos e fatores de crescimento epidérmico e proteínas da conexina juncional (ELVIN; YAN; MATUK, 2000).

Os folículos secundários aparecem no dia 210 da gestação em bovinos quando as células foliculares dos folículos primários sofrem divisão mitótica intensiva (RUSSE, 1983).

A progressão do folículo primário para secundário é caracterizada pela formação da segunda e terceira camada de células da granulosa, as células da teca são identificáveis em torno da membrana basal, ZP bem definida e o folículo contém uma fina rede capilar (FAIR, 2003). Nesta fase, os folículos já

alcançaram um diâmetro médio de 200 µm e se tornam responsivos às gonadotrofinas FSH e LH (LIMA-VERDE *et al.,* 2011). Neste momento, as células da granulosa apresentam uma extensiva rede de junções do tipo *gap*, que permitem a passagem de nutrientes, íons inorgânicos, segundo mensageiros e pequenos metabólitos entre as células (KIDDER; MHAWI, 2002).

2.1.2.2 Fase antral

A transição do folículo secundário para o folículo terciário é marcada pelo aparecimento de uma cavidade nas células da granulosa denominada antro, preenchida pelo líquido folicular, atingindo um diâmetro de 400 µm (ERICKSON, 1966; LEITÂO *et al.,* 2009). Os primeiros folículos antrais aparecem por volta dos 230 dias de gestação nos bovinos (RUSSE, 1983).

Durante a fase de transição, ocorre a produção de hialuronanos e proteoglicanos pelas células da granulosa que gera um gradiente osmótico e melhora a formação de líquido folicular (RODGERS; IRVING-RODGERS, 2010). Esse líquido serve como fonte importante de moléculas reguladoras, como as gonadotrofinas, os esteroides, os fatores de crescimento, as enzimas, as proteoglicanas e as lipoproteínas que são derivadas do sangue e das células que integram o folículo (VAN DEN HURK, 1997).

Entre as substâncias que promovem a formação de antro nos folículos bovinos, o GDF9 aumenta a expressão de versicano e perlecano, como consequência de uma interação positiva com o FSH (VASCONCELOS *et al*., 2013). Neste estádio, eles são denominados folículos terciários, pré-ovulatórios ou folículos de *Graaf*, possuindo células da granulosa cuboides em diversas camadas que adquire o primeiro receptor para LH, além de células da teca interna e externa, da lâmina basal e das células do *cumulus oophorus* (LEITÃO *et al.,* 2009).

Em taurinos, a capacidade ovulatória ocorre somente após os folículos alcançarem 10mm de diâmetro e em fêmeas de *Bos taurus indicus* ovulam com diâmetros inferiores (SARTORI *et al*., 2001).

Por fim, ao receber estímulos para desenvolvimento sincronicamente, em determinados períodos do ciclo estral e são conhecidos como ondas de crescimento folicular. Os folículos se tornam maduros e um montante que foram selecionados atinge o papel de dominante alcançando o status de folículo pré-ovulatório, possuindo este uma cavidade (antro) maior (LEITÃO *et al.,* 2009). Após o pico de LH, as células da teca interna começam a produzir progesterona que estimula localmente a síntese de uma enzima chamada colagenase. Esta enzima causa um desarranjo e digestão do colágeno na túnica albugínea levando ao aumento do fluido folicular que comprime os vasos sanguíneos, fazendo com que diminua e cesse o fluxo. Ao mesmo tempo, sob ação do LH e FSH, as células da teca interna na região do estigma perdem a sua continuidade e as uniões celulares do *cumulus oophorus* desintegram-se, ocorrendo o rompimento do folículo e liberação do ovócito e a atresia dos demais (MORAES *et al*., 2014).

Após a ovulação, a prostaglandina E2 (PGE2) sintetizada no ovário, auxilia na remodelação do folículo para corpo lúteo, através da ativação do plasminogênio. Este, por sua vez, é convertido em plasmina, que é uma enzima ativa responsável pela remodelação tecidual, ajudando na remodelação do folículo a corpo lúteo (SENGER, 2003).

## 2.2 Desenvolvimento e remodelação da matriz extracelular no folículo ovariano

### 2.2.1 Matriz extracelular

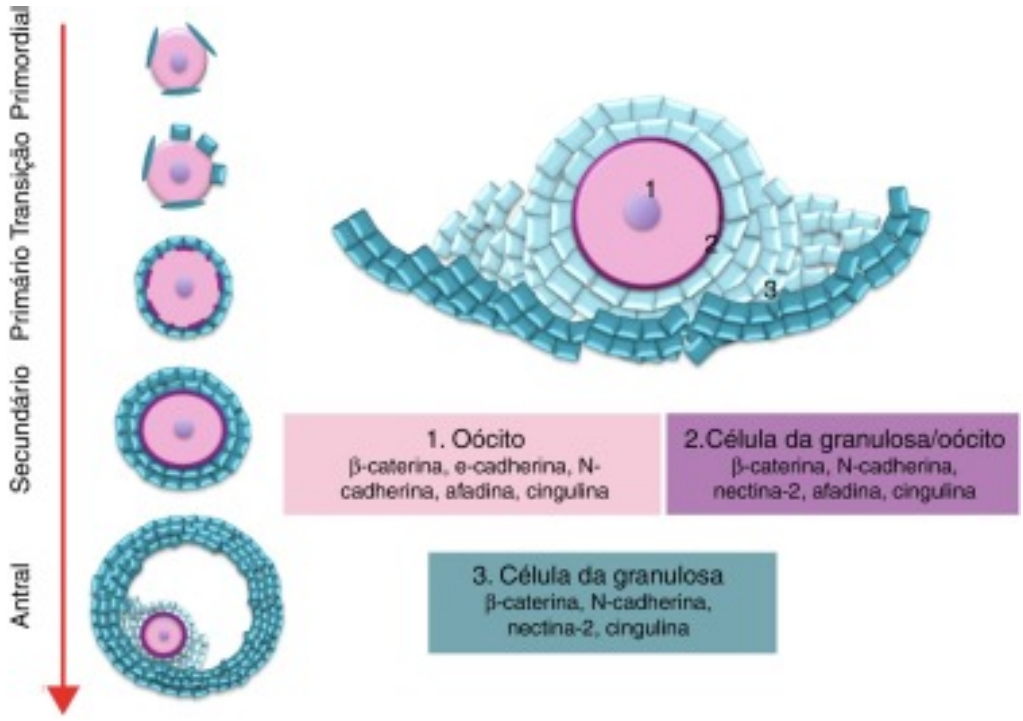
A matriz extracelular (MEC) é um conjunto de agregados supramoleculares constituídos por uma rede molecular que permite a comunicação entre as células que ocorre de forma parácrina, endócrina ou autócrina, como peptídeos, grandes proteínas inseridas na membrana celular, aminoácidos, nucleotídeos, esteroides, colágeno, glicoproteínas, proteoglicanos e glicosaminoglicanos que mantêm as células associadas possibilitando a organização dos tecidos e a sobrevivência celular (BULOW;

HOBERT, 2006) Principalmente composta por duas classes de macromoléculas: proteínas fibrosas (incluindo colágenos e elastina) e glicoproteínas (incluindo fibronectina, proteoglicanos e laminina) (MOUW *et al*., 2014).

Nos folículos existem vários compartimentos e MEC diferentes. Estes incluem a lâmina basal folicular, fluido folicular, zona pelúcida, membrana granulosa, *cumulus* e tanto a teca interna como teca externa em folículos antrais maiores, ou o estroma nos folículos primordiais e pré-antrais menores (HAY, 1991; LUCK, 1994).

A MEC apresenta papéis diferentes que incluem efeitos no comportamento celular (Figura 1), tais como: migração, divisão, diferenciação, morte celular e ancoragem celular. Todos esses comportamentos ocorrem no desenvolvimento folicular (HAY,1991). Variadas são as funções da MEC: Dinâmica dos fluidos de um tecido, fornecendo forças osmóticas, além de nutrientes, hormônios e outros sinais extracelulares necessários para alcançar a célula-alvo. Fornecer informações rígidas ou suporte mecânico elástico para tecidos. Apresenta capacidade de vincular fatores de crescimento (Figura 1) incluindo aqueles encontrados nos folículos.

Figura 1 - Folículo pré-ovulatório contém oócito circundado de células do *cumulus* e células da granulosa, que delimitam a cavidade antral circundadas pelas células da teca.




Fonte: HAY (1991); RODGERS *et al.* (2000).

O mecanismo utilizado pela MEC para suportar/melhorar a foliculogênese envolve o estabelecimento de folículos dominantes, capazes de bloquear o crescimento e induzir a atresia dos demais folículos, denominados subordinados (VAN DEN HURK; ZHAO, 2004). A dominância folicular envolve redução da secreção de FSH hipofisário, induzida pela inibina, e de hormônio liberador de gonadotrofina (GnRH) hipotalâmico induzida pelo estradiol, atuando na regressão dos folículos subordinados (GINTHER *et al*., 1996). Com a redução na concentração endógena de FSH, induzida pelo mecanismo de dominância, os folículos subordinados regridem, enquanto o dominante continua seu desenvolvimento.

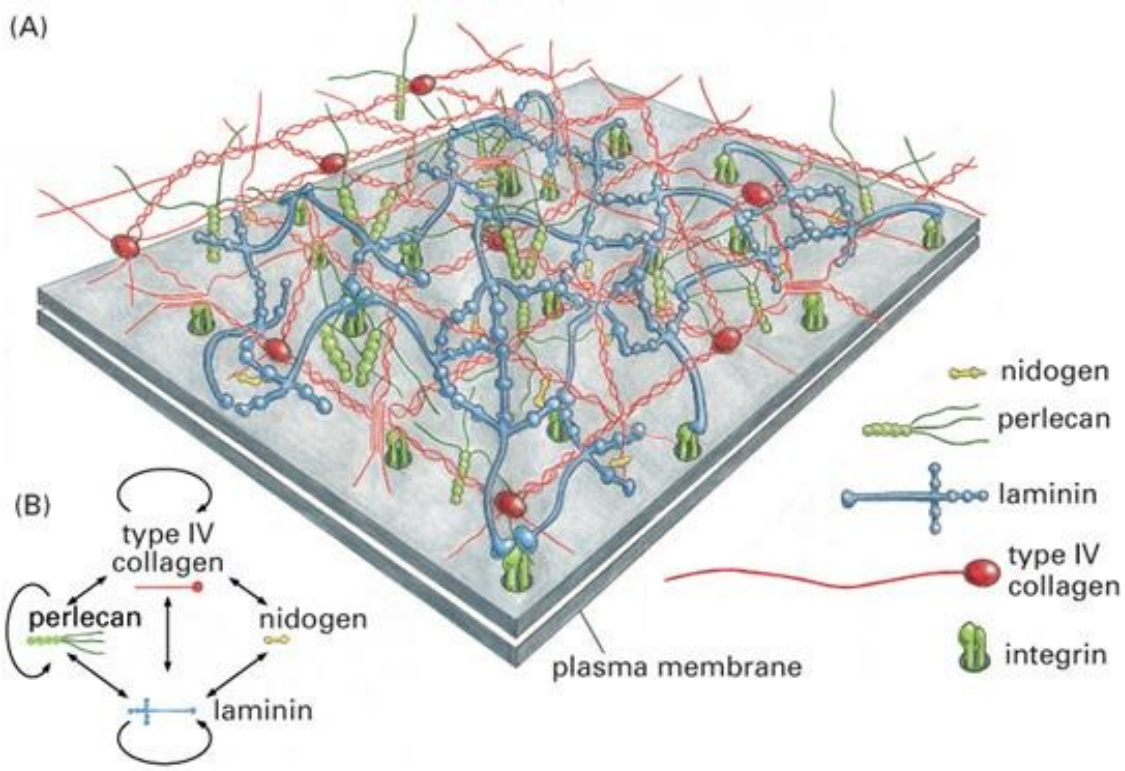
### 2.2.2 Lâmina basal

No ovário, a camada de célula da granulosa dos folículos ovarianos é envolvida por uma lamina basal, que os separa dos elementos do estroma circundante (VAN WEZEL; RODGERS, 1996). As lâminas basais são folhas especializadas de MEC que, nos epitélios, sustentam as células epiteliais e as separam do estroma adjacente (TIMPL; BROWN, 1996).

A lâmina basal exerce um importante papel onde influenciam a proliferação e diferenciação de células epiteliais, podem retardar seletivamente a passagem de moléculas e controlar fatores de crescimento para o interior do folículo (RODGERS *et al.,* 2000). No entanto, existem poucos estudos que tentam mostrar que essas mudanças na lâmina basal provocam mudanças no comportamento celular e tecidual durante o desenvolvimento folicular.

As lâminas basais são compostas por uma camada de glicoproteína (rede de colágeno tipo IV, entrelaçada com uma rede de laminina) e proteoglicanas secretadas pelas células epiteliais (Figura 2). Essa estrutura é estabilizada pela ligação de entactina / nidogênio ao colágeno e à laminina e por interações de baixa afinidade entre colágeno tipo IV e laminina (YURCHENCO; SCHITTNY, 1990; PAULSSON, 1992). Assim, cada molécula de colágeno tipo IV compreende três cadeias, sendo que até o momento, seis tipos diferentes de cadeia foram identificados. Potencialmente, qualquer combinação destes pode estar presente (HAY, 1991; ZHOU *et al.,* 1994). Considera-se que a composição única de cada lâmina basal contribui para as suas propriedades funcionais específicas.

Figura 2 - Desenho esquemático (A) rede formada por matriz extracelular e (B) estabilização das estruturas da lâmina basal.




Fonte: adaptado de Alberts *et al.* (2002).

Geralmente associada à porção inferior da lâmina basal, há uma camada de fibras reticulares (colágeno do tipo III), a lâmina reticular, que é secretada pelo tecido conjuntivo subjacente. A lâmina basal e a lâmina reticular compõem a membrana basal (MONTANARI, 2016). As lâminas basal e reticular mantêm-se unidas pela fibronectina (possui sítios para ligação de colágeno, heparina e receptores na membrana celular, que são as integrinas. Estas, por sua vez, se ligam aos filamentos de actina do citoesqueleto, permitindo uma influência mútua entre a célula e a matriz extracelular), uma glicoproteína de adesão; pelas fibrilas de ancoragem, de colágeno do tipo VII, e pelas microfibrilas, formadas pela glicoproteína fibrilina. Essas substâncias também são secretadas pelas células do conjuntivo. A membrana basal está ligada à matriz extracelular do tecido conjuntivo pelas fibrilas de ancoragem (RODGERS *et al.,* 2000; MONTANARI, 2016). A lâmina basal permite a adesão entre o epitélio e o tecido conjuntivo e é uma barreira de filtração seletiva para as substâncias que se movimentam entre esses dois tecidos. Ela influencia a

diferenciação e a proliferação das células epiteliais. Quando as células perdem o contato com a lâmina basal, elas morrem: sofrem apoptose. A lâmina basal serve ainda de apoio para a migração durante o desenvolvimento embrionário e a regeneração (MONTANARI, 2016).

### 2.2.3 Remodelação da matriz extracelular

A MEC está em constante remodelação e sua síntese e degradação acompanha a morfogênese e a regeneração Através das moléculas de adesão, a matriz extracelular ancora as células, permite a migração, estimula a proliferação, regula a diferenciação celular e influencia a transmissão de informações pela membrana plasmática (RODGERS,2000; KUMAR *et al.*, 2010).

Durante o desenvolvimento folicular, o remodelamento contínuo da parede folicular ocorre à medida que o folículo aumenta. Em particular, a rotatividade dos colágenos estruturais da teca externa, bem como a vasculatura da teca interna e estroma permite a expansão do folículo. A lâmina basal folicular também deve se expandir com a proliferação e diferenciação das células da granulosa (AMSTERDAM *et al.,* 1989; RICHARDSON *et al.,* 2000; LUCK, 1994). Existem poucos estudos que mostram essas mudanças na lâmina basal, no comportamento celular e tecidual durante o desenvolvimento folicular. Nos folículos de mamíferos, a área da superfície muda sua composição, amplia 19 vezes durante o desenvolvimento folicular, permitindo remodelamento contínuo da lâmina basal folicular (VAN WEZEL; RODGERS, 1996).

Os componentes da matriz extracelular dos ovários de mamíferos, mais comumente estudados: colágeno tipo I, colágeno tipo IV, laminina e fibronectina (BERKHOLTZ *et al.,* 2006). Segundo Rodgers *et al*. (1998), os colágenos a1 / a6 do tipo IV estão todos presentes em folículos primordiais. O colágeno tipo IV interage por meio de seus domínios terminais, a laminina participa na adesão das células à lâmina basal, enquanto que a fibronectina é importante nos processos de adesão, migração, crescimento e diferenciação

celular (RODGERS *et al.,* 2003). Portanto, a lâmina basal folicular é importante para manter a polaridade e a extensão da especialização das células granulosas (RODGERS *et al.,* 2000).

Os processos de mudança da MEC requerem controle discreto, pois o resultado final é uma expansão da matriz e não uma degradação total. O mecanismo preciso pelo qual isso ocorre não é bem compreendido, mas envolve simultaneamente degradação simultânea e síntese da matriz (SMITH *et al*., 1999). Durante o crescimento folicular, as alterações e reconstruções da membrana basal são facilitadas por enzimas chamadas metaloproteinases (MMPs) 1, 2, 9 e 14, que são secretadas pelas células tecais que degradam o colágeno tipo IV que é um dos principais componentes da membrana basal. A ação destas enzimas é controlada por inibidores teciduais de metaloproteinases (TIMPs) 1 e 2 que são responsáveis pela reconstrução da membrana basal durante a ovulação e formação de corpo lúteo. A membrana basal é reconstruída pela deposição dos seus principais componentes que são secretados pelas células da granulosa, da teca e do estroma ovariano (McCAFFERY *et al.,* 2000).

O equilíbrio entre regeneração e degradação da matriz extracelular é mantido, em parte, pela ação de enzimas proteolíticas extracelulares que são secretadas por células locais. Dentre estas se destacam as metaloproteinases da matriz (MMPs), as quais dependem da ligação do $Ca^{2+}$ ou $Zn^{2+}$ para sua atividade. Atualmente, a família das MMPs é composta por 25 tipos agrupados em quatro classes: colagenases (MMP-1, -8, -13), gelatinases (MMP-2 e MMP-9), estromalisinas e as MMPs típicas de membranas (MTMMPs) (STERNLICHT; WERB, 2001). Três mecanismos básicos atuam para assegurar o controle das MMPs: secreção da forma inativa (pro-enzima), confinamento por receptores de superfície celular e inibidores teciduais de metaloproteinases (TIMPs). O aumento de TIMPs atua coordenando as ações de MMPs, regulando a localização e extensão da remodelação da matriz extracelular durante o processo ovulatório em mamíferos (CURRY; OSTEEN, 2003). As MMPs desempenham um importante papel no processo ovulatório de diferentes grupos de vertebrados, atuando na ruptura folicular, fragmentação

da membrana basal e fibras conjuntivas associadas ao folículo ovariano (CURRY; OSTENN, 2003; OGIWARA *et al*., 2005). Em teleósteos, a distribuição e a ação proteolítica das MMPs foram descritas em fígado, músculos, brânquias e gônadas (MATSUI *et al.,* 2000; OGIWARA *et al*., 2005).

## 2.3 Dimensão fractal

A Dimensão Fractal (DF) de um objeto possui uma grande riqueza de informação em suas diferentes escalas espaciais e demonstra ser uma eficiente ferramenta para o reconhecimento de padrões (BACKES, 2006). A geometria euclidiana define e estuda formas ditas como perfeitas (pontos, retas, quadrados, círculos e polígonos). Contudo, isto não é suficiente para classificar algumas formas presentes na natureza. Esses objetos são conhecidos como sendo idealizações que não têm comprimento característico e nem tamanho abstoluto (ASSIS *et al.,* 2008). Essas formas possuem como base, regras simples de construção, mas que ao serem repetidas inúmeras vezes, geram figuras de uma complexidade espantosa (BACKES, 2006). Essas novas formas apresentam auto-semelhança em nível de escala, ou seja, o conjunto total é constituído por pequenas réplicas dele mesmo independente da escala utilizada para visualizá-lo (GULICK, 1992).

A metodologia foi denominada de metodologia *fractal,* palavra proveniente do latim *fractus* e *frangere*, que significa quebrar, fraturar (ASSIS *et al.,* 2008). A dimensão de um objeto fractal é um valor fracionário, pois este valor indica o grau de complexidade ou irregularidade que uma imagem possui, ou seja, o quanto do espaço físico ela ocupa (TRICOT, 1994).

Essa característica a torna uma ferramenta muito útil na comparação de duas formas fractais e possibilita ser empregada em organismos. No campo da biologia e medicina, este conceito vem sendo utilizado para quantificar mudanças dinâmicas e estruturais tanto em células como em tecidos (SMITH JUNIOR *et al.,* 1989). Bem como na quantificação do alinhamento e comprimento de fibra ou identificação de bordas e mudança na morfologia do citoesqueleto (DOAN *et al*., 2013).

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# Artigo Científico

# Fractal analysis and histomolecular phenotyping provides insights into extracellular matrix remodeling in the developing bovine fetal ovary

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## HIGHLIGHTS

1) Fractal dimension is a useful approach in evaluating tissue remodeling in the bovine fetal ovary.

2) The expression of collagen I and IV, metalloproteinases 2 and 14, and their inhibitors is regulated during bovine fetal ovary remodeling and is related to its fractal dimension.

**ABSTRACT**

Although studies have focused on extracellular matrix (ECM) remodeling during the formation and functioning of adult ovaries, there is no comprehensive understanding of the mechanisms controlling preantral follicle development in fetal bovine ovaries during gestation. Thus, to gain insights into ECM remodeling during initial ovarian development, we used fetal ovaries to quantify the fractal dimension (FD), total collagen, and relative mRNA abundance of genes related to ECM remodeling (*COL1A1, COL1A2, COL4A1, MMP2, MMP9, MMP14, TIMP1,* and *TIMP2*). For this, pairs of fetal ovaries were obtained from cows in a local abattoir at days 60, 90, 120, and 150 of gestation; one of each pair was submitted to RNA extraction for target transcript analysis, and the other was used for total collagen and FD evaluation. From day 120 total collagen appeared to occupy a greater area in the fetal ovary. The fractal analysis with picrosirius red staining shows higher at day 150 when compared with that on day 60. On the contrary, we found an inverse pattern when we used the hematoxylin and eosin staining approach. Concerning target gene expression, the relative abundances of *COL1A1, COL4A1, MMP2, MMP14, TIMP1,* and *TIMP2* mRNA were higher on day 150 when compared with that on day 60. We conclude that fractal analysis reflects the morphological changes occurring during structural organization of the fetal ovary and that the expression of genes related to ECM remodeling is modulated throughout gestation in bovine fetal ovaries.

Keywords: metalloproteinases, collagen, gene expression, preantral follicles, picrosirius, cattle.

## INTRODUCTION

The appearance of the preantral stages in the ovary has a species-specific temporal pattern. In cattle, all preantral stages manifest themselves in the fetal ovary during gestation [1]. Data on the specific time of appearance of each preantral category are divergent. Russe et al. [2] reported the appearance of primordial, primary, and secondary follicles at approximately 90, 140, and 210 days of gestation, respectively, whereas Tanaka et al. [3] reported, in the predominantly taurine fetuses, the appearance of the same stages of development at approximately 74, 91, and 120 days of gestation, respectively, with an increase in secondary follicle numbers up to 150 days of gestation. Castilho et al., [4] recently demonstrated a similar profile of the preantral follicular dynamics in bovine fetuses, predominantly of the Nellore breed, with the appearance of primordial follicles at 75, primary at 90, and secondary at 150 days of gestation.

The formation, development, and regression of these follicles requires considerable tissue remodeling, cell replication, and specialization, with the extracellular matrix (ECM) involved in many of these processes [5,6,7]. Rodgers et al, [8] was the first to identify type I collagen as the main component of bovine c*orpus luteum* (CL), representing about one-sixth of the luteal dry matter. Despite the detection of the protein and mRNA of type IV collagen in ruminant luteal tissue—the main component of the follicular basal lamina—its distribution pattern is still unknown. It

may be located in two possible areas: around the large luteal cells and in the basal lamina of the blood vessels, since this tissue has an extensive capillary weave [8]. The abilities of ECM to direct cell proliferation, differentiation, and function imply its remodeling in normal ovarian function. Specific ECM components are cleaved by matrix metalloproteinases (MMPs) whose activities are specifically inhibited by tissue metalloproteinase inhibitors (TIMPs) [9].

Although previous studies have been focused on ECM remodeling of adult ovaries, nothing is known about the mechanisms that promote and control the process during the preantral follicle formation in fetal bovine ovaries throughout gestation. To increase our knowledge of ECM remodeling in the fetal bovine ovary, the use of less subjective tools such as FD may allow quantification of tissue structural changes [10]. The FD aims to scale the spatial organization of an image from number fractals that describe the relation of free space and the self-similarity of the structure [11]. For this reason, FD is able to detect subtle morphological changes, transforming the complexity of the form into quantitative analytical data and reconciling structural features and functional quantitative measures [12].

In fact, Frisch et al. [11] have indicated fractal analysis as a consistent method when evaluating tissue remodeling of tendon collagen in rats exposed to injury. Zouein et al. [13] also evaluated the morphology and architecture of collagen in the dermis of mice through FD, proposing it as a preferred method for quantifying collagen in organs and tissues. These histological findings allow more precise analysis of the results and decrease errors from manual and subjective quantification [11], besides allowing a direct comparison with other studies, since it is a technique that allows the results to be free from evaluator bias [14].

Therefore, to elucidate the mechanisms involved in ovarian development in bovine fetuses and establishment of the follicular population during gestation, the present study was conducted to characterize the histomolecular phenotype of ECM remodeling through FD, the quantification of collagen, and the profiling of transcripts involved with ECM remodeling in ovaries of bovine fetuses associated with preantral follicle formation.

## MATERIALS AND METHODS

### *Tissues*

In accordance with the methods of Tanaka et al. (2001) and Castilho et al. (2014), 20 female fetuses at 60, 90, 120, and 150 days of gestation (n = 5/group), predominantly from Nellore cattle (*Bos taurus indicus*), were obtained from a local abattoir near the São Paulo State University campus in Assis city and were classified according to crown-rump lengths. Subsequently, one fetal ovary of each fetus was transported to the laboratory in TRIzol® Reagent for RNA total extraction and the other was fixed in methacarn solution (60% methanol, 30% chloroform, 10% acetic acid) for 2 h and held in 70% ethanol for histological analysis.

The tissues were dehydrated in ethanol and soaked in Paraplast (Oxford Labware, St. Louis, MO, USA). Sections of 4 μm thickness were obtained and stained with hematoxylin and eosin (HE) or picrosirius red staining (PSR), according to the standard laboratory histological protocol. The slides were analyzed, and the images were captured with a digital photomicroscope Axiophot II (Zeiss Jenaval, Jena, Germany) with a 40x objective. Fetal ovary sections stained with PSR were used to

quantify total collagen. The analysis was performed using the ImageJ software. Tissue FD was evaluated in fetal ovary sections stained with PSR or HE (n=10 images/slide; total 50 images per gestational age).

*Fractal Analysis*

For the FD analysis, the photographs were binarized for reading, and FD was estimated by the box-counting method using ImageJ. The software considered box-counting in two dimensions, allowing the quantification of the distribution of pixels within this space, not considering the texture of the image. The result of such analysis is that two images with the same distribution of pixels, one binarized and the other in gray levels, have the same FD. The analysis of the fractal histological slides was based on the relation between the resolution and the evaluated scale, and the result was quantitatively expressed as the FD of the object, as $FD = \text{Log } Nr/\text{Log } r^{-1}$, where, Nr is the quantity of equal elements needed to fill the original object and r is the scale applied to the object. Thus, FD was calculated using the ImageJ software set between 0 and 2, not distinguishing different textures.

*RNA extraction and expression of target genes*

Total RNA was extracted according to the manufacturer's protocol and stored at -80 °C (TRIzol, Invitrogen®). To prevent any contamination by genomic DNA that may interfere with the results, all samples of total RNA (400 ng/reaction) were incubated with DNAse I (1IU/µg; Invitrogen, São Paulo, Brazil) and then reverse-transcribed

using random primers according to the protocol provided with the High Capacity Kit (Applied Biosystem®, Foster City, CA)

The RT-qPCR analysis of each target and reference gene was performed on QuantStudio ™ 7 Flex equipment using the Power Sybr Green® (Applied Biosystems®) system, together with the corresponding bovine-specific primer oligonucleotides, previous published by [16] . The reactions were optimized to provide the maximum amplification efficiency for each gene. The specificity of each PCR product was determined by the analysis of the dissociation curve and fragment size confirmation by 1.5% agarose gel electrophoresis. Each sample was analyzed in duplicate and negative controls, which used water instead of cDNA, were run on each plate. To select the most stable reference gene, the amplification profiles of the genes: isomeryl peptidylprolyl isomerase A (*PPIA*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), and histone H2A (*H2AFZ*), were tested using the NormFinder software. The most stable reference gene for the fetal ovaries was *PPIA.* To quantify the relative expression, the ΔΔCt method (target genes / PPIA) with correction of efficiency for each target gene was followed, using a control sample as the calibrator [15].

*Statistical analysis*

The gestational age effects on total collagen, FD, and relative mRNA abundance of target genes were evaluated by One-way ANOVA and the differences were compared by the Tukey-Kramer test. All analyses were performed using the JMP software (SAS Institute Cary, NC) and the data are presented as mean ± SEM. Differences were considered significant when $p \leq 0.05$.

## RESULTS

Regarding the quantitative approach for histological evaluation of ECM remodeling, we found a greater amount of total collagen in bovine fetal ovaries at days 120 and 150 when compared with the amount on days 60 and 90 (p=0.033, figure 1). We found a higher FD of collagen fibers in PSR-stained slides from day 120 when compared with that from day 60 of gestation (p=0.025, figure 1). In contrast, the FD in the HE-stained slides that showed cytoplasm/nucleus relation, exhibited an inverse profile with lower levels of FD at 120 days of gestation when compared with those at 60 and 90 days (p=0.02, figure 2).

On evaluating the gene expression profiles related to fetal ovary ECM remodeling, we identified that relative mRNA abundance of *COL1A1* was greater at day 150 when compared with that at day 60 (p=0.0068) and *COL4A1* was upregulated at day 150 when compared with that in other days (p=0.035). However, the gestational age did not interfere with the abundance of *COL1A2* (p=0.32). The relative mRNA abundances of *MMP2* (p = 0.004) and *MMP14* (p = 0.037), and of *TIMP1* (p = 0.001) and *TIMP2* (p = 0.012) (figure 3) were higher at day 150 when compared with those on other days , but there was no difference in the *MMP9* mRNA abundance (p = 0.85).

## DISCUSSION

In order to broaden the understanding of the development and organization of fetal bovine ovary, we demonstrated for the first time that remodeling of ECM is

183 correlated with FD in the bovine fetal ovarian tissue. In addition, the findings presented
184 here indicate that fetal ovarian development is accompanied by extensive ECM
185 remodeling, and suggest that modification in the amount and distribution of collagen
186 could be partially explained by the transcriptional regulation of genes involved with
187 ECM changing (figure 4). Furthermore, as previously described for bovine CL, FD
188 analysis proves to be an effective approach in understanding the morphological changes
189 that occur during fetal ovary developmental [16].

190 Regarding FD as a useful tool to evaluate ECM remodeling of bovine fetal
191 ovaries, it is relevant to highlight the inverse relationship between FD in HE staining in
192 relation to that in PSR staining. These findings may indicate that lower FD values in HE
193 staining reflect an organized tissue, differentiating from a more functional tissue at 150
194 days of gestation, which would corroborate the same pattern already demonstrated in
195 bovine CL by Favero et al., 2019. On the contrary, the higher FD values in PSR staining
196 could be related to the greater amount of collagen and possibly reflect lower cellularity
197 and an increase in the collagen/cell ratio at this stage of bovine fetal ovarian
198 development. Together, these findings suggest that the use of FD could help better
199 standardize histological studies, thus allowing more accurate analysis of the results,
200 reduction of subjective quantification errors, and direct comparison among studies. FD
201 analysis is not a widely used approach to explore female reproductive tissue
202 remodeling, especially in cattle, which makes this tool promising as a new form of
203 accurate assessment technique to quantify temporal changes during folliculogenesis and
204 evaluate the impact of the reproductive cycle on mammalian females.

As described by Berkholtz et al., [7] collagen type I and IV, and other components of the ECM such as fibronectin and laminin affect the morphology, survival, proliferation, and steroidogenesis of granulosa cells, follicles, and ovaries. Consistent with the importance of collagen deposition and its distribution throughout the development of ovaries in bovine fetuses, we believe the highest amount of collagen at 150 days of gestation could be related to greater expression of genes encoding type I and type IV collagen, but not by type II. Furthermore, these findings reinforce that an increase in the collagen amount and its differential distribution in fetal ovaries at 150 days could play a role in future ECM remodeling during folliculogenesis in adult ovaries and have an impact on cell morphology, communication, proliferation, survival, and steroidogenesis in reproductive tissues [7].

MMPs and TIMPs, the regulators of ECM remodeling, are postulated to play a critical role during follicular development [15]. Indeed, MMPs are also responsible for the degradation of most ECM components during tissue remodeling, and are required for the growth and expansion of the follicle during the ovulatory process. On the contrary, the TIMPs are the tissue inhibitors of its action [17]. Although it has been described that *MMP1*, *MMP2*, and *MMP14* mRNA expression are not regulated during the follicular phase and development of the CL in adult ovaries [18], in the present study, we demonstrated a greater expression of *MMP2* and *MMP14* as well as *TIMP1* and *TIMP2* in fetal ovaries at 150 days of gestation.

Furthermore, our findings are contrary to observations on ovaries of adult female swine, where *MMP1*, *MMP2*, and *MMP9* expression in CL is always accompanied by the decrease in *TIMP1* and *TIMP2* expression [19]. This reinforces that tissue

remodeling of bovine fetal ovary involves a variety of MMP inhibitors, and further establishes that ECM remodeling during ovarian development of bovine fetuses is an intense, complex, and unique process where all MMP/TIMP systems are recruited and required to be highly expressed for the final development of fetal ovary.

In summary, we found that differential FD observed during fetal bovine ovarian development and preantral folliculogenesis is an effective method for evaluating ovarian tissue changes. In addition, we conclude that the remodeling of ECM during the development of the fetal bovine ovary is related to histomolecular alterations in the expression of collagens type I and IV, MMPs 2 and 14, and TIMPs 1 and 2, as well as in the amount and distribution of collagen fibers.

## STATEMENT OF INTEREST

We declare that there is no conflict of interests.

## FUNDING

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## FIGURE LEGENDS

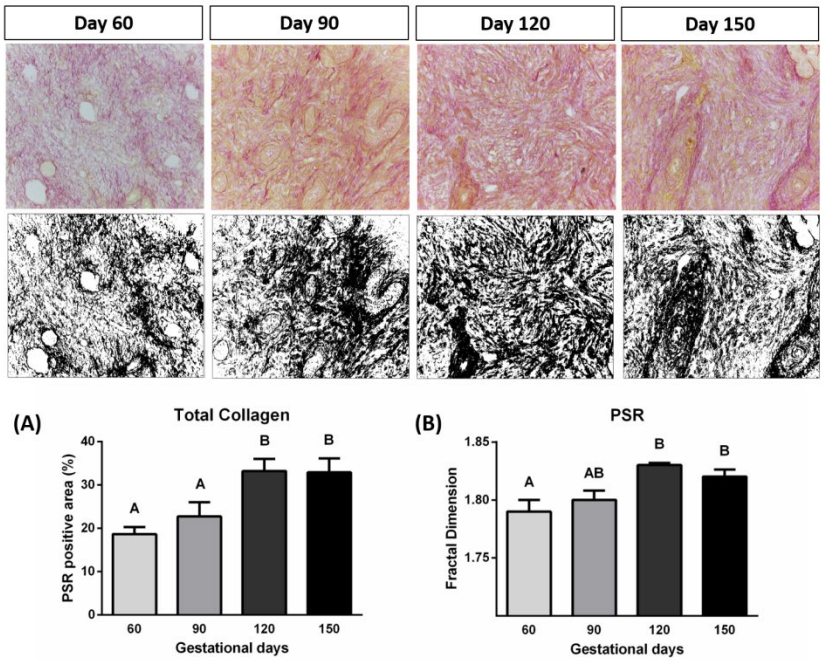
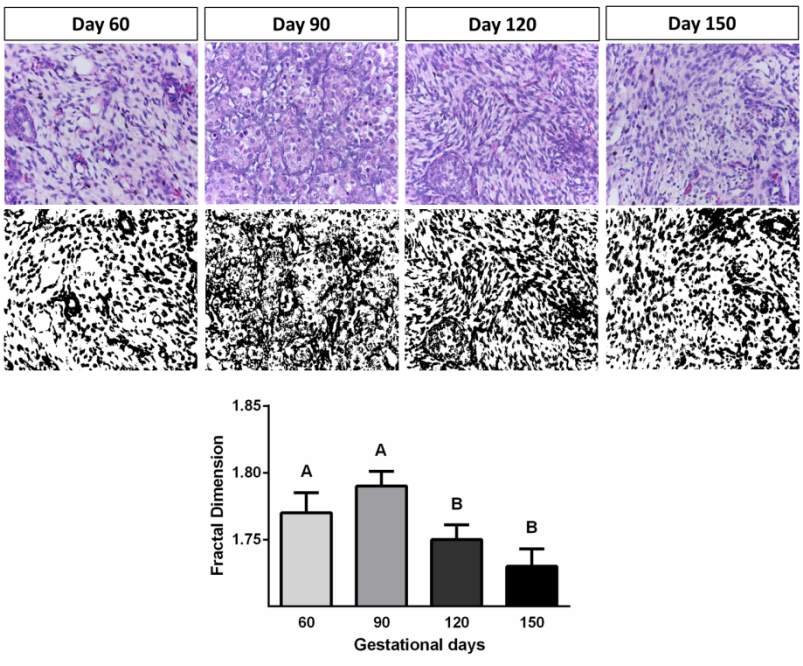
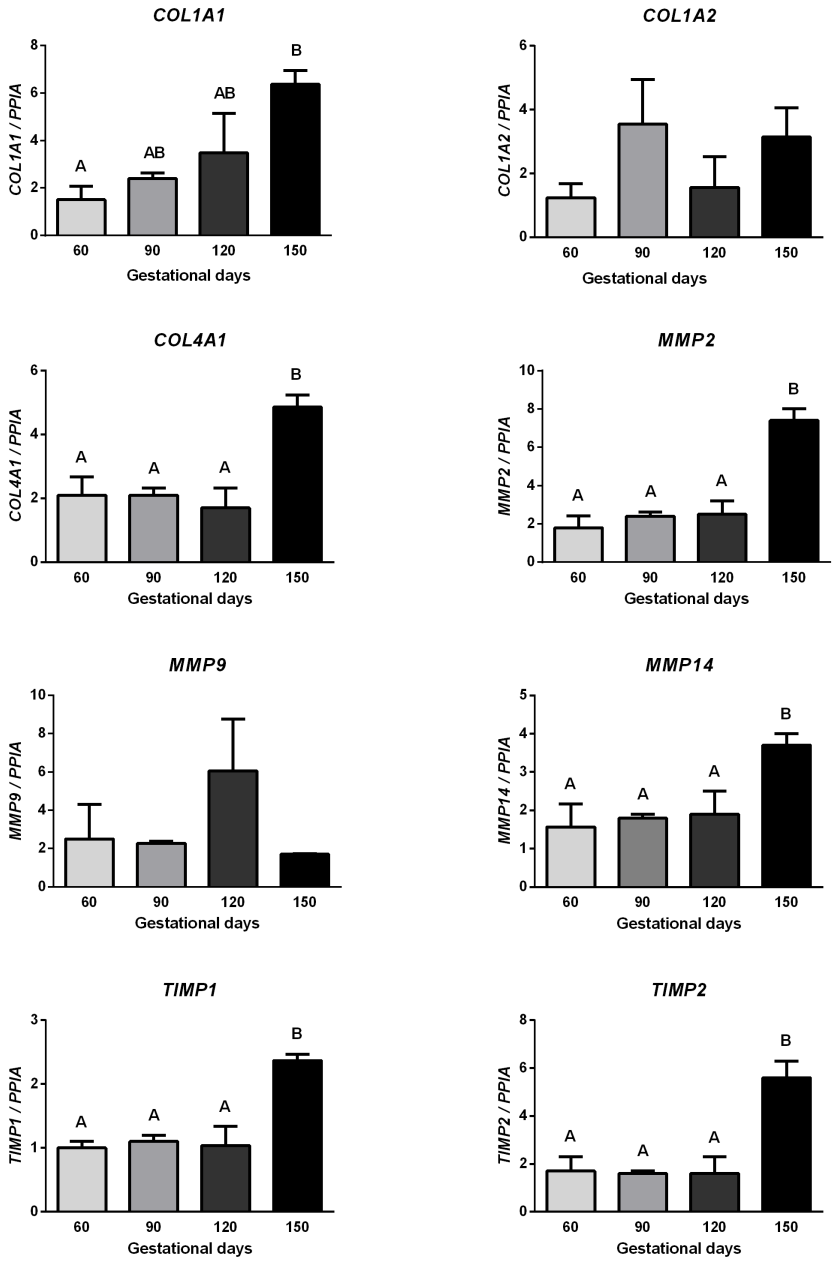



Figure 1. Gestational age effects on: **(A)** Quantification of total collagen in PSR-stained bovine fetal ovarian sections; **(B)** FD in staining in PSR-stained sections. The results can be quantitatively expressed as FD = (Log Nr / Log r-) and, for that reason, the dimension will always range between 0 and 2. Data are presented by mean ± SEM. Different letters (A; B) indicate a significant difference ($p \leq 0.05$).




260

261 Figure 2. Gestational age effects on FD in bovine fetal ovarian sections in HE staining.
262 The results can be quantitatively expressed as FD = (Log Nr / Log r-1), and for this
263 reason the dimension will always range between 0 and 2. Data are presented by mean ±
264 SEM. Different letters (A; B) indicate a significant difference ($p \leq 0.05$)




265

266 Figure 3. Gestational age effects on the differential mRNA relative abundance of
267 *COL1A1, COL1A2, COL4A1, MMP2, MMP9, MMP14, TIMP1,* and *TIMP2* during the
268 development of bovine fetal ovaries (n = 5 ovaries / gestational age). The expression
269 values were calculated relative to a calibrator sample using the ΔΔCt method with
270 efficiency correction. Data are presented as mean ± SEM. Bars with different letters (A,
271 B) show significant difference ($p \leq 0.05$).

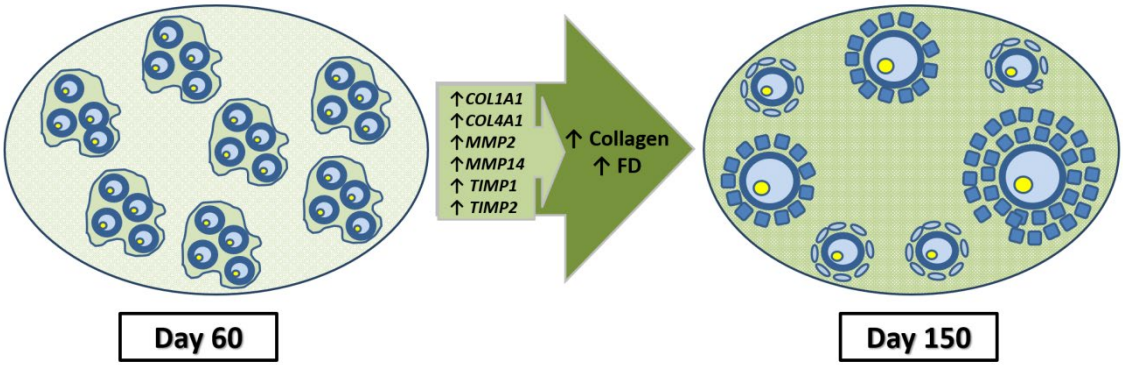



Figure 4. Biological model to gain insight about mechanisms involved with ECM development in bovine fetal ovary and establishment of the follicular population during gestation. At day 60, we can visualize the ovigerous cords spread over into ovarian stroma and a poor ECM remodeling, which could be partially explained by the lower collagen deposit (light green). On the contrary, when fetal ovary acquires preantral follicles (primordia characterized by flattened granulosa cells; primary follicle characterized by cuboidal granulosa cells; and secondary follicle by multilayered granulosa cells) until gestational day 150, we can observe a higher level of collagen (green), MMP and TIMP expression that reaches a thin tissular architecture, and the establishment of cortical or medullar area in bovine fetal ovary.

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## ANEXO- NORMAS DA REVISTA

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