UNIVERSIDADE DE LISBOA

FACULDADE DE MEDICINA VETERINÁRIA

NEW INSIGHTS ON THE INHIBITION OF NEUTROPHIL EXTRACELLULAR TRAPS ENZYMES IN EQUINE ENDOMETRIUM

ANA SOFIA PIRES AMARAL

Orientadores: Professora Doutora Graça Maria Leitão Ferreira Dias

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Tese especialmente elaborada para obtenção do grau de Doutor em Ciências Veterinárias na especialidade de Ciências Biológicas e Biomédicas

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"Happiness can be found even in the darkest of times,
if one only remembers to turn on the light."
Albus Dumbledore
J.K. Rowling

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Novas perspetivas na inibição das enzimas das redes extracelulares dos neutrófilos na edometrose equina

Resumo

Nas éguas, após inseminação natural ou artificial, é frequente ocorrer endometrite, caracterizada pela chamada de neutrófilos para o lúmen uterino. Os neutrófilos, além de libertarem grânulos de enzimas proteolíticas e citotóxicas, podem também libertar filamentos de DNA, histonas e várias enzimas para o meio extracelular originando as redes extracelulares dos neutrófilos (NETs). Embora a sua principal função seja o aprisionamento e o combate de agentes patogénicos, a persistência de NETs tem sido associada ao desenvolvimento de várias situações patológicas, de entre as quais fibrose. A elastase (ELA), catepsina G (CAT) e mieloperoxidase (MPO) são enzimas pró-fibróticas presentes nas NETs que induzem a produção de colagénio tipo I (COL1) na fibrose endometrial equina. As metalopeptidases da matriz (MMPs) são cruciais nesta remodelação da matriz extracelular. As prostaglandinas (PG)s E_2 e $F_{2\alpha}$ têm sido associadas a efeitos anti e pró-fibróticos. Neste trabalho foram utilizados explantes de endométrio equino tanto da fase folicular (FP) como da fase lútea-média (MLP) que foram tratados *in vitro* com as enzimas presentes nas NETs e seus inibidores durante 24 e 48h. Este trabalho teve como objetivos a avaliação da resposta dos explantes de endométrio equino: (i) à inibição da ELA pelo sal sivelestat de sódio (SIV) na transcrição de *COL1A2* e secreção de PGE_2 e $PGF_{2\alpha}$; (ii) ao efeito do tratamento com ELA e SIV na atividade das MMP-2/-9 e inibição pelo SIV no COL1 induzido pela ELA; (iii) ao efeito do tratamento com CAT e Inibidor I da Catepsina G (ácido β-acetofosfónico; INH) na atividade das MMP-2/9 e na inibição pelo INH no COL1 induzido pela CAT; e (iv) à inibição pela hidrazida de ácido 4-aminobenzóico (ABAH) no COL1 induzido pela MPO e os efeitos da MPO e ABAH na atividade da MMP-2/-9. Na FP, o tratamento com SIV reduziu a transcrição de *COL1A2* e a produção de $PGF_{2\alpha}$ pró-fibrótica mas aumentou a produção de PGE_2 anti-fibrótica. No tratamento com ELA e SIV, a expressão de MMPs variou com a fase do ciclo e a duração do tratamento. Na FP (24h) e MLP (24 e 48h), o SIV reduziu os transcriptos de *COL1A2* induzidos pela ELA. O INH reduziu o COL1 induzido pela CAT nas duas fases, às 48h. A MMP-2 parece estar envolvida numa resposta rápida à CAT, e a MMP-9 numa resposta tardia na FP. O ABAH diminuiu o COL1 induzido pela MPO na FP às 48h. A MMP-2 parece estar envolvida numa resposta aguda à MPO, e a MMP-9 numa resposta longa na FP. O uso de inibidores específicos das enzimas das NETs pode constituir a base para o desenvolvimento de potenciais fármacos passiveis de serem utilizados numa abordagem profilática ou terapêutica na endometrose equina.

Palavras-chave: elastase, catepsina G, myeloperoxidase, inibição, endometrose.

New insights on the inhibition of neutrophil extracellular traps enzymes in equine endometrium

Abstract

Mares physiologically develop a post-breeding endometritis characterized by a fast arrival of neutrophils into the uterine lumen. These neutrophils besides releasing granules of proteolytic and cytotoxic enzymes, may also deliver to the extracellular environment their DNA, histones and enzymes forming neutrophil extracellular traps (NETs). Besides trapping and fighting pathogens, NETs persistence has been also associated to the development of pathological conditions, such as fibrosis. The enzymes found in NETs, such as elastase (ELA), cathepsin G (CAT) and myeloperoxidase (MPO) act as pro-fibrotic factors in equine endometrial fibrosis, by inducing collagen type I (COL1) accumulation. Matrix metallopeptidases (MMPs) are crucial for this extracellular matrix remodeling. Prostaglandins (PG)s E_2 and $F_{2\alpha}$ have been described as possessing anti or pro-fibrotic effects. Equine endometrial explants from follicular phase (FP) or mid-luteal phase (MLP) were treated *in vitro* with ELA, CAT or MPO and their specific inhibitors for 24 or 48h. This work aimed to evaluate the explants response to: (i) ELA inhibition by sivelestat sodium salt (SIV) on *COL1A2* transcription and PGE_2 and $PGF_{2\alpha}$ secretion; (ii) ELA and SIV treatment on MMP-2 and MMP-9 activity, and the inhibitory effect of SIV on ELA-induced COL1; (iii) CAT and Cathepsin G inhibitor I (β-keto-phosphonic acid; INH) treatment on MMP-2 and MMP-9 activity, and the effect of INH on CAT-induced COL1 production; (iv) the inhibitory effect of 4-aminobenzoic acid hydrazide (ABAH) on MPO-induced COL1 and the effect of MPO and ABAH on MMP-2 and MMP-9 gelatinolytic activity. In FP, *COL1A2* transcription decreased in SIV-treated group, simultaneously with reduced pro-fibrotic $PGF_{2\alpha}$ and increased anti-fibrotic PGE_2 production. In ELA- and SIV-treated explants, MMPs expression depended on estrous cycle phase and time of treatment. Sivelestat inhibited ELA-induced *COL1A2* transcripts in FP (24 h) and MLP (24 h, 48 h). The effect of INH was observed on CAT-induced COL1 in both phases at 48h. The MMP-2 might be involved in an earlier response to CAT, while MMP-9 in a later response in FP. The inhibitory effect of ABAH on MPO-induced COL1 was detected in FP at 48h. Matrix metallopeptidase-2 appears to be involved in an acute response to MPO treatment in MLP and MMP-9 in FP in a prolonged MPO treatment. The use of specific inhibitors of ELA, CAT or MPO, might be the grounds for future development of specific drugs to be used as prophylaxis or therapy of endometrosis in the mare.

Keywords: elastase, cathepsin G, myeloperoxidase, inhibition, endometrosis

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ABAH	4- aminobenzoic acid hydrazide
ACT	α1-anti-chymiotrypsin
Akt	serine-threonine protein kinase
ANOVA	analysis of variance
B2M	beta-2-microglobulin
CAT	cathepsin G
CL	*corpus luteum*
COL	collagen
COL1	collagen type I
COL1A1	*collagen type 1 α1* gene
COL1A2	*collagen type 1 α2* gene
COL3	collagen type III
COL3A1	*collagen type 3 α1* gene
COPD	chronic obstructive pulmonary disease
CXCL	motif chemokine ligand
CXCR	chemokine receptor
DAMP	damage-associated molecular pattern
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	desoxirribonuclease
DTT	dithiothreitol
E2	estrogen
ECM	extracellular matrix
EDTA	thylenediaminetetraacetic acid
ELA	elastase
ELISA	enzyme-linked immunosorbent assay
EP	prostagalndin E_2 receptor
ERK	extracellular signal-regulated kinases
ESR	estrogen receptor
FP	follicular phase
FSH	follicle stimulating hormone
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GnRH	gonadotropin-releasing factor

HRP	horseradish peroxidase
ICAM	intracellular adhesion molecule
IL	interleukin
INH	Cathepsin G inhibitor I
LDH	lactate dehydrogenase
LFA	lymphocyte function-associated antigen
LH	luteinizing hormone
LTB4	lipid mediator leukotrienes B4
Mac-1	macrophage-1 antigen
MAPK	mitogen-activated protein kinases
MCP1	monocyte chemoattractant protein 1
miRNA	micro ribonucleic acid
MLP	mid-luteal phase
MMP	matrix metallopeptidase
MPO	myeloperoxidase
mRNA	messenger ribonucleic acid
MSCs	mesenchymal stem cells
NADPH	nicotinamide adenine dinucleotide phosphate
NET	neutrophil extracellular trap
OXT	oxytocin
P4	progesterone
PAMP	pathogen-associated molecular pattern
PAR	protease-activated receptor
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PG	prostaglandin
PGES	prostaglandin E_2 synthases
PGFS	prostaglandin $F_{2\alpha}$ synthases
PGH_2	prostaglandin H_2
PGIS	prostaglandin I_2 synthases
PGR	progesterone receptor
PI3K	phosphoinositide 3-kinase
PKC	protein kinase C
PMA	phorbol-myristate acetate
PRR	pattern recognition receptor
PTGS2	prostaglandin endoperoxidase synthases

qPCR	quantitative polymerase chain reaction
RIPA	radioimmunoprecipitation assay buffer
ROS	reactive oxygen species
RPL32	ribosomal protein L32
Serpin	monocyte neutrophil elastase inhibitor
SDHA	succinate dehydrogenase A complex, subunit A, flavoprotein
SDS	sodium dodecyl sulfate
SIV	sivelestat sodium salt
SLPI	secretory leucocyte protease inhibitor
SMAD	Sma and Mad related family
TCE	2,2,2-trichloroethanol
TGF	transforming growth factor
TIMP	tissue inhibitor of metallopeptidase
TLR	toll-like receptor
TNF	tumor necrosis factor
UV	ultra-violet
α-SMA	α smooth muscle actin
α1-PI	α1-proteinase inhibitor

Chapter I
General Introduction and Objectives

1. General Introduction

Among the reproductive problems affecting mares that contribute to infertility, equine endometrial fibrosis is considered a major cause. In equine endometrial fibrosis, also known as endometrosis, fibroblasts differentiate into myofibroblasts responsible for collagen (COL) fibers synthesis in extracellular matrix (ECM) leading to periglandular and/or stromal endometrial fibrosis with alterations of endometrial glands (Hoffmann et al. 2009a). If endometritis becomes persistent, it might trigger endometrosis development. Neutrophils are the most abundant immune cells, which arrive fast into the uterine lumen contributing to breeding-induced endometritis (Katila 1995). These immune cells play an essential role defending the host against the invading pathogens by phagocytosis, degranulation and generation of neutrophil extracellular traps (NETs). The release of enzymes and nuclear constituents by neutrophils contributes for NETs formation. Among those enzymes are elastase (ELA), cathepsin G (CAT) and, myeloperoxidase (MPO) (Brinkmann et al. 2004). The *ex vivo* presence of NETs was detected in endometrial mucus from mares with endometritis (Rebordão et al. 2014). Moreover, it was also found that, in the presence of bacteria that cause endometritis, the equine neutrophils have the capacity to form NETs (Rebordão et al. 2014). Besides the antimicrobial action of NETs and their components, ELA, CAT and MPO were also proven to be capable of inducing collagen type I (COL1) production *in vitro* in equine endometrial explants, suggesting the dual effect of enzymes found in NETs, since they also can also act as pro-fibrotic agents (Rebordão et al. 2018).

Some specific inhibitors of enzymes found in NETs have been used, showing beneficial effects is many diseases. Sivelestat sodium salt (SIV) is a selective ELA inhibitor that has shown beneficial effects on hindering fibrosis development, both in *in vitro* studies and clinical trials (Takemasa et al. 2012; Polverino et al. 2017). Cathepsin G Inhibitor I (β-keto-phosphonic acid; INH) is a selective inhibitor of CAT. This inhibitor exhibited anti-inflammatory properties and favorable results in the treatment of acute and chronic airway diseases (de Garavilla et al. 2005; Maryanoff et al. 2010). To inhibit MPO, 4-aminobenzoic acid hydrazide (ABAH) has been the most used inhibitor tested, both in acute inflammation and in fibrotic conditions (Pulli et al. 2015; Hair et al. 2017).

Matrix metallopeptidases (MMPs) are enzymes involved in ECM turnover. Some of the physiological actions of MMPs include cell proliferation, migration and differentiation, angiogenesis, apoptosis and tissue repair. Nevertheless, MMPs have also been associated to pathological conditions including fibrosis (Wang and Khalil 2018). The action of MMP-2 and MMP-9 in fibrogenesis has been controversial since they have been mentioned to either act as anti-fibrotic or as pro-fibrotic mediators. In equine endometrium, recent *in vitro* studies showed that MMP expression is affected by several mediators of inflammation (interleukins,

transforming growth factor β1 - TGFβ1 and prostaglandins - PGs) (Szóstek-Mioduchowska et al. 2019b, 2020a, 2020b), and differs among stages of endometrosis (Szóstek-Mioduchowska et al. 2020a).

Prostaglandin E_2 and $PGF_{2\alpha}$ act in inflammatory responses and have several functions on regulation of estrous cycle phase, and pregnancy establishment in the mare (Douglas and Ginther 1976; Zavy et al. 1978; Vanderwall et al. 1994). The treatment of equine endometrial explants with enzymes found in NETs, such as ELA, CAT and MPO, showed a decrease in PGE_2 concentration, suggesting a protective effect against endometrial fibrosis (endometrosis) by reduction of COL deposition in equine endometrium (Rebordão et al. 2019). When challenged with TGFβ1, a pro-fibrotic cytokine, equine epithelial cells reduced PGE_2 secretion (Szóstek-Mioduchowska et al. 2020b). However, in fibroblasts treated with $PGF_{2\alpha,}$ the MMPs and COL1 expression augmented, suggesting that prostaglandins may be involved in equine endometrial pathological remodeling (Szóstek-Mioduchowska et al. 2020b).

These issues were the main focus of this work. We have investigated the effect of the *in vitro* selective inhibition of ELA, CAT and MPO on COL1-induced by these enzymes in equine endometrial explants to address the possibility of hinder equine endometrosis. The effect of ELA and SIV treatment on PGs secretion was also assessed to evaluate the pro- or anti-fibrotic performance of PGE_2 and $PGF_{2\alpha}$ in equine endometrial tissues. In order to investigate the role of MMP-2 and MMP-9 in equine endometrosis, endometrial explants were treated with ELA, CAT and MPO and their selective inhibitors, to evaluate MMPs expression and gelatinolytic activity. This work evaluated the involvement of PGs and MMPs in equine explants response to pro- and anti-fibrotic agents. In addition, it was investigated if the use of NETs enzymes inhibitors would reduce COL1 output in endometrial tissues. In conclusion, data gathered in this work might contribute to develop a putative therapeutic approach to reduce endometrosis, responsible for large economical losses worldwide, due to mare infertility.

2. Objectives

The major objectives of this work were:

(1) To evaluate if the *in vitro* inhibition of ELA would affect *COL1A2* transcription and PGs secretion by endometrium, in different estrous cycle phases.

The developed work was published in *Reproduction of Domestic Animals*: Amaral A, Fernandes C, Lukasik K, Szóstek-Mioduchowska A, Baclawska A, Rebordão MR, Aguiar-Silva J, Pinto-Bravo P, Skarzynski DJ, Ferreira-Dias G. 2018. Elastase inhibition affects collagen transcription and prostaglandin secretion in mare endometrium during the estrous cycle. *Reprod Dom Anim*. 53:66–69. doi:10.1111/rda.13258.

(2) To assess the *in vitro* effect of SIV on inhibition of ELA-induced COL1 protein relative abundance in equine endometrial explants, and the effect of ELA and SIV on the expression and activity of MMP-2 and MMP-9.

The developed work was published in *Animals*: Amaral A, Fernandes C, Rebordão MR, Szóstek-Mioduchowska A, Lukasik K, Gawronska-Kozak B, Telo da Gama L, Skarzynski DJ, Ferreira-Dias G. 2020. The *in vitro* inhibitory effect of sivelestat on elastase induced collagen and metallopeptidase expression in equine endometrium. *Animals*. 10(5):863. doi:10.3390/ani10050863.

(3) To evaluate the *in vitro* effect of INH on CAT-induced COL1 protein relative abundance in equine endometrial tissues, and the effect of CAT and INH on the expression and activity of MMP-2 and MMP-9.

The developed work was published in *Frontiers in Veterinary Science*: Amaral A, Fernandes C, Morazzo S, Rebordão MR, Szóstek-Mioduchowska A, Lukasik K, Gawronska-Kozak B, Telo da Gama L, Skarzynski DJ, Ferreira-Dias G. 2020. The Inhibition of Cathepsin G on Endometrial Explants With Endometrosis in the Mare. *Front Vet Sci*. 7:582211. doi:10.3389/fvets.2020.582211.

(4) To investigate the *in vitro* capacity of ABAH to inhibit MPO-induced COL1 output in equine endometrial explants, and the effect of MPO and ABAH on the expression and gelatinolytic activity of MMP-2 and MMP-9.

The developed work was published in *Animals*: Amaral A, Fernandes C, Rebordão MR, Szóstek-Mioduchowska A, Lukasik K, Pinto-Bravo P, Telo da Gama L, Skarzynski DJ, Ferreira-Dias G. 2020. Myeloperoxidase inhibition decreases the expression of collagen and metallopeptidase in mare endometria under in vitro conditions. *Animals*. 11(1):208. doi:10.3390/ani11010208.

1. Neutrophils

Neutrophils are the most abundant immune cells constituting about 50-70% of all leukocytes in human blood (Selders et al. 2017; Mortaz et al. 2018; Peiseler and Kubes 2019). They play an essential role defending the host against invading pathogens, particularly bacteria and fungi (Kolaczkowska and Kubes 2013; Scapini et al. 2016; Hidalgo et al. 2019). Neutrophils kill microorganisms through phagocytosis, degranulation, and formation of neutrophil extracellular traps (NETs). The killing of pathogens in phagosomes occurs by the fusion of lysosomes granules that liberate cytotoxic proteins, peptides and enzymes into the phagolysosome (Cowland and Borregaard 2016), and activation of a membrane-bound nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase producing superoxide anions (O_2^-), which are metabolized into hydrogen peroxide (H_2O_2) and other reactive oxygen species (ROS) (Babior et al. 1973). In the latter process, the fusion of granules leads to degranulation and activation of NADPH-oxidase (Roos et al. 2003; Segal et al. 2005).

The neutrophil interaction with platelets and endothelial cells plays a key role in neutrophil tissue recruitment, generation of ROS and phagocytosis. Neutrophils reach the infection or inflamed site from the bloodstream and are mediated by the interaction of adhesion molecules on the neutrophil surface with their respective ligands on the vascular endothelium (Schmidt et al. 2013). In order to cross the endothelial barrier, neutrophils develop membrane extensions that stabilize neutrophil rolling despite the flowing blood (Sundd et al. 2012). Then, neutrophil β2 integrins (lymphocyte function-associated antigen, LFA-1; and macrophage-1 antigen, Mac-1) firmly adhere to endothelial cell intracellular adhesion molecule (ICAM)-1 and ICAM-2 and cross the endothelium barrier (Sundd et al. 2012; reviewed by Kazzaz et al. 2016). Platelets beyond acting on the blood loss also act as sentinels in infectious and inflammatory diseases (Rondina et al. 2013; Vieira-de-Abreu et al. 2012). This is fundamental for the recruitment of neutrophils to sites of inflammation. Examples of ligand/receptor pairs that mediate platelet/neutrophil interactions include P-selectin/ P-selectin glycoprotein ligand 1, ICAM-2)/LFA-1, and platelet glycoprotein Ib beta chain/Mac-1 (reviewed by Kazzaz et al. 2016).

The acute damaged tissue, releases a large variety of signals that are chemoatractive to neutrophils (Ley 2002; Gambardella and Vermeren 2013). The G protein-coupled receptors, Fc receptors, adhesion receptors, cytokine receptors, as well as pattern recognition receptors (PRRs) (Futosi et al. 2013) are some of the pro-inflammatory receptors expressed by neutrophils that can detect pro-inflammatory factors. The damaged cells and pathogens trigger the release of damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) respectively, which activate hydrogen peroxide (de Oliveira et al. 2016; Peiseler and Kubes 2019). Neutrophils become activated by DAMPs stimulated

chemokines, such as motif chemokine ligand 8 (CXCL8) that binds G-protein-coupled receptors (Furze and Rankin 2008) leading the affinity for ICAM-1 expressed on the endothelium to promote neutrophils adherence (Mortaz et al. 2018). Other pro-inflammatory factors such as cytokines, interleukin (IL)-1β and lipid mediator leukotrienes (LTB4) are stimulated by DAMPs from the surrounding tissues to attract other neutrophils. In infections, the release of PAMPs, immune cell like tissue residents and recruited inflammatory macrophages, T cells and dendritic cells are responsible for neutrophils recruitment (de Oliveira et al. 2016). In order to amplify neutrophil recruitment, both CXCL8 (stimulated by IL-1β and tumor necrosis factor α - TNFα), and LTB4 will continue to attract neutrophils from bloodstream (de Oliveira et al. 2016). When neutrophils cross the cell barrier, the neutrophil chemotaxis is inhibited (Wiemer et al. 2010), but shows other functions like oxidative burst (Nathan 1987), phagocytosis and degranulation (van der Poll et al. 1992). Degranulation consists in a rapid and precise neutrophil response to infection. They release molecules stored in intracellular granules, which regulate adhesion, transmigration, phagocytosis, and NETs formation (Ley et al. 2018).The matrix metallopeptidase (MMP)-9 is also produced by neutrophils and degrade DAMPs, reducing the additional recruitment of inflammatory cells (Cauwe et al. 2009). But, other studies report MMP-9 to enhance neutrophil chemotaxis by cleaving CXCL8 or by acting on collagen to release collagen-derived chemotactic peptides (Tester et al. 2007; Afonso et al. 2013). By the end of this process, neutrophils must be removed from the injured tissue. This can occur via apoptosis/necrosis and engulfment by machophages, through removal of neutrophil by reverse migration into the vascular vessels or by expulsion to the external environment (de Oliveira et al. 2016; Jorch and Kubes 2017; Wang et al. 2017; Peiseler and Kubes 2019). In mice, neutrophil elastase (ELA) promoted reverse transmigration from the extravascular tissue to the vascular lumen favors the dissemination of systemic inflammation (Colom et al. 2015), while cathepsin C deficient animals showed reduced reverse transmigration (Wang et al. 2017). It is evident that neutrophils contribute to healing, even though they are also associated to pathological conditions (Ley et al. 2018; Hidalgo et al. 2019).

1.1 Neutrophil extracellular traps

One of the mechanisms for neutrophils to fight pathogens is the capacity to form NETs. This process was firstly described in 2004 in response to infectious stimuli (Brinkmann 2004). These are complex networks of deoxyribonucleic acid (DNA) chromatin filaments coated with histones and enzymes released by neutrophils (Brinkmann 2004; Jorch and Kubes 2017; Papayannopoulos 2018, Neubert et al. 2020). Among these enzymes are elastase (ELA), cathepsin G (CAT), myeloperoxidase (MPO), leucocyte proteinase 3, lactoferrin, gelatinase, lysozyme C, calprotectin, neutrophil defensins and cathelicidins (Urban et al. 2009; Brinkmann

2018). The formation of NETs occurs as a response to a variety of stimuli like bacteria, virus, fungi, parasites, activated platelets, phorbol-myristate acetate (PMA), cytokines and mitogens (reviewed by Neubert et al. 2020). They have been described in many animal species such as mammalians, birds, fish, and invertebrates (reviewed by Brinkmann 2018). Neutrophil extracellular traps were already described in appendicitis in humans, endometritis in the mare, pyometra in the queen and bitch, peritoneal fluid of endometriosis human patients, mastitis in sheep, or skin infection in mice (Brinkmann 2004; Berkes et al. 2014; Rebordão et al. 2014, 2017; Pisanu et al. 2015; Halverson et al. 2015). Interestingly, the composition of NETs may vary depending on the stimulus. Dwyer et al. (2014) reported that *Pseudomonas aeruginosa* induced NETs containing 33 common proteins and 50 variable proteins. The enzymes found in NETs such as ELA and MPO show antimicrobial properties (Brinkmann 2004; O'Donoghue et al. 2013; Hoeksema et al. 2016), but also NET-associated DNA mediates pathogen killing by induction of bacterial lysis (Halverson et al. 2015). The release of NETs, which results in cell death is called "suicide NETosis" (Fuchs et al. 2007; Steinberg and Grinstein 2007). This process can also occur by maintaining neutrophils structurally intact, and in this case it is called "vital NETosis" (Jorch and Kubes 2017). The NETs formation involves activation of protein kinase C (PKC) with subsequent mitogen-activated protein kinases (MAPK) / extracellular signal-regulated kinases (ERK) signaling pathway (Hakkim et al. 2011). In response to PMA, microorganisms and parasites, some ROS-inducing receptors and kinases, such as MAPK/ERK, IL-1 receptor-associated kinase, PKC, phosphoinositide 3-kinase (PI3K) and protein kinase B (Akt) have been associated to NETosis (reviewed by Papayannopoulos 2018). Then NADPH-oxidase generated induces the production of ROS (Jorch and Kubes 2017; Brinkmann 2018). This stimulates protein-arginine deiminase 4 (an enzyme that converts arginine to citrulline on histones) and stimulates MPO to activate and translocate ELA to the nucleus where ELA modifies histones and disrupts chromatin. Thus, the decondensing of chromatin starts with MPO binding synergy with ELA (Papayannopoulos 2018). After, the nuclear membrane breaks and chromatin is released to the cytosol mixing with granular and cytosolic proteins, such as CAT, proteinase 3 and lactoferrin (Urban et al. 2009; Jorch and Kubes 2017). The "suicide NETosis" is triggered by ROS generation and lasts for up to 4 hours. The "vital NETosis" is a faster process, which does not require ROS production, leading to a rapid release of NETs within minutes after activation (Rochael et al. 2015; Jorch and Kubes 2017). It is proposed that in "vital NETosis", neutrophils release part of the nucleus without breaking the cell membrane (Yipp and Kubes 2013). By the ELA action on unfolding chromatin, a dilation of the nucleus occurs and forms a vesicle containing DNA and proteins. These vesicles fuse with the cell membrane allowing the release of the contents without cell lysis. These "survival" neutrophils can still perform other functions as chemotaxis, phagocytosis and killing bacteria (Jorch and Kubes 2017). Another study found that "vital NETosis" uses

mitochondrial DNA allowing for cell survival (Yousefi et al. 2009). In “vital NETosis”, the activation can be mediated by complement receptors and Toll-like receptor (TLR)2 ligands (with *Staphylococcus aureus)*, directly via TLR4 (with *Escherichia coli*) or indirectly via TLR4-activated platelets (reviewed by Jorch and Kubes 2017).

The primordial role of NETs is the antimicrobial activity. The size of microorganisms seems to influence the release of NETs, since small microorganisms are phagocytized and the largest ones are killed by NETs (Papayannopoulos 2018). The NETs structure entraps pathogens limiting their spread due to electrostatic interaction between the negatively charged DNA backbone and positively charged superficial bacterial compounds (Brinkmann and Zychlinsky 2007). The virulence factors of some pathogens are cleaved by ELA, CAT and proteinase 3 (Brinkmann 2004; Averhoff et al. 2008). The production of hypochlorous acid by MPO oxidizes the pathogen membrane (Klebanoff 2005; Parker and Winterbourn 2012). Other NETs constituents, such as lactoferrin and calprotectin, can restrict nutrient supply for microbes by chelating iron or sequestering zinc (Urban et al. 2009; Papayannopoulos and Zychlinsky 2009). However, microorganisms have already developed strategies to escape from NETs. They can inactivate NETs components, prevent NETs formation, or develop resistance to NETs components (reviewed by Zawrotniak et al. 2017; Papayannopoulos 2018). Besides the benefits of NETs, their uncontrolled release can injure tissues and be further associated to some autoimmune, thrombotic, metabolic and cancer diseases (Zawrotniak et al. 2017; Brinkmann 2018; Papayannopoulos 2018; He et al. 2018; Thålin et al. 2019; Snoderly et al. 2019; Bonaventura et al. 2020).

2. Fibrosis

The acute inflammatory responses are characterized by fast resolving vascular changes, edema, and neutrophil inflammation. When the inflammation lasts for months it becomes chronic, and tissue remodeling and repair processes occur simultaneously leading to fibrosis. Therefore, fibrosis results from a wound-healing that has gone out of control (Wynn 2007, 2008). Some factors can damage tissues, such as acute or chronic stimuli, infections, autoimmune reactions, toxins, radiation and mechanic injury. The dying cells and pathogens produce DAMPs and PAMPs, which induces the inflammatory response (Wynn and Vannella 2016). After an injury, the dead or damaged cells must be replaced. Neutrophils, macrophages, innate lymphoid cells, natural killer cells, B cells, T cells, fibroblasts, epithelial cells, endothelial cells and stem cells together drive the cellular response of tissue repair (Wynn 2008; Wynn and Vannella 2016). The regenerative phase occurs first, the injured cells are replaced by the same type cell leaving no traces, and fibrosis occurs when connective tissue replaces the normal parenchymal tissue. A persistent stimulus sustains the production of growth and

angiogenic factors, proteolytic enzymes and fibrogenic cytokines that stimulate the deposition of connective tissue. This process becomes pathological when persists, resulting in substantial remodeling and excessive deposition of the extracellular matrix (ECM) leading to formation of a permanent scar tissue. Depending on the localization and extension, it can lead to loss of organ architecture, organ failure or death (Wynn 2007, 2008; Zeisberg and Kalluri 2013). Neutrophils are recruited to damaged tissue by the injured endothelial and epithelial cells producing growth factors, cytokines and chemokines and by platelets aggregation. Consequently, neutrophils produce cytokines and chemokines that amplify the wound-healing response, stimulating the endothelial cells to surround the injury and form new vasculature (Wynn, 2007, 2008). Fibroblasts are cells that produce and secrete all the components of ECM like structural (collagen type I – COL1), adhesive proteins (fibronectin and laminin) and space filling ground substance (glycosaminoglycans and proteoglycans) (Kendall and Feghali-Bostwick 2014). These cells have the ability to be activated into myofibroblasts which are involved in the pathogenesis of fibrosis (Wynn and Ramalingam 2012; Zeisberg and Kalluri 2013; Wynn and Vannella 2016). In addition, fibroblasts exhibit phenotypic heterogeneity suggesting different origins, activation, localization and stage of fibrogenesis (Liu 2011). The COL1 is the most abundant protein in mammals and provides rigidity by its rope-shaped, triple-stranded helical tertiary protein structure supplying tensile strength and preventing overstretching. Fibroblasts are also responsible for ECM maintenance and reabsorption (Kendall and Feghali-Bostwick 2014).

In spite of the classical view that myofibroblasts derive from resident fibroblasts, more recent studies revealed that they can derive not only from fibroblasts but also from other cell types such as pericytes, adipocytes, epithelial, endothelial and mesenchymal cells (reviewed by Zent and Guo 2018). Myofibroblasts are the central cellular fibrosis mediators. These cells express α smooth muscle actin (α-SMA) that allows contractility, which differentiates them from fibroblasts. Myofibroblasts also produce ECM proteins, such as COL1 and MMPs (Zent and Guo 2018). Fibroblasts and platelets expresses TLRs, which recognize PAMPs leading to fibroblast differentiation into myofibroblasts. Activated-platelets also release (transforming growth factor (TGF)β1 and platelet-derived growth factor, which induces fibroblast paracrine activation. Even though, neutrophils, in order to kill the pathogens, release ROS during oxidative burst also contributing to fibrosis establishment (Van Linthout et al. 2014).

Macrophages are other immune cells involved in tissue repair. But a disturbance in its function can lead to abnormal repair and fibrosis as well. In fact, they produce pro-fibrotic TGFβ1, are the main source of MMPs involved in ECM degradation, and are able to recruit myofibroblasts (Van Linthout et al. 2014; Wynn and Vannella 2016).

2.1 Physiophatological mechanisms of fibrosis

The injured tissues release several pro-fibrotic mediator molecules that drive fibroblast activation. Between them, TGFβ1, and the altered secretion of MMPs and PGs are widely reported factors triggering fibrosis.

2.1.1 Transforming growth factor β1

Transforming growth factor β1 is produced by macrophages, neutrophils, platelets, as well as fibroblasts. Despite regulating tissue homeostasis and repair, immune and inflammatory responses, ECM deposition, cell differentiation and growth, TGFβ1 is also considered one of the major pro-fibrotic signals for myofibroblast differentiation and inductor of genes expression for ECM components including COL1 (Yang et al. 2010; Ueha et al. 2012; Zeisberg and Kalluri 2013; Seki and Brenner 2015). The TGFβ1 is overexpressed in all fibrotic tissues, and induces COL production in cultured fibroblasts, irrespective of their origin (Zeisberg and Kalluri 2013). The MAPK and PI3K are signaling pathways that promote cell proliferation induced by TGFβ1 (Kim et al. 2018).

In response to injury, COL1 and 3 are the major collagens whose expression is induced by TGFβ1 to reestablish the tensile strength and integrity of the tissue. Several proteins and micro ribonucleic acids (miRNAs) are highly regulated by TGFβ1 in the process of COL tissue deposition. These proteins acts in every stage of COL processing from expression, translation, secretion and deposition. Also, the control on miRNA expression by TGFβ1 tends to stabilize COL protein expression, and secretion in the ECM. The TGFβ1 activates MAPK/PI3K/Akt and Sma and Mad related family (Smad) 2/3 pathways, which induce *collagen type 1 α1* (*COL1A1)* and *collagen type 1 α2* (*COL1A2)* transcription. The COL is synthesized in the rough endoplasmic reticulum. It is secreted as procollagen that in turn is proteolytically processed as tropocollagen, which begins to self-associate into microfibrils. The final physical and stable form of COL also depends on TGFβ1-induced proteins, such as fibronectin, lysyl oxidases, and inhibitors of proteases: plasminogen activator inhibitor I and tissue inhibitor of metallopeptidase (TIMP) 1 and 3. These proteases inhibitors prevent the breakdown of recent and vulnerable deposited tropocollagen. In the end, collagen organization is controlled by biglycan and periostin proteins, also induced by TGFβ1 (Kim et al. 2018). The adhesive ECM protein fibronectin regulates the amount of active TGFβ, thus protecting tissue from overproduction of this growth factor (Kawelke et al. 2011). In addition, the proteoglycans of the ground ECM substance directly interact with TGFβ inhibiting or augmenting TGFβ (Kendall and Feghali-Bostwick 2014).

Recent studies in humans have demonstrated that TGFβ1 induced renal fibrosis by activation of myofibroblasts, excessive production and inhibition of degradation of ECM (Walton et al. 2017). In idiopathic pulmonary fibrosis, the TGFβ1 pathway activation leads to

disease progression (Epstein Shochet et al. 2020). Also in the liver, TGFβ1 has been demonstrated to induce fibrosis (Gupta et al. 2019).

In reproductive tract, TGFβ1 plays a role in preparation for implantation by promoting decidualization of endometrial stromal cells and for maternal support of embryo development in humans (Jones et al. 2006). There are also reports on TGFβ1 signaling that increase endometriotic lesions growth (Correa et al. 2016). The overexpression of TGFβ1 enhances the migration and invasive ability of ectopic endometrial cells (Liu, Yi, et al. 2019) and increases leiomyoma cell proliferation depending on estrous cycle phase (Arici and Sozen 2003).

2.1.2 Matrix metallopeptidases

Matrix metallopeptidases are endopeptidases, whose enzymatic activity is dependent on calcium and zinc. They are the major enzymes involved in ECM turnover. Collagen as one of the ECM components is highly involved in tissue remodeling and repair (Harvey et al. 2016; Wang and Khalil 2018). The MMP family includes at least 25 enzymes divided in four classes: collagenases, gelatinases, stromelysins and membrane type enzymes (Vandooren et al. 2013; Djuric and Zivkovic 2017). Among them, MMP-2 and MMP-9 are gelatinases that denature collagens (gelatins) and other ECM substrates (Vandooren et al. 2013; Djuric and Zivkovic 2017).

The MMPs are secreted to the extracellular environment or linked to cell membrane, as inactive pro-enzymes or zymogens (Nissinen and Kähäri 2014; Harvey et al. 2016). Their activity is regulated by gene transcription, protein production, pro-enzyme activation and activity inhibition (Sternlicht and Werb 2001; Harvey et al. 2016). The MMPs gene expression is stimulated by growth factors, cytokines, hormones cell-extracellular matrix and cell-cell interactions (Harvey et al. 2016), and regulated by MAPKs signaling pathway (Vandooren et al. 2013). Posttranscriptional regulation includes mRNA stability, protein translational efficiency, and regulation by miRNAs (Pardo et al. 2016; Djuric and Zivkovic 2017). The first step of MMP activation is the replacement of thiol group of cysteine by water (Nagase 1997). This activation can be induced by plasmin, mast cell proteases, chymases and tryptases, but once activated, MMPs are able to activate other pro-MMPs in a positive feedback loop (Djuric and Zivkovic 2017). After activation, MMPs bind the cell membrane and target their catalytic activity to specific substrates in the pericellular space (Vandooren et al. 2013). The activity of MMPs can be inhibited by TIMPs (Harvey et al. 2016; Djuric and Zivkovic 2017; Wang and Khalil 2018). They bind the active site of the MMPs hindering the access to ECM substrates. Among the four types of TIMPs, TIMP-1 is a specific inhibitor for MMP-9 (Vandooren et al. 2013), while TIMP-2 regulates MMP-2 activity (Giannandrea and Parks 2014). Despite the main action of TIMPs being the inhibitory effect on MMPs, they are also involved in cell growth-promoting, anti-apoptotic, steroidogenic and antiangiogenic activities, which are in part

independent of MMP inhibition. (Robert et al. 2016). Other natural inhibitors of MMPs have been reported: α 2-macroglobulin, cysteine-rich protein with Kazal motif and glycosyl phosphatidylinositol-anchored glycoprotein (Djuric and Zivkovic 2017; Wang and Khalil 2018).

Some of the physiological actions of MMPs include cell proliferation, migration and differentiation, angiogenesis, apoptosis and tissue repair (Wang and Khalil 2018). However, for the last years, MMPs have been linked to pathological conditions including fibrosis (Robert et al. 2016; Djuric and Zivkovic 2017; Wang and Khalil 2018). They were associated to the migration of fibrocytes in idiopathic pulmonary fibrosis (Pardo et al. 2016), and to myofibroblast activation in vascular fibrosis (Harvey et al. 2016). Giannandrea and Parks (2014) reported that MMP-2 has an anti-fibrotic role and MMP-9 has a pro-fibrotic effect in liver and kidney. In early stages of fibrosis of hepatic tissue, MMP-9 is capable of activating the TGFβ1 pathway, while in the later stages MMP-2 reduced COL1 relative abundance (Duarte et al. 2015). In pulmonary fibrosis, MMP-9 is linked to inflammatory-induced tissue remodeling, while MMP-2 may be associated with impaired tissue remodeling, leading to abnormal collagen deposition and interstitial fibrosis (Wang et al. 2011). While Churg et al. (2012) related MMP-2 and other MMPs to chronic obstructive pulmonary disease (COPD) pathogenesis, Tomaru et al. (2015) showed that MMP-2 had a protective effect in mice pulmonary fibrosis. Also, although cystic fibrosis patients exhibited increased serum levels of MMP-9 (Rath et al. 2014), MMP-9 was related to fibrosis resolution in mice liver (Feng et al. 2018). In addition, recent studies have linked both MMP-2 and -9 to fibrosis development, namely in bleomycin treated lungs of mice (Summer et al. 2019), and in human idiopathic pulmonary fibrosis (Todd et al. 2020).

Additionally, the enzymes found in NETs were already described to alter MMPs expression in fibrosis studies. Elastase activated pro-MMP-9 in cystic fibrosis in the lung (Voynow et al. 2008). Both CAT and ELA activated pro-MMP-2 in human tumor cells invasion (Shamamian et al. 2001). In a tumor murine model, CAT enhanced TGFβ signaling together with MMP-9 (Wilson et al. 2009b).The concomitant increased levels of MPO and MMP-2/-9 were also reported in rat temporomandibular joint inflammation (Nascimento et al. 2013), in inflamed human dental pulp tissue (Accorsi-Medonça et al. 2013) and in fat meal induced endothelial damage in humans (Spallarossa et al. 2008).

2.1.3 Prostaglandins

Prostaglandin E_2 and $PGF_{2\alpha}$, besides their role on inflammatory responses (Ricciotti and FitzGerald 2011), have several functions on the regulation of estrous cycle phase and pregnancy establishment in the mare (Douglas and Ginther 1976; Zavy et al. 1978; Vanderwall et al. 1994). From mid to late luteal phase in mares, the concentrations of $PGF_{2\alpha}$ increase because of its role in luteolysis (Douglas and Ginther 1976; Zavy et al. 1978). Also, the luteal cells treated with $PGF_{2\alpha}$ upregulated TGFβ suggesting that TGFβ is also involved in luteolysis

in mares (Galvão et al. 2018). Otherwise, PGE_2 is luteotropic, once prolonged the CL function in non-pregnant mares (Vanderwall et al. 1994). Prostaglandins are originate from arachidonic acid liberated from phospholipids, which is converted into PGH_2 by the action of prostaglandin endoperoxidase synthases (PTGS2). In turn, PGH_2 is converted into PGE_2 by the action of PGE_2 synthases (PGES), into $PGF_{2\alpha}$ by $PGF_{2\alpha}$ synthases (PGFS) and into PGI_2 by PGI_2 synthases (PGIS) (Simmons et al. 2004). Despite the involvement of PGs in the development of signs of inflammation being well known, their contribution to inflammation resolution is controversial (Ricciotti and FitzGerald 2011). Immune responses regulation, blood pressure, gastrointestinal integrity and fertility are biological processes where PGE_2 acts. It also acts in redness (by augmenting microvascular permeability), swelling and pain inflammatory signs (Funk 2001; Ricciotti and FitzGerald 2011). Four receptors have been described for PGE_2, designated EP1, EP2, EP3 and EP4 (Ricciotti and FitzGerald 2011). Some of the actions of $PGF_{2\alpha}$ occur in ovulation, luteolysis, uterine contraction, parturition, renal function, arteries contraction, myocardial dysfunction, brain injury and pain (reviewed by Ricciotti and FitzGerald 2011). Prostaglandin F receptor is the receptor for $PGF_{2\alpha}$ (Ricciotti and FitzGerald 2011). Despite the references of equine endometrial cells as the main source of uterine PGs (Szóstek et al. 2012; Galvão et al. 2013), also the equine myometrium was recently described as being able to produce PGE_2 and $PGF_{2\alpha}$. This enables the myometrium, independently from the endometrium, to also regulate the uterine functions during estrous cycle (Piotrowska-Tomala et al. 2020).

Besides the biological actions of prostaglandins, they are also linked to many diseases. For the last decades, both prostaglandins PGE_2 and $PGF_{2\alpha}$ have been linked to fibrosis establishment. For instance, many studies refer PGE_2 to shown an anti-fibrotic effect. It was reported that PGE_2 acts as an autocrine factor that controls cellular over-activation (Sokolova et al. 2005), can induce protective fibroblasts apoptosis (Huang et al. 2009), or decrease fibroblasts proliferation, migration and differentiation into myofibroblasts, thus reducing COL production (Kolodsick et al. 2003; Sokolova et al. 2005; White et al. 2005; Huang et al. 2007; Stratton and Shiwen 2010; Bozyk and Moore 2011; Ueha et al. 2012; Zhao et al. 2016). However, PGE_2 may also inhibit myofibroblast differentiation induced by TGFβ (Bozyk and Moore 2011). In fact, lung fibroblasts obtained from pulmonary fibrosis patients and bleomycin-induced pulmonary fibrosis in mice impaired the capacity to synthesize PGE_2 (Wilborn et al. 1995; Moore et al. 2000; Sokolova et al. 2005). Another study, reported that PGE_2 was found increased in fibroblasts from patients with severe fibrosing systemic sclerosis (Startton and Shiwen 2010). The PGE_2 have shown protective effects in bleomycin-induced pulmonary fibrosis (Dackor et al. 2011), by the use of anti-fibrotic noscapine mediated by EP2 (Kach et al. 2014). This prostaglandin also downregulated fibrosis in lungs via EP2 receptor (Kolodsick et al. 2003; Huang et al. 2007; White et al. 2005) and in skin (Zhao et al. 2016). The PGE_2

suppressed the phagocytic activity of neutrophils in bovine sperm in the oviduct (Marey et al. 2014), and inhibited NETosis in a mouse model (Shishikura et al. 2015) and also NETosis induced by a variety of stimuli (Domingo-Gonzalez et al. 2016). Recently, PGE_2 was shown to inhibit the conversion of human fibroblasts from patients with pulmonary fibrosis into myofibroblasts (Mukherjee et al. 2019).

The opposite pro-fibrotic effect is attributed to $PGF_{2\alpha}$, for example, in pulmonary fibrosis it was associated to disease severity and prognosis (Oga et al. 2009, 2013; Aihara et al. 2013). Also in cardiac fibrosis, increased COL1 and 3 through prostaglandin F receptor was associated to $PGF_{2\alpha}$ effects in cardiac fibroblasts (Ding et al. 2012), but also in an animal model (Ding et al. 2014). The TGFβ was reported to be stimulated by $PGF_{2\alpha}$ in bovine *corpus luteum* (Hou et al. 2008) and in skin fibrosis (Kanno et al. 2013). However, Oga et al. (2009) reported that the COL production in mice with pulmonary fibrosis was induced by $PGF_{2\alpha}$ via prostaglandin F receptor, since the deficiency of prostaglandin F receptor decreased fibrosis, independently of TGFβ. Also in human patients with knee osteoarthritis $PGF_{2\alpha}$ induced COL production, independently of TGFβ (Bastiaansen-Jenniskens et al. 2013).

2.2 Mechanisms leading to fibrosis progression

When fibrosis gets established, the mechanism which avoids tissue regeneration is fibroblast or myofibroblast persistence, rather than their activation. The persistence of fibroblasts is stimulated by continuous secretion of ECM components, growth factors and cytokines by activated fibroblasts self-perpetuating an autocrine stimulation of other fibroblasts (Kalluri and Zeisberg 2006). It seems that the combination of TGFβ1 and the contact with COL1 triggers an irreversible proximal tubular cells (a source of myofibroblasts) transdifferentiation (Yen et al. 2016). Fibroblast resistance was also promoted by TGFβ1 and enfothelin-1 via activation of focal adhesion kinase and PI3K/Akt pathways (Kulasekaran et al. 2009). Therefore, the inhibition of these kinases was able to attenuate pulmonary fibrosis in animal models (Lagares et al. 2012; Ding et al. 2013).

It was firstly described that when tissue repair is finished, the majority of myofibroblats undergo apoptosis (Hinz and Lagares 2020). In addition, TGFβ released by platelets or macrophages during inflammation may induce myofibroblast apoptosis by inducing cell death signaling pathways or inhibiting the pro survival-pathways (Zhang and Phan 1999). In fibrotic conditions, the persistence of myofibroblasts introduce the idea that these cells are apoptosis-resistant (Thannickal and Horowitz 2006; Fattman 2008). The stiffening of affected tissues promotes the biomechanical activation of TGFβ1 perpetuating fibrosis progression (Hinz et al. 2019). Additionally, in lung fibrosis, the ECM components deposition resulted in stiffness increase that leads to amplification of fibrosis (Liu et al. 2010; Booth et al. 2012). Myofibroblasts can also escape death by activating pro-survival mechanisms of autocrine production of

TGFβ1 that mediate resistance to apoptosis in lung fibroblasts (Lagares et al. 2010; Kulkarni et al. 2011). The PGE_2 was also described to induce fibroblast apoptosis (Huang et al. 2009, Maher et al. 2010), and downregulation of this PG contributes to myofibroblast apoptosis resistance in fibrotic disorders (Huang et al. 2008). Liu et al. (2010) also associated the mechanical stiffness stimuli and fibroblast activation to the suppression of PGE_2. Another mechanism of myofibroblasts to avoid apoptosis is the acquisition of a senescent phenotype (Hinz and Lagares 2020). Senescent myofibroblasts were identified in age-related idiopathic pulmonary fibrosis (Mora et al. 2017; Álvarez 2017; Cui et al. 2017), and upregulated TGFβ1 (Barnes et al. 2019). This mechanism was firstly developed to prevent tumor establishment but also might be involved in the fibrosis loop. For the last years, epigenetics has been referred as contributing to myofibroblasts phenotype regulation (Zeisberg and Kalluri 2013; Duong and Hagood 2018). A large number of epigenetic modifications triggers myofibroblast activation and differentiation into pathologic myofibroblast phenotypes leading to the progression of fibrosis (Duong and Hagood 2018). Epigenetics have been related to fibrosis in many organs, such as heart, liver, lungs, kidney, eye and systemic sclerosis (Xu et al. 2016; Nwosu et al. 2016; Tzouvelekis and Kaminski 2015; Chang et al. 2016; Kim, Park, et al. 2016; Bergmann and Distler 2017).

Once fibrosis is established in many organs, fibrotic diseases have a large impact in human health. Mostly atherosclerosis, but also pulmonary, liver and renal fibrosis are fibroproliferative disorders that account for about 45% of all deaths in humans (Nanchahal and Hinz 2016). Some other fibrotic associated diseases, such as endometriosis and abdominal adhesions, also affect over 10% of human population causing morbidity (Nanchahal and Hinz 2016).

3. Neutrophil extracellular traps in disease and fibrosis

Besides the benefits of NETs, they also drive the pathophysiologic development of many diseases, mainly fibrosis related ones. In thrombosis, NETs play a central role by promoting fibrin deposition and networks formation (Fuchs et al. 2010). In acute myocardial infarction, NETs show thrombogenic potential expressing functional tissue factor, and inducing platelet activation, which leads to thrombin generation (Stakos et al. 2015). Interestingly, NETs were found in a large amount in older coronary thrombi (Mangold et al. 2015) suggesting their involvement in an early thrombus dissolution process in coronary artery disease (de Boer et al. 2013). Also, NETs can be used to predict prognosis outcomes in patients with myocardial infarction, linking NETs to the occurrence of this disease and adverse cardiac events (Liu, Yang, et al. 2019).

In diabetes, neutrophils are able to infiltrate pancreas supporting the disease progression. In addition, high levels of glucose were reported to induce NETosis and also ELA was associated to diabetic retinopathy development (reviewed by Jorch and Kubes 2017; Bonaventura et al. 2020).

In autoimmune diseases, there are also a number reports about NETs contribution. In systemic lupus erythematosus, NETs are the main source of autoantigens. The NETolytic activity was decreased, but increased cell free DNA and MPO activity were found together in systemic lupus erythematous patients (Jeremic et al. 2019). Neutrophils from rheumatoid arthritis produced more NETs than healthy donors and stimulated the secretion of inflammatory cytokines contributing to pathogenesis of this disease (Ribon et al. 2019).

Rayes et al. (2019) demonstrated that circulating NET levels are increased in esophageal, gastric and lung cancer patients. Moreover, by inhibiting NETosis it was possible to reduce lung and liver metastasis.

In respiratory diseases, the involvement of NETs was also widely related, both to acute and chronic conditions. In acute pneumonia, the large amount of NETs formation, besides being vital for microbial defense, also increased the clinical instability risk prolonging hospital stay and mortality (Ebrahimi et al. 2018).

In an *in vitro* study, the treatment with fibrotic agents caused fibroblast differentiation into myofibroblasts, while the treatment with desoxirribonuclease (DNase), heparin or MPO inhibitor reduced this differentiation, indicating the involvement of NETs in the process of fibroblasts differentiation. The release of NETs may perpetuate tissue injury, and can be caused by either chronic or recurrent inflammation. Additionally, NETs components and the lack of NETs clearance by DNase or macrophages may perpetuate fibrosis progression (Chrysanthopoulou et al. 2014). It appears that in cystic fibrosis, the massive influx of neutrophils and NETs formation into the bronchioles increases mucus viscosity and provides a better environment for colonization of bacteria exacerbating the disease (Khan et al. 2019). Moreover, NETs contribute to inflammation and lung destruction, rather than their anti-microbial action (Law and Gray 2017). The involvement of NETs in fibrosis has been described in many organs, such as in the liver (Mirea et al. 2019), heart (Martinod et al. 2017) and kidney (reviewed by Salazar-Gonzalez et al. 2019).

Even though ELA might have the dominant proteolytic activity of NETs components, other enzymes rather than ELA, may also play this role on proteolysis (O'Donoghue et al. 2013), such as CAT and MPO. In fact, liver injury have been attributed to ELA released by NETs, in at least 80% of the causes, and DNase treatment did not remove all enzymes from the vascular wall (Kolaczkowska et al. 2015). Elastase is derived from azurophilic neutrophils granules and has the ability to degrade ECM components, such as elastin, COL and fibronectin (Korkmaz et al. 2008). Since ELA acts on ECM proteolysis it has been assumed that it can

play a role in degenerative and inflammatory diseases (Kawabata et al. 2000). Likewise, ELA was increased in neutrophils retrieved from the sputum of cystic fibrosis patients (Dittrich et al. 2018), and induced *in vitro* lung fibroblast proliferation and myofibroblast differentiation (Gregory et al. 2015).

When ELA was immune depleted from NETs derived from healthy human neutrophils, the remaining activity was attributed to CAT (O'Donoghue et al. 2013). Cathepsin G can also trigger the recruitment of inflammatory cells, thus contributing to self-propagating chronic inflammation (Maryanoff et al. 2010). The conversion from angiotensin I into II can be mediated by CAT (Lindberg et al. 1997; Owen and Campbell 1998; Helske et al. 2006). Since angiotensin II is a strong proliferative agent that contributes to tissue hypertrophy, fibrosis, and remodeling in chronic inflammatory diseases of the lungs (Orito et al. 2004), kidneys (Huang et al. 2003), and cardiovascular system (Nishimoto et al. 2001), the long-term exposition to CAT may lead to fibrosis establishment in *in vivo* systems. Indeed, CAT contributes to a greater extent to inflammation and fibrosis establishment in COPD in humans (Brehm et al. 2014). Furthermore, CAT action was associated with aortic stenosis remodeling and fibrosis (reviewed by Helske et al. 2006), renal fibrosis after ischemia (Shimoda et al. 2007) glomerulonephritis and renal failure (Cohen-Mazor et al. 2014), lung cystic fibrosis (Sedor et al. 2007; reviewed by Kosikowska and Lesner 2013; and Twigg et al. 2015), and fibrotic Dupuytren's disease in humans (Tan et al. 2018). In fact, in the pathophysiology of COPD, CAT may play a very important role (de Garavilla et al. 2005), justifying the recent development of diagnostic test that use CAT as a COPD marker (Gudmann et al. 2018).

Elastase and CAT derived from neutrophils are activators of MMP-9 (Vandooren et al. 2013). Moreover, MMPs, especially MMP-2 and MMP-9, increase the release of TGFβ1 that stimulates TIMPs, leading to the inhibition of ECM degradation and accumulation, vascular remodeling and vascular fibrosis (Harvey et al. 2016).

At the sites of inflammation, neutrophils, monocytes and macrophages release MPO that uses hydrogen peroxide to oxidase several substrates (Davies 2010) and interacts with ionic, atomic and molecular entities producing potent oxidants (Chapman et al. 2009). These oxidants are toxic to many microorganisms and play an important role on the immune defense (Hampton et al. 1998). However, the unregulated production of these oxidants can damage host cells and contribute to various diseases (Khan et al. 2018). Atherosclerosis, cancer, renal disease, lung injury, multiple sclerosis, Alzheimer's and Parkinson's disease have been linked to MPO oxidants (Klebanoff 2005; Khan et al. 2018). In addition, MPO is used as an oxidative stress marker in these diseases (Khan et al. 2018). In cystic fibrosis, lung damage is associated to MPO oxidation of methionine (Chandler et al. 2018). Moreover, mouse models have provided evidence that MPO can contribute to cardiac remodeling and myocardial fibrosis (Mollenhauer et al. 2017).

3.1 Enzymes signaling pathways

Protease-activated receptors (PARs) and TLRs are distinct transmembrane receptors involved in innate immune response to pathogens. The PARs are a family of four G protein-coupled receptors that detect host serine proteases and proteases derived from pathogens (Heuberger and Schuepbach 2019). These receptors are expressed by almost all cell types, thus controlling important physiological processes, including hemostasis, inflammation, pain, cellular proliferation and healing (Adams et al. 2011). Thrombin is able to activate PAR-1, PAR-3 and PAR-4, while trypsin activates PAR-2 and PAR-4. These proteases are able to cleave PARs at established sites with the extracellular N-terminal domains, and expose tethered ligands that stabilize conformations of the cleaved receptors. This mechanism activates the canonical pathway of G protein and/or β-arrestin-dependent signaling. Other proteases, different from thrombin and trypsin can also cleave PARs, but mainly at divergent sites which activates distinct signaling pathways, referred as biased signaling. The biased signaling shows unique physiopathological outcomes (Zhao et al. 2014). Elastase has been referred as being a biased agonist of PAR-1 inducing stress fiber formation, and endothelial barrier permeability through MAPK pathway (Ramachandran et al. 2011; Mihara et al. 2013). Besides the physiological effects, PARs also control pathological processes, such as inflammation associated disorders, fibrosis and cancer (Ungefroren et al. 2018). The thrombin-induced effects are prevented by CAT that cleaves PAR-1 into non-functional parts (Molino et al. 1995), but also induces chemoattractant signaling via PAR-1 (Wilson et al. 2009a). Both ELA and CAT can activate PAR-2 by disarming the receptor (Ramachandran et al. 2011). The ELA activation of PAR-2 may contribute to inflammatory diseases in which the receptor is involved, such as in ulcerative colitis (Morohoshi et al. 2006; Lohman et al. 2012). In addition, the activation of PAR-2 by ELA mediated inflammatory edema and mechanical hyperalgesia (Zhao et al. 2015), and joint inflammation and pain in mice (Muley et al. 2016, 2017). The functional relevance of cleavage of PAR-2 by CAT remains unknown (Zhao et al. 2014). Moreover, agonists of PAR-2 enhanced MPO release, ROS production and reduction of viral influenza gene transcription (Feld et al. 2013). Otherwise, CAT causes platelet secretion and aggregation mediated by PAR-4, triggering calcium mobilization in PAR-4-transfected fibroblast, this supports the hypothesis that CAT mediates neutrophil-platelet interaction at sites of vascular injury or inflammation (Sambrano et al. 2000; Faraday et al. 2013). Additionally, CAT triggers colon inflammation activating PAR-4, thus associating CAT to ulcerative colitis development (Dabek et al. 2009).

Some reports also associated PARs to the development of fibrosis. The mutual interaction between PAR-1/PAR-2 and TGFβ, forms a complex regulatory network that controls fibrosis and cancer (Ungefroren et al. 2018). Indeed, PAR-1 and PAR-2 activating proteases induce fibroblast migration, differentiation and ECM production in pulmonary fibrosis

(Lin et al. 2015a, 2015b). The involvement of ELA in fibroproliferative responses in pulmonary diseases occurs via proteinase-activated receptor PAR-1 (Suzuki et al. 2005). Elastase cleaves PAR-1 signaling through silencing PAR-1 calcium signaling and triggering MAPK pathways (Mihara et al. 2013). This signaling pathway seems to have the capacity of driving fibrotic responses by activating TGFβ1 and influencing myofibroblast differentiation (Scotton et al. 2009). Moreover, ELA binds to PAR-2 increasing mucus secretion in chronic inflammatory airway diseases (Zhou et al. 2012). The association of NETosis to PARs is now established. It was reported that NETs formation depended on the activation of PAR-2 by proteases derived from *Porphyromonas gengivalis* in human periodontitis. Also, NETs proteases as virulence factors antagonize the antibacterial activity of NETosis (Bryzek et al. 2019).

Another group of receptors involved in innate immune system are TLRs. They are a family of pattern recognition receptors that recognizes both PAMPs (Zang and Liang 2016) and DAMPs (Yu and Feng 2018). The TLRs are expressed in all innate immune cells like macrophages, neutrophils, dendritic cells, natural killer cells, mast cells, basophils and eosinophils (Delneste et al. 2007), as well as in non–immune cells such as fibroblasts and epithelial cells (Kawasaki and Kawai 2014). In response to their activation, TLRs stimulate signaling cascades of defense mechanisms to repair the damaged tissue (Wang et al. 2016) which in turns leads to the release of inflammatory cytokines and immune modulators (Wong et al. 2009). The presence of TLR2 and TLR4 were already confirmed in equine endometrial epithelial and stromal cells (Siemieniuch et al. 2016). Besides the regulatory effect in innate immune system, an imbalance in TLRs activation contributes to the development and progression of autoimmune, chronic inflammatory and infectious diseases and cancer (Huang and Pope 2009; Devaraj et al. 2011; Isaza-Correa et al. 2014; Jialal et al. 2014; Gao et al. 2017). In fact, TLR2 and TLR4 gene expression were up-regulated in endometria from mares suffering from subacute suppurative endometritis (Siemieniuch et al. 2016).

In 2016, Al-Khafaji and team (2016) demonstrated that neutrophil NADPH oxidase induces NETosis with the synergy action of TLR4. The association between ELA, TLR4 and inflammatory cytokines have been already established. Neutrophil ELA enhanced interleukins production via TLR4 in a study in human embryonic kidney cells (Devaney et al. 2003), and in a mice model of bacteria-induced pneumonia (Benabid et al. 2012; Domon et al. 2018). In addition, CAT was already linked to TLRs. In psoriatic lesions, the interferon production in plasmocytoid dendritic cells was stimulated by CAT, which activated TLR9 to sense free-cell DNA (Skrzeczynska-Moncznik et al. 2013). Likewise, in a mice model of induced glomerulonephritis, TLR2 and TLR9 enhanced MPO-induced autoimmunity (Summers et al. 2011).

4. Inhibition of enzymes found in NETs

The perfect enzyme inhibitor would be able to inhibit ELA, CAT and MPO with similar efficiency, controlling their proteolytic and oxidizing activities. It should also be resistant to oxidation and proteolysis, have small size to afford better access to enzymes when they are bound to ECM and to molecular or cellular components, be easily administered, and resist to *in vivo* degradation (Korkmaz et al. 2010; von Nussbaum and Li 2015).

4.1 Elastase inhibition

Because of the emerging evidences of the contribution of NETs components to fibrosis development, some proteases inhibitors have been tested for the last years. The first generation of ELA inhibitors are from endogenous source, namely, α1-proteinase inhibitor (α1-PI), elafin, secretory leucocyte protease inhibitor (SLPI), α1-anti-chymiotrypsin (ACT), α2-macroglobulin and monocyte neutrophil elastase inhibitor (Serpin) B1. The ELA activity is mainly regulated by α1-PI, SerpinB1 and SLPI (Korkmaz et al. 2010; Delgado-Rizo et al. 2017), but when the balance between protease and endogenous inhibitor fails it may lead to tissue damage. The endogenous inhibitors compromise the tertiary structure of not bounded ELA, contributing to inflammation resolution and preventing tissue damage (Bronze-da-Rocha and Santos-Silva 2018). However, these endogenous inhibitors has lack of stability under oxidative stress conditions in pathological conditions, and only ELA of non-adherent neutrophils remains sensitive to these inhibitors (Korkmaz et al. 2005; Dubois et al. 2012). Additionally, the host and microbial proteases can degrade these endogenous inhibitors (Guyot et al. 2008). The bounded-ELA and -CAT are catalytically active and resistant to inhibition by endogenous inhibitors, facilitating its exit from vasculature, tissues penetration and recruitment to inflammation sites. A dysregulation of the cell surface expression of these neutrophil enzymes has the potential to cause tissue destruction during inflammation (Owen et al. 1995). Moreover, ELA bounded to DNA released from neutrophils is insensitive to endogenous protease inhibitors (Belorgey and Bieth 1998). Although, some studies show the efficacy of these inhibitors. Inhalation of α1-PI by cystic fibrosis patients decreased ELA and pro-inflammatory neutrophil cytokine levels but had no effect on lung function (Griese et al. 2007). More recently, the intravenous administration of α1-PI to human patients showing severe α1-PI deficiency slows the progression of emphysema (Chapman et al. 2015). The imbalance between ELA and SLPI ratio in lugs is associated to tissue destruction. A novel administration strategy was recently proposed by entrapping SLPI inside albumin nanoparticles, as carriers, to avoid side effects (Tarhini et al. 2018).

The second-generation of ELA inhibitors (sivelestat - SIV and freselestat) are mechanism-based suicide and inhibits the release and membrane-bound of ELA (von

Nussbaum and Li 2015; Bronze-da-Rocha and Santos-Silva 2018). Sivelestat is a selective inhibitor of ELA, which inhibits the enzymatic action of ELA directly by a reversible 'acylation-deacylation' mechanism (Nakayama et al. 2002). However, the SIV mechanism of action is not fully understood. In bacterial infection in lung, it was proposed that SIV blocks ELA-induced disruption of pulmonary epithelial cells and prevents bacterial invasion into the bloodstream (Yanagihara et al. 2007; Domon et al. 2016). More recently, Domon et al. (2018) suggested that SIV abolishes the extracellular effect of ELA and subversion of host immune response, without impairing intracellular bacterial killing. The decreased levels of cytokines were associated with decreased bacteremia in SIV-treated mice, where ELA activity was reduced in broncho-alveolar fluid lavage (Domon et al. 2018). This inhibitor has shown beneficial effects on fibrosis impairment, either during *in vitro* studies or in clinical trials. Studies on the use of SIV have been focused on the responses to injury and inflammatory reactions, such as, acute inflammation in lungs (Tamakuma et al. 2004; Mikumo et al. 2017), liver (Soejima et al. 1999) and in pulmonary fibrosis where reduced COL deposition in mice was noted (Takemasa et al. 2012). In fact, SIV inhibited inflammatory cell recruitment and TGFβ1 activation in lungs, which is the putative mechanism for SIV modulatory action (Takemasa et al. 2012). In addition, SIV was reported to suppress neutrophil activation of pro-inflammatory mediators in mice liver ischemia/reperfusion injury (Uchida et al. 2010). It also avoided organ failure by inhibiting vascular permeability and reducing cytokine production in a porcine hepatectomy model of ischemia/reperfusion injury (Shimoda et al. 2019). Some other studies reported the benefits of SIV in lipopolysaccharide-induced lung injury in rat lungs (Yuan et al. 2014), reducing portal pressure associated with chronic liver diseases in mice (Hilscher et al. 2019), in bleomycin-induced pulmonary fibrosis in mice (Song et al. 2009; Takemasa et al. 2012), and to ameliorates sepsis-related kidney injury in rats (Li, Jia et al. 2016). Most recently, both SIV and SerpinB1 reduced lung neutrophil infiltration and pulmonary oxidative stress restoring pulmonary barrier function in a rats model (Yao et al. 2019). In humans, SIV is actually administered in acute lung diseases, to improve clinical condition and prognosis (Aikawa et al. 2011; Kido et al. 2016; Polverino et al. 2017). The administration of ELA to mice induced acute inflammation and pain in knee joints via activation of p44/42 MAPK pathway PAR-2-dependent, but the use of SIV reduced inflammation and pain (Muley et al. 2016). The same team performed another experiment and concluded that ELA and PAR-2 contributed to the development of joint inflammation and pain in induced osteoarthritis in mice, while SIV treatment reduced these signs (Muley et al. 2017). In a mice model of endotoxin-induced liver injury and partial hepatectomy, treatment with SIV improved the survival rate, liver function and reduced cytokine levels by nuclear factor kβ pathway through TLR4 (Kwon and Qiu et al. 2007). Even though TLR4 is involved in liver fibrosis and regeneration, SIV did not have any effect on TLR4 levels in an ischemia/reperfusion in liver of a porcine model, suggesting that SIV does

not act at cellular level (Shimoda et al. 2019). The comparison between the use of SIV and neutralization of TLR5 in pneumonia-induced mice revealed that both experiments reduced neutrophil recruitment, inflammation, mortality, and secretion of ELA into the airway (Jones-Nelson et al. 2018). In a study of ELA-induced endotoxic shock in mice, the inhibition of ELA by administration of nanoparticles loaded with SIV hindered NETs formation, reduced NETs-mediated vascular damage and alleviated the production of inflammatory cytokines, thus avoiding endotoxic shock. This study demonstrated a new way to improve SIV efficacy (Okeke et al. 2020). Althoug SIV may present some toxicity risks, the inhibition of the pro-fibrotic effects of ELA by SIV in several fibrotic diseases in a number species, altogether, substantiates the use of SIV as a potential therapeutic approach for equine endometrosis.

The third and fourth generation of ELA inhibitors, alvelestat and BAY-678 respectively, are nonreactive, reversible inhibitors originated from pyridine and dihydropyrimidone structure (von Nussbaum and Li 2015; Bronze-da-Rocha and Santos-Silva 2018). They show a very high specificity and no significant pharmacological interactions (von Nussbaum and Li 2015). Avelestat, administered orally, reduced inflammation and lung injury, but had a small effect on ELA activity and lung function (Elborn et al. 2012). However, it has shown benefits in abdominal aortic aneurysm in rats, reducing MPO, ELA and cell free DNA (Delbosc et al. 2016). The fifth inhibitors generation is composed by Bay-85-8501, which has pre-adaptive pharmacophores derived from the fourth generation (von Nussbaum and Li 2015). The inhibitor Bay-85-8501 shows excellent potency, selectivity and has improved the cardiac function in a rat model (von Nussbaum et al. 2016). Both avelestat and Bay-85-8501 are in clinical trials for the treatment of cystic fibrosis, COPD, bronquiectasia and pulmonary disease (von Nussbaum and Li 2015; Vergelli et al. 2017). Sirtinol is an inhibitor used in cancer and neurodegenerative diseases (Villalba and Alcain 2012) by acting on genes expression, metabolic regulation and cell apoptosis. Recently, in human neutrophils, sirtinol inhibited ELA without affecting neutrophil function showing potential application for inflammatory lung diseases treatment (Tsai et al. 2015). However, since the development of fibrosis may not be always ascribed to ELA action in all pathological conditions, the use of single ELA inhibitors is not always effective (Piccioni et al. 1992; Hirche et al. 2005). Some studies reported that naturally occurring inhibitors in tissues, such as α1-PI, preferentially target ELA leaving CAT and proteinase 3 free in the extracellular space (Ohbayashi 2002; Korkmaz et al. 2005, 2010). This situation can explain why selective ELA inhibitors can be insufficient in controlling neutrophil mediated damage in the airways (Ohbayashi 2002). In the interstitial space can exist a compartmentalization of these NETs proteases and their natural inhibitors, thereby creating a microenvironment that excludes high molecular weight inhibitors, such as α1-PI, and also can contain high concentrations of proteases relative to their inhibitors (Owen et al. 1995). So, the inhibitors that naturally exist in the tissues seem to leave these proteases uncontrolled. Likewise, the use of

a single selective inhibitor may reveal itself ineffective knowing that other NETs components are involved in diseases development, as well.

4.2 Cathepsin G inhibition

The inhibitors α1-PI, elafin, α2-macroglobulin; Serpins and SLPI are capable of inhibiting not only ELA, but also CAT and proteinase 3 (Kosikowska and Lesner 2013; von Nussbaum and Li 2015). Although, the close association of ELA and CAT with plasma membrane may confer resistance to physiological inhibitors, such as α1-PI (Kosikowska and Lesner 2013). The first described CAT inhibitors are peptide base inhibitors. Bovine pancreatic trypsin inhibitor, ACT and exogenous salivary protein of *Ixodes ricinus* (a parasite responsible for transmission of Lyme disease) are examples of peptide inhibitors (Krowarsch et al. 2003; Schmidt and Winter 2006; Chmelar et al. 2011). Recently, in a mice model, CAT inhibition by endogenous SerpinB1 and SerpinB6 prevented monocyte and neutrophil death induced by CAT. These endogenous inhibitors also regulate systemic inflammation (Burgener et al. 2019). From the phosphonic inhibitors group, β-keto-phosphonic acid (Cathepsin G Inhibitor I - INH) offers a promising therapeutic tool for chronic inflammatory conditions, such as asthma, COPD and arthritis (Kosikowska and Lesner 2013). Cathepsin G Inhibitor I is a potent, selective, reversible, competitive, non-peptide and small-molecule inhibitor of CAT and chymase. The ligand 1 of INH occupies the S1 and S2 subsites of CAT and chymase, with the 2-naphtyl in S1, the 1-naphtyl in S2 and the phosphonate group in a complex network of hydrogen bonds. The carboxamido-N-(naphthalene-2-carboxyl)piperidine group occupies the hydrophobic S3/S4 subsites (Greco et al. 2002; de Garavilla et al. 2005). This inhibitor could be used for the treatment of COPD and asthma in humans (de Garavilla et al. 2005; Maryanoff et al. 2010; Brehm et al. 2014). Additionally, INH exhibits anti-inflammatory activity in rat models of glycogen-induced peritonitis and lipopolysaccharide-induced airway inflammation (de Garavilla et al. 2005), and in airway inflammatory diseases dependent on CAT in animal models (Maryanoff et al. 2010). In a photoaging study in mice, INH inhibited CAT-mediated MMP-1 increase and reduced mRNA encoding COL and TIMP1, ameliorating ECM damage and MMP upregulation (Son et al. 2012). This inhibitor blocks the increase of TNFα and monocyte chemoattractant protein 1, both linked to airway hyperactivity (de Garavilla et al. 2005), and blocks neutrophilia (Abraham 2008). More recently, the inhibition of CAT by INH reduced myocyte death and improved cardiac remodeling after myocardial ischemia reperfusion injury by attenuating COL deposition (Hooshdaran et al. 2017), and decreases COL7 and MMP-13 levels and neutrophil infiltration in ultra-violet irradiation of mouse skin (Kusumaningrum et al. 2018)

Another inhibitors, such as boswellic acids, (used in traditional medicine to treat arthritis, ulcerative colitis, asthma and peptic ulcers), heparin, (besides being anti-inflammatory

have strong anti-coagulant properties), thiazolidines and aptamers (single-stranded RNA or DNA molecules with high affinity to their targets) have also been reported to inhibit CAT (reviewed by Kosikowska and Lesner 2013).

4.3 Myeloperoxidase inhibition

Ceruloplasmin is an endogenous potent MPO inhibitor that showed reduction of MPO activity in plasma of mice, providing evidence that can be a protective inhibitor against MPO oxidant production during inflammation (Chapman et al. 2013). Within the group of suicide irreversible inhibitors, benzoic acid hydrazides and 2-thioxanthine are MPO inhibitors with high potency. Oxidation of these inhibitors by MPO promotes inactivation, either by destruction or covalent modification of the enzyme's heme prosthetic groups (Forbes et al. 2013). The compound 2-thioxanthine reduced NETs activation, ROS production, and attenuated *in vitro* neutrophil-mediated endothelial cell damage production, and reduced *in vivo* kidney damage in glomerulonephritis in a murine model (Antonelou et al. 2020). In mouse models of vascular inflammation and atherosclerosis, oral administration of 2-thioxanthine improved endothelial function by reducing MPO activity (Cheng et al. 2019). Also, this inhibitor, showed positive effects attenuating obesity and liver damage, but had no direct effect improving cardiac function (Piek et al. 2019). However, the most common experimentally used inhibitor of MPO is 4-aminobenzoic acid hydrazide (ABAH) (Kettle 1997; Lazarević-Pasti et al. 2015). Recent studies show that ABAH reduced MPO-dependent hepatocyte death in a steatohepatitis mice model (Pulli et al. 2015), MPO activity in acute stroke in mice (Kim, Wei, et al. 2016), and inhibited MPO in pulmonary cystic fibrosis sputum (Hair et al. 2017). The ABAH mechanism of action is not well known yet. Some authors proposed a mechanism of action, where MPO oxidizes ABAH to a radical that reduces the enzyme to its ferrous intermediate by destroying the MPO heme group. Ferrous MPO reacts with hydrogen peroxide to give irreversible inactivation (Kettle et al. 1997, Burner et al. 1999). Engelmann et al. (2000) reported that ABAH inhibits hydrogen peroxide-induced apoptosis of leukemia cells, without altering catalase, glutathione oxidase activities, and superoxide production by neutrophils. Recent findings about ABAH mechanism of action proved that inhibition is due to hydrolysis of the ester bond between MPO and heavy chain glutamante 242 residue and the heme ring, freeing the heme linked light chain MPO subunit from the larger remaining heavy chain portion. So, the destruction of heme ring does not occur by tracking the heme prosthetic group (Huang et al. 2015).

The reversible inhibitors compete with MPO substrates by occupying the heme-binding pocket, blocking the enzyme's oxidizing capacity, without permanent changes to the enzyme production (Forbes et al. 2013; Lazarević-Pasti et al. 2015). Salicylhydroxamic acid was identified as reversible MPO inhibitor (Forbes et al. 2013; Lazarević-Pasti et al. 2015), but

showed a poorer inhibition of MPO, than ABAH (Kettle et al. 1995). Also melatonin, tryptophan, serotonin, flavonoids and resveratrol are described as MPO inhibitors. Moreover, nonsteroidal anti-inflammatory drugs are being tested as inhibitors of MPO (Lazarević-Pasti et al. 2015; Galijasevic 2019).

Another MPO inhibitors showed promising results. The inhibitor PF-1355, decreased MPO activity in mouse myocardial infarction model improving ventricular function and remodeling (Ali et al. 2016). Non-toxic N-acetyl lysyltyrosylcysteine amide restores blood-brain barrier integrity in autoimmune encephalomyelitis in mice, by inhibiting MPO (Zhang et al. 2016). In cystic fibrosis sputum, peptide inhibitor of complement C1 showed similar inhibition of MPO comparing to ABAH (Hair et al. 2017). Another recent mechanism-based inhibitor, PF-06282999, was found to alter the inflammatory tone of atherosclerotic lesions in mice, but did not affect the atherosclerotic plaque (Roth Flach et al. 2019).

5. Estrous cycle in mares

Mare is a polyestrous of long days breeder meaning that seasonal reproductive activity is regulated by a positive photoperiod, being stimulated by long days and short nights (Ginther 1974; Kooistra and Ginther 1975; Palmer and Guillaume 1992). When daylight hours increase (in the spring and summer), the secretion of melatonin decreases, which in turn, stimulates the release of gonadotropin-releasing factor (GnRH) in the hypothalamus (Strauss et al. 1979; Grubaugh et al. 1982; Kilmer et al. 1982). The GnRH stimulates the adenohypophysis to synthesize follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Johnson 1986; Cleaver et al. 1991; George et al. 2004). These gonadotropins are transported to the ovary where they specifically exert their function (Irvine and Alexander 1993; George et al. 2004). The FSH acts on the granulosa cells of the preovulatory follicle stimulating the growth, follicular maturation and estrogen (E2) biosynthesis. On the theca cells, LH is involved in oocyte maturation, ovulation, establishment and maintenance of *corpus luteum* (CL) and in the synthesis of progesterone (P4). The ratio of LH/FSH reaching the circulation is influenced by GnRH pulse frequency and by the physiologic feedback from both ovarian steroids released from the ovaries, these events determine the estrous cycle in the mare (Sharp and Davies 2011; Evans et al. 2011; Velez et al. 2012). The synthesis and release of FSH are stimulated by low-frequency GnRH pulses while the synthesis and release of LH are stimulated by GnRH high-frequency pulses (Irvine and Alexander 1993). During diestrus, P4 suppresses the high frequency of GnRH release and during estrus, and E2 increases GnRH pulse frequency (Raz and Aharonson-Raz 2012). The increase of FSH in the spring transition period causes the development of small follicular waves, which lead to the development of multiple follicles from 6 to 21 mm of diameter, that regress simultaneously in the absence of a dominant follicle

(Freedman et al. 1979; Ginther et al. 2004a; 2004b). However, in the end of spring period, larger follicular waves occurs and a set of follicles develops. Most of the follicles will reach atresia but one of them reaches a larger size than the others – the preovulatory follicle (Driancourt et al. 1982; Michael Lacker et al. 1987; Aurich 2011). The increasing E2 concentrations by this follicle induce LH synthesis, which leads to the first ovulation, indicating the onset of the ovulatory season at which fertile cyclical activity begins (Aurich 2011).

The breeding season runs approximately from April to October in the northern hemisphere (Ginther 1974). The spring transitional period ranges from 30 to 90 days. A dominant follicle between 20 mm and 30 mm develops simultaneously with smaller follicles greater than 15mm (Aurich 2011). Before ovulation, 1-3 anovulatory follicular waves develop (Donadeu and Watson 2007). The occurrence of surges in circulating LH is the most important factor to end the transitional phase and re-iniciate the ovulatory activity (Aurich 2011). The estrous cycle is longer at the beginning of spring (April or May), than at the end of the breeding season. The decreased influence of photoperiod and elevated temperatures in late summer promote the onset of autumn transition period from October to December (Sharp and Ginther 1975). In the seasonal anestrus, the FSH concentration reaches the prolonged nadir value causing a poor LH surge resulting in anovulation (Irvine et al. 2000). A dominant follicle does not develop and only a few follicles have a diameter bigger than 15 mm (Aurich 2011). During winter, the release of high concentrations of melatonin reduce GnRH release that is not enough to stimulate the secretion of gonadotropins FSH and LH (Garcia and Ginther 1976; Alexander and Irvine 1991; revised by Aurich 2011).

Some exogenous factors such as age, reproductive state, nutrition, body condition (leptin) or environmental temperature also affect the seasonal reproductive activity in mares (Ferreira-Dias et al. 2005; Aurich 2011). So, in a large horse population, a proportion of mares continue to cycle throughout the year (Hafez and Hafez 2000; Ferreira-Dias et al. 2005; Davies Morel et al*.* 2010; Aurich 2011).

The repetitive sequence of events that prepare the mare for conception is the equine estrous cycle, which lasts an average of 21 days. There are four stages of mare's estrous cycle, classified according to the changes in steroid hormones concentration and endometrial structural and functional events. The follicular phase (FP) also called estrous or ovulatory phase (day 16-17 to 21 – ovulation day, the latter usually being called day 0), early luteal phase (day 1 to 4), mid luteal phase (MLP, around day 8) and late luteal phase (day 12 to 15) (Aurich 2011).

5.1 Estrus

The FP is identified by the presence of different stages of the development of follicles and the increase of E2 secretion. During estrus, which corresponds to the FP, the mare is

sexually receptive to the stallion, and the genital tract is prepared to accept and transport sperm and an oocyte to the site of fertilization (Crowel-Davies 2007; Ginther et al. 2008; Raz and Aharonson-Raz 2012). The E2 is the main steroid in this phase and commands the physiological events that occurs in female body and uterus. The uterine wall thickens, muscular tone increases and vascularity becomes greater. The cervix is relaxed and open (Aurich 2011). Kenney (1978) described endometrial histological changes during the FP, when the endometrial glands proliferate and become active and the lamina propria is highly edematous with reduced gland density and a loosely woven appearance of the stroma. The presence of neutrophils in the stroma reveals inflammation, even though they can also be found in marginate venules and capillaries, physiologically.

One or two follicular waves develop during estrus and are associated with a FSH surge that reaches a peak when the largest follicle attains 13 mm in diameter (Gastal et al. 1997). The FSH concentration diminishes enabling the dominant follicle to grow. The pre-ovulatory follicle grows and reaches approximately 40 mm, or even more (Ginther et al. 2008).

In the mare, the LH levels increase during estrus, by the positive feedback exerted by the pre-ovulatory follicle E2 and reach the peak one day after ovulation (Hafez and Hafez 2000), but it is preceded by biological LH activity shortly before ovulation (Alexander and Irvine 1982).

Estrogen receptors (ESR) consist of two predominant isoforms of nuclear receptors, ER1 and ER2. In mare endometrium, they are upregulated by E2 as well as the P4 receptors (PGR) (Hartt et al. 2005). The dominant E2 receptor is ESR1 playing a major role in uterotrophic effects of E2 (Weihua et al. 2000). The ESR2 has also been described in the mare (Honnens et al. 2011) and suggested to modulate the uterotrophic effects of ESR1 (Weihua et al. 2000) and to attenuate the transcriptional activity of ESR1 in the uterus (Large and DeMayo 2012). High endometrial levels of *ESR1*, *ESR2* and *PGR* messenger ribonucleic acid (mRNA) and proteins detected in luminal and glandular epithelia and stromal cells have been reported in mares during estrus (Watson et al. 1992; Hartt et al. 2005; Honnens et al. 2011; Gebhardt et al. 2012; Silva et al. 2014).

5.2 Diestrus

During diestrus, when a CL is presented in the ovary (luteal phase) the mare is not receptive to the stallion. The ruptured ovulatory follicle develops into a CL that secretes P4 and, in the nonpregnant mare, it regresses 14-15 days after ovulation. The P4 is the main steroid influencing this phase. Its actions leads to a decrease in uterine wall thickness, myometrial tone and endometrial gland activity. The cervix becomes firmer and is tightly closed (Aurich 2011). The endometrial gland density increases due to decreased stromal edema and the increased tortuosity of glands (Kenney 1978). After ovulation, the concentration of P4

increases (Ferreira-Dias and Mateus 2003; Roberto da Costa et al. 2005) from early to mid-luteal phase (Van Niekerk et al. 1975; Aguilar et al. 2006). In the absence of pregnancy, the luteal phase culminates with the lysis of CL induced by endometrial prostaglandin (PG)$F_{2\alpha}$ and decreased concentrations of P4 (Crowell-Davis 2007; Ginther et al. 2008).

In mice, there are two PGR iso-forms, the PGR-A plays a major role mediating the actions of P4 in the uterus and ovaries, while PGR-B is more important in the development of the mammary gland (Mulac-Jericevic 2000; Mulac-Jericevi et al. 2003). When circulating P4 levels are high, endometrial expression of ESR and PGR are inhibited. In mare's endometrium, from mid to late diestrus, the concentration of ESR and PGR receptor protein decreases (Watson et al. 1992). Also, abundance of *ESR1* and *PGR* mRNA decreases from days 0 to 11 of estrous cycle and increases by late diestrus (McDowell et al. 1999; Honnens et al. 2011; Gebhardt et al. 2012; Silva et al. 2014). On day 11 and 14 of mare estrous cycle, ESR and PGR expression decreased in stroma and in deeper glandular epithelia and they were not detected in luminal epithelium (Hartt et al. 2005). On days 14 to 16 of mare estrous cycle luteolysis occurs (Ginther et al. 2007).

During pregnancy, gene transcription for ESR and PGR receptors decreases with increasing days of gestation and sustained P4 concentration (Watson et al. 1992; McDowell et al. 1999) controlled by a negative feedback mechanism. More recently, during diestrus, Nelis et al. (2015) described a high P4 concentration in oviductal tissue and fluid ipsilateral to the ovary when ovulation occurred. Although, a downregulation of PGR occurred possibly due to negative feedback mechanism (Hai et al. 1977; Nelis et al. 2015).

6. Equine endometrial fibrosis

6.1 Endometritis contributes to chronic endometrial changes

Endometritis is an acute or chronic inflammation of the endometrium and considered as a major cause of subfertility/infertility in mares. Air, urine, semen, bacteria, fungi or yeasts are capable of inducing an endometrium reaction. After breeding, mares develop a physiological transient breeding-induced endometritis. The semen-induced uterine inflammation is characterized by a fast arrival of neutrophils into the uterine lumen (Kotilainen et al. 1994; Katila 1995). The influx of inflammatory cells in the mare's uterus empowers the inflammatory reaction, resulting in the removal of unnecessary spermatozoa, contaminating bacteria, and debris introduced in the uterus (Troedsson et al. 1993; Troedsson 2006). This response starts about 30 minutes after natural mating or artificial insemination (Katila 1995), and is limited to the endometrium, without hematological changes detected (Tuppits et al. 2014). The breeding-induced endometritis resolves within 24-48h in healthy mares, that are considered "resistant" mares, *i.e.* not prone to endometritis. Likewise, the mares more prone

to endometritis are considered “susceptible” (LeBlanc et al. 1994; Troedsson et al. 1993). Other factors may be present in susceptible mares contributing to the difficulty in cleaning inflammation. The older mares and parity are predisposing factors to persistent endometritis (Woodward et al. 2012). Also, poor vulvar conformation, pneumovagina, pendulous uterus, cervical fibrosis secondary to a traumatic birth are considered other risk factors that limit uterine clearance (Ricketts 1999; LeBlanc et al. 1995, 1998; LeBlanc and Causey 2009; Pycock 2009, Scoggin 2015). Degenerative changes, as abnormal myometrium, periglanular fibrosis, vascular degeneration, lymphangiectasia, scarring and atrophy of endometrial folds also triggers the delayed bacteria clearance (LeBlanc and Causey 2009).

The etiology, diagnosis and pathogenesis of acute and chronic mare endometritis have been largely studied (Ferreira-Dias et al. 1994; Nielsen 2005; Hoffmann et al. 2009a; Szóstek et al. 2013, Woodward et al. 2013). In the infectious endometritis, pathogenic or opportunistic bacteria and fungi access to the uterus during breeding. *Streptococcus equi* subspecies *zooepidemicus*, *Escherichia coli, Pseudomonas aeruginosa, Klebsiella spp., Proteus and Corynebacterium* among others are major causes of equine endometritis, (LeBlanc et al. 2007; Wittenbrink et al. 2008).

In the presence of *Escherichia coli* and *Streptococcus equi* subspecies *zooepidemicus* (Rebordão et al. 2014), or in contact with equine semen (Alghamdi and Foster 2005; Alghamdi et al. 2009), the equine–neutrophils produced NETs in the mare endometrium. The induced NETs trap spermatozoa, but the DNase present in seminal plasma can degrade these NETs and free entagled sperm cells. Interestingly, seminal plasma proteins are highly selective suppressing spermatozoa-induced NETs, but not those bacteria-induced NETs (Alghamdi and Foster 2005). The mechanism of neutrophils to fight bacteria causing endometritis include not only phagocytosis but also the entrapment of bacteria by NETs.

If the inflammation/infection becomes chronic, it leads to severe, progressive and irreversible fibrosis of equine endometrium (endometrosis). The long-standing influx of neutrophils into the endometrium may contribute to chronic degenerative changes, culminating in fibrosis. This condition impairs endometrial function and future pregnancies, causing infertility (Hoffmann et al. 2009a). Indeed, the treatment of equine endometrial explants with enzymes found in NETs (ELA, CAT and MPO) act as pro-fibrotic factors, by inducing COL production (Rebordão et al. 2018).

6.2 Equine endometrosis

Kenney (1992) introduced the term endometrosis, meaning occurrence of fibrotic and degenerative changes in mare endometrium. Nowadays, endometrosis might be described as an active or inactive periglandular and/or stromal endometrial fibrosis with alterations of glands within the fibrotic foci (Hoffmann et al. 2009a). Single glands and/or glandular nests can be

affected (Kenney 1978). It has been referred that the degree of endometrosis increases with age, but without connection with the number of foalings (Ricketts and Alonso 1991). In fact, according to Doig et al. (1981), Ricketts and Alonso (1991) and Hoffmann et al. (2009a) endometrosis is more associated to age than parity because aged maiden mares can also develop endometrosis without being exposed to semen or pregnancy. In our laboratory, we observed that young maiden mares may also present endometrosis. The genetic predisposition can justify this finding, causing disruption in endometrial inflammation and repair mechanisms, which results in permanent activation of COL synthesis by mediators released by local and infiltrating immune cells (Oddsdóttir 2007). So, age, repeated pregnancies, parturition, chronic inflammation and endocrine problems seem to drive endometrosis severity (Hoffmann et al. 2009a). In endometrosis, the endometrium undergoes structural changes. There are progressive focal proliferation of the endometrial glands that gather in nests surrounded by numerous fibrous layers, as the process goes on. Inside the nests, there are cysts with decreasing number of normal endometrial glands (Katkiewicz et al. 2007). Lymphatic lacunae and reduction in the number of uterine glands and atrophy can also be observed (Kenney 1978). Impaired uterine clearance (LeBlanc and Causey 2009), repeated endometritis (Doig et al. 1981), aging and multiple pregnancies (Ricketts and Alonso 1991) have been described as triggering factors of endometrosis. In order to predict the capacity of mares to conceive, Kenney (1978) developed an endometrium classification, later modified by Kenney and Doig (1986), based on the quality of glands and lymphatic vessels, fibrotic changes and inflammatory cells. The mares can be classified in four categories that are listed in Table 1.

Table 1: Standard score classification system for histologic changes in equine endometrium, according to Kenney and Doig (1986).

Category	Structural changes in endometrium	% of altered glands	Expecting foaling rate (%)
I	Normal and healthy, active and well distributed glands, little to no inflammatory cells	-	80-90%
IIA	Mild, scatted inflammation and fibrosis around individual branches, lack of glandular nests, slight to moderate inflammatory changes, lymphatic lacunae, partial endometrial atrophy	10-35%	50-80%
IIB	Moderate scattered inflammation and fibrosis, 2-4 fibrotic nests of gland, inflammatory and lymphatic changes are widespread, diffuse and moderately severe	35-60%	10-50%
III	Dilated glands surrounded by layers of fibrotic cells, 5 or more fibrotic nests, diffuse and severe inflammatory changes, severe lymphatic lacunae	>60%	10%

At the initial stage of endometrosis, fibroblasts differentiate into myofibroblasts responsible for the synthesis of collagen fibers, ECM deposition, and ultimately leading to

endometrial periglandular fibrosis (Hoffmann et al. 2009b; Szóstek-Mioduchowska et al. 2019a). When endometrosis gets established, it is characterized by abundant fibrosis, lack cilia and cell boundaries, and present more degenerative cell structures and few organelles (Ferreira-Dias et al. 1994, 1999). Thus, these histological changes are the culprit of a decrease in pregnancy rates in the mare (Kenney 1978; Liepina and Antane 2010).

More recently, Hoffmann et al. (2009a, 2009b) graded endometrosis as active, destructive or non-destructive fibrosis. The differences can be observed by the morphology of stromal cells involved in fibrotic foci (Hoffmann et al. 2009b). Excessive ECM accumulation, dilated glands with extensive epithelial degeneration, large number of α-SMA myofibroblasts, loss of normal architecture and invasion of the glandular lumen by stromal cells are characteristics of destructive endometrosis (Hoffmann et al. 2009b). Active stromal cells with active synthesis of COL and ECM deposition occur in active endometrosis, whereas inactive endometrosis shows metabolically inactive stromal cells. The cycle-associated endocrinological changes do not determine the activity of stromal cells (Hoffmann et al. 2009a).

The predominance of COL type has been controversial. According to Lunelli et al. (2013), the endometrial samples examined of category I and II, from estrus and diestrus, had a predominance of COL3. However, COL1 predominates relative to COL3 in endometrial periglandular fibrosis (Porto 2006). More recently, a study revealed that the COL1 fibers were prevalent in inactive and/or destructive endometrosis, while COL3 fibers were predominant in active and/or non-destructive endometrosis with the concomitant presence of periglandular myofibroblasts (Costa 2015). In a healthy endometrium, the first collagen to be synthesized is COL3, which in turn is gradually replaced by COL1 following the development of fibrotic lesions ,(Masseno 2009; Costa 2015). Moreover, in severe endometrosis COL1 predominates, in contrast to the dominance of COL3 in healthy endometria with little alterations (Pinto-Bravo et al. 2018).

The expression of ESR and PGR receptors in fibrotic tissues has been controversial. Aupperle et al. (2000) reported that their expression in fibrotic glands were lower than in healthy areas. Also, the fibrotic areas appear to be independent of the endocrine uterine control mechanisms because a cycle-asynchronous staining for ESR and PGR in the stromal cells of periglandular fibrosis was noted by Hoffmann et al. (2009a) and Lehmann et al. (2011). The stromal cells are maldifferentiated in endometrosis allowing them to release paracrine signals that are unable to reflect the actual estrous cycle state (Hoffmann et al. 2009a). Although, a mild increase of the epithelial ESR and PGR was observed in active non-destructive fibrotic foci, whereas a decreased expression of these receptors was seen in all other types of endometria, mainly in severe destructive fibrosis (Hoffmann et al. 2009a). On the contrary, Lunelli et al. (2013) found no differences in both receptors expression in fibrotic endometrium.

The degenerative changes may favor the thickening of vessel walls in a phenomenon called angiosclerosis. This situation is associated to lower perfusion in older mares with increasing parturition rates (Grüninger et al. 1998). In one study, two thirds of the mares with endometrosis have angiosclerosis (Hoffmann et al. 2006), but in another report, no correlation between endometrosis and angiosclerosis was found (Lehmann et al. 2011). Moreover, the elastofibrosis of vessels increase with age and are closely related to progression of endometrial fibrosis (Hanada et al. 2014). The mares presenting elastofibrosis during both estrus and diestrus showed lower levels of uterine perfusion, and could impair endometrial glands development, decrease uterine clearance, induce post-breeding endometritis, and hinder the development of the conceptus and overall fertility (Esteller-Vico et al. 2015).

Endometrosis also affects the uterine secretion pattern within the fibrotic loci reducing fertility. In barren mares, there was lower expression of uteroglobin and uterocalin. These proteins were detected in the fibrotic areas, especially in mares suffered from moderate destructive endometrosis (Lehmann et al. 2011). Deficiency of uterocalin contributes to early embryonic death, once it supplies proteins to the embryo (Crossett et al. 1998). Uteroglobin protects the embryo from mare's immune system and its deficiency leads to embryonic loss (Zhang et al. 2000). In destructive endometrosis, both protein levels are decreased (Hoffmann et al. 2009b).

Endometrosis has a wide range of reproductive implications. Mares suffering from destructive endometrosis, have a higher frequency of endometritis, which are associated to impaired function of endometrial glands in the physiological clearance of the endometrium (Hoffmann et al.,2009a). In addition, mares with a higher category grade more frequently retain fluid inside the uterine lumen after insemination (Woodward et al. 2012). The loss of epithelium and uterine mucus blanket in chronic inflamed endometria predisposes to persistent uterine infection (LeBlanc and Causey 2009; LeBlanc 2010). Fibrosis reduces the number of healthy glands, which in turn decrease the exchange of nutrients and metabolic products via placenta, reducing the chance of a viable conceptus (Kenney 1978). In fact, endometrial alterations due to fibrosis development lead to insufficient secretory activity and pregnancy failure, delayed placental development, retarded fetal growth or abortion (Kenney 1978; Hoffmann et al. 2009a; Lehmann et al. 2011).

6.3 Physiopathological mechanisms of endometrosis

Regardless of the extensive research on mare endometritis and endometrosis, the physiopathological mechanism involved in fibrosis establishment in equine endometrium is not fully understood. It is dependent on many factors, which may lead to ECM components deposition. Some pathways have been studied in equine endometrial fibrosis pathogenesis, such as prostaglandins (Rebordão et al. 2019; Szóstek-Mioduchowska et al. 2020b), TGFβ1

(Szóstek-Mioduchowska et al. 2019a), interleukins, such as IL-1α, IL-1β, IL-6, IL-10 (Szóstek et al. 2013; de Holanda et al. 2019; Szóstek-Mioduchowska et al. 2019b) and enzymes found in NETs (Rebordão et al. 2018).

6.3.1 Transforming growth factor β1

In spite of regulation of cell growth, development and tissue remodeling, TGFβ1 is also involved in the pathogenesis of fibrosis in many organs, as well as in equine endometrosis. The role of TGFβ1 in the pathogenesis of equine endometrosis has not been widely investigated. Though, an older study found no differences in the expression of *TGFβ1* mRNA levels between endometrial categories (Cadario et al. 2002). However, the concentration of TGFβ1 increased with the score of endometrial fibrosis, suggesting its involvement in fibroblast activation into α-SMA myofibroblasts (Ganjam and Evans 2006). Nonetheless, Kiesow et al. (2011) found the expression of both isoforms of TGFβ to be reduced in stromal cells within fibrotic foci, possibly due to disturbed hormonal stimulation or stromal synthesis disorders. Recently, in order to determine TGFβ1 involvement in equine endometrosis, Szóstek-Mioduchowska and collaborators (2019a) treated equine explants with TGFβ1 and found increased α –SMA, COL1, COL3 and fibronectin protein relative abundance. The growth factor TGFβ1 induced myofibroblast differentiation, increased ECM component secretion from fibroblasts and stimulated fibroblast proliferation, suggesting its involvement in equine endometrosis (Szóstek-Mioduchowska et al. 2019a). Also in equine fibroblasts and epithelial cells, TGFβ1 affects MMPs and TIMPs expression, suggesting that TGFβ1 is a regulator of equine endometrial remodeling (Szóstek-Mioduchowska et al. 2020a).

6.3.2 Matrix metallopeptidases

Matrix metallopeptidases are involved in fibrosis development, but their regulation and involvement in equine endometrosis is still relatively unknown. A study in equine endometrium reported that the active form of MMP-2 was increased in mare fibrotic endometrium in diestrus (Walter et al. 2005). During bacterial and breeding-induced acute equine endometritis, MMP-2 and -9 are involved in inflammatory response and COL remodeling and subsequent establishment of endometrial fibrosis (Oddsdóttir et al. 2008). In stromal cells of endometrial foci, MMP-2 increased possibly due to the progressive destruction of the glandular basal lamina (Kiesow et al. 2011). Although, Aresu et al. 2012 reported that there were no changes in MMP-2 or -9 expression between normal and fibrotic equine endometrium, another study found out that MMP2 transcription was upregulated in endometrial fibrosis (Centeno et al. 2018). The most recent *in vitro* studies in equine endometrium showed that MMP expression is affected by several mediators of inflammation (interleukins, TGFβ1 and prostaglandins) (Szóstek-Mioduchowska et al. 2019b, 2020a, 2020b), and differs among stages of

endometrosis (Szóstek-Mioduchowska et al. 2020a). The equine endometrial explants treated with cytokines linked inflammation to endometrosis development by cytokines modulation on ECM components expression, MMPs and TIMPs (Szóstek-Mioduchowska et al. 2019b). In equine fibroblasts and epithelial cells treated with TGFβ1, the expression of MMPs and TIMPs are altered during endometrosis, being TGFβ1 a regulator of endometrial ECM remodeling via MMPs and TIMPs in equine endometrial cells (Szóstek-Mioduchowska et al. 2020a).

6.3.3 Prostaglandins

An increasing number of studies have shown that PGs have multiple regulatory actions in tissue remodeling and fibrosis. Equine endometrial samples of different endometrial fibrosis categories were evaluated revealing changes in mRNA of PG synthases and in PG secretion in the development of fibrosis during the estrous cycle. These changes cause disorders on estrous cycle and early embryo losses (Szóstek et al. 2012). Also, the endometrial explants treated with interleukins regulated PG secretion during the progression of endometrosis and may affect embryo implantation (Szóstek et al. 2013). Nevertheless, another study found no differences on PG secretion during the development of fibrosis, while PGE_2 production was up-regulated in mares with subclinical endometritis and fibrosis (Gajos et al. 2015). In equine endometrium explants, challenged with enzymes found in NETs, such as ELA, CAT and MPO, PGE_2 showed a protective effect against endometrosis, mediated mainly by EP2 receptor. This can lead to a reduction of COL deposition in equine endometrium (Rebordão et al. 2019). In response to TGFβ1, both luteal cells (Galvão et al. 2018) and equine epithelial cells reduced PGE_2 secretion (Szóstek-Mioduchowska et al. 2020b). However, $PGF_{2\alpha}$ treatment increased MMPs and COL1 expression by fibroblasts, suggesting that PGs may be involved in equine endometrial pathological remodeling (Szóstek-Mioduchowska et al. 2020b).

6.3.4 Neutrophil extracellular traps

Currently, it is well established that NETs play a role in many fibrotic diseases in several species and organs. Recently, it was described the capacity of equine neutrophils to release NETs when in contact with equine spermatozoa (Alghamdi and Foster 2005; Alghamdi et al. 2009). For the first time, Rebordão et al. (2014) showed that NETs were present *ex vivo* in endometrial mucus from mares with bacterial endometritis. Moreover, it was also showed that equine neutrophils have the capacity to form NETs *in vitro* in the presence of bacteria that causes endometritis (*Streptococcus equi* subspecies *zooepidemicus*, *Escherichia coli* or *Staphylococcus capitis*), or even in the absence of bacteria, when stimulated by PMA. The expression of ELA and MPO in *ex vivo* NETs was also confirmed in this study. The authors concluded that NETs formation, an alternative to phagocytosis mechanism of neutrophils, is an important way to fight bacteria in endometritis, and may be considered for future therapeutic

targets (Rebordão et al. 2014). Additionally, increased MPO levels were detected in uterine lumen of cycling mares with endometritis (Parrilla-Hernandez et al. 2015). More recently, the involvement of NETs enzymes, ELA, CAT and MPO, in the development of endometrosis was the grounds for being considered as pro-fibrotic factors (Rebordão et al. 2018). The equine endometrial explants increased COL1 relative protein abundance and *COL3A1* transcription after treatment with ELA, CAT or MPO. The protease ELA increased COL1 protein relative abundance independently of fibrotic category and estrous cycle phase. The effect of CAT was dependent of endometrium category, as well as estrous cycle phase since it enhanced COL1 protein relative abundance in category IIB/III of FP endometria. The response to MPO treatment appears to be also hormone-dependent because it induced COL1 in I/IIA FP explants, while in type IIB/III the effects were noticed in both estrous cycle phases. Follicular phase might be more susceptible to these pro-fibrotic effects, although MLP may also be susceptible to fibrogenic mediators present in NETs (Rebordão et al. 2018).

6.4 Endometrosis treatment

There is no currently available effective therapy to treat endometrosis in mares. However, for the last three decades, a number of therapies have been proposed. Some treatments, such as mechanical curettage has shown to improve the degree of chronic degenerative endometritis in 80% of the treated mares. In addition, 60% of the mares increased pregnancy rates. Although, the prognosis was worse in older mares, where curettage had no effect (Ricketts 1985).

Dimethyl sulfoxide (DMSO) due to its anti-inflammatory properties was proposed to reduce fibrosis. In fact, an intrauterine administration of 10-30% DMSO reduced chronic inflammatory cell infiltrates and periglandular fibrosis in 30% of tested mares, with no harmful histological changes. However, the treatment did not improve the pregnancy rates comparing to saline-treated mares (Ley et al. 1989).

Kerosene was also used to treat endometrosis (Bracher et al. 1991). The uterine irrigation with 250-500 mL of kerosene caused uterine edema lasting for 1-2 days and after results in expulsion of retained excretions, improving fertility rates. The application of this therapy brings rather a short-term effect, as half of the mares which were pregnant after treatment with kerosene miscarried later (Allen 1993). Otherwise, in a recent study, the treatment using kerosene showed no effect on endometrial histopathology grade (Podico et al. 2020).

One of the first inhibitors of NETs described was DNase and is currently clinically available for the treatment of cystic fibrosis in humans (Fuchs et al. 1994; Papayannopoulos et al. 2011). It is known that DNase fails to clear all the components of NETs, as histones, ELA or CAT, and they can still induce tissue injury (Kolaczkowska et al. 2015). However, DNase

was approved for the treatment of cystic fibrosis showing improving lung function (Jones and Wallis 2003). In a recent study, the use of DNase in bacterial meningitis reduced the bacterial load, confirming that NETs reduced bacterial clearance in central nervous system (Mohanty et al. 2019).

Another approach to treat endometrosis was the use of stem cells. Mesenchymal stem cells (MSCs) have been used because of their immunomodulation activity and capacity to regenerate tissues. The equine adipose tissue-derived MSCs were successfully incorporated by a method similar to artificial insemination, and were widely distributed in the uterus of mares with endometrosis. At 7 and 21 days, the MSCc were detected by fluorescence in the uterine body and horns, but not after 60 days. This can be explained by the fast division of these cells. Likewise, the MSCs were incorporated in clusters in periglandular and glandular tissue, suggesting that MCSc proliferate within the endometrium (Mambelli et al. 2013). In another study, the intrauterine transplantation of equine adipose tissue-derived MSCs induced a positive remodeling of the endometrial tissue in mares with endometrosis until day 60. The MSCs modulated the expression of α-SMA, which was no longer observed at day 7 in uterine glands, suggesting the use of MSCs as therapy in endometrosis (Mambelli et al. 2014). In contrast, despite of adipose-derived stem cells ability to be incorporated in endometrial periglandular tissue and single glands, there were an increase in pro-inflammatory interleukins. The balance between pro- and anti-inflammatory, lytic and fibrotic environment was very subtle (Falomo et al. 2015). Alvarenga et al. (2016) successfully administered by endometrial injection, autologous bone marrow MSCs, opening the clinical trials for the use of these stem cells, as well. In 2017, the presence of MSCs were firstly identified in equine endometrium, offering a promising new therapeutically approach not only for endometrial regeneration but also for other tissues (Rink et al. 2017). The same cells were autologously infused in healthy uterine horns of early diestrus mares and were identified after 6, 12 and 24h in the uterine lumen but not in endometrial tissue, limiting the promising results (Rink et al. 2018). Interesting, in a recent study of intra-ovarian injection of MSCs from donors, besides being well tolerated, only altered the gene expression, but did not improved ovarian function in aged mares. These findings do not support the use of MSCs as treatment for age-related ovarian dysfunction in mares (Grady et al. 2019).

1. Elastase inhibition affects collagen transcription and prostaglandin secretion in mare endometrium during the estrous cycle

Adapted from:

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1.1 Abstract

We have shown that bacteria induce neutrophil extracellular traps (NETs) in mare endometrium. Besides killing pathogens, NETs may contribute for endometrosis (chronic endometrium fibrosis). Since elastase (ELA) is a NETs component that regulates fibrosis and prostaglandin (PG) output, the aim was to evaluate if inhibition of ELA would affect collagen 1 (*COL1A2*) transcription and PGs secretion by endometrium explants, in different estrous cycle phases. Follicular-FP (n=8) and mid luteal–MLP (n=7) phases explants were cultured for 24 or 48h with medium alone (Control), ELA (0.5µg/mL, 1µg/mL), sivelestat - ELA inhibitor (SIV, 10µg/mL), or ELA (0.5µg/mL, 1µg/mL) + SIV (10µg/mL). *COL1A2* gene transcription was done by qPCR and PGE_2 and $PGF_2\alpha$ determination in culture medium by ELISA. In FP, at 24h, ELA0.5 increased *COL1A2* transcription ($P < 0.001$) but its inhibition (ELA0.5 + SIV) decreased *COL1A2* transcription ($P < 0.01$) and $PGF_2\alpha$ production ($P < 0.05$). Also, ELA0.5 + SIV or ELA1 + SIV raised PGE_2 production ($P < 0.01$). At 48h, ELA1 increased *COL1A2* transcription ($P < 0.01$) and $PGF_2\alpha$ production ($P < 0.001$), but its inhibition (ELA1 + SIV) decreased these actions ($P < 0.01$; $P < 0.05$, respectively). Besides, ELA1 + SIV incubation increased PGE_2 ($P < 0.05$). $PGF_2\alpha$ also augmented with ELA0.5 ($P < 0.001$), but lowered with ELA0.5 + SIV ($P < 0.01$). In MLP, ELA0.5 up-regulated *COL1A2* transcription (24h, $P < 0.01$; 48h, $P < 0.001$), but ELA0.5 + SIV decreased it (24h, $P < 0.05$; 48h, $P < 0.001$). At 48h, incubation with ELA1 also increased *COL1A2* transcription and $PGF_2\alpha$ production ($P < 0.05$), but $PGF_2\alpha$ production decreased with ELA1 + SIV incubation ($P < 0.05$). PGE_2 production was higher in ELA1 + SIV incubation ($P < 0.05$). Therefore, ELA inhibition may reduce the establishment of mare endometrial fibrosis by stimulating the production of anti-fibrotic PGE_2 and inhibiting pro-fibrotic $PGF_2\alpha$.

Keywords: elastase, mare, endometrium, fibrosis, elastase inhibitor

1.2 Introduction

We have shown that in mares with bacterial endometritis, neutrophils are able to induce neutrophil extracellular traps (NETs) locally in endometrium (Rebordão et al. 2014). At the infection site, NETs components (histones, elastase, cathepsin G, myeloperoxidase), besides binding and killing microorganisms (Brinkmann 2004; von Nussbaum and Li 2015), may also contribute for mare endometrial fibrosis. This pathology, known as endometrosis, is characterized by chronic deposition of collagen in the endometrium and is ascribed to mare infertility (Kenney and Doig 1986, Lehmann et al. 2011). Indeed, enhanced collagen type I (COL1) production after *in vitro* exposure of mare endometrial explants to NETs components has been observed (Rebordão et al. 2018). Actually, ELA was the NETs component that enhanced *in vitro* COL 1 production the most in mare endometrial explants (Rebordão et al. 2018).

Besides NETS and pro-fibrotic cytokines, prostaglandin (PG) E_2 and $PGF_2\alpha$ may provide additional pathways in fibrogenesis. While PGE_2 triggers anti-fibrotic actions, $PGF_{2\alpha}$ can induce fibrosis in lungs (Olman 2009). Since NETs persistence has been related to fibrogenesis (Korkmaz et al. 2010), inhibition of NETs enzymes might be a therapeutic approach. In fact, sivelestat sodium salt, an elastase (ELA) inhibitor (SIV), has prevented bleomycin-induced pulmonary fibrosis in mice (Takemasa et al. 2012). Thus, we hypothesized that by inhibiting ELA in mare endometrium, COL1 development would reduce and contribute for fibrosis impairment. Therefore, the aim of this study was to evaluate if inhibition of ELA would affect: (i) *collagen type I α1* (*COL1A2*) transcription; and (ii) PGs secretion by endometrium, in different estrous cycle phases.

1.3 Materials and Methods

Uteri and blood were collected *post-mortem* from cyclic mares, euthanized according to European Legislation (EFSA, AHAW/04–027). Mare's estrous cycle phase determination was based on plasma progesterone concentration and macroscopic assessment of ovarian structures (Roberto da Costa et al. 2007). Endometria explants, obtained from follicular (FP; n = 8) and mid luteal phases (MLP; n =7), were incubated for 24h or 48h (Rebordão et al. 2018), as follows: (i) Control – culture medium alone; (ii) Elastase (ELA; 0.5, 1µg/mL; A6959, Applichem GmbH, Germany); (iii) ELA inhibitor: sivelestat sodium salt (SIV; 10µg/mL; sc-361359; Santa Cruz Biotechnology, USA); or with (iv) ELA(0.5, 1µg/mL) + SIV (10µg/mL). After incubation, explants and culture medium were kept frozen.

After RNA isolation and cDNA synthesis from incubated explants, *COL1A2* and ribosomal protein *(RPL32*; as reference gene), were used for qPCR studies. Primers sequences are shown (Table 2). qPCR data were analyzed as described (Rebordão et al. 2018).

Table 2: Primers used in quantitative polymerase chain reaction (qPCR) in experimental work 1.

Gene (Accession number)	Sequence 5'-3'	Amplicon
COL1A2 **(XM_001492939.3)**	Forward: CAAGGGCATTAGGGGACACA	196
	Reverse: ACCCACACTTCCATCGCTTC	
RPL32 **(XM_001492042.6)**	Forward: AGCCATCTACTCGGCGTCA	144
	Reverse: GTCAATGCCTCTGGGTTTCC	

COL1A2 – collagen type 1 alpha2; *RPL32* - ribosomal protein L32.

Prostaglandins in culture medium were determined by Enzyme Immunoassay Kits (PGE_2 ELISA kit, ADI-901-001, Enzo, USA; $PGF_2\alpha$ ELISA kit, ADI-901-069, Enzo). The standard curve for PGE_2 ranged from 39-2,500 pg/mL and the intra- and inter-assay coefficients of variation (CV) were 7.4 and 4.1 %, respectively. For $PGF_2\alpha$, the standard curve ranged from 3-50,000 pg/mL and the intra- and inter-assay CV were 5.9 and 4.3 %, respectively.

Explants viability was assessed by lactate dehydrogenase (LDH) activity by a colorimetric assay kit (ab102526, Abcam, UK). Tissue viability was calculated as described (Schäfer et al. 2010).

Data analysis was performed using GraphPAD PRISM (Version 6.00, 253 GraphPAD Software, San Diego, CA, USA). One-way analysis of variance followed by Tukey's multiple comparisons test was used to compare endometrial explants viability, *COL1A2*, PGE_2 and $PGF_2\alpha$ results. Significance was defined as $P < 0.05$.

1.4 Results

Viability of explants after 1h, 24h and 48h incubation was 95.19±0.7%, 92.64±1.2% and 88.17±2.75, respectively. Differences were found between 1h-48h and 24h-48h incubation ($P < 0.0001$).

In FP, at 24h, ELA0.5 increased *COL1A2* transcription ($P < 0.001$) comparing to control, while its inhibition (ELA0.5 + SIV) decreased it ($P < 0.01$). Also, SIV lowered *COL1A2* transcription with respect to ELA0.5 ($P < 0.001$; Fig. 1A). At 48h, ELA1 increased *COL1A2* transcription ($P < 0.01$) regarding control, which decreased when ELA1 was inhibited (ELA1 + SIV; $P < 0.01$). Again, sivelestat alone decreased gene transcription comparing to ELA1 ($P < 0.05$; Fig. 1B).

In MLP, ELA0.5 upregulated *COL1A2* transcripts compared to control at 24h and 48h ($P < 0.01$), but ELA0.5 + SIV decreased them ($P < 0.05$). At 48h, incubation with ELA1 also increased *COL1* transcription relative to control ($P < 0.05$; Fig. 1C, 1D).

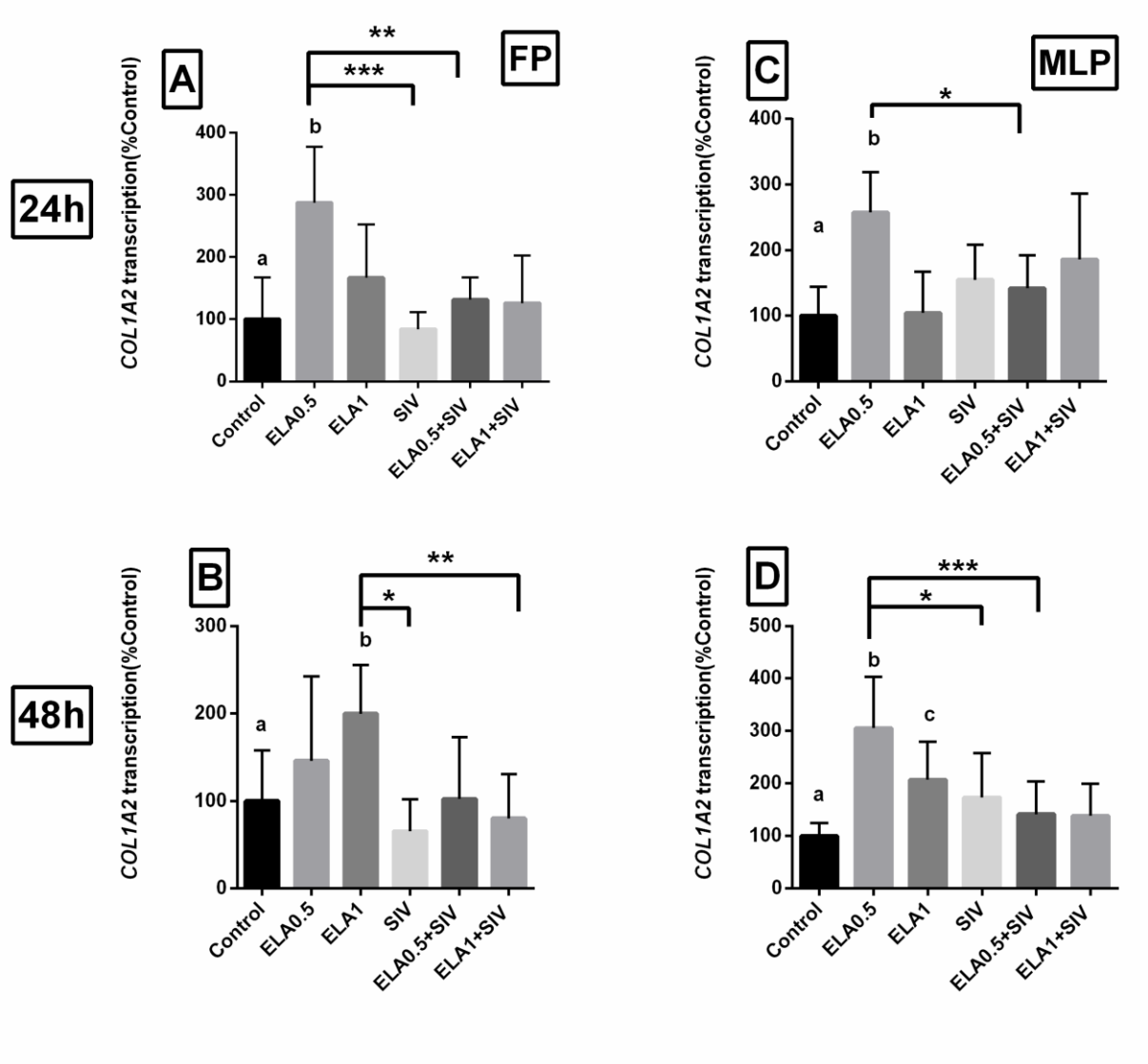


Figure 1: Transcription of *COL1A2* in mare endometrium in follicular-FP (n=8) and mid luteal–MLP (n=7) phases explants cultured for 24 or 48h with medium alone (Control), ELA (0.5µg/mL, 1µg/mL), sivelestat - ELA inhibitor (SIV, 10µg/mL), or ELA (0.5µg/mL, 1µg/mL) + SIV (10µg/mL). FP: A–24h; B–48h; MLP: C–24h; D–48h. All values are expressed as percentage of change from control (non-treated tissues). Bars represent mean ± SD. Significant differences relative to control are depicted by different superscripts (A: a-b, *P* < 0.001; B and C: a-b, *P* < 0.01; D: a-b, *P* < 0.001; a-c, *P* < 0.05). Asterisks indicate significant differences between treatments (P* < 0.05; ***P* < 0.01; ****P* < 0.001).**

In FP, ELA0.5 increased $PGF_{2\alpha}$ production comparing to control (*P* < 0.001) at 48h, but its inhibition (ELA0.5 + SIV) decreased it at 24 and 48h (*P* < 0.05). At 48h, ELA1 also increased $PGF_2\alpha$ production (*P* < 0.001), with respect to control, but decreased with ELA1 + SIV incubation (*P* < 0.05). At 24h, PGE_2 production raised with ELA0.5 + SIV related to control, to ELA0.5 (*P* < 0.01) and to SIV (*P* < 0.05). At 48h, ELA1 + SIV up-regulated PGE_2 production versus control (*P* < 0.05; Fig. 2A, 2B, 3A, 3B). In MLP, while no differences were detected at

24h for PGE_2 and $PGF_2\alpha$ production, at 48h, ELA1 increased $PGF_2\alpha$ comparing to control ($P < 0.05$), and a decrease in $PGF_{2\alpha}$ was found when sivelestat was added (ELA1 + SIV; $P < 0.05$). Also, at 48h, PGE_2 dropped with ELA1 and SIV in respect to control ($P < 0.05$), but increased with ELA1 + SIV compared to ELA1 and SIV ($P < 0.05$; Fig. 2C, 2D, 3C, 3D).

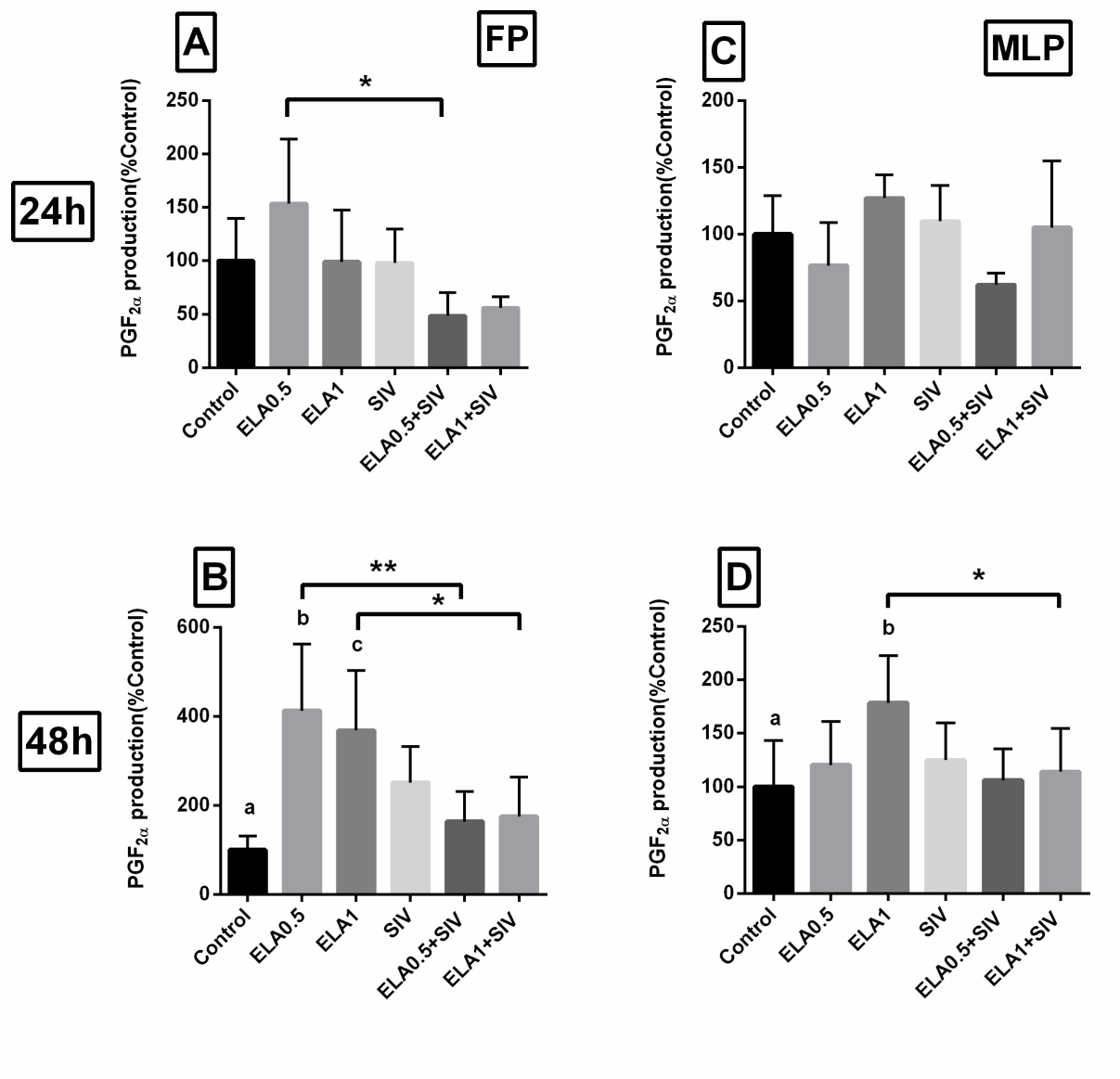


Figure 2: Production of $PGF_{2\alpha}$ by mare endometrium in follicular-FP (n=8) and mid luteal–MLP (n=7) phases explants cultured for 24 or 48h with medium alone (Control), ELA (0.5µg/mL, 1µg/mL), sivelestat - ELA inhibitor (SIV, 10µg/mL), or ELA (0.5µg/mL,1µg/mL)+SIV (10µg/mL). FP: A–24h; B–48h; MLP: C–24h; D–48h. All values are expressed as percentage of change from control (non-treated tissues). Bars represent mean ± SD. Significant differences relative to control are depicted by different superscripts (B: a-b and a-c, $P < 0.001$; D: a-b, $P < 0.05$). Asterisks indicate significant differences between treatments (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

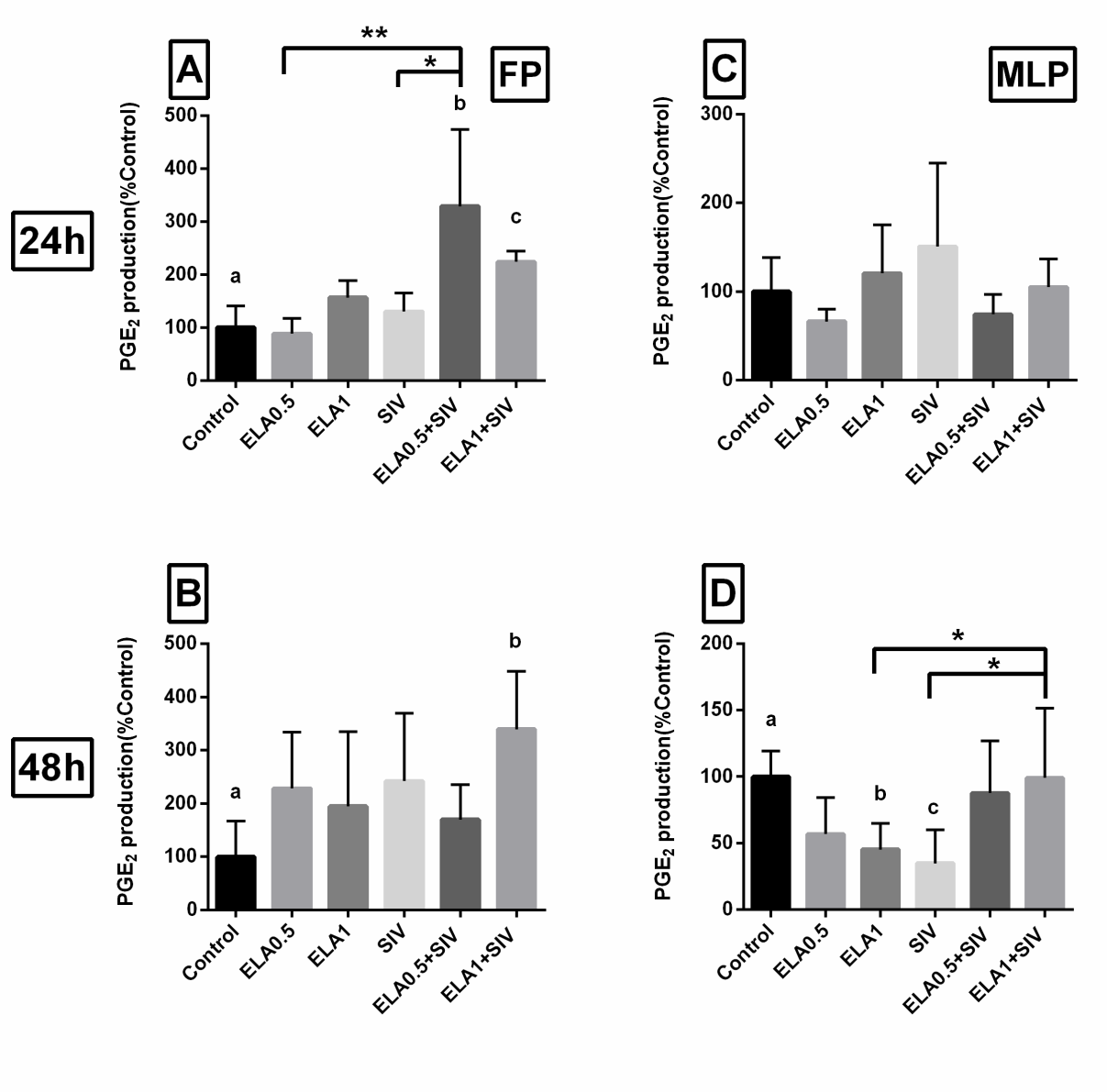


Figure 3: Production of PGE_2 by mare endometrium in follicular-FP (n=8) and mid luteal–MLP (n=7) phases explants cultured for 24 or 48h with medium alone (Control), ELA (0.5µg/mL, 1µg/mL), sivelestat - ELA inhibitor (SIV, 10µg/mL), or ELA (0.5µg/mL, 1µg/mL)+SIV (10µg/mL). FP: A–24h; B–48h; MLP: C–24h; D–48h. All values are expressed as percentage of change from control (non-treated tissues). Bars represent mean ± SD. Significant differences relative to control are depicted by different superscripts (A: a-b and a-c, *P* < 0.01; B: a-b, *P* < 0.05; D: a-b and a-c *P* < 0.05). Asterisks indicate significant differences between treatments (P* < 0.05; ***P* < 0.01; ****P* < 0.001).**

1.5 Discussion

In injured tissue, NETs persistence may lead to chronic inflammation, and ultimately to fibrosis by activation of myofibroblasts (Chrysanthopoulou et al. 2014). In mare endometrium, we have shown that ELA increased COL1 *in vitro* production (Rebordão et al. 2018), as referred in other studies in humans (O'Donoghue et al. 2013). In our present and previous studies, endometrial explants challenged with ELA, showed increased *COL1A2* transcription, both in FP and MLP. Also in lung fibrosis in humans, ELA promoted myofibroblast differentiation (Gregory et al. 2015). Our present data suggest that inhibition of ELA resulted in a reduction of *COL1A2* transcripts in both phases of the estrous cycle. Likewise, in a bleomycin-induced pulmonary fibrosis model in mice, with increased neutrophil ELA levels, sivelestat also inhibited fibrotic changes and inflammatory cell count including neutrophils (Takemasa et al. 2012).

The pro-fibrotic role ascribed to $PGF_{2\alpha}$ in lung (Olman et al. 2009), and in mare endometrium (Rebordão et al. 2016) was also detected in the present work. In FP, this effect was reduced by sivelestat, which was able to inhibit both *COL1A2* transcription and pro-fibrotic $PGF_{2\alpha}$ production. However, in MLP a similar result was only detected at 48h incubation.

In lungs, an anti-fibrotic effect of PGE_2 has been described (Bozyk and Moore 2011), as well as in mare endometrium (Rebordão et al. 2016). Sivelestat increased PGE_2 and lowered *COL1A2* transcripts in FP endometrium. Our results suggest that by inhibiting ELA, mare endometrial fibrosis may be impaired, likely mediated by PG production. Actually, it is in the FP, when the mare is in estrus and breeding occurs that the endometrium is more prone for neutrophil infiltration, endometritis establishment and subsequent NETs formation. Therefore, the action of sivelestat might be a putative therapeutic means to fight endometritis/endometrosis. However, further studies should be carried out to better elucidate the role of sivelestat on fibrosis treatment.

2. The in vitro inhibitory effect of sivelestat on elastase induced collagen and metallopeptidase expression in equine endometrium

Adapted from:

Amaral A, Fernandes C, Rebordão MR, Szóstek-Mioduchowska A, Lukasik K, Gawronska-Kozak B, Telo da Gama L, Skarzynski DJ, Ferreira-Dias G. 2020. The In Vitro Inhibitory Effect of Sivelestat on Elastase Induced Collagen and Metallopeptidase Expression in Equine Endometrium. *Animals*. 10(5):863. doi:10.3390/ani10050863.

2.1 Abstract

Neutrophil extracellular traps (NETs) fight endometritis, and elastase (ELA), a protease found in NETs, might induce collagen type I (COL1) accumulation in equine endometrium. Metallopeptidases (MMPs) are involved in extracellular matrix balance. The aim was to evaluate the effects of ELA and sivelestat (selective elastase inhibitor) on MMP-2 and MMP-9 expression and gelatinolytic activity, as well as the potential inhibitory effect of sivelestat on ELA-induced COL1 in equine endometrium. Endometrial explants from follicular (FP) and mid-luteal (MLP) phases were treated for 24 or 48 h with ELA, sivelestat, and their combination. Transcripts of *COL1A2*, *MMP2*, and *MMP9* were evaluated by qPCR; COL1 protein relative abundance by Western blot, and MMP-2 and MMP-9 gelatinolytic activity by zymography. In response to ELA treatment, there was an increase in *MMP2* mRNA transcription (24 h) in active MMP-2 (48 h), both in FP, and in *MMP9* transcripts in FP (48 h) and MLP (24 h) ($P < 0.05$). Sivelestat inhibited ELA-induced *COL1A2* transcripts in FP (24 h) and MLP (24 h, 48 h) ($P < 0.05$). The sivelestat inhibitory effect was detected in *MMP*9 transcripts in FP at 48 h ($P < 0.05$), but protease activity was unchanged. Thus, MMP-2 and MMP-9 might be implicated in endometrium fibrotic response to ELA. In mare endometrium, sivelestat may decrease ELA-induced COL1 deposition and hinder endometrosis development.

Keywords: endometrosis, mare, elastase, sivelestat, collagen, metallopeptidases, endometrium, neutrophil extracellular traps (NETs)

2.2 Introduction

After breeding, mares develop a transient physiological endometritis, which resolves shortly in healthy uteri. The semen-induced uterine inflammation is characterized by a fast arrival of neutrophils into the uterine lumen (Kotilainen et al. 1994; Katila 1995). The influx of inflammatory cells in the mare's uterus empowers the inflammatory reaction, resulting in the removal of unnecessary spermatozoa, contaminating bacteria, and debris (Troedsson et al. 1993; Troedsson 2006). In addition, active neutrophils at the inflammation site also release their DNA and cytoplasm proteins to the extracellular environment, such as histones, and enzymes as elastase (ELA), cathepsin G (CAT), and myeloperoxidase (MPO), forming neutrophil extracellular traps (NETs) (Brinkmann 2004; Jorch and Kubes 2017). Equine neutrophils produce NETs in the mare endometrium in the presence of *Escherichia coli* and *Streptococcus equi* subspecies *zooepidemicus* (Rebordão et al. 2014), or in contact with equine semen (Alghamdi and Foster 2005; Alghamdi et al. 2009). However, the enzymes found in NETs might also induce a pro-fibrotic response in the endometrium of mares susceptible to chronic endometritis (endometrosis), characterized by the accumulation of collagen type I (COL1), which may link these enzymes to endometrosis pathogenesis (Rebordão et al. 2018; Amaral et al. 2018).

After tissue injury, for extracellular matrix (ECM) reorganization, and especially in the presence of continuous stimuli, the parenchymal tissue is replaced by connective tissue components, such as interstitial COL1 (Wynn 2007). If the balance between ECM synthesis and degradation fails, it leads to fibrosis and to an increase in ECM components deposition and/or a reduction of its degradation. Metallopeptidases (MMPs) are proteases involved in ECM balance maintenance. Among them, MMP-2 and MMP-9 are enzymes that denature collagens (gelatins) and other ECM substrates (Vandooren et al. 2013). However, it has been documented that MMPs can have both stimulatory or inhibitory effects in fibrosis and can act differently among organs (Giannandrea and Parks 2014). MMP-2 and MMP-9 are also related to the migration of fibrocytes in idiopathic pulmonary fibrosis (Pardo et al. 2016), as well as to myofibroblast activation in vascular fibrosis (Harvey et al. 2016). In the liver and kidney, MMP-2 appears to have an anti-fibrotic effect and MMP-9 has a pro-fibrotic role (Giannandrea and Parks 2014). In fact, in the early stages of fibrosis in hepatic tissue, MMP-9 is capable of activating the TGFβ1 pathway, while in the later stages of established fibrosis MMP-2 reduced COL1 relative abundance (Duarte et al. 2015). It has also been suggested that, in pulmonary fibrosis, MMP-9 is linked to inflammatory-induced tissue remodeling, while MMP-2 may be associated with impaired tissue remodeling, leading to abnormal collagen deposition and interstitial fibrosis (Wang et al. 2011). Our studies showed that the endometrial expression of MMPs and their tissue inhibitors (TIMPs) is altered at the different stages of endometrosis, and in response to interleukins (Szóstek-Mioduchowska et al. 2019b, 2020a).

Elastase is a serine protease that has been reported to be increased in neutrophils retrieved from the sputum of cystic fibrosis patients (Dittrich et al. 2018), and to induce in vitro lung fibroblast proliferation and myofibroblast differentiation (Gregory et al. 2015). Recently, we have found that ELA induced *COL1A2* mRNA transcripts (Rebordão et al. 2018; Amaral et al. 2018) and COL1 relative abundance (Rebordão et al. 2018) in equine endometrium explants, suggesting ELA´s involvement in the development of equine endometrosis.

The use of sivelestat sodium salt (SIV), which is a selective inhibitor of ELA retrieved from neutrophils, has shown beneficial effects on fibrosis impairment, either during *in vitro* studies or in clinical trials. Sivelestat has been reported to reduce pulmonary deposition of COL and fibrosis in mice (Takemasa et al. 2012), and to diminish the in vitro *COL1A2* transcription in equine endometrium (Amaral et al. 2018). In addition, SIV administration in human patients with acute lung injury has improved their clinical condition and prognosis (Aikawa et al. 2011; Kido et al. 2016). Altogether, the inhibition of the pro-fibrotic effects of ELA by SIV in several fibrotic diseases in a number species substantiate the use of SIV as a potential therapeutic approach for equine endometrosis. Therefore, the rationale for this study was to evaluate whether COL1 production could be restrained when mare endometrium was challenged by the protease ELA found in NETs. Thus, the aim of this *in vitro* study was to evaluate the inhibitory effect of SIV on ELA induced COL1 protein relative abundance in equine endometrial explants, and the effect of ELA and SIV on the expression and activity of MMP-2 and MMP-9.

2.3 Materials and Methods

2.3.1 Animals and Tissue Collection

The mares used in the present study were healthy, as determined by official veterinary inspection, and presented ovarian cyclicity. These mares were used for meat production for human consumption. They were handled and euthanized at horse abattoirs in Poland, according to the European (EFSA, AHAW/04–027) mandates. From April 2017 to September 2018, uteri were retrieved post-mortem from follicular phase (FP; n = 8) and mid-luteal phase (MLP; n = 7) mares. Prior to euthanasia, peripheral blood samples from the jugular vein were collected into heparinized tubes (Monovettes, Sardtedt, Numbrecht, Germany). Progesterone (P4) plasma concentrations were further determined to confirm the phase of the estrous cycle, firstly based on ovarian structures evaluation immediately after slaughter, as previously described (Roberto da Costa et al. 2007). Briefly, presence of a follicle >35 mm diameter, absence of an active corpus luteum (CL), and plasma P4 concentration <1 ng/mL were characteristic of mares in the FP. In contrast, in the MLP, a well-developed CL was associated with follicles between 15 and 20 mm diameter and a plasma P4 concentration >6 ng/mL (Roberto da Costa et al. 2007). The uteri were immersed in ice-cold Dulbecco's modified Eagle's medium (DMEM) F-12 Ham medium (D/F medium; 1:1 (*v*/*v*); D-2960; Sigma, St Louis,

MO, USA), supplemented with 100 µg/mL streptomycin (S9137; Sigma), 100 IU/mL penicillin (P3032; Sigma), and 2 µg /mL amphotericin (A2942; Sigma). After collection, uteri and blood were transported on ice to the laboratory, within 1 h. All the collected uteri were confirmed for the absence of endometritis, as previously described Rebordão et al. 2018, 2019).

2.3.2 *In Vitro* Endometrial Explant Culture

The uteri were washed in phosphate-buffered saline (PBS) with 100 µg/mL streptomycin (S9137; Sigma) and 100 IU/mL penicillin (P3032; Sigma), and the ipsilateral horn to the active ovary was open and strips of endometrium were detached from myometrium using scissors. Two endometrial samples were immersed in 4% buffered formaldehyde for histological evaluation and endometrial classification. Endometria were histologically graded according to Kenney and Doig´s classification (Kenney and Doig 1986), based on the extent of inflammation and/or fibrosis, as category I, IIA, IIB, or III, corresponding to minimum, mild, moderate, or severe lesions of endometrial fibrosis, respectively. In order to group and normalize the samples, only mare endometria classified as grade IIA or IIB were considered in this study. Thereby, the variation due to endometrium category was excluded from this experiment.

The endometrial strips were placed in phosphate-buffered saline (PBS) with 100 µg/mL streptomycin (S9137; Sigma) and 100 IU/mL penicillin (P3032; Sigma) in a petri dish on ice. Endometrial explants (20–30 mg/well) from FP or MLP were placed in 1 mL of DMEM culture medium supplemented with 0.1% (*w*/*v*) bovine serum albumin (BSA; 735078; Roche Diagnostics, Mannheim, Germany), 100 µg/mL streptomycin (S9137; Sigma), 100 IU/mL penicillin (P3032; Sigma), and 2 µg /mL amphotericin (A2942; Sigma), in a single well in a 24-well sterile cell culture plate (Eppendorf, #0030 722.116) for 1 h, at 37 °C, 5% CO_2 in a humidified atmosphere (Biosafe Eco-Integra Biosciences, Chur, Switzerland) with gentle shaking (150 rpm), as described (Rebordão et al. 2018). After 1 h treatment, the culture medium was replaced, and explants were further treated for 24 h or 48 h, as follows: (i) vehicle (control)—culture medium alone; (ii) elastase (ELA; 0.5 µg/mL; A6959, Applichem GmbH, Germany); (iii) ELA inhibitor: sivelestat sodium salt (SIV; 10 µg/mL; sc-361359; Santa Cruz Biotechnology, USA); (iv) ELA (0.5 µg/mL) + SIV (10 µg/mL); (v) transforming growth factor beta β1 (TGFβ1; 10 ng/mL; GF111; Merck, Darmstadt, Germany), used as a positive control for the assessment of fibrogenic capacity on endometrial explants, as established before (Rebordão et al. 2018; Szóstek-Mioduchowska et al. 2019a); or (vi) oxytocin (OXT; 10^{-7} M), a positive control for prostaglandin (PG) secretion - validation of proper secretory function of endometrial explants in long-term culture (Nash et al. 2008; Szóstek et al. 2013). The ELA inhibitor (SIV) was added at the time of culture medium replacement, while proteases present in NETs were only added 1 h later, to give the inhibitor time to bind. Each treatment was applied

in quadruplicate. After incubation, explants were placed in RNAlater® (R901, Sigma) at 4 °C, overnight. Explants and conditioned culture media were stored at −80 °C. The culture media for PG determination was collected into a 1% stabilizer solution of 0.3 M ethylenediaminetetraacetic acid (EDTA; E5134, Sigma) and 1% aspirin (A2093; Sigma) to prevent PG degradation.

The ELA dose-response assessment was based on a previous study where 0.5 µg/mL proved to induce the release of TGFβ1, a fibrotic marker, and production of COL1 in equine endometrial explants (Rebordão et al. 2018). In addition, the concentration of ELA used is within the range of the concentrations found in physiological and inflammatory processes and has been used in other *in vitro* assays (Voynow et al. 2008). In order to determine the most adequate concentration of SIV, a dose-response trial was carried out based on previous *in vitro* studies that used SIV (0.01, 0.1, 1, 10, and 100 µg/mL) (Misumi et al. 2006; Amaral et al. 2018). In the preliminary work, 10 µg/mL was the optimal concentration of SIV, which was able to inhibit ELA by reducing *COL1A2* transcripts in mare endometrium (Amaral et al. 2018). This SIV concentration provoked an inhibitory effect on *COL1A2* transcription that remained for 24 h, but after the 48 h treatment, this effect was reduced. Therefore, 10 µg/mL of SIV was added again to the culture medium at the end of the 24 h treatment, with explants undergoing a total of 48 h of treatment.

2.3.3 Viability of Endometrial Explants

The viability of endometrial samples was determined based on PG secretion in conditioned culture medium and on lactate dehydrogenase (LDH) activity. Prostaglandin $F_{2\alpha}$ in culture medium was determined by an enzyme immunoassay kit ($PGF_{2\alpha}$ ELISA kit - ADI-901-069, Enzo), according to the manufacturer's instructions. The standard curve ranged from 3 to 50,000 pg/mL and the intra-and inter-assay coefficients of variance (CVs) were 5.9% and 4.3%, respectively. The outputs of $PGF_{2\alpha}$ were used to check the secretory capacity of the non-treated and OXT-treated tissues, suggesting that the endometrial explants contain functional cells (Nash et al. 2008; Szóstek et al. 2013). The LDH activity was assessed by a colorimetric assay kit (ab102526, Abcam, UK) according to the manufacturer's procedures. The enzyme LDH converts pyruvate into lactate with concomitant inter-conversion of NADH, whose concentration was measured. Extracellular LDH activity was measured in explant conditioned culture media (1 h, 24 h, and 48 h incubation) after a 1:100 dilution in the kit assay buffer. For the measurement of intracellular LDH, 10 mg of the incubated explants (1 h, 24 h, and 48 h) was homogenized using a disruptor (TissueLyser II; Qiagen, Madrid, Spain) in 250 µL kit assay buffer and diluted 1:200 times in the same buffer. The LDH activity was read spectrophotometrically (FLUOstar OPTIMA Microplate Reader; BMG Labtech; Ortenberg; Germany) in a kinetic mode at 450 nm wavelength, at 37 °C, for 1 h. Since the point at which

the cell membrane is damaged, and LDH is released to the extracellular environment, explant viability was calculated from the quotient of the intracellular LDH activity and the total activity (extracellular plus intracellular LDH) (Schäfer et al. 2010).

2.3.4 Quantitative Real-Time Polymerase Chain Reaction (qPCR)

Total RNA was extracted using TRI Reagent® (T9424; Sigma) according to the manufacturer's instructions. The quantification of RNA was performed using the Nanodrop system (ND 200C; Fisher Scientific, Hamton, PA, USA) and its quality was assessed by visualization of 28S and 18S rRNA bands after electrophoresis through a 1.5% agarose gel and red staining (41,003; Biotium, Hayward, CA, USA). Reverse transcription was carried out with M-MLV reverse transcriptase enzyme (M5313; Promega; Madison, USA) from 1000 ng total RNA in a 20 µL reaction volume using oligo(dT) primer (C1101; Promega).

Specific primers for *COL1A2, MMP2, MMP9*, and the reference gene ribosomal protein L32 (*RPL32*) were previously designed by us using Primer3 Software and Primer Express (Applied Biosystems, Foster City, CA, USA) (Rebordão et al. 2018). The primers used are listed in Table 3. The genes glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), succinate dehydrogenase A complex, subunit A, flavoprotein (*SDHA*), beta-2-microglobulin (*B2M*), and *RPL32* were tested to determine which should be used as reference gene. In PCRs with efficiencies approaching 100%, the amount of internal reference gene relative to a calibrator (fold change between two Ct values) is given by the following equation: Fold difference = $2^{-\Delta Ct}$. At a reaction efficiency of 100%, one cycle (expressed as Ct in qPCR) corresponds to a twofold change (Dheda et al. 2004). As *RPL32* was the most stable internal control gene in our experimental conditions (less than twofold changes between different biological conditions) (Dheda et al. 2004), it was used as the reference gene throughout the study.

After primer concentrations optimization in a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Warrington, UK), target and reference genes were run simultaneously, and all the reactions were performed in duplicate on a 96-well plate (4306737; Applied Biosystems). Products of PCR were run on a 2.5% agarose gel to confirm specificity, and relative mRNA data were quantified as described (Zhao and Fernald 2005; Rebordão et al. 2018).

Table 3: Primers used in quantitative real-time polymerase chain reaction (qPCR) in experimental work 2.

Gene (Accession Number)	Sequence 5′-3′	Amplicon
COL1A2 **(XM_001492939.3)**	Forward: CAAGGGCATTAGGGGACACA	196
	Reverse: ACCCACACTTCCATCGCTTC	
MMP2 **(XM_001493281.2)**	Forward: TCCCACTTTGATGACGACGA	115
	Reverse: TTGCCGTTGAAGAGGAAAGG	
MMP9 **(NM_001111302.1)**	Forward: GCGGTAAGGTGCTGCTGTTC	177
	Reverse: GAAGCGGTCCTGGGAGAAGT	
RPL32 **(XM_001492042.6)**	Forward: AGCCATCTACTCGGCGTCA	144
	Reverse: GTCAATGCCTCTGGGTTTCC	

COL1A2—collagen type 1 α2; *MMP2*—matrix metallopeptidase 2; *MMP9*—matrix metallopeptidase 9; *RPL32*—ribosomal protein L32.

2.3.5 Western Blot Analysis

Relative protein abundance of COL1 was assessed by Western blot using a stain-free total protein loading control. The tryptophan present in proteins produces an ultraviolet (UV) reaction with trihalo compounds present in 2,2,2-trichloroethanol (TCE; 808610; Merck) used to stain acrylamide gels, which can be visualized as a fluorescent signal in a transilluminator (Ladner et al. 2004; Gilda and Gomes 2013). Endometrial explants were minced and placed on ice-cold RIPA buffer (50 mM Tris-HCl, pH 7.4, 50 mM EDTA, 150 mM NaCl, and 1% Triton X-100) supplemented with a protease inhibitor (cOmplete Mini Protease Inhibitor Cocktail Tablets, 1 tablet per 10 mL of buffer; Roche, Basel, Switzerland) and briefly disrupted (TissueLyser II, Qiagen). After protein extraction, Bradford reagent (500-0006; Bio-Rad, Hercules, CA, USA) was used for determination of protein concentration. Afterwards, 30 µg of protein in 2× Laemmli Loading Buffer (62.5 mM Tris-HCl, pH 6.8 containing 2% SDS, 25% glycerol, 0.01% bromophenol blue) was prepared. Then, the reducing agent dithiothreitol (DTT) was added fresh to obtain a final concentration of 50 mM. Denaturation of proteins was accomplished by heating at 95 °C for 5 min and then cooling on ice for 10 min. The samples were loaded on an 8% acrylamide gel (MB04501; Nzytech, Lisbon, Portugal) with 0.5% (*v*/*v*) TCE incorporated in gel (Ladner et al. 2004) using a Mini-PROTEAN® Vertical Tetra Cell system (Bio-Rad). Just before transfer to a nitrocellulose membrane (GE10600001; Amersham™ Protran® Western blotting membranes, nitrocellulose pore size 0.2 µm, roll W × L 300 mm × 4 m; GE Healthcare; Chicago, IL, USA), the gels were exposed for 1 min to UV light at ChemiDoc XRS + System (Bio-Rad). After transfer (Mini-Trans® Blot, Bio-Rad), the membranes were also exposed for 1 min to UV light to obtain the final image to use in the normalization channel. An image of gels after transfer was also kept, ensuring that the transfer

occurred effectively. The membranes were incubated overnight, at 4 °C with the primary antibody against COL1 (1:1000 diluted; 20121; Novotec, Lyon, France), as previously described and validated (Rebordão et al. 2018). Afterwards, the membranes were incubated with the secondary antibody horseradish peroxidase (HRP)-conjugated anti-rabbit (1:20,000 diluted; P0448; DakoCytomation, Carpinteria, CA, USA) for 1.5 h at room temperature. The COL1 protein relative abundance was visualized using luminol enhanced chemiluminescence (Super Signal West Pico, 34077; Thermo Scientific, Waltham, MA, USA) and image acquisition was performed by ChemiDoc XRS + System (Bio-Rad). A standard sample (30 µg) of mixed endometrial explants was loaded in all gels in a single lane, in order to normalize all bands in the same membrane and to compare bands between membranes. Relative abundance of COL1 protein was analyzed using Image Lab 6.0 (Bio-Rad) software and by creating a multichannel protocol, which allowed the lanes' detection in stain-free total protein membrane image and bands' detection on chemiluminescence image after incubation with the antibodies. The software calculated the normalization factor and volume of target protein, and the values were adjusted for variation in the protein load (Posch et al. 2013). The use of a protein loading control has been questioned owing to its possible instability in certain samples (Gilda and Gomes 2013), variations ascribed to experimental conditions, and the saturation of the chemiluminescent signal from the loading control proteins (Comajoan et al. 2018). Some studies refer to these proteins as not being suitable as a loading control (Gilda and Gomes 2013; Comajoan et al. 2018). Therefore, a better solution is to use a stain-free total protein loading control as it measures the real amount of protein loaded and considers the real differences among samples (Posch et al. 2013). In our preliminary studies, using equine endometrial tissue, this blot normalization technique was shown to produce cleaner images providing an improving normalization (data not shown).

2.3.6 Zymography

The most simple, sensitive, and effective method to analyze MMPs is zymography. It allows the proteins to separate by electrophoresis under denaturing and non-reducing conditions in a polyacrylamide gel containing gelatin to detect proteases, namely gelatinases MMP-2 and MMP-9, which degrade gelatin. As in Western blot analysis, zymography normalization was done using a stain-free total protein loading control. The protein content of culture medium supernatant from the explants cultured was measured using the Bradford method. The general protocol followed was previously described (Manuel and Gawronska-Kozak 2006). Thus, 40 µg of protein in 2× sample buffer (62.5 mM Tris-HCl pH 6.8, 25% glycerol, 4% SDS, and 0.01% bromophenol blue) was loaded, without heating or reduction, to an 8% polyacrylamide gel (MB04501; Nzytech) containing 0.1% gelatin and 0.1% SDS. To verify MMPs' molecular weight, MMP-2 and MMP-9 standards were loaded (Recombinant

Human MMP2 Protein, CF -902-MP-010 and Recombinant Human MMP-9 Western Blot Standard Protein—WBC018; R&D Systems, Minneapolis, MN, USA) in all gels. SDS-PAGE electrophoresis was conducted in Mini-PROTEAN® Vertical Tetra Cell system (Bio-Rad). The gels were then washed with 2.5% Triton X-100 for 40 min and incubated in the development solution (50 mM Tris–HCl buffer pH 7.5, 200 mM NaCl, 0.02% Triton X-100, and 5 mM $CaCl_2$) for 16 h at 37°C. After that, gels were incubated in 10% (*v*/*v*) TCE in a 1:1 methanol/water mixture for 10 min. As TCE can inhibit gelatinases activity, it should not be incorporated in gels (Raykin et al. 2017). Thus, gels were exposed for 5 min to UV light at ChemiDoc XRS + System (Bio-Rad), and then washed in distilled water to remove the TCE solution before staining (50% methanol, 10% acetic acid, and 0.1% Coomassie brilliant blue) for 30 min, and destained in the same solution in the absence of dye, until clear bands were visible. In a way to normalize all lanes and bands in the same gel and compare each gel with all the gels obtained in the experiment, a standard sample (40 µg) of mixed culture medium was loaded in all gels in a single lane. Image Lab 6.0 (Bio-Rad) software was used to analyze MMP-2 and MMP-9 by creating a multichannel protocol, which enabled lane detection in stain-free total protein gel image, and band detection on Coomassie staining image. The software calculated the normalization factor and volume of target protein, and the values were adjusted for variation in the protein load. The use of a stain-free total protein normalization and Coomassie staining is a better way to normalize and overcome variations on the protein loaded in each sample. Besides, this normalization method avoids variations between different experimental conditions and between gels (Raykin et al. 2017).

2.3.7 Statistical Analysis

Statistical analysis of the viability data and TGFβ1 fibrogenic assay was performed using GraphPAD PRISM (Version 6.00, 253 GraphPAD Software, San Diego, CA, USA). One-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test was used to compare endometrial explants viability ($PGF_{2\alpha}$ concentration and LDH activity assay), and the effect of TGFβ1 treatment. These data are shown as mean ± SEM and the results were considered significant at $P < 0.05$.

The response variables evaluated in the experimental work were *COL1A2*, *MMP2*, and *MMP9* transcription measured by qPCR; COL1 protein relative abundance by Western blot; as well as MMP-2 and MMP-9 activity evaluated by zymography in both pro- and active forms. Compliance with normality after various transformations was assessed visually and using the Kolmogorov–Smirnov test in Proc Univariate function of SAS v. 9.4 (SAS Institute Inc., Cary, NC, USA). As many of these variables did not have a normal distribution, the square root and logarithmic transformation were tested, and the best transformation for a given variable was chosen for further analysis. In a preliminary analysis, each transformed response variable was

analyzed with the PROC GLM of SAS, as a function of the various treatments that resulted from the combination of the use of ELA, use of SIV, estrous cycle phase, and time of treatment, for a total of 16 treatment combinations. The least squares means for the various treatment combinations were compared with the PDIFF option of PROC GLM, assuming $P < 0.05$ as the threshold of significance, and the means were back transformed to the original scale for graphical presentation. In a second analysis, the factorial nature of the treatment combinations was evaluated, by considering the main effects of the factors above plus their two-, three-, and four-way interactions, allowing the comparison of specific treatment combinations. The results of COL1 relative abundance protein, *COL1A2*, *MMP2*, and *MMP9* mRNA are shown as median with interquartile range. The MMP-2 and MMP-9 gelatinolytic activity results are shown as least square means ± SEM. The graphs were performed using GraphPAD PRISM. Back-transformed SEM are presented as 95% confidence interval.

2.4 Results

2.4.1 Validation of the Viability of Long-Term Endometrial Explant Culture

A preliminary experiment aimed to verify whether COL1 increases when the endometrial explants are exposed to TGFβ1, a fibrogenic agent. Treatment with TGFβ1 increased *COL1A2* transcription at both phases and times of treatment (FP: 24 h - $P < 0.0001$, 48 h - $P < 0.001$; MLP: 24 h - $P < 0.05$, 48 h - $P < 0.01$; Table 4), and augmented COL1 protein relative abundance in FP at 24 h ($P < 0.001$) and MLP at 24 and 48 h ($P < 0.001$; Table 4).

Table 4: The effect of transforming growth factor beta β1 (TGFβ1) (10 ng/mL) on *COL1A2* mRNA transcription and COL1 protein relative abundance in follicular phase (FP) and mid-luteal phase (MLP) equine endometrial explants treated for 24 h or 48 h, relative to control (non-treated explants). Results are presented as fold-change means ± SEM. Different superscript letters indicate statistical differences between respective columns (within estrous cycle phases and times of treatment).

Estrous Cycle Phase	**FP**				**MLP**			
Time of Treatment	**24 h**		**48 h**		**24 h**		**48 h**	
Treatment	**Control**	**TGFβ1 (10 ng/mL)**	**Control**	**TGFβ1 (10 ng/mL)**	**Control**	**TGFβ1 (10 ng/mL)**	**Control**	**TGFβ1 (10 ng/mL)**
***COL1A2* transcription (fold increase)**	0.66 ± 0.06 [a]	0.97 ± 0.04 [b]	1.02 ± 0.86 [a]	1.82 ± 0.25 [b]	1.00 ± 0.24 [a]	2.75 ± 0.47 [b]	1.00 ± 0.24 [a]	3.86 ± 0.48 [b]
COL1 protein (fold increase)	1.34 ± 0.05 [a]	1.93 ± 0.12 [b]	1.37 ± 0.05 [a]	1.33 ± 0.05 [a]	0.71 ± 0.54 [a]	1.06 ± 0.01 [b]	0.58 ± 0.02 [a]	0.87 ± 0.004 [b]

The viability of endometrial explants determined by LDH activity after 1 h, 24 h, or 48 h incubation is listed in Table 3. Differences were found between 1 h and 48 h, and between 24 h and 48 h incubation ($P < 0.001$; Table 5). The results were independent of estrous cycle phase.

Table 5: Lactate dehydrogenase (LDH) activity measured in conditioned culture medium of equine endometrial explants after 1 h, 24 h, or 48 h incubation. Explants' viability was calculated from the quotient of the intracellular LDH activity and the total activity (extracellular plus intracellular LDH). Results are presented as means ± SEM. Different superscript letters indicate statistical differences within time of incubation.

Time of Incubation	LDH Activity (%)
1 h	94.3 ± 0.9 [a]
24 h	92.6 ± 0.5 [a]
48 h	89.0 ± 0.6 [b]

In addition, $PGF_{2\alpha}$ secretion by endometrial explants after treatment with OXT increased compared with non-treated tissues at 24 h ($p > 0.01$) and 48 h ($p > 0.05$; Table 6). These results were independent of estrous cycle phase.

Table 6: The effect of oxytocin (OXT) on prostaglandin (PG) $F_{2\alpha}$ secretion in equine endometrial explants after 24 h or 48 h. Results are presented as means ± SEM. Different superscript letters indicate statistical differences within the different time of treatment.

Time of Treatment	24 h		48 h	
Treatment	Control	OXT (10−7 M)	Control	OXT (10−7 M)
$PGF_{2\alpha}$ secretion (ng/mg)	7.3 ± 0.8 [a]	16.0 ± 1.3 [b]	7.6 ± 0.9 [a]	14.0 ± 3.2 [b]

There were significant interactions between treatments, time of treatment, and estrous cycle phase. All data are shown in Table 7.

Table 7: Levels of significance (*P* values) for 2- and 3-way interactions between estrous cycle phases, treatment time, and elastase (ELA) or sivelestat (SIV) treatments in the analyses of relative transcript of target genes, COL1 protein relative abundance and gelatinolytic activity of MMP-2 and -9. The results were considered significant at *P* < 0.05 and are highlighted in yellow color.

Interaction	*COL1A2*	COL1	*MMP2*	Pro-MMP-2	Active MMP-2	*MMP9*	Pro-MMP-9	Active MMP-9
ELA x SIV	<0.0001	0.4035	0.1016	0.735	0.7368	0.0002	0.544	0.0728
ELA x treatment time	0.2078	0.6172	0.6266	0.0345	0.6855	0.8901	0.5405	
ELA x estrous cycle phase	0.9161	0.5926	0.1627	0.1991	0.4638	0.0342	0.8451	0.7948
SIV x treatment time	0.9928	0.2083	0.7208	0.449	0.7423	0.1817	0.4907	
SIV x estrous cycle phase	0.1506	0.2031	0.8992	0.0967	0.4069	0.0063	0.6047	0.1537
Time of treatment x estrous cycle phase	0.037	0.0121	0.0144	0.0646	0.0005	0.1068	0.891	
ELA x SIV x treatment time	0.2803	0.1868	0.8579	0.1713	0.5834	0.4412	0.9695	
ELA x SIV x estrous cycle phase	0.0056	0.7039	0.7636	0.7116	0.4531	0.225	0.3224	0.5971
ELA x treatment time x estrous cycle phase	0.2111	0.8787	0.3928	0.1144	0.072	0.1472	0.7899	
SIV x treatment time x estrous cycle phase	0.4026	0.02	0.0998	0.1949	0.8512	0.6236	0.9003	

COL1A2 - collagen type 1 α2; COL1 – collagen type I protein; *MMP2* - matrix metallopeptidase 2; *MMP9* - matrix metallopeptidase 9.

The differences found between estrous cycle phases (FP vs. MLP) within each treatment and treatment time are listed in Table 8.

Table 8: Listed significant differences of the same treatments between the follicular phase (FP) and mid-luteal phase (MLP) of the estrous cycle, within each treatment time.

Evaluated Variables	Treatment Comparisons	P Value	Figures
***COL1A2* transcription**	ELA 48h FP *vs* ELA 48 h MLP	$P < 0.001$	4A, 4B
COL1 protein relative abundance	SIV 48h FP *vs* SIV 48 h MLP	$P < 0.05$	4C, 4D
	ELA + SIV 48 h FP *vs* ELA + SIV 48 h MLP	$P < 0.001$	
***MMP2* transcription**	ELA + SIV 24 h FP *vs* ELA + SIV 24 h MLP	$P < 0.05$	6A, 6B
***MMP9* transcription**	SIV 24 h FP *vs* SIV 24 h MLP	$P < 0.01$	6C, 6D
	SIV 48 h FP *vs* SIV 48 h MLP	$P < 0.01$	
	ELA 24 h FP *vs* ELA 24 h MLP	$P < 0.0001$	
	ELA + SIV 24 h FP *vs* ELA + SIV 24 h MLP	$P < 0.0001$	
	ELA + SIV 48 h FP *vs* ELA + SIV 48 h MLP	$P < 0.01$	
Pro-MMP-2 activity	ELA 48 h FP *vs* ELA 48 h MLP	$P < 0.001$	7A, 7B
	ELA + SIV 48 h FP *vs* ELA + SIV 48 h MLP	$P < 0.05$	
Active MMP-2 activity	ELA 48 h FP *vs* ELA 48 h MLP	$P < 0.001$	7A, 7B

COL1A2 - collagen type 1 α2; COL 1 – collagen type I; *MMP2* - matrix metallopeptidase 2; *MMP9* - matrix metallopeptidase 9; ELA – elastase; SIV – sivelestat sodium salt; FP – follicular phase; MLP – mid-luteal phase.

2.4.2 Inhibitory Effect of Sivelestat on ELA-Induced COL1

Endometrial explants treated with ELA increased *COL1A2* mRNA transcription in FP after 24 h ($P < 0.0001$; Figure 4A), and in MLP after 24 h ($P < 0.01$; Figure 4B) and 48 h ($P < 0.0001$; Figure 4B), compared with the respective control group. However, the combination of ELA and SIV reduced *COL1A2* mRNA, when related to the respective ELA-treated group (FP 24 h: $P < 0.01$; MLP 24 h: $P < 0.05$; MLP 48 h: $P < 0.001$; Figure 4A, B). In ELA-treated explants, *COL1A2* transcripts also increased when compared with the SIV-treated group in FP at 24 h ($P < 0.0001$, Figure 4A), and in MLP at 48 h ($P < 0.01$; Figure 4B). In addition, in FP endometrium treated with ELA for 48 h, COL1 protein relative abundance increased when compared with the SIV-treated group and ELA + SIV-treated group ($P < 0.01$; Figure 4C; 5).

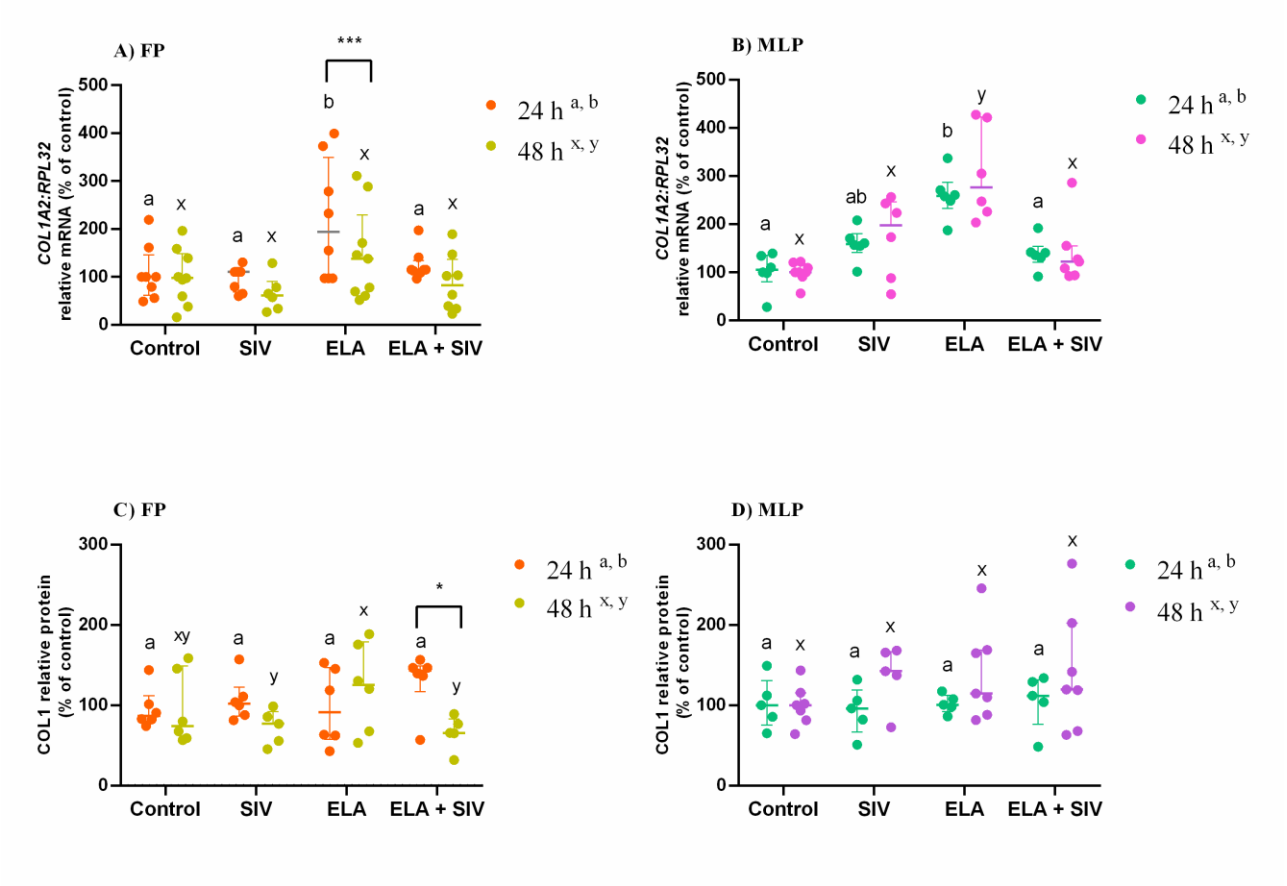
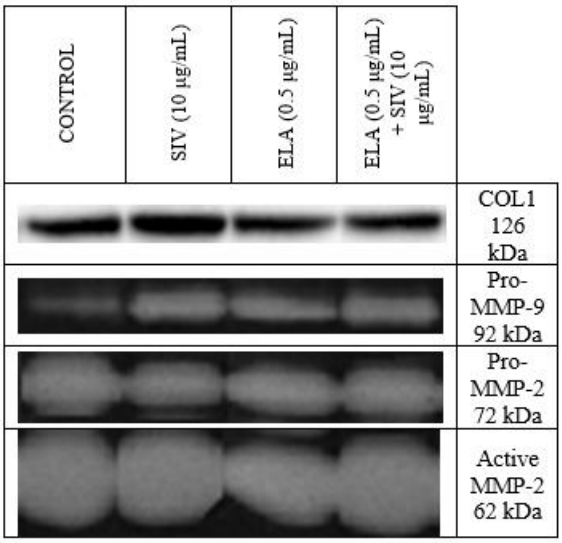
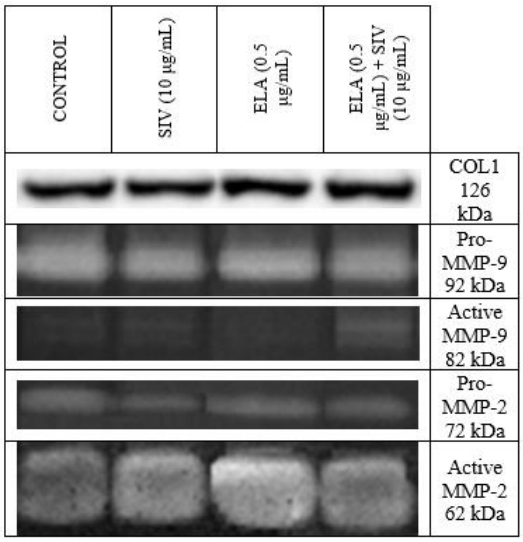


Figure 4: Relative collagen type I (*COL1A2*) mRNA transcription (A, B) and protein (COL1) relative abundance (C, D) in follicular phase (FP) and mid-luteal phase (MLP) mare endometrial explants treated for 24 or 48 h with medium alone (control), elastase (ELA: 0.5 µg/mL), sivelestat (SIV: 10 µg/mL), or ELA (0.5 µg/mL) + SIV (10 µg/mL). Data are shown as median with interquartile range. Results were considered significant at *P* < 0.05. Different superscript letters indicate significant differences between treatments within each treatment time (a,b—24 h; x,y—48 h). Asterisks indicate statistical differences between times of treatment for the same treatment (P* < 0.05; ****P* < 0.001).**

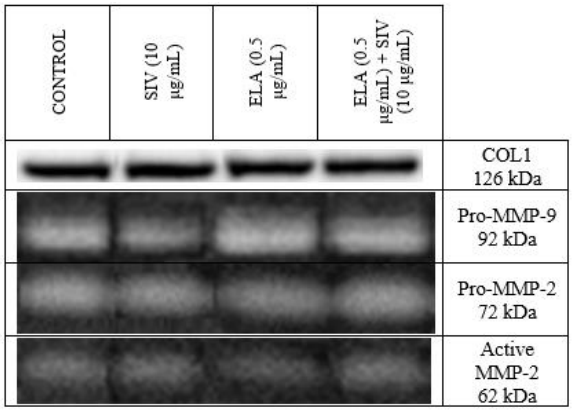
In addition, ELA highly stimulated *COL1A2* transcripts at 24 h of treatment in FP mare endometria, when compared with the 48 h treatment (Figure 4A). In addition, at 48 h, the inhibitory effect of SIV on ELA induced-COL1 protein relative abundance in FP explants was higher compared with 24 h treatment (Figure 4C; 5).



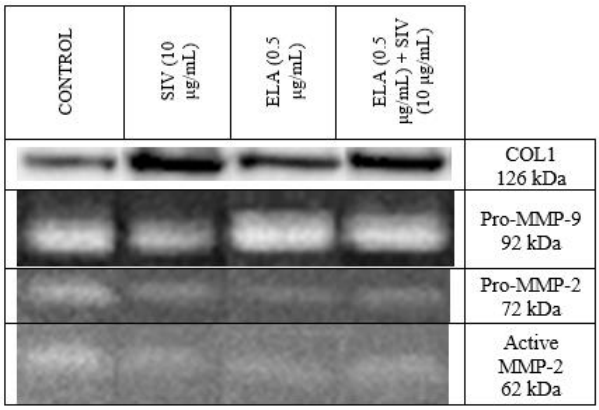
A –24h FP endometrium explants



B – 48h FP endometrium explants



C – 24h MLP endometrium explants



D – 48h MLP endometrium explants

Figure 5: Representative panels of type I collagen (COL1) western blotting and pro- and active form of MMP-2 and MMP-9 zymograms in mare endometrium in follicular phase (FP) or mid-luteal phase (MLP) treated for 24h or 48h with elastase (ELA; 0.5 µg/mL), ELA inhibitor: sivelestat sodium salt (SIV; 10µg/mL) or ELA (0.5 µg/mL) + SIV (10µg/mL). A - 24h treatment of FP endometrium explants; B – 48h treatment of FP endometrium explants, C – 24h treatment of MLP endometrium explants; and D – 48h treatment of MLP endometrium explants.

2.4.3 The Effect of ELA and SIV on MMP expression

Transcription levels of *MMP2* mRNA in endometrial explants were augmented in FP at 24 h with ELA and ELA + SIV- treated group compared with control ($P < 0.01$; $P < 0.05$ respectively; Figure 6A).

The transcripts of *MMP9* were upregulated in FP explants treated with ELA for 48 h, when compared with control ($P < 0.05$; Figure 6C). However, treatment with the combination of ELA + SIV reduced *MMP9,* when compared with the respective ELA-treated group ($P < 0.05$; Figure 6C). In MLP endometria, all treatments upregulated *MMP9* mRNA at 24 h ($P <$

0.01; Figure 6D). The transcripts of *MMP9* in ELA-treated explants were increased at 24 h in MLP with respect to 48 h in the same estrous cycle phase (Figure 6D).

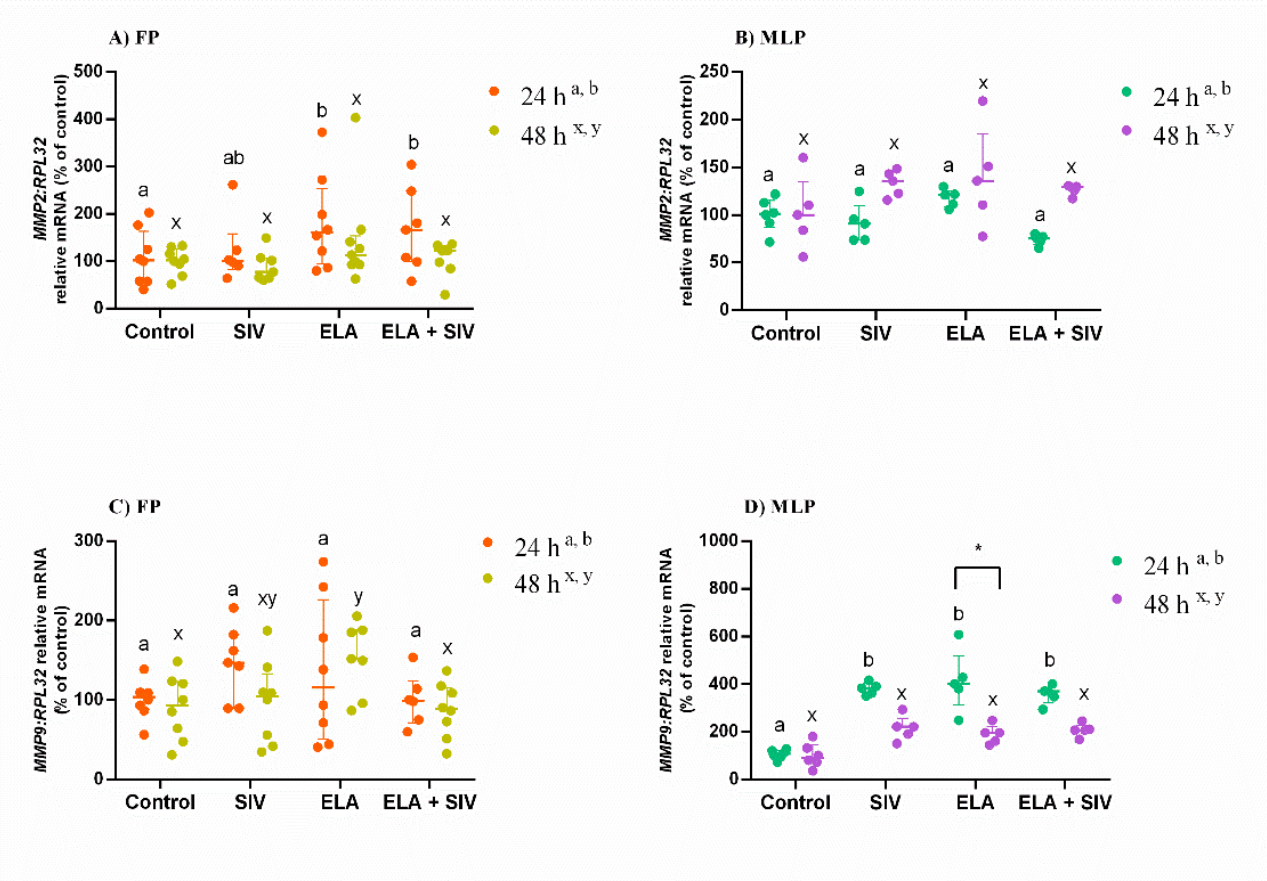


Figure 6: Relative mRNA transcription of matrix metallopeptidase 2 (*MMP2*) (A, B) and *MMP9* (C, D) in follicular phase (FP) and mid-luteal phase (MLP) mare endometrial explants treated for 24 or 48 h with medium alone (control), elastase (ELA: 0.5 µg/mL), sivelestat (SIV: 10 µg/mL), or ELA (0.5 µg/mL) + SIV (10 µg/mL). Data are shown as median with interquartile range. Results were considered significant at *P* < 0.05. Different superscript letters indicate significant differences between treatments within each treatment time (a,b—24 h; x,y—48 h). Asterisks indicate statistical differences between times of treatment for the same treatment (P* < 0.05).**

The activity of pro-MMP-2 increased in MLP endometrial tissue treated for 24 h with ELA, when compared with SIV alone, while after 48 h, the activity subsided in explants treated with ELA compared with control ($P < 0.05$; Figure 7B). However, ELA increased the gelatinolytic activity of MMP-2 active form in FP endometrium after 48 h of treatment when compared with control ($P < 0.05$; Figure 5; 7A).

Differences between 24 and 48 h of treatment were found regarding the activity of pro- and active form of MMP-2 in MLP tissues. Thus, the 24 h treatment of endometrial explants with ELA induced the highest activity (Figure 7B). Nevertheless, it was after a 48 h treatment with ELA that the active form of MMP-2 in FP endometrial explants showed the highest activity

(Figure 5; 7C, 7D). In the active form of MMP-9, only FP explants treated for 48 h showed gelatinolytic activity (Figure 5; 7A).

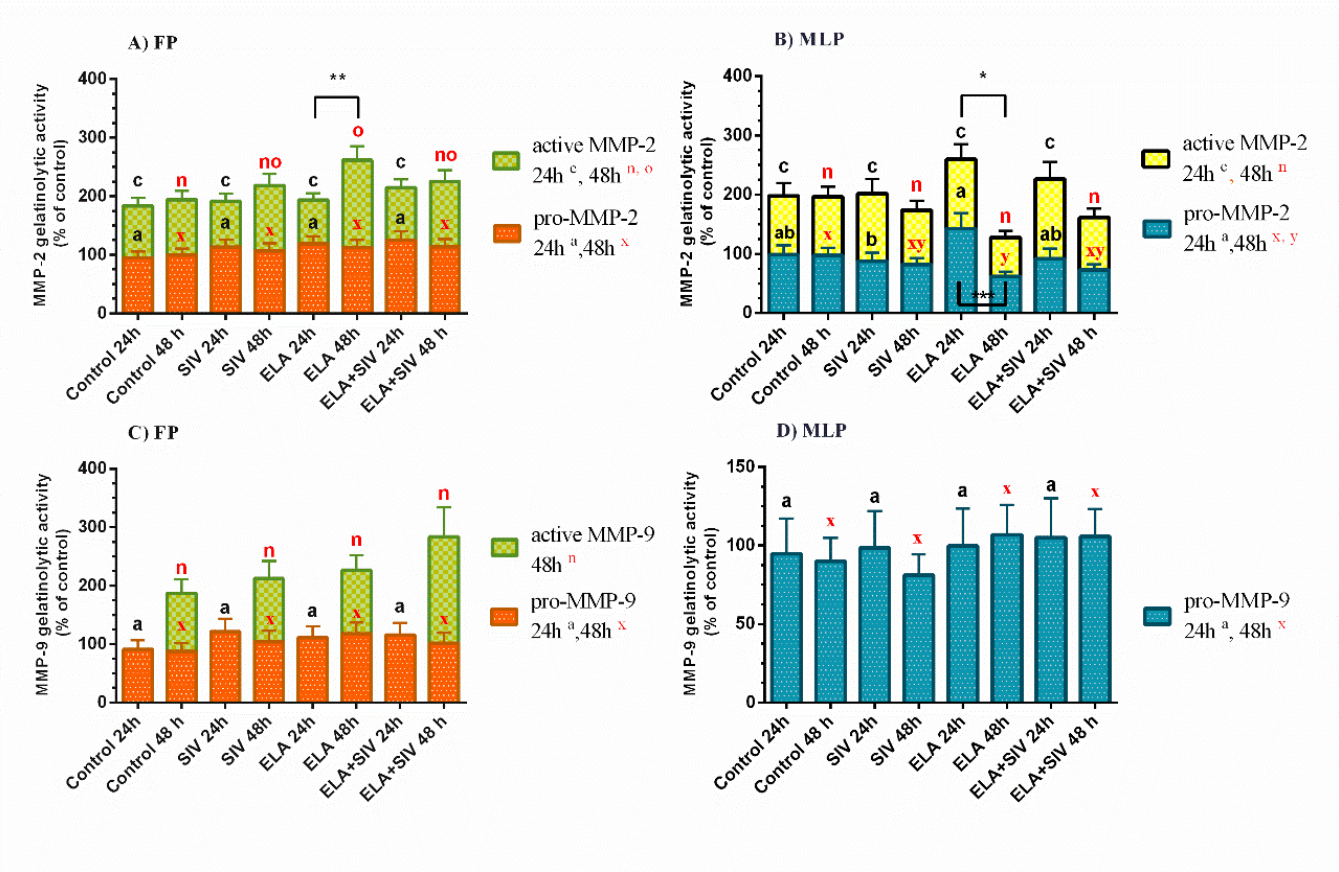


Figure 7: Relative gelatinolytic activities of MMP-2 (A, B) and MMP-9 (C, D) in follicular phase (FP) and mid-luteal phase (MLP) mare endometrial explants treated for 24 or 48 h with medium alone (control), elastase (ELA: 0.5 µg/mL), sivelestat (SIV: 10 µg/mL), or ELA (0.5 µg/mL) + SIV (10 µg/mL). All values are expressed as percentage of change from control (non-treated tissues). Bars represent least square means ± SEM and results were considered significant at *P* < 0.05. Different superscript letters indicate significant differences between treatments within each treatment time. Asterisks indicate statistical differences between different treatment times for the same treatment, and for the same form of MMP (P* < 0.05; ***P* < 0.01; ****P* < 0.001).**

2.5 Discussion

The present study showed that ELA is capable of inducing *COL1A2* mRNA transcription by mare endometrial tissue *in vitro*, in both FP and MLP. This work, reinforced by our previous experiments, strengthens the hypothesis that ELA, as a pro-fibrotic protease, may play a role in the pathogenesis of endometrosis (Rebordão et al. 2018; Amaral et al. 2018). These data are in agreement with our previous study by Rebordão et al. (2018) on endometria with moderate to severe lesions (Kenney and Doig IIB/III category) characteristics of endometrosis, where ELA was also capable of stimulating COL1 protein relative abundance. As a follow-up of those results, SIV was tested here as a specific ELA inhibitor.

In a porcine hepatectomy model of ischemia/reperfusion injury, SIV was reported to avoid organ failure by inhibiting vascular permeability and reducing cytokine production (Shimoda et al. 2019). Studies on the use of SIV have been focused on the response to injury and inflammatory reactions, such as lipopolysaccharide-induced lung injury in rat lungs (Yuan et al. 2014), reduced portal pressure associated with chronic liver diseases in mice (Hilscher et al. 2019), and bleomycin-induced pulmonary fibrosis in mice (Song et al. 2009; Takemasa et al. 2012). One should bear in mind that SIV has been largely reported as being administered to humans, mainly in acute lung diseases, to improve their clinical condition (Polverino et al. 2017). In fact, SIV acts by inhibiting the inflammatory cell recruitment and TGFβ1 activation in lungs, which is the putative mechanism for SIV modulatory action (Takemasa et al. 2012). Therefore, we hypothesized that, inhibiting ELA, it would be possible to reduce COL1 deposition, and thus preventing fibrosis establishment at the course of endometrosis in mares. In fact, the inhibitory effect of SIV on ELA-induced *COL1A2* transcripts was observed in FP and MLP equine endometrium, reinforcing our preliminary results (Amaral et al. 2018). Thus, SIV might be a helpful inhibitor of ELA induced COL1 production in equine endometrium by reducing *COL1A2* gene transcription, and its use in fighting fibrosis may be considered as a putative therapeutic approach.

In the present work, the protein COL1 relative abundance did not follow the gene transcription pattern. The SIV inhibitory effect on ELA-induced COL1 protein relative abundance was only detected in FP explants treated for 48 h with ELA. Thus, it is likely that endometrium from FP, which is endogenously primed with estrogens, is more responsive to SIV treatment to impair COL production than the endometrium under the endogenous influence of progesterone in the MLP. It has been common to use mRNA transcription to predict the relative abundance of corresponding proteins, but the relative abundance of protein may not occur in proportion to their mRNA. Post-transcriptional, translational, and degradation regulation contributes to protein relative abundance at least as much as the transcription itself. The protein relative abundance should focus on the rates of protein production and turnover, and how this can change among different cellular conditions (Vogel and Marcotte 2012). This model can fit in COL deposition in fibrosis, which is a chronic, progressive, and irreversible process. Possibly, the endometrium tries to prevent fibrosis establishment by increasing COL degradation as much as possible. Furthermore, as the high level of COL production needs 5000 times more mRNA than for the average protein, this process can take several days to induce an abundant level of COL protein, in contrast to the minutes needed to induce the synthesis of an average protein (Schwarz 2015). Despite high levels of transcription or translation, the most abundant proteins are often related to a slow translation, but very stable at a high final concentration (Vogel and Marcotte 2012). Therefore, our experimental time window can be too short for the resultant COL protein production to be detected.

The turnover of COL and remodeling of ECM are regulated by MMPs, which are involved in protein degradation and in regulatory functions in inflammation and immunity (Giannandrea and Parks 2014). The knowledge on MMP-2 and MMP-9 regulatory mechanisms facing a fibrotic stimulus is an important way to understand the pathogenesis of endometrosis. As a matter of fact, depending on the severity of endometrosis, the response to cytokine stimulation on MMP-2 and MMP-9 secretion by equine endometrial explants differed, which may associate them with endometrial microenvironment modifications that favor fibrosis establishment (Szóstek-Mioduchowska et al. 2019b). In the present study, endometria with mild/moderate endometrosis lesions (category IIA/IIB) showed different *MMP2* and *MMP9* mRNA levels and protein activity in response to ELA or SIV treatments, either alone or combined, depending on the treatment length. Those previous results (Szóstek-Mioduchowska et al. 2019b) are consistent with ours, where MMPs' expression seems to be different depending on estrous cycle phase and time of treatment. These findings suggest that hormonal changes and duration of the stimulus can affect the endometrial response. The protease ELA was capable of inducing *MMP9* mRNA transcription in FP endometrium at 48 h, and in MLP explants at 24 h. It has been reported that ELA activates pro-MMP-9 in cystic fibrosis in the lung (Voynow et al. 2008). In fact, the gelatinolytic activity of MMP-9 pro-enzyme was detected in equine endometrial explants, even though unchanged, while the active form was only observed in FP after a 48 h treatment, also unaltered. Regarding *MMP2* transcription, ELA treatment was also capable to induce a positive response in FP endometrium at 24 h, and in MMP-2 enzyme activity only at 48 h treatment time. It is worth mentioning that these enzymes are secreted to the extracellular environment or linked to cell membrane as inactive proenzymes (Nissinen and Kähäri 2014), and their activity is regulated by transcription, protein production, and activation of latent enzymes (Sternlicht and Werb 2001). This might explain the fact that the enzyme activity did not follow the gene transcription pattern. Nothnick (2008) noted that *MMP9* transcripts may be present in high levels in the uterus of mice, but translation may be repressed, preventing protein and subsequent MMP-9 activity, with MMP-9 expression also being regulated by ovarian steroids. Taking our results into account, as the estrous cycle phase influenced the endometrial explant response to ELA and SIV treatment, it may be suggested that ovarian steroids in the mare can be implicated in MMPs' secretion, as shown for mice (Nothnick 2008). Metallopeptidases, independent of their proteolytic function, seem to be associated with TGFβ1 activation (Yu and Stamenkovic 2000; D'Angelo et al. 2001; Iida and McCarthy 2007; Kobayashi et al. 2014), activation of other MMPs (Overall 2002), myofibroblast differentiation (Dayer and Stamenkovic 2015), and cell proliferation (D'Angelo et al. 2001; Hattori et al. 2009; Overall 2002; Kobayashi et al. 2014; Dayer and Stamenkovic 2015), thus enhancing fibrosis. However, further studies are needed to confirm their action in the development of endometrial fibrosis in mare.

Despite decades of research on the treatment of endometrosis, no efficient therapy has been found. Even though claims have been made on the anecdotal use of intrauterine infusion of kerosene to treat endometrosis (Bracher et al. 1991), no effect on the endometrium histopathology grade was noted (Podico et al. 2020). In humans, for the treatment of pulmonary fibrotic conditions, SIV has been administered intravenously (Aikawa et al. 2011; Polverino et al. 2017). Likewise, knowledge transfer from the use of this ELA´s specific inhibitor for the treatment of fibrosis in humans could be applied to the horse. Thus, the novel findings from the present *in vitro* study might pave the way for testing the *in vivo* use of SIV in mares to prevent or hinder COL deposition in the endometrium. Specifically, in mares susceptible to post-breeding endometritis, associated with a prolonged inflammatory reaction and neutrophil influx into the uterus, SIV might be a potential therapeutic means to be tested *in vivo* against ELA induced fibrosis establishment. Therefore, this drug may be also beneficial to use in mares, either at the initial stages of fibrosis development, as well as in those showing full-fledged severe endometrosis. However, close caution should be taken, as SIV´s mechanisms of action, doses, as well pharmacokinetics (absorption, distribution, metabolism, excretion, and bioavailability) in horses are unknown. Moreover, different routes of administration, either intravenously or locally by uterine lavage, should be considered. NETs induced fibrosis development in mare endometrium is a complex process wherein many different enzymes are involved (Rebordão et al. 2014, 2018). Rather than ELA, we have shown that other enzymes found in NETs, such as CAT and MPO, also induce COL1 protein relative abundance in equine endometrial explants (Rebordão et al. 2018). As such, because COL deposition in mare endometrium exposed to NETs may result from the effect of many of their enzymes, the use of a combination of different inhibitors of ELA, CAT, and MPO is a promising therapeutic approach to be considered.

2.6 Conclusions

The present data support the hypothesis that the protease ELA present in NETs is capable of inducing *COL1* mRNA transcription in equine endometrium and might be an important player in the regulatory cascade of the pathogenesis of endometrosis in mares. This fibrogenic action is inhibited by ELA selective inhibitor SIV, which may provoke a reduction in COL1 production by the mare endometrium. Moreover, further studies are needed to understand the cellular mechanisms and pathways leading to endometrosis, and the process in which MMP-2 and MMP-9 are involved. The complexity of equine endometrosis suggests that effective therapeutic interventions may require the administration of more than one agent, capable of inhibiting fibrosis in a nonspecific way. The promising results of the present work might be the basis for future development of putative therapeutic means to impair endometrosis.

3. The inhibition of cathepsin G on endometrial explants with endometrosis in the mare

Adapted from:

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3.1 Abstract

Although enzymes found in neutrophil extracellular traps (NETs) have antimicrobial properties, they also stimulate collagen 1 (COL1) production by the mare endometrium, contributing for the development of endometrosis. Cathepsin G (CAT), a protease present in NETs, is inhibited by specific inhibitors, such as Cathepsin G Inhibitor I (β-keto-phosphonic acid; INH). Metallopeptidases (MMPs) are proteases involved in the equilibrium of the extracellular matrix. The objective of this study was to investigate the effect of CAT and INH (a selective CAT inhibitor) on the expression of MMP-2 and MMP-9 and on gelatinolytic activity. In addition, the putative inhibitory effect of INH on CAT-induced COL1 production in mare endometrium was assessed. Endometrial explants retrieved from mares in follicular phase (FP) or mid-luteal phase (MLP) were treated for 24 or 48h with CAT, inhibitor alone, or both treatments. In explants, transcripts (qPCR) of *COL1A2*, *MMP2* and *MMP9*, as well as the relative abundance of COL1 protein (western blot), and activity of MMP-2 and MMP-9 (zymography) were evaluated. The protease CAT induced COL1 expression in explants, at both estrous cycle phases and treatment times. The inhibitory effect of INH was observed on *COL1A2* transcripts in FP at 24h treatment, and in MLP at 48h ($P < 0.05$), and on the relative abundance of COL protein in FP and MLP explants, at 48h ($P < 0.001$). Our study suggests that MMP-2 might also be involved in an earlier response to CAT, and MMP-9 in a later response, mainly in FP. While the use of INH reduced CAT-induced COL1 endometrial expression, MMPs might be involved in the fibrogenic response to CAT. Therefore, in mare endometrium, the use of INH may be a future potential therapeutic means to reduce CAT induced COL1 formation, and to hamper endometrosis establishment.

Keywords: endometrosis, cathepsin G, cathepsin G inhibitor, fibrosis, metallopeptidases

3.2 Introduction

In the endometrium, the innate and adaptive immune mechanisms, which rely on a complex network of key components (mainly growth factors/cytokines, immune cells and epithelial and stromal cells), modulate integrated interactions between the endocrine system and the immune system. As such, they regulate uterine physiological function, and provide protection against pathogens (Skarzynski et al. 2020; Hickey et al. 2011; Sheldon et al 2017). Disruption of those immune-endocrine mediated mechanisms may lead to endometrial dysfunction, and ultimately to fibrogenesis and infertility (Hickey et al. 2011; Sheldon et al 2017).

A transient breeding-induced endometritis is a normal process to remove bacteria and the excess of spermatozoa from the uterus, causing an increase of neutrophils influx to the uterine lumen, which in turn increases the uterine inflammatory reaction (Katila 1995; Troedsson 2006; LeBlanc and Causey 2009). If the inflammation becomes chronic, the persistent influx of neutrophils towards the endometrium prompts to chronic degenerative alterations, ending in endometrosis (endometrial fibrosis) (Hoffmann et al. 2009a). However, impaired uterine clearance (LeBlanc and Causey 2009), repeated endometritis (Doig et al. 1981), aging and multiple pregnancies (Ricketts and Alonso 1991) have been described as triggering factors of equine endometrosis. Equine endometrial fibrosis is a progressive and irreversible severe fibrotic disorder in the endometrium (Hoffmann et al. 2009a; Kenney 1978, 1992), causing subfertility/infertility. At the initial stage of endometrosis, fibroblasts differentiate into myofibroblasts responsible for the synthesis of collagen fibers, extracellular matrix (ECM) deposition, and ultimately leading to endometrial periglandular fibrosis (Hoffmann et al. 2009a; Szóstek-Mioduchowska et al. 2020b). Thus, these histological changes are the culprit of a decrease in pregnancy rates in the mare (Kenney 1978; Liepina and Antane 2010).

The presence of bacteria or semen in the equine endometrium (Alghamdi and Foster 2005; Alghamdi et al. 2009; Rebordão et al. 2014) induces neutrophil migration from blood to the uterus to fight the infection. These neutrophils release proteins and components from the nucleus that form “neutrophil extracellular traps” (NETs) extracellularly (Brinkmann 2004; Alghamdi and Foster 2005; Rebordão et al. 2014). Enzymes present in NETs, namely cathepsin G (CAT), elastase (ELA) or myeloperoxidase (MPO), possess strong antimicrobial properties, aiding on killing bacteria in the extracellular environment. However, their persistence may lead to chronic inflammation and degenerative changes in equine endometrium (Rebordão et al. 2018). Increased collagen type I (COL1) in mare endometrial explants challenged with NETs components have been described previously (Rebordão et al. 2018; Amaral et al. 2018, 2020a).

Cathepsin participates to a greater extent to inflammation and fibrosis establishment in chronic obstructive pulmonary disease (COPD) in humans (Brehm et al. 2014). Also, CAT

action was associated with aortic stenosis remodeling and fibrosis (Helske et al. 2006), renal fibrosis after ischemia (Shimoda et al. 2007) glomerulonephritis and renal failure (Cohen-Mazor et al. 2014), lung cystic fibrosis (Sedor et al. 2007, reviewed by Kosikowska and Lesner 2013; Twigg et al.2015), and fibrotic Dupuytren's disease in humans (Tan et al. 2018). Cathepsin G Inhibitor I (β-keto-phosphonic acid; INH) is a small non-peptide molecule that in a selective, potent, and reversible manner inhibits CAT. This inhibitor could be used for the treatment of COPD and asthma in humans (Brehm et al. 2014; de Garavilla et al. 2005; Maryanoff et al. 2010). Additionally, INH exhibits an anti-inflammatory action in rats with glycogen-induced peritonitis and lipopolysaccharide-induced inflammation of the airways (de Garavilla et al. 2005), and in airway inflammatory diseases dependent on CAT in animal models (Maryanoff et al. 2010).

Matrix metallopeptidases (MMPs) are involved in ECM balance and in endometrial tissue remodeling (Wang and Khalil 2018). These enzymes have the capability to degrade ECM structural components, such as collagen (Salamonsen 2003). In the equine endometrium, during bacterial and breeding-induced acute endometritis, MMP-2 and -9 are engaged in the inflammatory reaction and COL modification (Oddsdóttir et al. 2008). But, if an alteration in the regulation of these MMPs or a prolonged exposure to inflammation occurs, it leads to deposition of COL and subsequent establishment of endometrial fibrosis (Oddsdóttir et al. 2008). In our recent *in vitro* studies on equine endometrium, MMP expression was affected by mediators of inflammation, such as interleukins, transforming growth factor (TGF)β1 and prostaglandins (PG) (Szóstek-Mioduchowska et al. 2019b, 2020a, 2020b), differs among stages of endometrosis (Szóstek-Mioduchowska et al. 2020a) and might be implicated in fibrotic response to ELA (Amaral et al. 2020a).

It has been known that ELA and CAT enzymes released by neutrophils are capable to destroy the ECM, stimulating leukocyte migration and inducing tissue remodeling (Owen and Campbell 1999; de Garavilla et al. 2005). Our previous studies reported them as being also associated with endometrial fibrosis establishment (Rebordão et al. 2018; Amaral et al. 2018, 2020a). In fact, ELA, CAT and MPO appear to act as pro-fibrotic factors in mare endometrosis (Rebordão et al. 2018). The inhibition of ELA using sivelestat, a specific ELA inhibitor, provoked a downregulation of *COL1A2* mRNA transcription (Amaral et al. 2018, 2020a). Among enzymes present in NETs, the one that shows the predominant proteolytic activity is ELA. Nevertheless, when ELA is immune depleted from NETs derived from healthy human neutrophils, the remaining activity was attributed to CAT (O'Donoghue et al. 2013). Moreover, in the pathophysiology of chronic obstructive pulmonary disease in humans, CAT seems to play a particularly important role (de Garavilla et al. 2005), justifying the recent development of diagnostic test that use CAT as COPD marker (Gudmann et al. 2018). Thus, the importance

of studying inhibitors of other enzymes present in NETs, such as CAT, is imperative for the development of putative therapeutic measures for the control of fibrosis.

Since CAT (present in NETs) and MMPs appear to be involved in the development of equine endometrosis (Rebordão et al. 2018; Amaral et al. 2020a), we have decided to investigate putative potential ways of fighting this condition by impairing fibrosis formation. The rationale of this work was to assess if COL1 output was diminished when equine endometrium was exposed to specific inhibitors of CAT. Therefore, the objective of this *in vitro* study was to investigate the INH inhibitory action on the relative abundance of CAT-induced COL1 protein in explants of mare endometrium. In addition, the influence of CAT and INH on MMP-2 and MMP-9 expression and gelatinolytic activity was assessed.

3.3 Materials and Methods

3.3.1 Animals

From April to September, at an abattoir in Poland (Rawicz), uteri and jugular venous blood were randomly retrieved *post-mortem* from cyclic mares destined for meat production, according to the European (EFSA, AHAW/04–027) legislation. Mare's average age was 12 years old. The official veterinary inspection certified that those mares were healthy, and their meat was safe for human consumption. Estrous cycle phase of each mare was determined based on ovarian and uterine features, and on progesterone plasma concentration, as previously described (Rebordão et al. 2018; Roberto da Costa et al. 2007). Thus, mares which presented a follicle >35 mm diameter, absence of an active *corpus luteum*, and plasma progesterone concentration <1 ng/mL were classified as being in the follicular phase. In contrast, the existence of a well-developed *corpus luteum* associated with the presence of follicles with a diameter between 15 and 20 mm, and plasma progesterone concentration >6 ng/mL were the grounds for considering those mares in the mid-luteal phase. For the present study, follicular phase (FP; n = 8), and mid-luteal phase (MLP; n = 7) endometria were used. After collection, uteri, and jugular venous blood in ethylenediaminetetraacetic acid (EDTA) tube were transported on ice to the laboratory. The uteri were placed in ice-cold Dulbecco's modified Eagle's medium (DMEM) F-12 Ham medium (D/F medium; 1:1 (v/v); D-2960; Sigma-Aldrich, St Louis, MO, USA), supplemented with antibiotics, such as penicillin (100 IU/mL; P3032; Sigma-Aldrich) and streptomycin (100 µg/mL; S9137; Sigma-Aldrich), and an antimycotic drug - amphotericin (2 µg /mL; A2942; Sigma-Aldrich). All the mares´ uteri used were examined for the absence of endometritis, both macroscopically and microscopically. The macroscopic exam enabled the visualization of increased mucus production or altered endometrial surface color in the presence of endometritis. The microscopic evaluation of the eventual presence of bacteria and/or neutrophils in the endometrium was accomplished by collecting the cells with a sterile swab, rolled on a glass slide, and colored with Diff-Quick stain (Rebordão et al. 2018,

2019). To perform the histological and endometrial classification (Kenney and Doig 1986) two endometrial samples from each uterus were immersed in 4% buffered paraformaldehyde. Changes in mare endometrium were assessed as described by Kenney and Doig (Kenney and Doig 1986). Regarding the amount of endometrial inflammation and/or fibrosis, endometria were classified as I, IIA, IIB or III categories, according to Kenney and Doig (Kenney and Doig 1986). Slight to scattered inflammation (endometritis), or mild fibrosis (endometrosis) or mild lymphatic lacunae can be found in Kenney and Doig´s category IIA. In category IIB there might be moderate inflammation, but mostly moderate fibrosis that can be multifocal or diffuse, or moderate lymphatic lacunae (Kenney and Doig 1986; Schöniger and Schoon 2020). Although, in this study, only endometria with mild to moderate fibrotic lesions (IIA or IIB category) were used, avoiding endometria with inflammation (endometritis). Besides, no category III endometria were used to exclude possible variations due to increased endometrial fibrotic lesions.

3.3.2 *In vitro* endometrial explant culture

Strips (around 0.5 cm width by 2-3 cm length) of endometrium from the ipsilateral horn to the active ovary were detached from the myometrium after the uterus was washed in PBS with streptomycin (100 µg /mL; S9137; Sigma-Aldrich) and penicillin (100 IU/mL; P3032; Sigma-Aldrich) added.

For explant culture experiments, strips of endometrium were put in ice-cold PBS supplemented with antibiotics (as above) in a Petri dish. Then, the endometrium strips were washed with PBS supplemented with antibiotics and endometrial explants, cut, and blotted with a filter paper. The explants weighting 20-30 mg each were placed in a single well of a sterile 24-well cell culture plate (Eppendorf, #0030 722.116) with 1mL of DMEM culture medium with bovine serum albumin (0.1% (w/v BSA; 735078; Roche Diagnostics, Mannheim, Germany), streptomycin (100 µg/mL; S9137; Sigma-Aldrich), penicillin (100 IU/mL; P3032; Sigma-Aldrich) and amphotericin (2 µg/mL; A2942; Sigma-Aldrich). The endometrial explants were pre-incubated at 38ºC, in a 5% CO_2 humidified atmosphere (Biosafe Eco-Integra Biosciences, Chur, Switzerland), for 1h, and submitted to 150 rpm gentle shaking, as described previously (Rebordão et al. 2018). Afterwards, culture medium was replaced, and equine endometrial explants were treated for 24h or 48h, as follows: (i) vehicle (negative control) – culture medium alone; (ii) cathepsin G (CAT; 1 µg/mL; A6942, Applichem GmbH, Germany); (iii) cathepsin G inhibitor I (INH; 1 µg/mL; β-keto-phosphonic acid; $C_{36}H_{33}N_2O_6P$, sc-221399; Santa Cruz Biotechnology, USA); and (iv) CAT (1µg/mL) + INH (1µg/mL). Each treatment was performed in quadruplicate. The INH was added after 1h of pre-incubation, at the time of culture medium replacement, to allow time for the inhibitor to bind. Protease CAT was added 1h later. In studies undergoing a total of 48h, after 24h treatment, 1 µg/mL of IHN was added

once again to the culture medium, since in the pre-trial its inhibitory effect only remained for 24h and waned at 48h treatment. At the end of each treatment time, explants were collected and placed in RNAlater (R901, Sigma-Aldrich), while conditioned media were collected and stored at -80ºC. In a previous study, as a positive control for COL expression, endometrial tissue response to a fibrotic stimulus was assessed by adding TGFβ1 (a pro-fibrotic cytokine) to tissue culture medium (Amaral et al. 2020a). To assess viability, the explants were also incubated with oxytocin (OXT), as described before (Amaral et al. 2020a).

As shown by our previous work, when dose assessment was determined (Rebordão et al. 2018), the use of 1 µg/mL of CAT proved to induce the expression of fibrotic marker - TGFβ1. A dose-response pilot experiment was performed to assess the most suitable concentration of INH, based in other previous *in vitro* studies (Reich et al. 2009). The INH was tested using 0.01, 0.1, 1, 10 and 100 µg/mL and the optimal concentration that inhibited *COL1A2* transcription was 1 µg/mL (data not shown).

3.3.3 Assessment of endometrial explants viability

The assessment of endometrial explant viability was based on lactate dehydrogenase (LDH) activity as described before (Amaral et al. 2020a) and on OXT-induced $PGF_{2\alpha}$ secretion in conditioned culture medium. The $PGF_{2\alpha}$ secretion was determined by using an enzyme immunoassay kit (ADI-901-069, Enzo), according to the manufacturer's instructions.

3.3.4 Quantitative Real-Time polymerase chain reaction (qPCR)

Total RNA from equine endometrial treated explants was extracted using TRI Reagent® (T9424; Sigma-Aldrich), as indicated by the manufacturer. After, RNA quantification and quality evaluation were performed, as described previously (Amaral et al. 2020a). Specific primers for the reference gene ribosomal protein L32 (*RPL32*) and for *COL1A2, MMP2* and *MMP9* were earlier referred (Amaral et al. 2020a). The reference gene *RPL32* was the most stable internal control, already determined in a previous study (Dheda et al. 2004; Amaral et al. 2020a). All the reactions for target and reference genes were performed in duplicate, on a 96 well plate (4306737; Applied Biosystems) and run in a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Warrington, UK). To confirm specificity, the PCR products were run on a 2.5% agarose gel, and relative mRNA data were quantified, using the qPCR miner algorithm. Briefly, the average of the cyclic threshold (Cq) and the primer efficiency level (E) for each sample were related using the [1/(1þE)Cq] equation. Afterwards the expression levels of the target genes were normalized against the reference gene (Zhao and Fernald 2005).

3.3.5 Western blot analysis

Protein relative abundance of COL1 was determined by western blot technique using a non-staining total protein loading control as previously described (Ladner et al. 2004; Amaral et al. 2020a). The membranes were incubated overnight, at 4ºC with the primary antibody against COL1 (1:1,000 diluted; 20121; Novotec, Lyon, France), as previously defined (Rebordão et al. 2018). The secondary antibody used was Horseradish peroxidase (HRP)-conjugated anti-rabbit (1:20,000; P0448; DakoCytomation, Carpinteria, CA, USA) incubated at room temperature for 1.5h. Visualization of the relative abundance of COL1 protein was accomplished by luminol enhanced chemiluminescence (Super Signal West Pico, 34077; Thermo Scientific, Waltham, MA, USA). For band normalization in each membrane, and to allow band comparison between membranes, a standard sample of a blend of endometrial explants (30 µg) was loaded in a single lane, in all gels. Image acquisition and band normalization were performed, as described (Amaral et al. 2020a; Posch et al. 2013).

3.3.6 Zymography

The activity of MMP-2 and MMP-9 on gelatin gel was assessed by zymography, through a non-staining total protein loading control, as previously described (Manuel and Gawronska-Kozak 2006; Raykin et al. 2017; Amaral et al. 2020a). Gels and samples of culture medium supernatant were handled, as referred by Amaral et al. (2020a). In all gels, molecular weight determination was made using Recombinant Human MMP-2 Protein, CF (902-MP-010; R&D Systems, Minneapolis, USA), and Recombinant Human MMP-9 Western Blot Standard Protein (WBC018; R&D Systems). In order to normalize and compare gels, a standard sample (40 µg) of mixed culture medium was also loaded. The images detection and MMPs gelatinolytic activity were determined as already reported (Amaral et al. 2020a). Briefly, using Image Lab 6.0 (Bio- Rad) software, the lanes were detected in a non-staining total protein gel image and the bands corresponding to MMP-2 and MMP-9 activity were detected on the Coomassie staining image. The normalization factor and volume of target protein were calculated by the software and then the values were adjusted for variation in the protein load.

3.3.7 Statistical analysis

The variables assessed in this study were *COL1A2, MMP2* and *MMP9* transcription, COL1 protein relative abundance and gelatinolytic activity of both pro- and active forms of MMP-2 and MMP-9. The Kolmogorov-Smirnov test in Proc Univariate function of SAS v. 9.4 (SAS Institute Inc.) and visual examination were used to check data normality. The square root and logarithmic transformations were achieved because some of the variables did not show a normal distribution, and the best transformation method was chosen. At first, the response variables were analyzed by PROC GLM of SAS, as a function of the different treatments:

combination of the use of CAT, use of INH, estrous cycle phase, and incubation time, in a total of 16 treatment combinations. Using the PDIFF option of PROC GLM the least square means of the treatments combinations were compared, and the results were considered significant as $P < 0.05$. For data plotting, the means were back transformed to the original scale. Afterwards, the two-, three- and four-way interactions of the treatment combinations were also performed. In Figures 8 and 10, the results of relative abundance of COL1 protein, *COL1A2*, *MMP2* and *MMP9* transcripts are depicted as median with interquartile range. In Figure 11, gelatinolytic activity data for MMP-2 and MMP-9 are shown as least square means ± SEM. The graphs presented were built using GraphPAD PRISM.

3.4 Results

3.4.1 Long-term viability of explants from equine endometrium

As shown before by Amaral et al. (2020a), *COL1A2* transcription and protein relative abundance of COL1 were up regulated in response to TGFβ1 treatment. About viability data, no difference was found in LDH activity between 1h and 24h treatment times, but a slight decrease was shown at 48h, regardless of estrous cycle phase. Besides, mare endometrial tissues treated with OXT augmented $PGF_{2\alpha}$ secretion at both estrous cycle phases and treatment times (Table 9).

Table 9: The effect of oxytocin (OXT, 10^{-7} M) on prostaglandin (PG) $F_{2\alpha}$ secretion from equine endometrial explants in follicular phase (FP) and mid-luteal phase (MLP) treated for 24 h or 48 h, relative to control (non-treated explants). Results are presented as means ± SEM. Different superscript letters indicate statistical differences between respective columns (within estrous cycle phases and times of treatment). The results were considered significant at $P < 0.05$.

Estrous Cycle Phase	FP				MLP			
Time of Treatment	**24 h**		**48 h**		**24 h**		**48 h**	
Treatment	**Control**	**OXT (10^{-7} M)**	**Control**	**OXT (10^{-7} M)**	**Control**	**OXT (10^{-7}M)**	**Control**	**OXT (10^{-7} M)**
$PGF_{2\alpha}$ secretion (ng/mg)	4.43 ± 1.3 ^{a}	7.71 ± 2.8 ^{b}	6.30 ± 1.3 ^{a}	13.9 ± 6.2 ^{b}	8.50 ± 1.6 ^{a}	13.2 ± 2.3 ^{b}	6.4 ± 0.9 ^{a}	11.0 ± 1.5 ^{b}

3.4.2 The effect of INH on CAT-induced COL1

The Table 10 lists the interactions among treatments, time of treatment and estrous cycle phase.

Table 10: Levels of significance (*P* values) for 2-, 3- and 4-way interactions between estrous cycle phases, treatment time, and cathepsin G (CAT) or Cathepsin G Inhibitor I (INH) treatments in the analyses of relative transcript of target genes, COL1 protein relative abundance and gelatinolytic activity of MMP-2 and -9. The results were considered significant at *P* < 0.05 and are highlighted in yellow color.

Interaction	*COL1A2*	COL1	*MMP2*	Pro-MMP-2	Active MMP-2	*MMP9*	Pro-MMP-9	Active MMP-9
CAT x INH	0.0002	0.0237	0.0002	0.0989	0.2229	0.0246	0.522	0.0484
CAT x treatment time	0.8625	0.0247	0.0034	0.5608	0.0029	0.583	0.9213	.
CAT x estrous cycle phase	0.0282	0.9823	0.887	0.2488	0.4438	0.417	0.6549	0.7101
INH x treatment time	0.5519	<.0001	0.4835	0.2351	0.5119	0.0525	0.7105	.
INH x estrous cycle phase	0.5877	0.2437	0.0438	0.3968	0.0987	0.409	0.8159	0.5089
Time of treatment x estrous cycle phase	0.0002	0.0711	0.2474	0.2057	0.7389	0.0079	0.2558	.
CAT x INH x treatment time	0.5701	0.0161	0.4812	0.4717	0.0141	0.6212	0.3652	.
CAT x INH x estrous cycle phase	0.1657	0.1039	0.1516	0.3425	0.2843	0.8317	0.755	0.1491
CAT x treatment time x estrous cycle phase	0.0085	0.6088	0.4743	0.8015	0.0926	0.291	0.7341	.
INH x treatment time x estrous cycle phase	0.9312	0.2582	0.694	0.7429	0.0478	0.611	0.2875	.
CAT x INH x treatment time x estrous cycle phase	0.0365	0.944	0.3722	0.4534	0.645	0.4925	0.7734	.

Abbreviations: *COL1A2* - collagen type 1 α2; COL1 – collagen type I protein; *MMP2* - matrix metallopeptidase 2; *MMP9* - matrix metallopeptidase 9

The differences found between estrous cycle phases (FP vs. MLP) within each treatment and treatment times are listed in Table 11.

Table 11: Listed significant differences of the same treatments between the follicular phase (FP) and mid-luteal phase (MLP) of the estrous cycle, within each treatment time.

Evaluated variables	Treatment comparison	*p* value	Figures
***COL1A2* transcription**	CAT 48h FP vs CAT 48h MLP	*P* < 0.0001	8A, 8B
	CAT + INH 48h FP vs CAT + INH 48h MLP	*P* < 0.05	
COL1 protein relative abundance	CAT + INH 48h FP vs CAT + INH 48h MLP	*P* < 0.05	8C, 8D
***MMP9* transcription**	CAT + INH 48h FP vs CAT + INH 48h MLP	*P* < 0.05	10C, 10D
Pro-MMP-2 activity	CAT 24h FP vs CAT 24h MLP	*P* < 0.05	11A, 11B
Active MMP-2 activity	CAT 48h FP vs CAT 48h MLP	*P* < 0.01	11A, 11B

COL1A2 - collagen type 1 α2; COL 1 – collagen type I; *MMP2* - matrix metallopeptidase 2; *MMP9* - matrix metallopeptidase 9; CAT – cathepsin G; INH – Cathepsin Inhibitor I; FP – follicular phase; MLP – mid-luteal phase.

The treatment with CAT elevated *COL1A2* transcripts in FP endometrial explants at 24h (*P* < 0.01; Fig. 8A), and in MLP tissue at 48h (*P* < 0.0001; Fig. 8B) relative to the respective control group. Nevertheless, the combination of CAT and INH downregulated *COL1A2* transcripts compared to the corresponding CAT-treated groups (FP 24h: *P* < 0.01; MLP 48h: *P* < 0.001; Fig 8A and 8B). In MLP, at 48h, the transcription also increased in CAT-treated explants regarding INH-treated group (*P* < 0.001; Fig. 8B).

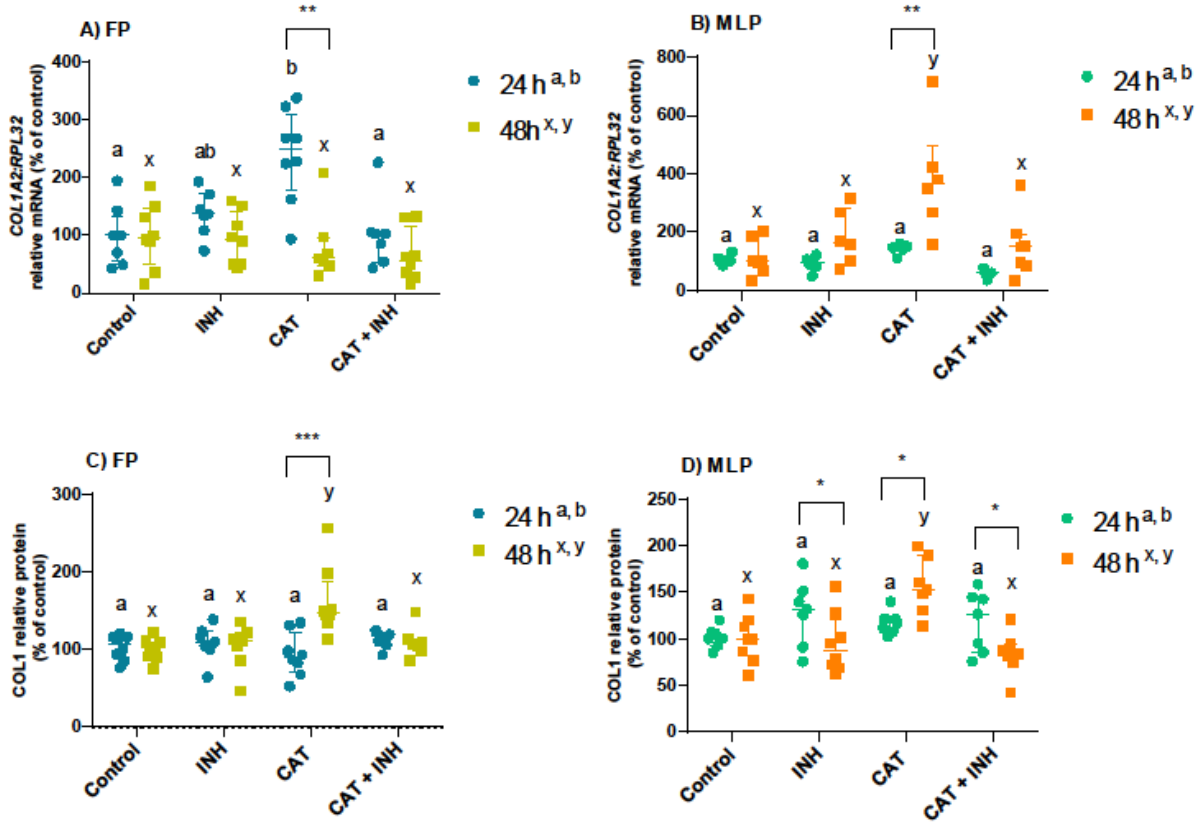
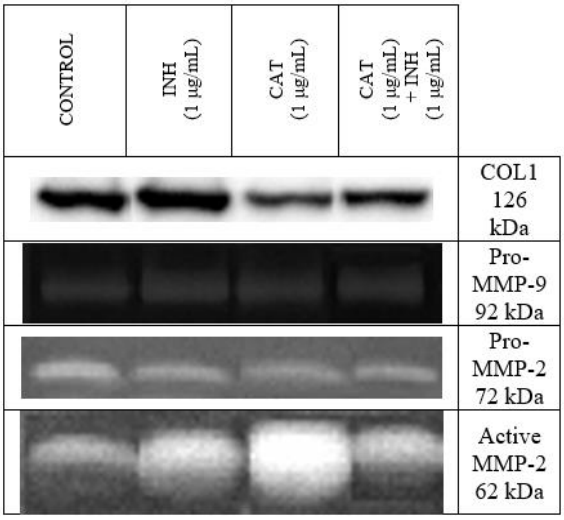
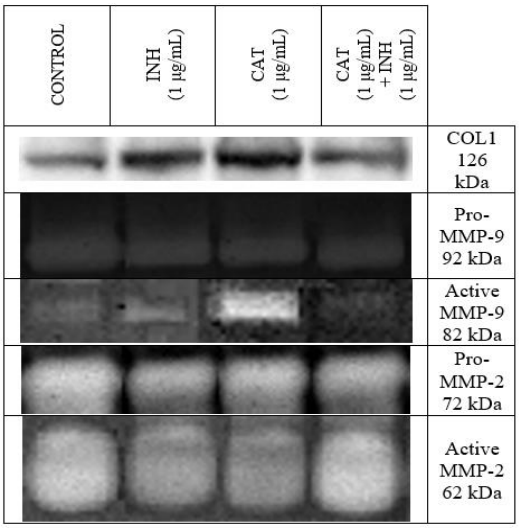


Figure 8: Relative collagen type I (*COL1A2*) mRNA transcription (A, B) and protein (COL1) relative abundance (C, D) in follicular phase (FP) and mid-luteal phase (MLP) mare endometrial explants treated for 24 or 48h with culture medium alone (Control), cathepsin G inhibitor I (INH: 1 µg/mL), cathepsin G (CAT: 1 µg/mL) or CAT (1 µg/mL) + INH (1 µg/mL). Data are shown as median with interquartile range. Results were considered significant at *P* < 0.05. Different superscript letters indicate significant differences between treatments within each treatment time (a,b- 24h; x,y- 48h). Asterisks indicate statistical differences between times of treatment for the same treatment (P* < 0.05; ***P* < 0.01; ****P* < 0.001).**

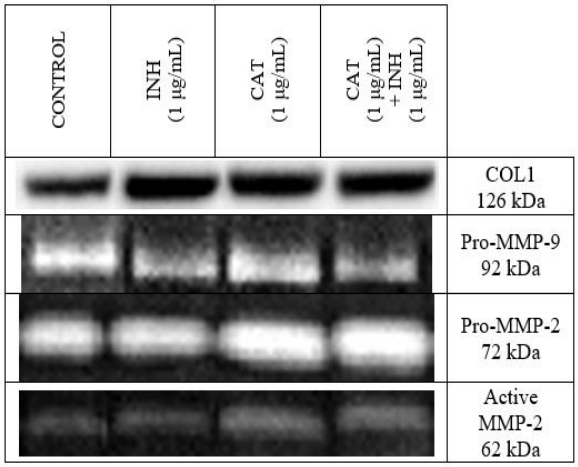
In CAT-treated tissues, COL1 protein relative abundance increased in the longest period of treatment both in FP (*P* < 0.01; Fig 8C) and MLP explants (*P* < 0.001; Fig. 8D, 9) relative to control group. The association of CAT and INH reduced protein relative abundance after 48h treatment both in FP (*P* < 0.01; Fig. 8C) and MLP explants (*P* < 0.001; Fig. 8D; 9) compared to respective CAT-treated groups. Explants treated with CAT, also elevated COL protein relative abundance at 48h, both in FP (*P* < 0.01; Fig. 8C), and in MLP endometria (*P* < 0.001; Fig. 8D, 9), when compared to respective INH-treated group.



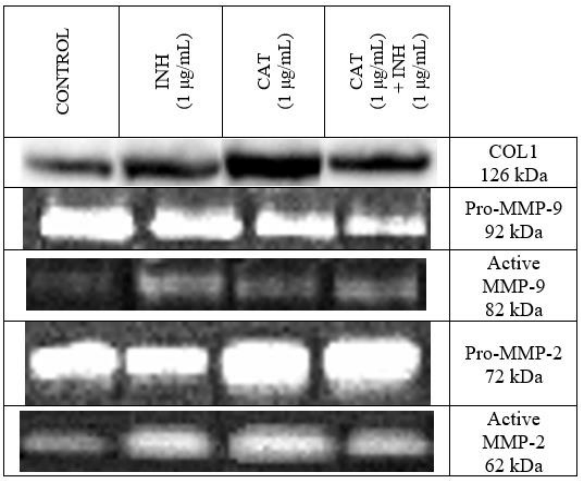
A – 24h FP endometrium explant



B – 48h FP endometrium explant



C – 24h MLP endometrium explant



D – 48h MLP endometrium explant

Figure 9: Representative panels of type I collagen (COL1) western blotting and pro and active form of MMP-2 and MMP-9 zymograms in mare endometrium in follicular phase (FP) or mid-luteal phase (MLP) treated for 24h or 48h with cathepsin G (CAT 1 µg/mL), CAT inhibitor I (INH; 1µg/mL) and CAT (1µg/mL) + INH (1µg/mL). A - 24h treatment of FP endometrium explants; B – 48h treatment of FP endometrium explants, C – 24h treatment of MLP endometrium explants; and D – 48h treatment of MLP endometrium explants.

At 24h, in FP, in CAT-treated group *COL1A2* mRNA transcription was higher when compared to 48h (Fig. 8A), although the protein relative abundance was higher at 48h (Fig. 8C). But, in MLP tissues, CAT treatment up-regulated *COL1A2* transcripts (Fig. 8B), and COL1 protein relative abundance (Fig. 8D) at 48h when compared to 24h. Also, in MLP at 48h, the COL1 protein relative abundance was reduced in INH-treated and CAT+INH-treated groups when compared to 24h treatment (Fig. 8D, 9).

3.4.3 Evaluation of CAT and INH effect on MMP expression

The *MMP2* transcript levels increased in CAT-treated explants in FP at 24h compared to its respective control group ($P < 0.001$) and INH-group ($P < 0.05$; Fig. 10A). But, when those explants were submitted to the combination of CAT and INH, there was a reduction in *MMP2* mRNA, comparing to the respective CAT-treated tissues ($P < 0.01$; Fig. 10A). In the same estrous cycle phase, but after 48h treatment, CAT+INH treatment reduced *MMP2* transcripts in relation to CAT-treated group ($P < 0.01$; Fig. 10A), which was not increased when compared to control. In MLP explants, at 24h, CAT treatment augmented *MMP2* mRNA when related to respective control ($P < 0.05$; Fig. 10B).

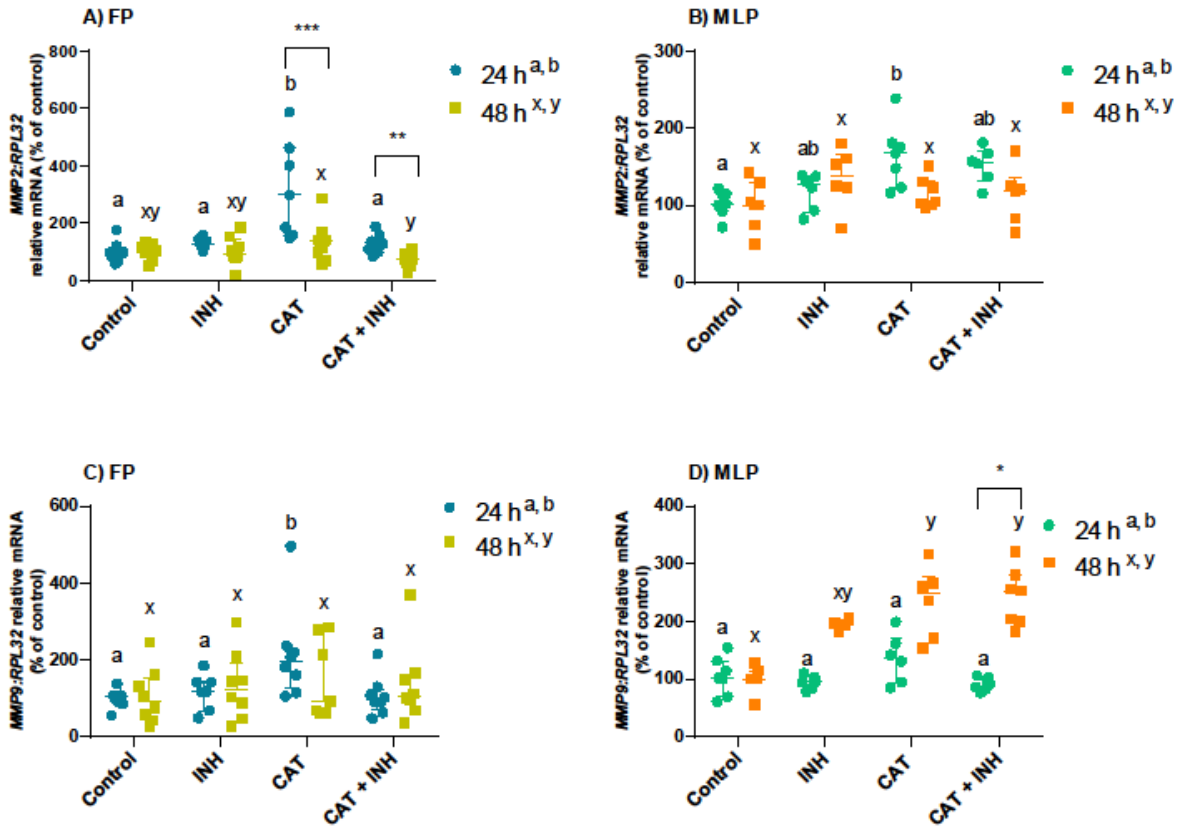


Figure 10: Relative mRNA transcription of *MMP2* (A, B) and *MMP9* (C, D) in follicular phase (FP) and mid-luteal phase (MLP) mare endometrial explants treated for 24 or 48h with culture medium alone (Control), cathepsin G inhibitor I (INH: 1 µg/mL), cathepsin G (CAT: 1 µg/mL), or CAT (1 µg/mL) + INH (1 µg/mL). Data are shown as median with interquartile range. Results were considered significant at $P < 0.05$. Different superscript letters indicate significant differences between treatments within each treatment time (a,b- 24h; x,y- 48h). Asterisks indicate statistical differences between times of treatment for the same treatment (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

In the FP, at 24h, the CAT treatment was able to increase *MMP9* mRNA levels in endometrial explants with respect to the respective control group ($P < 0.01$; Fig. 10C), and INH-treated group ($P < 0.05$; Fig. 10C). However, the CAT+INH-treated explants reduced *MMP9* transcripts compared to CAT-treated group ($P < 0.05$; Fig. 10C). At 48h, MLP endometrium treated with CAT up-regulated *MMP9* transcription ($P < 0.05$), which further increased with CAT + INH treatment ($P < 0.01$; Fig. 10D) compared to non-treated group.

In FP, the treatments of CAT and CAT + INH increased *MMP2* transcripts at 24h in comparison to 48h (Fig. 10A). In contrast, in MLP endometrium, in explants treated for 48h, the combination of CAT and INH augmented *MMP9* transcripts with respect to 24h treatment ($P < 0.05$; Fig. 10D).

The analysis of the pro-form of MMP-2 gelatinolytic activity has shown that INH-treated and CAT-treated groups decreased its activity in FP at 24h ($P < 0.05$; Fig. 9, 11A). Nevertheless, in FP endometrial explants treated for 24h with CAT and combination of CAT+INH, the gelatinolytic activity of MMP-2 active form was up-regulated with respect to control group ($P < 0.001$; $P < 0.05$ respectively; Fig 9, 11A). The active MMP-2 gelatinolytic activity was augmented in MLP tissues treated for 24h with CAT compared to control group ($P < 0.05$; Fig. 11B). At 48h, in MLP, CAT treatment increased active MMP-2 gelatinolytic activity comparing to INH-treated group ($P < 0.05$; Fig. 9, 11B).

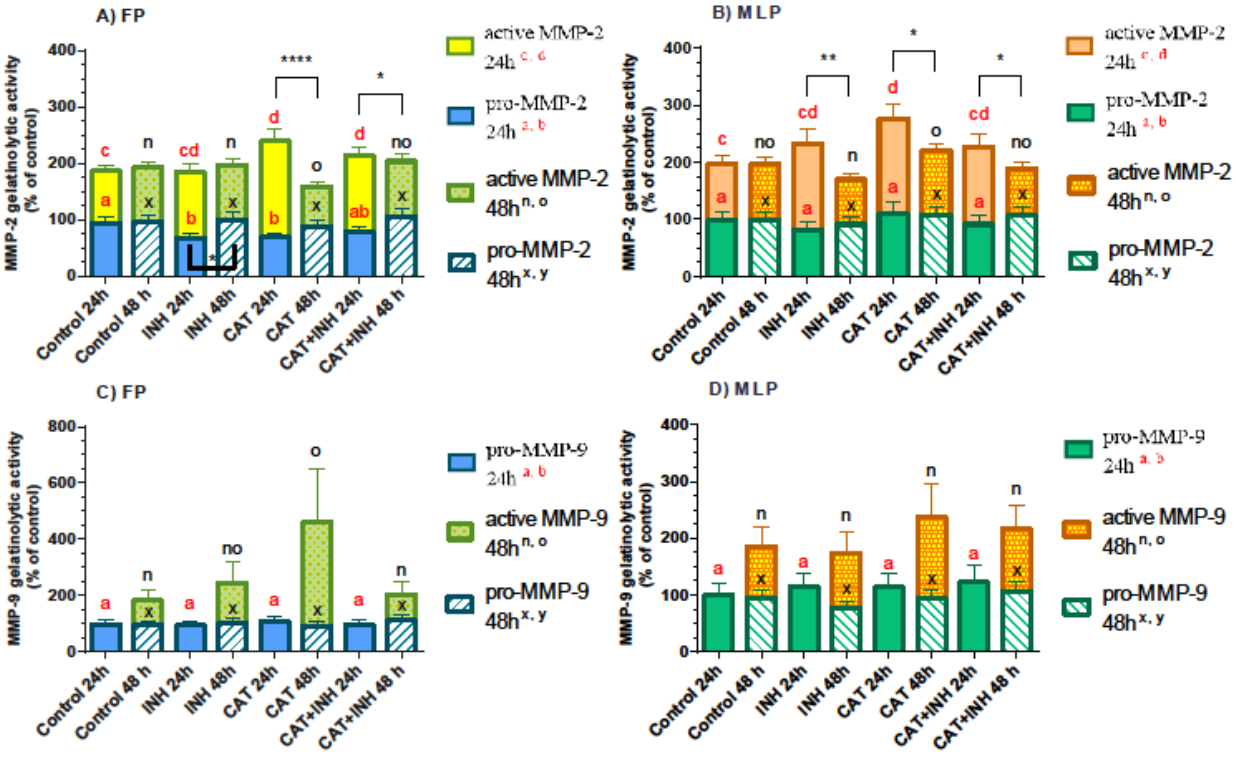


Figure 11: Relative gelatinolytic activities of MMP-2 (A, B) and MMP-9 (C, D) in follicular phase (FP) and mid-luteal phase (MLP) mare endometrial explants treated for 24 or 48h with culture medium alone (Control), cathepsin G inhibitor I (INH: 1 µg/mL), cathepsin G (CAT: 1 µg/mL), or CAT (1 µg/mL) + INH (1 µg/mL). All values are expressed as percentage of change from control (non-treated tissues). Bars represent least square means ± SEM and results were considered significant at *P* < 0.05. Different superscript letters indicate significant differences between treatments within each treatment time. Asterisks indicate statistical differences between different treatment times for the same treatment, and for the same form of MMP (P* < 0.05; ***P* < 0.01; *****P* < 0.0001).**

The gelatinolytic activity of MMP-9 active form was detected in both estrous cycle phases, but only at 48h treatment (Fig. 11C, 11D). In FP explants, treated with CAT, the active MMP-9 gelatinolytic activity increased comparing to control group (*P* < 0.05; Fig. 11C), and was reduced in CAT+INH-treated tissues, in comparison to the respective group treated with CAT (*P* < 0.05; Fig. 9, 11C).

The gelatinolytic activity of pro-MMP-2 enzyme in INH-treated group was downregulated at 24h in FP explants (Fig. 9, 11A). The stimulatory effect of CAT was higher in FP at 24h than at 48h on active MMP-2 gelatinolytic activity (Fig. 11A), and the combination of CAT and INH reduced the gelatinolytic activity at 48h comparing to 24h treatment (Fig. 9,

11A). In MLP endometrium, all treatments up-regulated the active gelatinolytic activity of MMP-2 at 24h, compared to 48h (Fig. 9, 11B).

3.5 Discussion

In the present study, CAT induced COL1 expression in explants of mare endometrium, at FP and MLP in a time dependent manner. The *COL1A2* mRNA results show that CAT acts as a pro-fibrotic protease, mainly in FP, as a response to a shorter stimulus, and in MLP as a response to a longer stimulus. During the FP, endogenous estrogen thickens the uterine wall and increases uterine muscular tone and vascularization. The cervix is relaxed and opens (Aurich 2011). The endometrial glands also proliferate and the lamina propria becomes highly edematous (Kenney 1978). The mare endometrium is more prone to inflammation and more reactive at estrus, which might explain why the explants obtained at the FP, under the influence of estrogens, were reactive to CAT after a short time of stimulation. Moreover, a longer time of CAT exposition was needed to increase expression of COL1 at the protein level. The COL1 protein relative abundance was increased by CAT only at 48h in both estrous cycle phases.

One of the aims of this study was to evaluate if by inhibiting CAT using a specific inhibitor (IHN) it would be possible to reduce CAT-induced COL1 relative abundance in equine endometrium. This inhibitor blocks the increase of monocyte chemoattractant protein 1 (MCP1) and tumor necrosis factor α (TNFα), both linked to airway hyperactivity (de Garavilla et al. 2005), and blocks neutrophilia (Abraham 2008). We showed in our study that the inhibitory effects of IHN was detected in the longest treatment time, corresponding to the increased COL1 relative abundance induced by CAT treatment. To the best of our knowledge, this is the first study describing that by inhibiting CAT, it is possible to reduce COL1 relative abundance in equine endometrium *in vitro*. Therefore, we suggest that this treatment could be a possible approach to prevent the formation of endometrosis. In fact, INH offers a promising therapeutic strategy in chronic inflammatory conditions, such as asthma or COPD (Kosikowska and Lesner 2013). Future *in vivo* studies are crucial to test this hypothesis. Currently, despite the therapies proposed to treat equine endometrosis, there is no routinely available effective treatment (Buczkowska et al. 2014; Schöniger and Schoon 2020). Several therapeutic approaches, as mechanical curettage or intrauterine application of chemical agents (kerosene, DMSO, isotonic salt) or mesenchymal stem cells have been studied (Ley et al. 1989; Keller et al. 2006; Mambelli et al., 2014). Nevertheless, they caused rather short-term beneficial effects, and/or did not improve pregnancy rates (Ley et al. 1989; Keller et al. 2006; Mambelli et al., 2014).Thus, the need for evaluating the *in vivo* efficacy of INH in the treatment of equine endometrosis associated to NETs is imperative. Indeed, our findings may be the grounds for further *in vivo* trials for INH testing.

Fibrosis is the result of a disruption of the balance of the ECM, with increased synthesis and deposition of ECM components, and decreased degradation of those ECM products (Harvey et al. 2016). The MMPs have been considered as being part of the highly regulated systems that control this ECM turnover (Vandooren et al. 2013). An increase in the active form of MMP-2 has been reported in mare endometrosis (Walter et al. 2005), although other works showed no changes in MMP-2 or -9 expression between normal and fibrotic equine endometrium (Aresu et al. 2012). Another study done by Centeno et al. (2018) found that *MMP2* transcription was upregulated in endometrial fibrosis. Moreover, we have recently reported an upregulation of MMP-2 and MMP-9 levels in mare endometrial tissue with mild to moderate lesions, as well as an increase of MMP-9 levels in fibroblasts and epithelial cells challenged by TGFβ1 (Szóstek-Mioduchowska et al. 2020a). In other tissues, CAT has previously been capable to activate pro-MMP-2 in human tumor cells invasion (Shamamian et al. 2001), and together with MMP-9 may enhance TGFβ signaling in a tumor murine model (Wilson et al. 2009a, 2009b). The inconsistency between MMP expression found in normal and fibrotic equine endometrium may be explained by the fact that fibrotic changes, as in other tissues *e.g* lungs (Vukmirovic and Kaminski 2018), are diffuse. The collected tissue may not always reflect the entire condition of the fibrotic organ and thus, might not fully address the cellular and spatial heterogeneity of fibrosis. Additionally, since endometria at different stages of fibrosis were obtained *post mortem* from different mares, it was not feasible to evaluate the evolution of the fibrogenic process individually. This may have affected the results, and thus could also explain the inconsistent pattern found. However, despite these limitations, also observed in other tissues, understanding of the molecular pathways and the expression of various factors involved in equine endometrosis, is rather important, by unravelling changes associated with this pathological condition.

In our study, the gelatinolytic activity of MMP-2 active form in endometrial explants increased in response to CAT treatment after the shortest treatment time (24h), at both estrous cycle phases. Nevertheless, this pro-fibrotic effect of CAT was diminished with INH addition in FP tissue treated for 24h. Apparently, MMP-2 appears to be involved in an immediate response, perturbing ECM balance. So, MMP-2 can mediate an acute response to a CAT induced inflammation, regardless of the estrous cycle phase.

In FP endometrial explants, the gelatinolytic activity of MMP-9 active form increased with CAT treatment and was inhibited by INH at 48h. This suggests MMP-9 involvement, especially in FP equine endometrium, remodeling the fibrogenic response to a prolonged exposition to CAT.

Elevated levels of MMPs in the endometrium may also indicate a cellular response to an altered ECM balance, as part of the normal regulation of MMP expression. In fact, the main role attributed to MMPs is their action on the turnover and degradation of ECM substrates.

Regulation of MMPs activity takes place at the stages of gene transcription, protein production, activation of pro-enzymes, and inhibition of the active enzymes by tissue inhibitor of matrix metallopeptidases (TIMPs) or α2-macroglobulin (Sternlicht and Werb 2001). Many of these factors can contribute to the differences found between gene transcription, pro-enzyme and active form of MMP-2 and MMP-9. In addition, MMP-9 may be regulated by ovarian steroids, which can explain why this enzyme activity differed according to various estrous cycle phases (Nothnick 2008). Many mechanisms are involved in the response to CAT pro-fibrotic stimulus, and more studies are necessary to unravel the role of MMPs, either in healthy or fibrotic endometrium.

3.6 Conclusions

Even though our previous (Amaral et al. 2018, 2020a) and present results suggest that ELA and CAT are pro-fibrotic factors, and are involved in equine endometrial fibrosis establishment, the study of other causes, including the role of other enzymes found in NETs is vital to fully understand the mechanisms of endometrosis pathogenesis. The use of a selective CAT inhibitor was effective on the reduction of COL1 expression. Therefore, these novel data may contribute to the development of a new prophylactic or therapeutic approach for endometrosis. Although, the use of a broad-spectrum enzyme inhibitor or specific selective inhibitors combined may be needed to obtain a strong and more effective inhibitory effect. MMP-2 might be involved in an earlier response to CAT, independent of estrous cycle phase, and MMP-9 in a later response, mainly in the FP.

4. Myeloperoxidase inhibition decreases the expression of collagen and metallopeptidase in mare endometrium under in vitro conditions

Adapted from:

Amaral A, Fernandes C, Rebordão MR, Szóstek-Mioduchowska A, Lukasik K, Pinto-Bravo P, Telo da Gama L, Skarzynski DJ, Ferreira-Dias G. 2020. Myeloperoxidase inhibition decreases the expression of collagen and metallopeptidase in mare endometria under in vitro conditions. *Animals*. 11(1):208. doi: 10.3390/ani11010208.

4.1 Abstract

Neutrophils can originate neutrophil extracellular traps (NETs). Myeloperoxidase (MPO) is a peroxidase found in NETs associated to equine endometrosis and can be inhibited by 4-aminobenzoic acid hydrazide (ABAH). Metallopeptidases (MMPs) participate in extracellular matrix stability and fibrosis development. The objectives of this *in vitro* work were to investigate in explants of mare's endometrium, (i) the ABAH capacity to inhibit MPO-induced collagen type I (COL1) expression; and (ii) the action of MPO and ABAH on the expression and gelatinolytic activity of MMP-2/-9. Explants retrieved from the endometrium of mares in follicular or mid-luteal phases were treated with MPO, ABAH, or their combination, for 24 or 48h. The qPCR analysis measured the transcription of COL1A2, MMP2, and MMP9. Western blot and zymography were performed to evaluate COL1 protein relative abundance and gelatinolytic activity of MMP-2/-9, respectively. Myeloperoxidase elevated COL1 relative protein abundance at both treatment times in follicular phase ($P < 0.05$). The capacity of ABAH to inhibit MPO-induced COL1 was detected in follicular phase at 48h ($P < 0.05$). The gelatinolytic activity of activated MMP-2 augmented in mid-luteal phase at 24h after MPO treatment, but it was reduced with MPO+ABAH treatment. The activity of MMP-9 active form augmented in MPO-treated explants. However, this effect was inhibited by ABAH in the follicular phase at 48h ($P < 0.05$). By inhibiting the pro-fibrotic effects of MPO it might be possible means to reduce the development of endometrosis. Metallopeptidase-2 might be involved in an acute response to MPO in the mid-luteal phase, while MMP-9 might be implicated in a prolonged exposition to MPO in the follicular phase.

Keywords: endometrosis, myeloperoxidase, 4-aminobenzoic acid hydrazide, fibrosis, metallopeptidases.

4.2 Introduction

Myeloperoxidase (MPO) is an enzyme that is expressed by several immune cells, as neutrophils, monocytes, and macrophages (Nicholls and Hazen 2005; Liu et al. 2015). Neutrophils are the first leucocytes acting on the defense against microbial attacks (Klebanoff 2005; Teng et al. 2017) and are able to degranulate, and release their DNA and some enzymes that possess antimicrobial properties. Thus, they form neutrophil extracellular traps (NETs). Some proteases, such as cathepsin G and elastase, and the peroxidase MPO are released by NETs to fight bacteria (Brinkman 2004). Among these enzymes, MPO has been described to be the most abundant one in neutrophils (Segal 2005). It uses the bacteria-induced hydrogen peroxide to produce chloramine and hypochlorite, which are toxic products for bacteria (Klebanoff 2005; Nauseef 2014).

After mating or artificial insemination, the sperm induces inflammation with a rapid influx of neutrophils into the uterus, which in turn leads to a physiological transient breeding-induced endometritis (Kotilainen et al. 1994; Katila 1995). This inflammatory response results in the elimination of needless spermatozoa, contaminating bacteria, and debris introduced in the uterus (Troedsson et al. 1993; Troedsson 2006). The process of NETs formation in equine endometrium has already been demonstrated by the contact of equine neutrophils with semen (Alghamdi and Foster, 2005; Alghamdi et al. 2009) or with bacteria associated to endometritis (Rebordão et al. 2014). But, besides the antimicrobial properties of NETs components, they may also contribute to the development of some pathological conditions (Manda et al. 2014). High concentrations of MPO in uterine lavage of mares was already related with endometritis (Parrilla-Hernandez et al. 2014), even though this was not demonstrated in cows with endometritis (Nazhat et al. 2018). In our previous studies, we have also identified the involvement of NETs enzymes in the establishment of endometrosis (Rebordão et al. 2018; Amaral et al. 2018, 2020a, 2020b). Endometrosis is a fibrotic, progressive, and degenerative condition, mainly diagnosed on the grounds of the paramount deposition of extracellular matrix (ECM) components, such as collagen, in mare endometrium (Kenney 1992; Hoffmann et al. 2009). In fact, the treatment of equine endometrial explants with NETs enzymes induced collagen type I (COL1) expression (Rebordão et al. 2018; Amaral et al. 2018, 2020a, 2020b). In humans, elevated levels of MPO in cystic fibrosis sputum have been associated with the severity of lung disease (Sagel et al. 2012; Sly et al. 2013). Moreover, this enzyme has also been associated to liver fibrosis (Beard et al. 2006; Pulli et al. 2015).

The 4-aminobenzoic acid hydrazide (ABAH), among many MPO inhibitors tested, has been the most investigated (Kettle 1997; Lazarević-Pasti et al. 2015). Recent studies have shown that ABAH reduced MPO-dependent mice hepatocyte death (Pulli et al. 2015), decreased the activity of MPO in acute stroke in mice (Kim, Wei et al. 2016) and inhibited MPO in cystic fibrosis sputum (Hair et al. 2017).

Metallopeptidases (MMPs) are a group of enzymes that mediate ECM turnover. Metallopeptidase-2 and -9 are endopeptidases which denature ECM substrates, such as collagens (gelatins) (Vandooren et al. 2013). In addition, the expression and activity of MMPs are affected by ovarian hormones in human endometrial tissue remodeling during the estrous cycle phases (Wang and Khalil 2018), but their role in fibrosis establishment is still uncertain. Comparing the normal and fibrotic equine endometrium, Aresu et al. (2012) found no alteration in MMP-2/-9 expression. However, MMP-2 transcription (Centeno et al. 2018) and its active form (Walter et al. 2005) were up-regulated in mare endometrosis. Our latest *in vitro* studies in equine endometrium revealed that MMP expression is altered by mediators of inflammation (interleukins, transforming growth factor (TGF)β1 and prostaglandins (Szóstek-Mioduchowska et al. 2019b, 2020a, 2020b), and could contribute for the fibrotic response to elastase (Amaral et al. 2020a) and cathepsin G (Amaral et al. 2020b).

Taking in consideration that equine endometrial explants treated with enzymes found in NETs induced COL1, it could be proposed that NETs enzymes play a role in equine endometrial fibrosis establishment. Thus, the rationale was to investigate if by inhibiting NETs enzymes it would reduce the MPO induced COL1. This way, we proposed to evaluate if a selective inhibitor of NETs enzyme MPO would be effective in reducing MPO pro-fibrotic effect, as it has been shown in other organs and species. Our previous in vitro findings in mare endometrial explants, showed that COL1 induced by elastase and cathepsin G was reduced by the use of their selective inhibitors (Amaral et al. 2020a, 2020b). We have hypothesized that by inhibiting MPO, the in vitro production of COL1 by mare endometrial explants would be reduced. Thus, the objectives of this in vitro work were to investigate in explants of mare's endometrium, (i) the ABAH capacity to inhibit MPO-induced collagen type I (COL1) expression; and (ii) the action of MPO and ABAH on the expression and gelatinolytic activity of MMP-2/-9.

4.3 Materials and Methods

4.3.1 Mares and tissue retrieval

Uteri from cyclic mares (n=14) intended for meat production were collected *post-mortem* at an abattoir (Rawicz, Poland) within 10–15 min of mares´ euthanasia, in agreement to the European (EFSA, AHAW/04–027) legislation. As confirmed by the official veterinary inspection carried out by the official veterinary, the mares used in this study showed no signs of illness. For further progesterone (P4) analysis, blood from the jugular vein was withdrawn into ethylenediaminetetraacetic acid (EDTA) tubes. For each mare, estrous cycle phase was determined according to uterine and ovarian evaluation, and confirmed by P4 concentration in plasma (Roberto da Costa et al. 2007; Rebordão et al. 2018). For this study, mid-luteal phase (MLP; n = 6) and follicular phase (FP; n = 8) endometria were immediately transported to the laboratory, on ice. As previously reported, mare uteri were placed in ice-cold Dulbecco's

modified Eagle's medium (DMEM) F-12 Ham medium (D/F medium; 1:1 (v/v); D-2960; Sigma-Aldrich, St Louis, MO, USA), supplemented with100 IU/mL penicillin (P3032; Sigma-Aldrich), 2 µg /mL amphotericin (A2942; Sigma-Aldrich) and 100 µg/mL streptomycin (S9137; Sigma-Aldrich). Only endometria without endometritis were included in the study, as previously referred (Rebordão et al. 2018, 2019). After samples were collected, two pieces of endometrium were immersed in 4% buffered paraformaldehyde for the histopathological classification of the endometrium (Kenney and Doig 1986). The endometrial samples were classified as I, IIA, IIB or III categories based on the extent of inflammation and/or fibrosis, according to Kenney and Doig (1986). Only endometria presenting mild to moderate histopathological lesions (IIA or IIB category) were used in this experiment, in order to exclude the variation due to endometrial fibrotic grade.

4.3.2 *In vitro* culture of endometrial explants

Endometrial tissue preparation and culture were performed as reported before (Amaral et al. 2020a). The explants were pre-incubated for 1h, at 38ºC, 5% CO_2 in a humidified atmosphere chamber (Biosafe Eco-Integra Biosciences, Chur, Switzerland) in 24-well cell culture sterile plates (Eppendorf, #0030 722.116) with 1mL of DMEM culture medium supplemented with 0.1% (w/v) bovine serum albumin (BSA; 735078; Roche Diagnostics, Mannheim, Germany), 100 µg/mL streptomycin (S9137; Sigma-Aldrich), 100 IU/mL penicillin (P3032; Sigma-Aldrich) and 2 µg/mL amphotericin (A2942; Sigma-Aldrich),.with gentle shaking (150 rpm). Afterwards, endometrial explants were further treated in new culture medium for 24h or 48h, as follows: (i) vehicle (negative control) – culture medium alone; (ii) myeloperoxidase (MPO; 0.5 µg/mL; orb81997; Biorbyt, Cambridge, UK); (iii) 4-aminobenzoic hydrazide, an MPO inhibitor (ABAH; 10 µg/mL; $C_7H_9N_3O$, sc-204107; Santa Cruz Biotechnology, USA); (iv) MPO (0.5 µg/mL) + ABAH (10 µg/mL); or (v) oxytocin (OXT; 10^{-7} M), a prostaglandin (PG) secretion positive control (Nash et al. 2008; Szóstek et al. 2013). Thus, in the present study, OXT treatment was a means to determine explant viability by assessing endometrium in vitro capacity to secrete $PGF_{2\alpha}$ throughout the incubation time. A fibrogenic assay using TGFβ1 was previously carried out as a positive control for COL expression (Amaral et al. 2020a). Each treatment was carried out in quadruplicate. The ABAH was added 1h later of pre-incubation, when the culture media were replaced, while MPO was added 1h later to allow binding of the inhibitor. In studies lasting for 48h, 10 µg/mL of ABAH were furthered added after the first 24h of treatment, since in the pre-trial its inhibitory effect persisted only for 24h and subsided afterwards. In the end, explants (in RNAlater, R901, Sigma-Aldrich) and conditioned culture media were stored at -80°C. For PG analysis a 1% stabilizer solution of 0.3M EDTA (E5134, Sigma-Aldrich) and 1% aspirin (A2093; Sigma-Aldrich) was added to the culture medium to prevent degradation before storage at -80°C.

The expression of TGFβ1, as a fibrotic indicator (Rebordão et al. 2018), was induced by 0.5 µg/mL of MPO in a dose assessment assay. In addition, a dose-response trial, found in other in vitro studies (Kettle et al. 1997; Forbes et al. 2013), using 0.01, 0.1, 1, 10 and 100 µg/mL ABAH, showed that the optimum concentration that inhibited COL1A2 mRNA MPO-induced was 10 µg/mL (data not shown).

4.3.3 Endometrial explants Viability assay

The endometrial explant viability was evaluated by lactate dehydrogenase (LDH) activity and by PGF2α secretion in conditioned culture medium, as described (Amaral et al. 2020a). The data of viability of endometrial explants are presented in Supplementary material, Figure S1 and S2.

4.3.4 Total RNA extraction, synthesis of cDNA and qPCR

TRI Reagent® (T9424; Sigma-Aldrich) was used to perform the extraction of total RNA, following to the guidelines provided by the manufacturer. The evaluation of both RNA quality and quantity, as well as cDNA synthesis was done as already reported (Amaral et al. 2020a). The primer sequences for *COL1A2*, *MMP2*, *MMP9* and for the reference gene ribosomal protein L32 (*RPL32*) were previously determined, as well as the reference gene validation (Dheda et al. 2004; Amaral et al. 2020a). The target and reference gene reactions were run simultaneously, in duplicate, on a 96 well plate (4306737; Applied Biosystems) and run in a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Warrington, UK). The specificity of the qPCR products was performed, as described (Zhao and Fernald 2005; Amaral et al. 2020a).

4.3.5 Western blot analysis

Equine endometrial explants were processed as described before (Amaral et al. 2020a). Collagen type I protein relative abundance was determined by the non-staining total protein loading control as reported before (Amaral et al. 2020a). The primary against COL1 antibody (1:1,000 diluted; 20121; Novotec, Lyon, France) was incubated overnight, at 4ºC as previously described and validated (Rebordão et al. 2018). The secondary antibody utilized was Horseradish peroxidase (HRP)-conjugated anti-rabbit (1:20,000; P0448; DakoCytomation, Carpinteria, CA, USA) incubated at room temperature for 1.5h. Imaging of COL1 protein relative abundance was achieved by luminol enhanced chemiluminescence (Super Signal West Pico, 34077; Thermo Scientific, Waltham, MA, USA). For band normalization and comparison between membranes, a standard sample (30 µg) of a mix of explants was loaded in all gels in a single lane. Image Lab 6.0 (Bio-Rad) software and a multichannel protocol were used to analyze COL1 relative abundance in lanes in non-staining

total protein membrane image. After antibodies incubation, the band was detected on chemiluminescence image. The target protein volume was software-calculated using a normalization factor allowing the adjustment of variability of the protein loaded (Posch et al. 2013; Amaral et al. 2020a).

4.3.6 Zymography

The MMP-2 and MMP-9 gelatinolytic activity was assessed by zymography, as described before (Manuel and Gawronska-Kozak 2006). Normalization of zymograms was accomplished using a non-staining total protein loading control (Raykin et al. 2017). The explant culture supernatant was processed as described (Amaral et al. 2020a). The molecular weight determination was made using Recombinant Human MMP-2 Protein, CF (902-MP-010; R&D Systems, Minneapolis, USA), and Recombinant Human MMP-9 Western Blot Standard Protein (WBC018; R&D Systems). To relate all the gels, a single lane of a standard sample (40 µg) of mixed culture medium was loaded. A multichannel protocol in Image Lab 6.0 (Bio-Rad) software was used for the detection of lanes in non-staining total protein gel image and bands on Coomassie staining image. Volume of target protein, as well as the normalization factor, were calculated, and the values adjusted for protein load variation (Raykin et al. 2017; Amaral et al. 2020a).

4.3.7 Statistical analysis

Data normality was evaluated visually and by the test of Kolmogorov-Smirnov in Proc Univariate of SAS v. 9.4 (SAS Institute Inc.). The viability data were assessed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test (GraphPAD PRISM, Version 6.00, 253 GraphPad Software, San Diego, CA, USA). These results are displayed as mean ± SEM and determined significant at $P < 0.05$. The evaluated variables consisted of *COL1A2*, *MMP2* and *MMP9* transcription, relative COL1 protein abundance and gelatinolytic activity of MMP-2/-9. Since some variables did not present a normal distribution, the square root and logarithmic transformation were used to transform these data for further analysis. In the first analysis, the PROC GLM of SAS was used to analyze each response variable to different treatments: combination of the effect of MPO, the use of ABAH, estrous cycle phase, and time of treatment, resulting in 16 treatment combinations in total. The least square means of the treatment combinations were compared (using the PDIFF of PROC GLM), and results were significant at $P < 0.05$. To perform the graphical presentation, the means were back transformed. After, the two-, three- and four-way interactions, were also analyzed.

4.4 Results

4.4.1 Viability of endometrial explants

The data of viability of endometrial explants are presented in Figure 12 and 13.

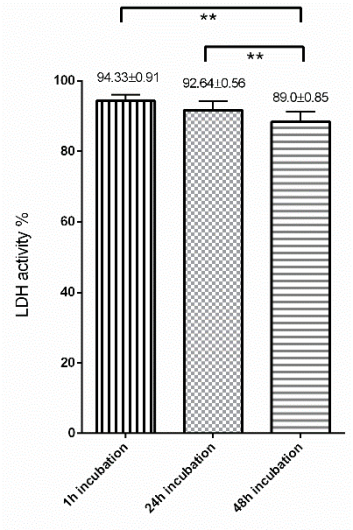


Figure 12: Lactate dehydrogenase (LDH) activity measured in conditioned culture medium of equine endometrial explants after 1h, 24h or 48h incubation. Explants viability was calculated from the quotient of the intracellular LDH activity and the total activity (extracellular plus intracellular LDH) (Amaral et al. 2020a). Results are presented as means ± SEM. Asterisks indicate statistical differences within time of incubation. Statistical differences were found between 1h - 48 h, and 24h - 48h (*P* < 0.01). The results were independent of estrous cycle phase.

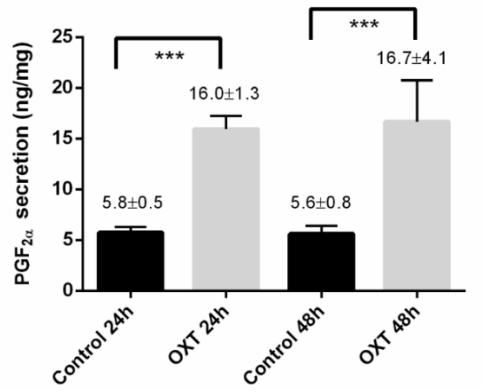


Figure 13: The effect of oxytocin (OXT) on prostaglandin (PG)$_{F2\alpha}$ secretion in equine endometrial explants after 24h or 48h. Results are presented as means ± SEM. Asterisks indicate statistical differences within the different time of treatment. Endometrial explants treatment with OXT increased PGF$_{2\alpha}$ secretion at 24h and 48h (*p* > 0.001) comparing to control (non-treated tissues). These results were independent of estrous cycle phase.

4.4.2 The effect of ABAH on the inhibition of COL1 induced by MPO

The interactions found between treatments, estrous cycle phase and time of treatment are listed in Table 12.

Table 12: Levels of significance (*P* values) for 2-, 3- and 4-way interactions between estrous cycle phases, treatment time, and myeloperoxidase (MPO) or 4-aminobenzoic hydrazide (ABAH) treatments in the analyses of relative transcript of target genes, COL1 protein relative abundance and gelatinolytic activity of MMP-2 and -9. The results were considered significant at *P* < 0.05 and are highlighted in yellow.

Interaction	*COL1A2*	COL1	*MMP2*	Pro-MMP-2	Active MMP-2	*MMP9*	Pro-MMP-9
MPO x ABAH	0.0982	0.1554	0.2915	0.216	0.0187	0.0013	0.5735
MPO x treatment time	0.9082	0.1717	0.6797	0.5799	0.7226	0.0245	0.4546
MPO x estrous cycle phase	0.0292	0.0331	0.4127	0.6731	0.6847	0.0011	0.5019
ABAH x treatment time	0.5321	0.3425	0.9631	0.2552	0.1017	0.3115	0.423
ABAH x estrous cycle phase	0.0284	0.0611	0.2415	0.3873	0.8572	<.0001	0.6101
Time of treatment x estrous cycle phase	0.0006	0.1814	0.6529	0.007	0.044	0.0572	0.0131
MPO x ABAH x treatment time	0.4935	0.497	0.8888	0.4357	0.9494	0.3593	0.0926
MPO x ABAH x estrous cycle phase	0.0034	0.0245	0.8832	0.153	0.1756	0.4073	0.8236
MPO x treatment time x estrous cycle phase	0.0079	0.491	0.7107	0.5299	0.2599	0.0875	0.8082
ABAH x treatment time x estrous cycle phase	0.8928	0.0689	0.655	0.1988	0.1769	0.3967	0.7737
MPO x ABAH x treatment time x estrous cycle phase	0.1748	0.8249	0.6073	0.0065	0.0199	0.0856	0.0309

Abreviations: *COL1A2* - collagen type 1 α2; COL1 – collagen type I protein; *MMP2* - matrix metallopeptidase 2; *MMP9* - matrix metallopeptidase 9

In Table 13 are shown the differences of the same treatments between the FP and MLP within each treatment time.

Table 13: Listed significant differences of the same treatments between the follicular phase (FP) and mid-luteal phase (MLP) of the estrous cycle, within each treatment time.

Evaluated variables	Treatment comparison	P value	Figures
***COL1A2* transcription**	MPO 24h FP vs 24h MLP	$P < 0.0001$	14A, 14B
COL1 protein relative abundance	MPO 24h FP vs 24h MLP	$P < 0.01$	14C, 14D
	MPO 48h FP vs 48h MLP	$P < 0.01$	
***MMP9* transcription**	ABAH 24h FP vs 24h MLP	$P < 0.001$	16C, 16D
	ABAH 48h FP vs 48h MLP	$P < 0.05$	
	MPO 24h FP vs 24h MLP	$P < 0.01$	
	MPO + ABAH 24h FP vs 24h MLP	$P < 0.0001$	
	MPO + ABAH 48h FP vs 48h MLP	$P < 0.0001$	
Pro-MMP-2 activity	MPO 24h FP vs 24h MLP	$P < 0.001$	17A, 17B
Active MMP-2	MPO 24h FP vs 24h MLP	$P < 0.01$	17A, 17B

COL1A2 - collagen type 1 α2; COL 1 – collagen type I; *MMP2* - matrix metallopeptidase 2; *MMP9* - matrix metallopeptidase 9; MPO – myeloperoxidase; ABAH – 4-aminobenzoic hydrazide; FP – follicular phase; MLP – mid-luteal phase.

In figure 14, the relative mRNA transcription and protein abundance of COL1 results are presented as median with interquartile range. Likewise, in figure 16, the transcription of MMP2/9 is shown as median with interquartile range. However, in figure 17, the results of MMP-2/-9 gelatinolytic activities are presented as least square means ± SEM. These figures were drawn in GraphPAD PRISM.

The *COL1A2* transcripts increased in MPO treated explants in FP at 24h and 48h when compared to control group ($P < 0.0001$; $P < 0.05$ respectively; Fig. 14A) and to ABAH-treated group ($P < 0.0001$; $P < 0.05$ respectively; Fig. 14A). However, the use of MPO and ABAH treatments, when combined, impaired *COL1A2* mRNA levels comparing to the corresponding MPO-treated tissues (FP: 24h – $P < 0.001$; 48h – $P < 0.01$; Fig. 14A). In MLP endometrial explants treated for 24h, the MPO treatment lowered the transcription regarding the control group ($P < 0.05$; Fig. 14B). In FP endometrial explants treated with MPO, *COL1A2* transcription was higher at 24h than at 48h (Fig. 14A). Nevertheless, in MLP explants the transcription was higher at 48h comparing to 24h (Fig. 14B) and was also increased with MPO + ABAH treatment (Fig 14B).

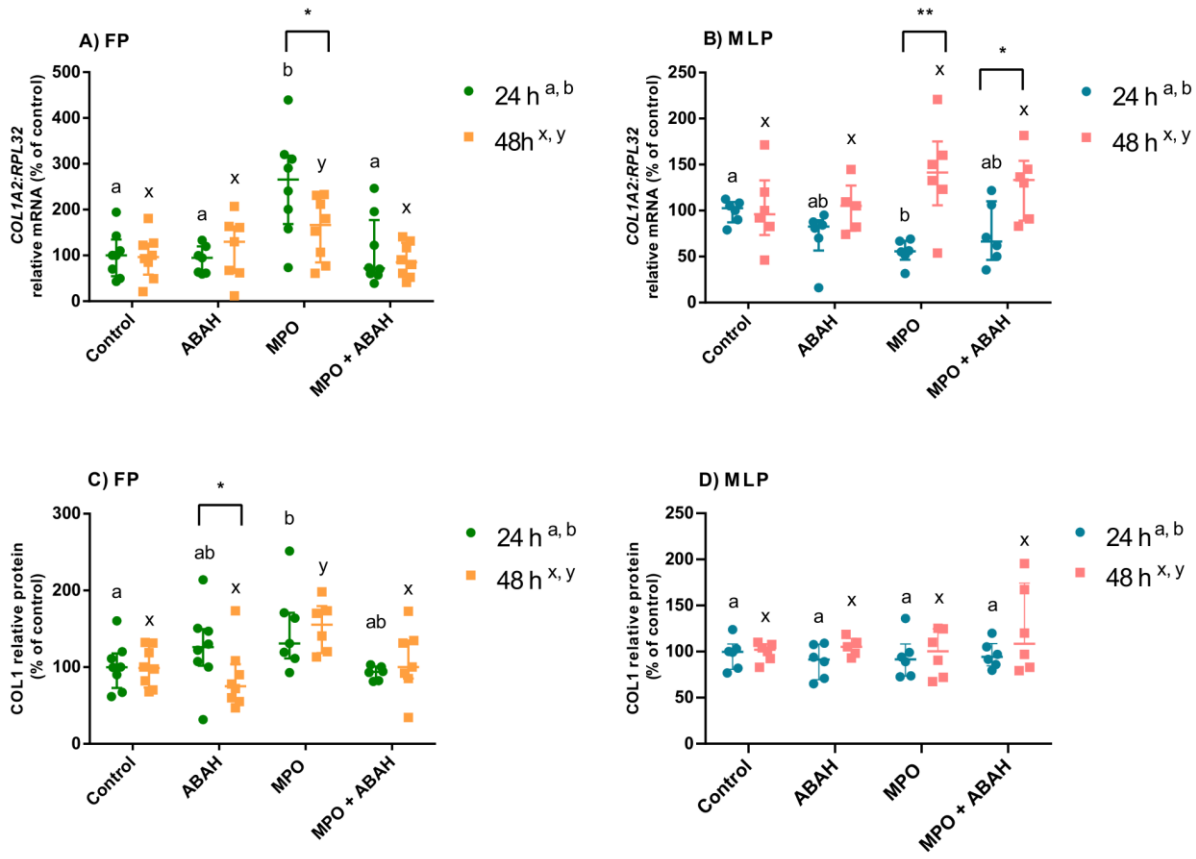
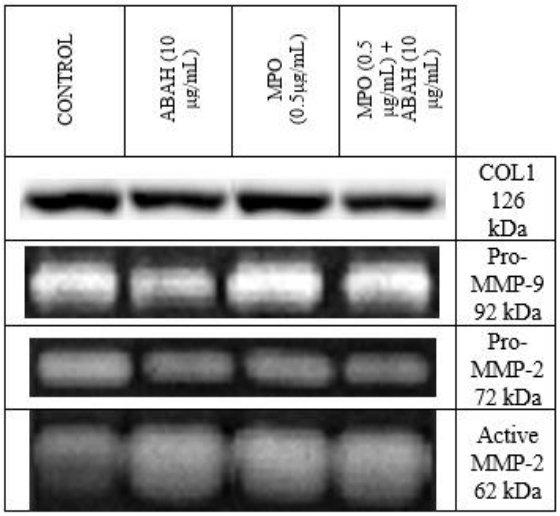
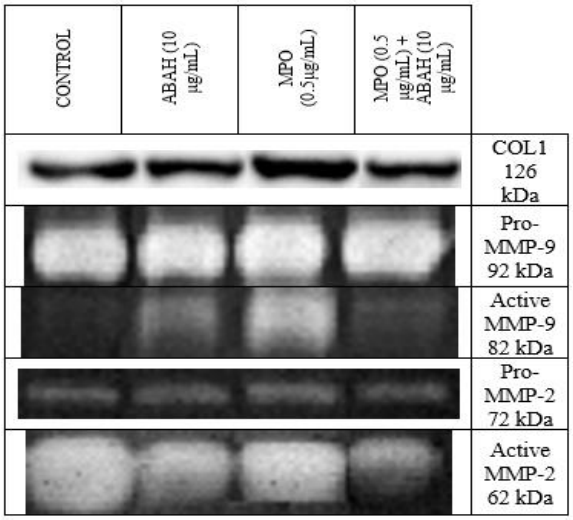


Figure 14: Relative mRNA transcription of type I collagen (*COL1A2*) (A, B) and relative abundance of COL1 protein (C, D) in mare endometrial explants from follicular phase (FP) and mid-luteal phase (MLP) treated with culture medium only (Control), myeloperoxidase (MPO: 0.5 µg/mL), 4-aminobenzoic hydrazide (ABAH: 10 µg/mL), or MPO (0.5 µg/mL) + ABAH (10 µg/mL) for 24 or 48h. Results are presented as median with interquartile range. Significance was determined at *P* < 0.05. The differences among treatments with the same treatment time are signaled by distinct superscript letters (a, b- 24h; x,y- 48h). The differences among times of treatment for the identical treatment are shown by asterisks (P* < 0.05; ***P* < 0.01).**

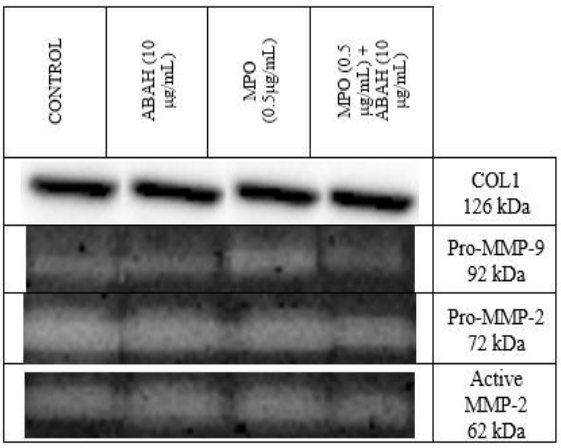
In MPO-treated explants, COL1 protein increased in the FP, both at 24h (*P* < 0.01; Fig. 14C) and 48h (*P* < 0.001; Fig. 14C), with respect to control group and ABAH group, but only in the longest treatment time (*P* < 0.0001; Fig. 14C). The inhibitory effect of ABAH was detected in FP at 48h regarding the group treated with MPO (*P* < 0.01; Fig. 14C, 15). In the FP, COL1 protein relative abundance was greater in ABAH-treated explants at 24h when compared to 48h treatment (Fig. 14C, 15). There were no differences in COL1 protein between treatments or treatment times in the MLP explants (Fig. 14D).



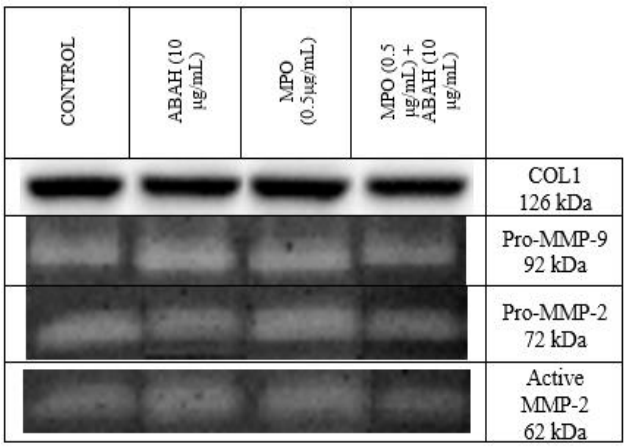
A – 24h FP endometrium explant



B – 48h FP endometrium explant



C – 24h MLP endometrium explant



D – 48h MLP endometrium explant

Figure 15: Representative panels of type I collagen (COL1) western blotting and pro and active form of MMP-2 and MMP-9 zymograms in mare endometrium in follicular phase (FP) or mid-luteal phase (MLP) treated for 24h or 48h with myeloperoxidase (MPO 0.5 µg/mL), 4-aminobenzoic hydrazide (ABAH; 10 µg/mL) or MPO (0.5µg/mL) + ABAH (10µg/mL). A - 24h treatment of FP endometrium explants; B – 48h treatment of FP endometrium explants, C – 24h treatment of MLP endometrium explants; and D – 48h treatment of MLP endometrium explants.

4.4.3 The effect of MPO and ABAH on MMP expression

The transcription of *MMP2* was unchanged at both treatment times and estrous cycle phase (Fig. 16A and 16B). In FP endometrial explants, at both treatment times, the transcript levels of *MMP9* increased in MPO treated groups relative to the respective non-treated explants ($P < 0.01$; Fig. 16C) and to the ABAH-treated set (24h - $P < 0.0001$; 48h - $P < 0.01$; Fig. 16C). However, the MPO + ABAH combined treatment reduced *MMP9* mRNA compared to MPO-treated groups (FP: 24h – $P < 0.01$; 48h - $P < 0.0001$; Fig. 16C). The ABAH treatment decreased *MMP*-9 mRNA regarding non-treated group at 24h in FP endometria ($P < 0.05$; Fig.

16C). In MLP tissues treated for 24h, all the treatments up-regulated *MMP9* transcription relative to control (ABAH – $P < 0.05$; MPO and MPO + ABAH – $P < 0.001$; Fig. 16D). Also, the MPO and MPO + ABAH treatments increased *MMP9* mRNA comparing to ABAH-treated group ($P < 0.001$; Fig. 16D). At 48h, in MLP equine explants, all the treatments increased *MMP9* transcript levels as well, compared to control tissues (ABAH – $P < 0.01$; MPO – $P < 0.05$; MPO + ABAH– $P < 0.0001$; Fig. 16D). The *MMP9* transcription was higher in MLP tissues MPO-treated at 24h than at 48h (Fig. 16D).

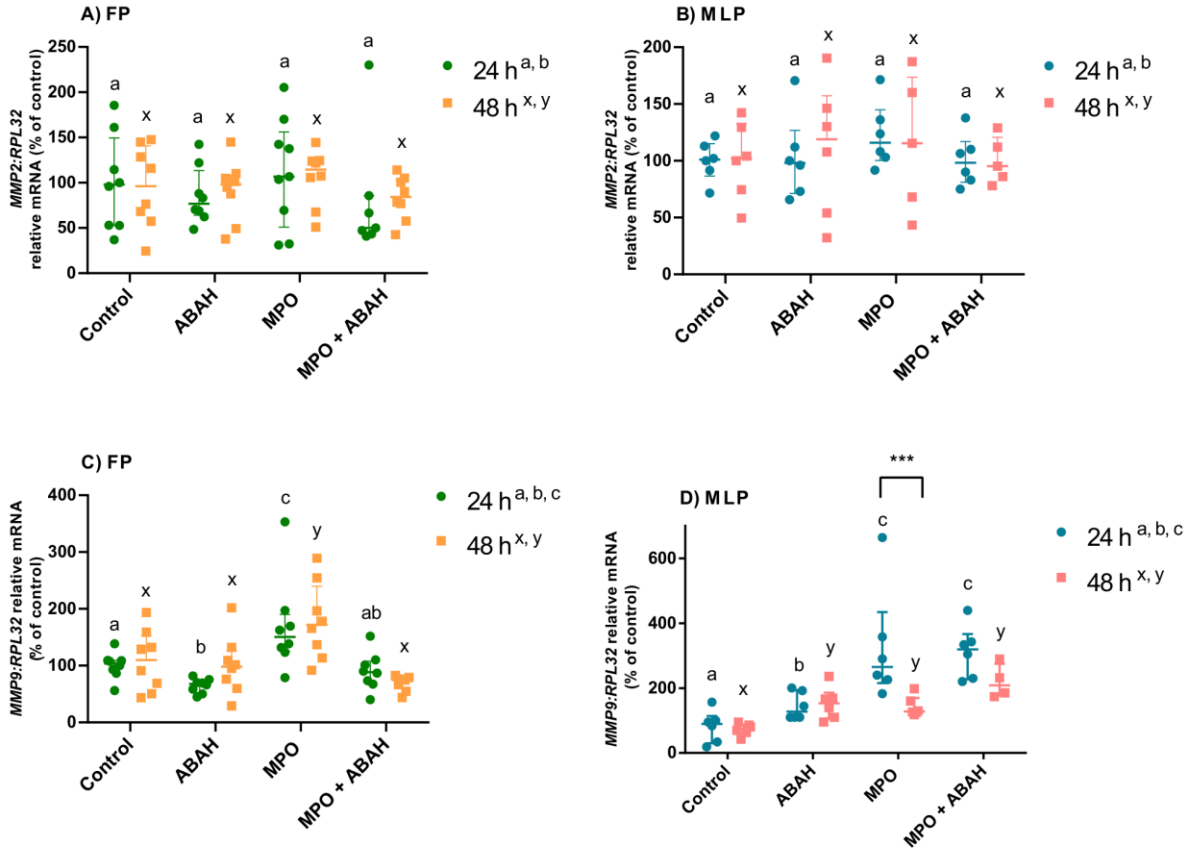


Figure 16: Transcription of *MMP2* (A, B) and *MMP9* (C, D) relative mRNA in mare endometrial explants from follicular phase (FP) and mid-luteal phase (MLP) treated with culture medium only (Control), myeloperoxidase (MPO: 0.5 µg/mL), 4-aminobenzoic hydrazide (ABAH: 10 µg/mL), or MPO (0.5 µg/mL) + ABAH (10 µg/mL) for 24 or 48h. Results are presented as median with interquartile range. Significance was determined at P < 0.05. The differences among treatments with the same treatment time are signaled by distinct superscript letters (a,b,c- 24h; x,y- 48h). The differences among times of treatment for the identical treatment are depicted by asterisks (*P < 0.001).**

In FP explants at 48h, the MPO + ABAH treatment reduced gelatinolytic activity of active MMP-2 comparing to MPO-treated group ($P < 0.05$; Fig. 15, 17A), which did not increase compared to control. The gelatinolytic activity of pro- ($P < 0.05$; Fig. 17B) and active ($P < 0.01$; Fig. 17B) MMP-2 increased in MLP explants at 24h with MPO treatment comparing to control. However, the combined treatment of MPO + ABAH reduced in comparison to MPO-treated group (pro-MMP-2: $P < 0.01$; active MMP-2: $P < 0.05$; Fig. 15, 17B).

The pro-MMP-2 gelatinolytic activity increased at 48h in FP with MPO treatment with respect to 24h (Fig. 17A), while in MLP both pro- and active MMP-2 gelatinolytic activities increased at 24h relative to 48h (Fig. 15, 17B).

The MMP-9 active form gelatinolytic activity was only identified in FP at 48h treatment and MPO treatment up-regulated it comparing to control group ($P < 0.01$; Fig. 17C). Nevertheless, MPO + ABAH combination reduced the activity of active MMP-9, with respect to MPO-treated explants ($P < 0.01$; Fig. 15, 17C). The analysis of pro-MMP-9 gelatinolytic activity showed its decrease after MPO + ABAH of MLP explant treatment for 24h, comparing to MPO-treated group ($P < 0.05$; Fig. 17D), which did not differ from its respective control.

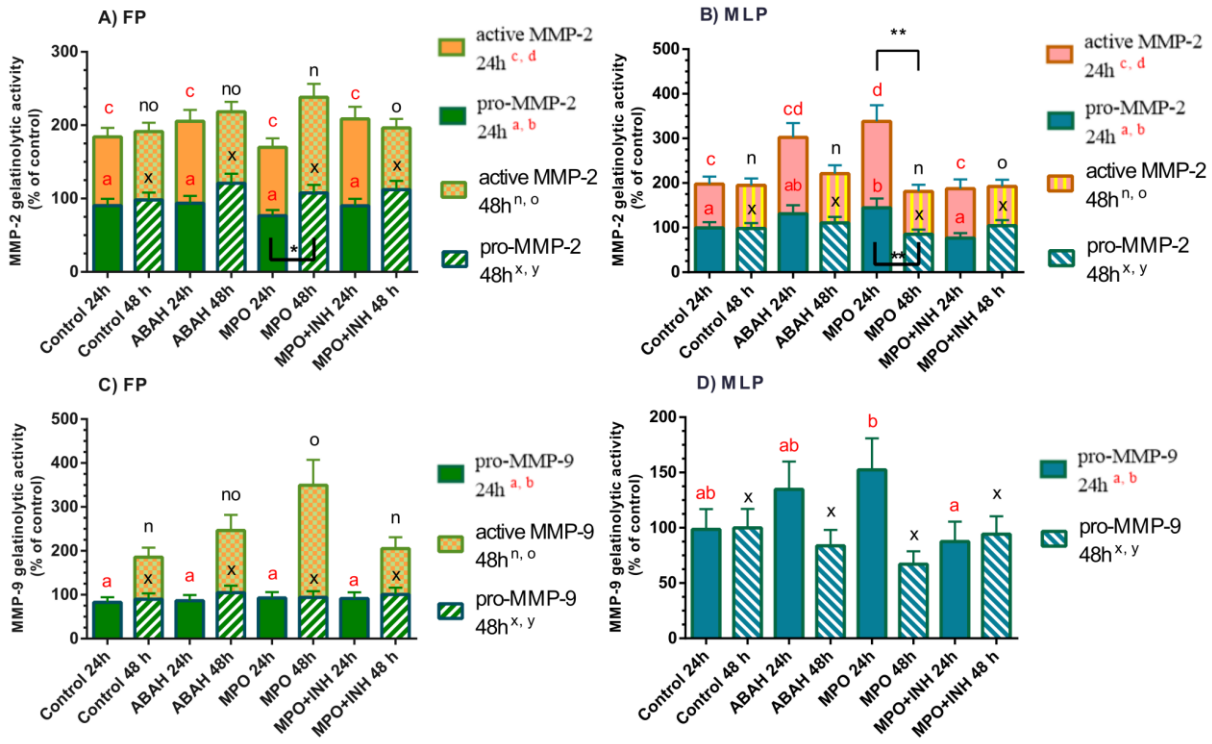


Figure 17: Relative MMP-2 (A, B) and MMP-9 (C, D) gelatinolytic activities in mare endometrial explants from follicular phase (FP) and mid-luteal phase (MLP) treated with culture medium only (Control), myeloperoxidase (MPO: 0.5 µg/mL), 4-aminobenzoic hydrazide (ABAH: 10 µg/mL), or MPO (0.5 µg/mL) + ABAH (10 µg/mL) for 24 or 48h. Data of the least square means ± SEM are shown in bars as percentage of change from control. Significance was determined at *P* < 0.05. The differences among treatments with the same treatment time are signaled by distinct superscript letters. The differences among times of treatment for the identical treatment and MMP form are presented by asterisks (P* < 0.05; ***P* < 0.01).**

4.5 Discussion

Since the discovery that neutrophils release NETs secondary to the contact with bacteria (Rebordão et al. 2014), and semen (Alghamdi and Foster, 2005; Alghamdi et al. 2009) in mare endometrium and that the enzymes found in NETs act as prof-fibrotic factors in mare endometrosis (Rebordão et al. 2018), we aimed to investigate if it would be feasible to reduce *in vitro* COL production induced by these enzymes, by specifically inhibiting them (Amaral et al. 2018, 2020a). In our study, MPO treatment elevated the transcripts of *COL1A2* mRNA and COL1 relative protein abundance at both times of incubation only in FP equine endometria. It is in the FP, under the influence of estrogens, the mare endometrium is more exposed to the invading bacteria, since the cervix is relaxed and open (Aurich 2011). Nevertheless, it appears that mares with healthy endometria are capable of defeating bacteria during this phase of the estrous cycle. As such, after induced endometritis with *Escherichia coli* infusion, mares

presented lower positive bacteriological cultures and neutrophil counts in endometrial swabs when infused at estrus, rather than at diestrus (Marth et al. 2016). But, it is worth noting that it is in the FP, that *Streptococcus* equi subspecies *zooepidemicus* (bacteria which cause endometritis in the mare), attach the most to mare endometria with endometrosis (Ferreira-Dias et al. 1994). Nevertheless, in spite of the mechanisms for defeating bacteria during estrus, a study on gene expression in biopsies from healthy equine endometrium at different times of the estrous cycle showed that in the FP the transcription of *COL1A1* increases until the ovulation day (Gebhardt et al. 2012). In contrast, in the luteal phase, gene expression of *COL1A1* is lowered (Gebhardt et al. 2012). Similarly, in women stromal endometrial cells treated *in vitro* with estrogen, showed an increase in the deposition of collagen, while progesterone stimulation resulted in an increase in the breakdown of collagen (Stenbäck 1989). These studies might explain why the MPO induced collagen response is only observed in the FP. These findings agree with Rebordão et al. work (2018), where MPO elevated COL1 production in FP type I/IIA endometria. Some studies in other tissues also linked MPO to tissue damage. Human cystic fibrosis (Chandler et al. 2018) and atrial fibrosis (Friedrichs et al. 2012), as well as liver fibrosis in a mice model (Pulli et al. 2015) have been associated to MPO-induced tissue injury. In addition, stellate cells from liver were activated by MPO leading to an in vitro up-regulation of COL1 via the fibrogenic factor TGFβ1 (Pulli et al. 2015). In equine endometrial explants (Rebordão et al. 2018; Amaral et al. 2020a) and fibroblasts (Szóstek-Mioduchowska et al. 2019a), TGFβ1 was linked to endometrosis by increasing COL1 production. We postulated that MPO may act via TGFβ1 also in the equine endometrium.

The inhibitory action of ABAH on MPO-induced COL1 was detected in FP on mRNA expression at both times of treatment, and on protein relative abundance at the longest treatment time. To date, we have shown for the fisrt time that by inhibiting MPO *in vitro*, it is possible to reduce COL1 relative abundance in equine endometrium. Although, the ABAH mechanism of action is not well known yet. Some authors proposed a mechanism of action where ABAH is oxidized by MPO to a radical that reduces MPO to its ferrous intermediate by destroying the MPO heme group. Ferrous MPO reacts with hydrogen peroxide originating an irreversible inactivation (Kettle et al. 1997; Burner et al. 1999). However, data from *in vitro* studies cannot be directly extrapolated to in vivo treatments (Fabian et al. 2019). Nevertheless, in vitro systems are a faster approach to predict fibrogenic potential by monitoring the response to pro-fibrotic modulators (Clippinger et al. 2016). Thus, the usage of ABAH as a prophylactic and/or therapeutic means for equine endometrial fibrosis, needs further evaluation for its in vivo use.

The proteolysis of the ECM appears to be a crucial occurence on the inflammatory process, and therefore the fibrotic process, as well. Increased synthesis/deposition and decreased degradation of ECM components leads to fibrosis (Harvey et al. 2016). The MMPs

are enzymes involved in this ECM turnover (Vandooren et al. 2013). In equine explants, MMP-2 and MMP-9 secretion differed when challenged with cytokines, depending on the severity of endometrosis, which may link them to modifications in the endometrium that predispose to fibrosis development (Szóstek-Mioduchowska et al. 2019b). It was demonstrated that TGFβ1 treatment increased MMP-9 secretion in mare endometrial fibroblasts and epithelial cells and that the endometrial MMP expression changes at different categories of endometrosis (Szóstek- Mioduchowska et al. 2020a). In other tissues, the concomitant increased levels of MPO and MMP-2/-9 were also reported in rat temporomandibular joint inflammation (Nascimento et al. 2013), in inflamed human dental pulp tissue (Accorsi-Medonça et al. 2013) and in fat meal induced endothelial damage in humans (Spallarossa et al. 2008).

In our study, the activated MMP-2 gelatinolytic activity increased in MLP explants at 24h in response to MPO treatment, and was reduced with the treatment combination of MPO + ABAH. This may suggest that MMP-2 seems to be implicated in an *in vivo* acute reaction to a MPO-induced inflammation, in MLP endometria. In addition, MMP-9 active form gelatinolytic activity augmented with MPO treatment but was inhibited by ABAH at 48h in FP endometrial explants. This could suggest MMP-9 participation, particularly, in FP, in a reaction to a continued exposure to MPO.

In our previous works, in response to elastase treatment, MMPs expression also differed according to the estrous cycle phase and treatment time, suggesting that the endometrial response is affected by hormonal variations and by the length of the stimulus (Amaral et al. 2020a). Moreover, cathepsin G treatment increased MMPs gelatinolytic activity mainly in follicular phase endometrial explants and reduced with additon of a cathepsin G inibitor (Amaral et al. 2020b). The MMPs output differs is dependent on the stimulus, phase of estrous cycle and duration of the treatment. So, further *in vitro* and *in vivo* studies are crucial to understand the role of MMPs either in healthy or fibrotic endometrium.

Therefore, future works should consider to test *in vivo* a combination of elastase, cathepsin G and MPO inhibitors or the use of a single inhibitor capable of hindering all the pro-fibrotic effects of those enzymes found in NETs, in mare endometrium. Based on this study, MMP-2 appears to be involved in a fast in vitro response to MPO treatment in MLP endometrial explants. In contrast, MMP-9 seems to be released by FP equine endometrial explants after a prolonged exposure to MPO.

4.6 Conclusions

Our findings reinforced the knowledge about MPO pro-fibrotic effects in equine endometrium. Myeloperoxidase induced COL1 and MMP-2/-9 activity in vitro in equine endometrium, and ABAH was shown to inhibit MPO-induced COL1 expression, as well as the activity of MMP-2/-9 induced by MPO. These data should be considered when studying endometrosis development and the attempt to fight this disease by inhibiting pro-fibrotic enzymes found in NETs. However, cautious should be taken by not extrapolating these *in vitro* study results on the use of ABAH as an *in vivo* therapeutic approach to prevent endometrosis.

1. General Discussion

The complete physiopathological mechanisms involved in equine endometrosis are not well known yet. However, some pathways have been studied and associated to the pathogenesis of equine endometrial fibrosis. Indeed, some authors already reported the effect of PGs, cytokines, TGFβ1, interleukins or enzymes found in NETs on endometrosis development (Szóstek et al. 2013; Rebordão et al. 2018, 2019; Szóstek-Mioduchowska et al. 2019a, 2019b; de Holanda et al. 2019).

The present work has focused on the effect of enzymes found in NETs on COL1 expression in equine endometrial explants, with further inhibition of this effect. Moreover, the involvement of PGs and MMPs in this process was also studied.

In a previous study, the equine endometrial explants increased COL1 relative protein abundance and *COL3A1* transcription in response to treatment with ELA, CAT or MPO (Rebordão et al. 2018). In the present work, all the enzymes studied induced COL1 expression by endometrial explants (Figure 18). The treatment with ELA induced *COL1A2* mRNA transcription, but did not affect COL1 protein relative abundance by endometrial tissue in both estrous cycle phases. In spite of high levels of transcription or translation, some proteins are related to a slow translation, but very stable at a high final concentration (Vogel and Marcotte 2012), which might explain the absence of COL1 protein relative abundance increase in response to ELA treatment. In turn, *COL1A2* mRNA results demonstrate that CAT acts as a pro-fibrotic protease, mainly in the follicular phase, as a response to a shorter stimulus, and in mid-luteal phase as a response to a longer stimulus. The COL1 protein relative abundance was increased by CAT only at 48h, in both estrous cycle phases, suggesting that COL1 protein production needs a longer time of CAT exposition. The treatment with MPO augmented *COL1A2* mRNA transcription and COL1 relative protein abundance at both times of incubation, but only in equine endometria retrieved in the follicular phase.

In the follicular phase the uterine wall thickens, muscular tone increases and vascularity becomes greater, the cervix is relaxed and opens (Aurich 2011). Thus, the estrogen influence makes the mare endometrium more reactive at estrus and more prone to inflammation. These physiological characteristics of the follicular phase, might explain why the explants were more reactive to CAT after a short time stimulation and why the MPO induced collagen response was only observed in the follicular phase. Nonetheless, in mid-luteal phase explants treated for 24h with MPO, there was a decrease in *COL1A2* transcription, suggesting that progesterone might control the reduction of MPO pro-fibrotic effects in mid-luteal phase. Some studies have demonstrated that there is a loss of estradiol and progesterone receptors in fibrotic endometrium turning it independent of uterine physiological endocrine control (Hoffmann et al. 2009a; Lehmann et al. 2011). However, progesterone has also shown

protective effects by reducing MPO activity in inflammatory or ischemic disorders (Aksoy et al. 2014; Keshavarzi et al. 2018). Early administration of progesterone inhibited lesion growth and conserved the estradiol and progesterone receptors expression in an endometriosis mouse model (Li, Adur et al. 2016). The least effect of pro-fibrotic enzymes found in NETs in mid-luteal phase in comparison with follicular phase may also be explained by the progesterone protective effect, through the preservation of estradiol and progesterone receptors.

Our results are in agreement with those reported by Rebordão et al. (2018), where the equine endometrial explants response to ELA treatment was also independent of estrous cycle phase. Although, the response to CAT and MPO might depended on estrous cycle phase, since these enzymes enhanced COL1 protein relative abundance in follicular phase, in I/IIA and IIB/III categories, after MPO and CAT treatment, respectively. Follicular phase might predispose to increase susceptibility to ELA, CAT and MPO pro-fibrotic effects, although mid-luteal phase may also be susceptible to fibrogenic mediators present in NETs (Rebordão et al. 2018).

Both PARs and TLRs are distinct transmembrane receptors involved in innate immune response to pathogens. The NETs enzymes were already reported to trigger physiological actions, but also to contribute to pathological conditions by cleaving PARs receptors (Morohoshi et al. 2006; Lohman et al. 2012; Mihara et al. 2013; Feld et al. 2013; Faraday et al. 2013; Muley et al. 2017). The PARs receptors may also drive fibrotic responses through the TGFβ pathway, influencing myofibroblast differentiation (Scotton et al. 2009; Lin et al. 2015a, 2015b; Ungefroren et al. 2018). Additionally, also the TLRs showed an involvement in autoimmune, chronic inflammatory and cancer diseases, beyond the physiological effect in the immune system (Huang and Pope 2009; Devaraj et al. 2011; Isaza-Correa et al. 2014; Jialal et al. 2014; Gao et al. 2017). Thus, the enzymes found in NETs were already reported to drive pathological responses by cleaving TLRs (Summers et al. 2011; Benabid et al. 2012; Skrzeczynska-Moncznik et al. 2013; Domon et al. 2018; Shimoda et al. 2019). It seems that NETs enzymes bind and exert their functions in various receptors, such as PARs and TLRs, triggering a complex combination of signaling pathways. More studies are needed to investigate and confirm which pathways mediate enzyme's pro-fibrotic effects. A complete knowledge on how enzymes trigger fibrotic responses is essential to develop an ideal inhibitor.

Elastase is the neutrophil protease most investigated and that shows the dominant proteolytic activity. Indeed, we have shown its involvement in equine endometrosis, and its pathological importance. Although, when ELA was immune depleted from NETs derived from human neutrophils, the remaining activity was attributed to CAT (O'Donoghue et al. 2013). In the present work, also CAT and MPO showed pro-fibrotic effects, by inducing COL1 protein relative abundance production by endometrial tissues. Many factors contribute to protein production, such as transcription, post-transcription, translation and degradation. Likewise,

protein production and turnover balance can change among different cellular conditions (Vogel and Marcotte 2012). This can justify how quickly COL1 production can be induced and may vary among stimuli from different enzymes. It may be proposed that other pro-fibrotic factors, such as TGFβ1, may be induced quickly or may act in synergy with some enzymes, rather than with others. Those findings suggest that other enzymes than ELA may be involved in a faster fibrotic response and provide further information to be considered, when studying the NETs contribution to endometrosis, as well as an attempt to fight this disease.

One of the main objectives of this experimental work was to evaluate the capacity of specific inhibitors on NETs enzymes inhibition in a perspective of equine endometrosis treatment (Figure 18). Animals already possess naturally occurring endogenous inhibitors of enzymes. But, clearly, these inhibitors are inefficient controllers of enzymes. In addition, these inhibitors are poorly stable under oxidative stress conditions, only the non-adherent enzymes are sensitive to them and they can be degraded by pathogens (Owen et al. 1995; Korkmaz et al. 2005; Guyot et al. 2008; Dubois et al. 2012; Kosikowska and Lesner 2013). The inhibitors used in this study were chosen based on previous promising reports (Kettle 1997; de Garavilla et al. 2005; Takemasa et al. 2012).

The inhibitory effect of SIV on ELA-induced *COL1A2* transcripts was observed in both estrous cycle phases. The inhibitory effect of INH was detected at 48h, corresponding to the increased COL1 relative abundance induced by CAT treatment. The effect of ABAH on the inhibition of MPO-induced COL1 was detected in follicular phase on *COL1A2* mRNA expression, at both times of treatment, and on protein relative abundance at the longest treatment time. To the best of our knowledge, this is the first study showing that by inhibiting ELA, CAT and MPO using specific inhibitors, namely, SIV, INH and ABAH, it is possible to reduce *COL1A2* transcription and COL1 relative protein abundance in equine endometrium. These inhibitors must be considered in future studies to be tested as preventive and therapeutic tools to defeat equine endometrosis. In addition, SIV is actually administered in acute lung diseases in humans, to improve clinical condition and prognosis (Aikawa et al. 2011; Kido et al. 2016; Polverino et al. 2017). Sivelestat is a selective inhibitor of ELA which inhibits the enzymatic action of ELA directly by a reversible ‘acylation-deacylation’ mechanism (Nakayama et al. 2002). This inhibitior did not have any effect on TLR4 levels in an ischemia/reperfusion in liver of a porcine model, suggesting that SIV does not act at cellular level (Shimoda et al. 2019). The CAT inhibitor INH acts by binding to CAT reversibly (Greco et al. 2002; de Garavilla et al. 2005) its use showed promising clinical results for the treatment of COPD and asthma (de Garavilla et al. 2005; Maryanoff et al. 2010; Brehm et al. 2014). The ABAH has also shown promising results in acute diseases, such as steatohepatitis, acute stroke in mice, and in pulmonary cystic fibrosis (Pulli et al. 2015; Kim, Wei et al. 2016; Hair et

al. 2017). The most acceptable ABAH mechanism of action is that ABAH is oxidized by MPO, destroying MPO (Kettle et al. 1997).

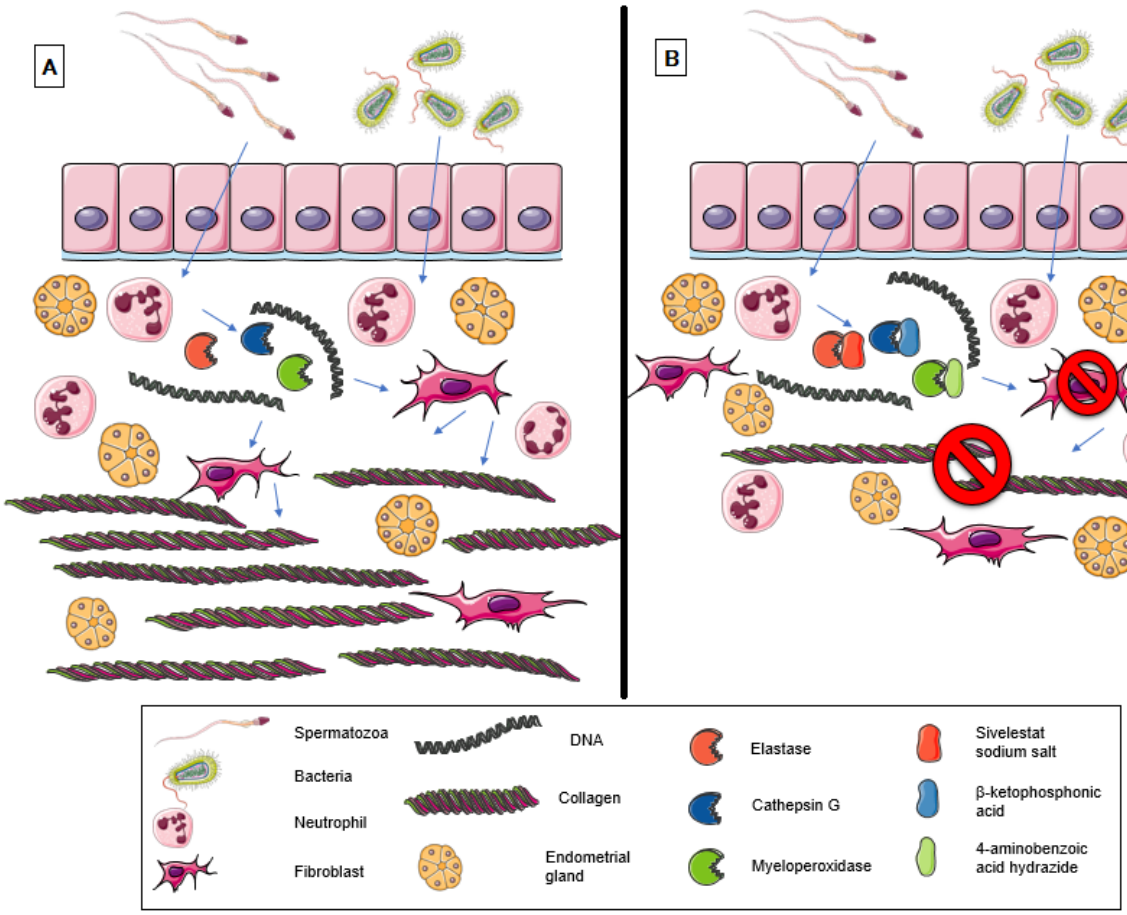


Figure 18: The pro-fibrotic effect of neutrophil extracellular traps (NETs) enzymes (elastase, cathepsin G and myeloperoxidase) promoting fibroblasts collagen secretion and further deposition in lamina propria in equine endometrium (A). Our *in vitro* results showed that by inhibiting elastase, cathepsin G or myeloperoxidase by the action of sivelestat sodium salt, β-ketophosphonic acid or 4-aminobenzoic acid hydrazide, respectively, it was possible to reduce collagen secretion and deposition in the equine endometrium, thus hindering endometrosis development (B).

Knowing that there are other specific NETs enzymes inhibitors, it will be interesting to investigate and compare their ability to inhibit ELA, CAT and MPO in the equine endometrium. Moreover, assuming that these enzymes may act by activating TGFβ pathway, it will be also interesting to unravel the effect of a TGFβ inhibitor on COL1-induced by NETs enzymes. This inhibitor could even inhibit all the pro-fibrotic enzymes effect in a combined approach.

Actually, some molecules are being tested for the treatment of fibrosis. Noscapine is an alkaloid used as an antitussive in humans for decades, presenting low toxicity (Karlsson et al. 1990). Because its action on microtubules and myofibroblast differentiation, noscapine

effects have been investigated in cancer (Quisbert-Valenzuela and Calaf 2016) and fibrosis. Indeed, noscapine proved to be an effective anti-fibrotic agent both *in vitro* and *in vivo* in bleomycin-induced pulmonary fibrosis in mice, acting through EP2 in pulmonary fibroblasts (Kach et al. 2014). In equine endometrium explants, noscapine has also shown the capacity to inhibit NET enzyme-induced COL1, suggesting its putative potential to be used in endometrosis treatment (Amaral et al. 2019).

Other aim of this study was to evaluate the MMPs response when endometria were challenged with NETs enzymes, and their respective inhibitors. In a response to ELA and SIV treatment, endometrial tissues showed different *MMP2* and *MMP9* mRNA levels and protein gelatinolytic activity, either alone or combined, depending on the treatment length. These findings suggest that endogenous hormonal changes and duration of the stimulus can affect the endometrial response. The gelatinolytic activity of MMP-2 active form in endometrial explants increased in response to CAT treatment at 24h, in both estrous cycle phases but decreased with INH addition in follicular phase tissue. Apparently, MMP-2 appears to be involved in an immediate response, perturbing ECM balance. Therefore, MMP-2 can mediate an acute response to a CAT induced inflammation, regardless of the estrous cycle phase. In follicular phase endometrial explants, the gelatinolytic activity of MMP-9 active form increased with CAT treatment, and was inhibited by INH at 48h. This suggests MMP-9 involvement, especially in follicular phase equine endometrium, remodeling the fibrogenic response to a prolonged exposition to CAT. In mid-luteal phase explants treated for 24h with MPO, the gelatinolytic activity of MMP-2 active form increased, and was reduced with the treatment combination of MPO + ABAH. This suggests that MMP-2 can be involved in an acute response to a MPO-induced inflammation, in mid-luteal phase endometria. The gelatinolytic activity of MMP-9 active form increased with MPO treatment and was inhibited by ABAH at 48h in follicular phase endometrial explants. This suggests the MMP-9 involvement in follicular phase equine endometrium remodeling in a response to a prolonged exposition to MPO. The MMPs response to CAT and MPO treatment show some similarities. The MMPs activity is dependent on transcription, protein production, and activation of latent enzymes (Sternlicht and Werb 2001). This fact justifies the different MMPs outputs in response to NETs enzymes and their inhibitors, and that MMPs expression can be influenced by many factors. In fact, MMPs appear to be associated with TGFβ1 activation (Yu and Stamenkovic 2000; D'Angelo et al. 2001; Iida and McCarthy 2007; Kobayashi et al. 2014), myofibroblast differentiation (Dayer and Stamenkovic 2015), and cell proliferation (Hattori et al. 2009; Kobayashi et al. 2014), thus enhancing fibrosis. Additionally, in a recent report in equine endometrial cells, TGFβ1 is a regulator of endometrial ECM remodeling via MMPs and TIMPs (Szóstek-Mioduchowska et al. 2020a). Also in equine endometrial explants, cytokines modulate MMPs and TIMPs, making a

connection between inflammation and endometrosis development (Szóstek- Mioduchowska et al. 2019b).

Our study also demonstrated the putative pro-fibrotic effect of $PGF_{2\alpha}$ and anti-fibrotic effect of PGE_2. Indeed, the concentration of $PGF_{2\alpha}$ in culture medium reduced with SIV treatment in follicular phase at both treatment times and in mid-luteal phase only at 48h. In contrast, the concentration of PGE_2 secreted by endometrial explants augmented with SIV treatment comparing to ELA-treated group in follicular phase at 24h and in mid-luteal phase at 48h. Although, this study only contemplated the use of ELA or SIV, on endometrial tissues PGs secretion on the culture medium. It would be useful to study the effect of other NETs enzymes and their inhibitors as well as gene transcription of PG receptors and synthases to investigate the PG pathways potentially involved in fibrosis. The anti-fibrotic action of PGE_2 was also demonstrated by other studies. In a previous study carried out in our laboratory, the PGE_2 output by equine endometrial explants treated with NETs enzymes showed a protective effect against endometrosis, suggesting that it can lead to reduction of COL deposition in equine endometrium (Rebordão et al. 2019). Moreover, the treatment with PGE_2 increased MMPs and COL1 expression by fibroblasts, suggesting that prostaglandins may be involved in equine endometrial pathological remodeling (Szóstek-Mioduchowska et al. 2020b).

The major limitation of an *in vitro* study is the extrapolation to an *in vivo* organism. We are looking forward to carry out *in vivo* studies and confirm the obtained promising results. Our results show that by inhibiting enzymes found in NETs it will be possible to reduce COL1 output in equine endometrium with further reduction of endometrosis development. Despite the physiopathological mechanism of fibrosis establishment being not fully understood yet, the present work contributes to broaden the knowledge about endometrosis etiology and therapeutics. Although fibrosis is considered an irreversible condition, some authors have also reported the possibility of reversal of fibrosis (Rangarajan et al. 2018; Itaba et al. 2019), introducing a new opportunity to revert fibrotic alterations.

2. Conclusions

The results reached with this work contribute for a better understanding of the mechanisms involved in equine endometrosis. The enzymes found in NETs, PGs and MMPs play a role on equine endometrial fibrosis development. However, the use of enzymes inhibitors revealed to be capable of reducing the pro-fibrotic effects of these poteases found in NETs. Considering the obtained results of this work, it is possible to achieve the following conclusions:

1) The protease ELA present in NETs is capable of inducing *COL1A2* mRNA in equine endometrium *in vitro*, while SIV showed inhibitory effects of this pro-fibrotic action of ELA.

2) The COL1 relative protein abundance was induced by NETs protease CAT in equine endometrial explants. However, when the explants were treated with INH, a CAT inhibitor, this effect was reduced.

3) The treatment of endometrial tissues with MPO induced COL1 relative protein abundance, suggesting that this peroxidase acts as pro-fibrotic factor. Nevertheless, the use of its specific inhibitor, ABAH, reduced MPO-induced COL1.

4) Endometria from the follicular phase showed to be more susceptible to pro-fibrotic effects of enzymes found in NETs, such as CAT and MPO.

5) Metallopeptidases may be implicated in tissue response to ELA and SIV treatment. Although, the MMPs transcription and gelatinolytic activity depended on estrous cycle phase and length of treatment.

6) In response to CAT and INH treatments of equine endometrial tissue, MMP-2 might be involved in an earlier response, independent from estrous cycle phase, and MMP-9 in a later response, mainly in the follicular phase.

7) The MMP-2 might be implicated in an acute response to MPO and ABAH treatments in mid-luteal phase endometrial tissues, whereas MMP-9 seems be involved in follicular phase in a prolonged treatment time.

8) Prostaglandin $F_{2\alpha}$ shows an *in vitro* pro-fibrotic effect and PGE_2 an anti-fibrotic in equine endometrial explants when challenged with ELA and SIV treatments.

3. Future perspectives

1) By inhibiting ELA with SIV, it will be possible to reduce endometrosis establishment.

2) The INH inhibitor, by inhibiting pro-fibrotic effects of CAT, showed that would be possible to hinder endometrosis.

3) The MPO inhibitor, ABAH, impaired the MPO pro-fibrotic effect, showing that by inhibiting MPO it will be possible to decrease endometrosis development.

In conclusion, the use of specific inhibitors of ELA, CAT or MPO, might be the grounds for future development of specific drugs to be used as prophylaxis or therapy of endometrosis in the mare.

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