UNIVERSIDADE DE LISBOA
FACULDADE DE MEDICINA VETERINÁRIA

INVOLVEMENT OF HORMONES, CYTOKINES AND ANGIOGENIC FACTORS ON MARE OVIDUCT PHYSIOLOGICAL FUNCTION AND FIBROSIS

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2018
Lisboa

Dedicated to my father

Acknowledgments

The work presented here is a culminate of a long journey, which was only possible to achieve, with the contribution, help, orientation, friendship and even love of some very important persons that I have the honor to refer to and express my gratitude:

First and foremost, my supervisor, Professor Graça Ferreira-Dias, who was also my supervisor during my Masters degree. Her friendship, altruistic personality, dedication to work, constant determination to help those who surround her, with constant incentives at the most difficult moments, the amazing capacity and acumen to correct us at the right moment, justifies the title of "Best Supervisor of the World", as my college Maria Rosa Rebordão had previously baptized her. I will always be most grateful and she will always be an example for me.

My sincere thanks to my Co-Supervisor, Professor Rosário Plácido Roberto da Costa. From the first moment that I started my work in the Coimbra College of Agriculture, he was always my mentor, partner and my big friend. Since 1999, he has always encouraged me to continue my academic course, which I'm very grateful for. Also, his contribution on the correction of the present Thesis was essential.

To my colleague, my consultant, and my big friend Doctor Maria Rosa Rebordão, who started this adventure with me, and always helped me every time I requested her advice. I'm sure we will keep working together in this area of research, which will give me enough time to pay back all the dinners that I owe you!!

To my college Ana Amaral, for all the help, friendship and time spent with me, encouraging me in difficult times. Thank you so much "Anocas!".

To Carina Fernandes, for her tremendous kindness and willingness to help.

To Sofia Morazzo, Cátia Pereira and Mariana Baptista, for their friendship and precious discussions about our works.

To Dr. Paula Serrão, for her friendship and assistance in laboratory work.

To Doctor António Galvão, who taught me the first steps about genetic research work. The constant good mood and laughter when he was around, are missed by us. His guidance in the first moments of my work, was very important.

To Doctor Elisabete Silva, whose advice was extremely important, whenever technical problems occurred. Her encouragement and friendship were very important.

To the colleagues who hosted me in the Institute of Animal Reproduction and Food Research, of Polish Academy of Sciences, specially Professor Dariusz Skarzynski and Ania Szóstek-

Mioduchowska. Their receptivity and teachings were very important and for that I am very grateful.

To the colleagues in the Department of Animal Science, at the Coimbra College of Agriculture, for their friendship, and special helps during my academic work, so that I could continue preforming my experimental works in Lisbon.

A special thanks to my colleague Maria Alexandra Oliveira, my office mate, who is truly my "sister at work".

To the Coimbra College of Agriculture/Instituto Politécnico de Coimbra, for having allowed me the possibility of carrying out this work as a valuable personal and professional experience.

To Mrs. Conceição Cravo and Engineer Isabel Herder, for their kindness, friendship and contribution during my experimental work performed in Coimbra.

To Dr. Pedro Pessa, my old friend, for his friendship and valued help.

To my colleagues Diana Lapa and Ricardo Campos, my back up support, my friends, my right and left arm, who have given me the crucial support during our clinical work, when I was not able to be present. Thank you so much! I'm afraid I will never be able to reward you…

To my clients/friends who always understood my unavailability to help them in their needs as it would be satisfactory.

To my friends, especially Filipe Gonçalves and Rogério Vieira, for their good friendship, who in different ways so helped me during all these years. Also, a special word of gratitude for his friendship goes to my departed friend Pedro Faria, who passed away two years ago, and whom I regret for not being able to visit him more often during his most difficult period...

Finally, a word for those who were my "left ventricle", pumping me the strength to continue this long journey: to all my family. The last eight years were difficult for us; important loved ones of ours left us... and others joined us... many nights our babies asked me to go sleep with them... maybe many times I was not there, when I was needed... but I'm sure we will be able to compensate the lost time, the lost games, the lost bedtimes stories!

Although the last person I am referring is not amongst us, he was very important for whom I am today. To him, I owe my passion for horses, my love for veterinary science and my satiety for research: my father!

Financial support

This work was supported by:

(i) - Fundação para a Ciência e Tecnologia (FCT), Portugal:

- PTDC/CVT/121805/2010; Coordinator: Prof. Graça Ferreira-Dias
- PTDC/CVT-REP/4202/2014; Coordinator: Prof. Graça Ferreira-Dias
- Joint Projects under the Agreement on Scientific Cooperation between Ministry of Sciences and Higher Education of Poland and the Portugal Foundation for Science and Technology (FCT), for: 2011-2012; 2013-2014; 2015-2016; 2017-2018; coordination of Prof. Graça Ferreira-Dias (FMV/UL, Portugal) and Prof. Dariusz Skarzynski, Department of Reproductive Immunology, Institute of Animal Reproductive and Food Research of PAS, Olsztyn, Poland.

(ii) - European project present by Polish Academy of Sciences, Olsztyn, Poland, under the European Commission CORDIS, Seventh Framework Program (FP7):

- REFRESH-FP7-264103, (2010-2013); Coordinator: Prof. Mariusz Piskula.

(iii) - National Polish Research Center, Poland:

- NRC 2011/02/A/NZ5/00338; Coordinator: Prof. Dariusz Skarzynski

(iv)- Centre for Interdisciplinary Research in Animal Health (CIISA):

- "*New Insights in Equine Oviduct Function*" – 5/CIISA. Projecto CIISA. "*New Insights in Equine Oviduct Function*" – 5/CIISA. Duração: 1 Janeiro de 2016 a 31 de Dezembro de 2016.
- Equine Oviduct Specific Glycoprotein – Is there a Cross Talk with Sperm Cells in the Oviduct?" Projecto 15/CIISA. Duração: 1 Janeiro de 2017 a 31 de Novembro de 2017.

THESIS TITLE: Involvement of hormones, cytokines and angiogenic factors on mare oviduct physiological function and fibrosis

Abstract

The oviduct is a very important organ of the female reproductive system, as it plays a crucial role in providing the ideal conditions for the final preparation of the gametes for fertilization and to support the early embryo development. This work contributed to: (i) clarify the role of ovarian steroid hormones, oxytocin (OXT) and TNFα on the modulation of oviduct prostaglandin secretion; (ii) relate the expression of angiogenic growth factors during angiogenesis, with oviductal function; (iii) evaluate the expression of OVGP1 throughout the estrous cycle in the mare; (iv) and to investigate the expression of collagen in equine oviduct and its correlation with endometrial fibrosis and possible pathways involved. *Post-mortem* tissues were used for experimental works, such as histological stains, immunohistochemistry (IHQ), western blot analysis, qPCR evaluation, enzyme immunoassay, oviduct epithelial cells and tissue explants *in vitro* culture. In equine oviduct, ESR1, ESR2, PGR, OXTR, PTGES and AKR1C3 mRNA and protein expression was estrous cycle dependent and varied with oviduct portions. Ovarian steroid hormones, OXT and TNFα stimulation of $PGF_{2\alpha}$ and/or PGE_2 production also depended on estrous cycle dependent and changed in the different portions of oviduct. In addition, protein and mRNA expression of FGF, VEGF and their receptors differed throughout the estrous cycle and between oviduct portions and agreed with changes in microvascular density and/or oviductal secretory function. Oviduct glycoprotein 1 (*OVGP1*) transcription presented differences throughout the oviduct portions, and in different phases of estrous cycle. A higher expression was observed in isthmus and during follicular phase ($P < 0.05$). Also in the follicular phase, OXT and TNFα upregulated *OVGP1* transcription; in the early luteal phase, estradiol (E_2), while in mid luteal phase, it was progesterone (P_4) that stimulated its transcription. However, OVGP1 *in vitro* production was not dependent of E_2, P_4, OXT or TNF treatments in any oviduct portion. Furthermore, sperm cells also up-regulated OVGP1 production, in isthmus, in early luteal phase ($P < 0.05$). *COL1* and *COL3* transcription in isthmus was correlated with the correspondent transcription in the endometrium. Particularly in isthmus, *AKR1C3* was implicated with collagen transcription ($P < 0.05$). Collagen transcription in isthmus was correlated with MMPs transcription in endometrium ($P < 0.05$). Thus, in the mare, endometrium fibrosis appears to reflect collagen deposition in the oviduct.

Key words: Mare, oviduct, hormonal regulation, angiogenesis, oviductin, fibrosis

TÍTULO DA DISSERTAÇÃO: Envolvimento hormonal, de citoquinas e fatores angiogénicos na função fisiológica e na fibrose do oviducto.

RESUMO

O oviduto é um órgão muito importante uma vez que proporciona as condições adquadas à preparação final dos gâmetas, fertilização e desenvolvimento embrionário no início da gestação. Os principais objetivos deste trabalho consistiram em: (i) clarificar a função das hormonas esteroides ováricas, ocitocina e do TNFα na regulação do funcionamento do oviduto; (ii) relacionar a densidade vascular com a expressão de fatores angiogénicos; (iii) avaliar a expressão da OVGP1 ao longo do ciclo éstrico da égua; e (iv) investigar a expressão do colagénio no oviduto equino relacionando-a com a fibrose peri-glandular do endométrio e possíveis vias envolvidas. Os tecidos obtidos pós-morte foram utilizados em diferentes procedimentos tais como preparações histológicas, imunohistoquímicos, WB e PCR semi-quantitativo. ensaios imunoenzimáticos e culturas *in vitro* de células epiteliais do oviduto e de explantes. A expressão proteica e de mRNA de ESR1, ESR2, PGR, OXTR, PTGES e AKR1C3 mRNA foram influenciadas pela fase do ciclo éstrico e pela porção do oviducto. A estimulação da produção de $PGF_{2\alpha}$ e de PGE_2 pelas hormonas esteroides ováricas, ocitocina e pelo TNFα foi também dependente da fase do ciclo éstrico e da porção do oviducto. A expressão proteica e génica do FGF, VEGF e dos seus recetores no oviducto da égua, variou ao longo do ciclo éstrico, entre as porções de oviduto e coincidiu com as alterações verificadas na densidade microvascular e/ou com a função secretora do oviduto. A OVGP1 apresentou diferenças na transcrição entre as porções do oviducto e ao longo do ciclo éstrico e uma expressão proteica superior no istmo e durante a fase folicular ($P < 0.05$). Os espermatozoides estimularam a produção de OVGP1 no istmo durante a fase lútea inicial ($P < 0.05$). A transcrição de COL1 e COL3 mostrou uma correlação com a sua transcrição no endométrio, bem como a expressão proteica apresentou o mesmo padrão no endométrio de éguas classificado na categoria III de Kenney. Particularmente no istmo, a *AKR1C3, o ALK5 e o TGFβII,* parecem estar implicados com a transcrição de colagénio ($P < 0.05$). Além disso, a transcrição de *COL1* e *COL3* no istmo foi correlacionada com a transcrição de MMPs do endométrio ($P < 0.05$). Assim sendo, a fibrose no endométrio da égua parece estar relacionada com a deposição de colagénio no oviduto.

Palavras-chave: Égua, oviducto, regulação hormonal, angiogénese, oviducto fibrose

TÍTULO DA DISSERTAÇÃO: Envolvimento hormonal, de citoquinas e fatores angiogénicos na função fisiológica e na fibrose do oviducto.

Resumo alargado

A criação de cavalos de aptidão desportiva é hoje em dia de grande importância dada a procura que o desporto equestre exige. A Europa é provavelmente o continente com uma maior produção de cavalos, sendo acompanhada de muito perto por alguns países da América nomeadamente Brasil, EUA e a Argentina. Além disto, a criação de cavalos assume particular importância em zonas rurais, dado a relevância que representa na sustentabilidade das populações.

O sucesso da atividade reprodutiva é fundamental para a criação de cavalos de desporto e disso é bom exemplo a grande importância que é prestada a este tema pelos maiores criadores de cavalos de desporto, os quais habitualmente possuem laboratórios clínicos muito bem equipados.

Bem como as qualidades seminais, o estado reprodutivo da égua é de grande importância. Além de ovários e útero, o oviduto é um órgão que também apresenta funções de grande importância no sucesso reprodutivo, pois ambos os gâmetas terão que percorrer e permanecer algum tempo neste órgão, visto ser neste local onde irá ocorrer a fertilização e ainda o desenvolvimento inicial do embrião. O oviduto é um órgão que fazendo a ligação anatómica entre o ovário e o útero, está sob a ação das hormonas esteróides ováricas (estradiol - E_2, progesterona – P_4), prostaglandinas (PGs) e oxitocina (OXT). Além da regulação hormonal, outros agentes também têm um papel no controlo da sua função tais como os fatores angiogénicos. No fluido do oviduto existem componentes semelhantes aos que se encontram no plasma sanguíneo, enquanto outros são produtos de secreção específicos deste órgão, tais como glicoproteínas específicas do oviduto (OVGP1).

O presente trabalho engloba quatro capítulos distintos, tais como:

i. Estudo da ação das hormonas esteroides ováricas, OXT e TNFα na função do oviduto equino;
ii. Estudo das alterações da expressão proteica e génicas dos fatores angiogénicos FGFs, VEGF e respetivos recetores, relacionando-as com as diferenças na densidade microvascular do oviduto;
iii. Estudo das alterações da expressão proteica e génica da glicoproteína 1 específica do oviducto (OVGP1) ao longo do ciclo éstrico da égua e possíveis fatores envolvidos na sua secreção;

iv. Estudo da possível existência da deposição de colagénio no oviducto da égua, sua possível correlação com a fibrose no endométrio e possíveis vias envolvidas na acumulação do colagénio.

Assim, foram recolhidos *pós-mortem*, aparelhos reprodutivos de éguas. As estruturas ováricas e as concentrações plasmáticas de P_4 foram usadas na determinação da fase do ciclo éstrico de cada uma das éguas em estudo. O endométrio foi classificado histologicamente segundo a escala de Kenney (1978). Por microscopia de luz polarizada foi determinada a proporção de colagénio do tipo I e do tipo III em endométrios e respetivos ovidutos corados com picrosirius red (PSR). Os ovidutos recolhidos foram sujeitos a vários procedimentos, tais como a avaliação de transcrição de mRNA de vários genes por PCR em tempo real (qPCR); a determinação da expressão proteica por western blot (WB) e imunohistoquímica (IHQ); a observação da ultraestrutura por microscopia eletrónica de transmissão (SEM); e a realização de ensaios *in vitro* de células do oviduto epiteliais (OEC) e de explantes.

Num primeiro estudo foi avaliada a expressão génica, por qPCR, e proteica por WB, do recetor da progesterona (PGR), dos recetores do estradiol (ESR), do recetor da oxitocina (OXTR), da síntase da PGE_2 (PTGES) e da $PGF_{2\alpha}$ (AKR1C3). A estrutura das células epiteliais foi avaliada por microscopia de varrimento (SEM). Foram estudados os efeitos de tratamentos de estradiol (E_2), progesterona (P_4), OXT e fator de necrose tumoral (TNF) na produção de PGE_2 e $PGF_{2\alpha}$. Num segundo estudo foi determinada a expressão génica do fator de crescimento de fibroblastos (FGF) 1 e 2, dos seus recetores 1 e 2 (*FGFR1* e *FGFR2*), do fator de crescimento do endotélio vascular (*VEGF*) e do seu recetor 2 (*KDR*); foi também determinada a expressão proteica do FGFR1, FGFR2 e do KDR; bem como as alterações da densidade microvascular do oviduto equino. Foram avaliados os efeitos do tratamento de E_2, P_4, OXT e TNF na transcrição do *FGFR1*, *FGFR2*, *KDR* e do recetor 1 do VEGF (*FLT1*). No terceiro estudo foram estudados a expressão génica e proteica da OVGP1 e os efeitos do tratamento de E_2, P_4, OXT e TNF na transcrição da *OVGP1* e na secreção de OVGP1. A influencia da presença de espermatozoides em co-cultura com explantes de oviduto na secreção de OVGP1 foi investigada. No quarto trabalho foi estudada a transcrição de colagénio tipo I (*COL1*) e tipo III (*COL3*) no oviduto equino, bem como a expressão proteica do COL1. Foi também considerada a proporção de áreas ocupadas por COL1 e COL3 visualizadas por microscopia de luz polarizada tanto no endométrio como no oviduto das mesmas éguas, previamente agrupadas de acordo com a sua classificação endometrial de Kenney (1978). Foram também consideradas a correlação da transcrição de *COL1* e *COL3* entre o oviducto e o endométrio; a correlação da transcrição de *COL1* e *COL3*, no oviducto, com diversos agentes potencialmente indutores de fibrose; bem como a correlação entre a transcrição de *COL1* e *COL3* no oviduto e no endométrio de diversos

agentes com influência no desenvolvimento de fibrose. O efeito dos tratamentos de E_2, P_4, OXT e TNF na transcrição e na expressão proteica de colagénio tipo I foi igualmente determinado. Enquanto a transcrição de *ESR1*, *ESR2*, *PGR*, *OXTR*, e *PTGES* foi superior na ampola, no caso da *AKR1C3*, foi superior no infundíbulo. Nenhum dos recetores do E_2 apresentou diferenças na sua transcrição ao longo do ciclo éstrico, enquanto o PGR apresentou maior transcrição na fase folicular e o OXTR, a PTGES e a AKR1C3 na fase lútea inicial. A expressão proteica foi superior na fase folicular no caso do PGR, ESR1, ESR2 e OXTR, enquanto a PTGES e a AKR1C3 não apresentaram diferenças. Por imunohistoquímica observou-se a expressão de OXTR no estroma da submucosa das pregas do oviduto, enquanto as restantes proteínas apresentaram expressão nas células epiteliais da mucosa. A SEM evidenciou uma maior abundância de células não secretoras, enquanto a densidade de cílios e das células ciliadas foi maior na ampola na fase folicular, do que no infundíbulo. A produção de PGE_2 e de $PGF_{2\alpha}$ por parte de células epiteliais e de explantes, sob a influência de E_2, P_4, OXT e TNF variou consoante a fase do ciclo éstrico e/ou a porção do oviduto.

O oviduto apresentou uma maior densidade microvascular no istmo, durante a fase folicular. A transcrição de FGF1, FGF2, VEGF, FGFR2 e KDR foi superior no istmo, enquanto no FGFR1, não apresentou diferenças. O FGFR1 revelou uma maior expressão proteica no istmo, enquanto ao longo do ciclo éstrico, no infundíbulo a expressão foi superior nas fases folicular e lútea inicial e na ampola foi superior na fase lútea inicial. O FGFR2 não apresentou diferenças entre as porções. Contudo, ao longo do ciclo éstrico, a sua expressão foi superior na fase lútea média, no infundíbulo, e na fase folicular na ampola e istmo. O KDR também apresentou maior expressão no istmo e enquanto o infundíbulo apresentou maior expressão na fase lútea inicial, na ampola a maior expressão verificou-se na fase folicular. Em explantes da ampola o E_2 estimulou a transcrição de *FGFR1*, *FGFR2*, *FLT1* e *KDR* na fase lútea inicial, enquanto a P_4 e a OXT também estimularam a transcrição de *FGFR1* na fase lútea inicial enquanto a P_4 estimulou a transcrição de *FLT1* na fase lútea média. A expressão de ligando e recetores do grupo dos FGFs e do VEGF, coincidem ora com a maior densidade microvascular do istmo ora com os principais fenómenos que ocorrem no oviducto durante a fase folicular e lútea inicial. Enquanto a transcrição de *OVGP1* foi superior na fase lútea inicial e na ampola, comparativamente com o infundíbulo, apenas na ampola se voltaram a verificar diferenças, com maior transcrição também na fase lútea inicial. A expressão proteica foi superior no istmo e em todas as regiões, durante a fase folicular. Os espermatozoides estimularam a produção de OVGP1 pelos explantes na fase lútea inicial. O COL1 e o COL3 apresentaram uma maior transcrição no istmo. As éguas com uma maior fibrose peri-glandular no endométrio,

apresentaram um padrão muito semelhante entre as áreas ocupadas pelo COL1 e pelo COL3 entre o istmo e o endométrio. Existe uma correlação significativa entre a transcrição de *COL1* e *COL3*, entre o istmo e o endométrio. Também se verifica uma correlação significativa entre a transcrição de *COL1* e *COL3* com alguns genes conhecidos pela sua ação pro-fibrótica, nomeadamente *AKR1C3*, *ALK5*, *TGFβRII*, *MMP2* e *MMP9*. A transcrição de *COL1* e *COL3* no istmo também apresentou uma correlação significativa com a transcrição de genes pro-fibróticos no endométrio, nomeadamente TNF, MMP2 e MMP9. Em conclusão, o oviducto da égua é uma estrutura complexa com a capacidade de produção de proteínas específicas, tal como OVGP1 e de expressar fatores angiogénicos sob a ação endócrina ovárica, com especificidades entre as diferentes porções do oviduto. Além disso, a fibrose no endométrio equino parece estar relacionada com a deposição de colagénio no oviduto, em especial no istmo.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

AI - artificial insemination

AIJ - ampullary-isthmic junction

AKR1C3 - aldo-keto reductase family 1-member C3

ALK - activin like kinase receptors

ANOVA - analysis of variance

AQP - aquaporins

AU - arbitrary units

B2M - beta-2-microglobulin

BSA - bovine serum albumin

CA - *corpus albicans*

Ca++ - calcium ion

CaMKII - calcium-calmodulim protein kinase II

CATSPER1 - cation channel sperm associated 1

CD31 - cluster of differentiation 31

cDNA – complementary deoxyribonucleic acid

Cf - *confer*

CH - *corpus hemorragicum*

CKD - chronic kidney disease

CL - *corpus luteum*

COC - cumulus-oocyte complex

COL1 - type I collagen

COL3 - type III collagen

CONT - control

COX-II - cyclo-oxygenase II

Ct - cyclic threshold

DCM - diabetes cardiomyopathy

DMEM - Dulbecco's Modified Eagle's medium

DNA - deoxyribonucleic acid

E - primer efficiency level

E_2 - estradiol

EC - endothelial cells

ECM - extracellular matrix

EDTA - ethylenediamine tetraacetic acid

EGF - epidermal growth factor

EIA - enzyme immunoassay

ELP - early luteal phase

EP - prostaglandin E2 receptors

ESR - estrogen receptors

et al - *et alia*

FGF - fibroblast growth factor

FGFR - fibroblast growth factor receptor

F-IPF - fibroblast in Idiopathic Pulmonary Fibrosis

FLT1 - fms related tyrosine kinase 1

FOXJ1 - forkhead box protein J1

FP - prostaglandin $F_{2\alpha}$ receptor

g - gram

GAPDH - glyceraldehyde 3-phosphate dehydrogenase

GDM - gestational diabetes mellitus

GnRH - gonadotrophin releasing hormone

hCG - human chorionic gonadotropin

HCO_3 - bicarbonate

HRP - horseradish peroxidase

ID50 - coefficient of variation

ie - *id est*

IgG - immunoglobulin G

IHC - immunohistochemistry

IL - interleukin

IP_3 - inositol-1,4,5-trisphosphate

IPF - idiopathic pulmonary fibrosis

IVF - *in vitro* fertilization

kDa - kilodalton

KDR - kinase insert domain receptor

LH - luteinizing hormone

mL - mililiter

MLP - mid-luteal phase

MMP - matrix metalloproteinase

mRNA - messenger ribonucleic acid

MRPL32 - mitochondrial ribosomal protein L32

NADPH - nicotinamide adenine dinucleotide phosphate

NETs - neutrophil extracellular traps

NO - nitric oxide

OEC - oviduct epithelial cell

OGPs - ostrogen-dependent glycoproteins

OVGP1 - oviduct-specific glycoprotein

OXT - oxytocin

OXTR - oxytocin receptor

P - probability value

P_4 - progesterone

PAX8 - paired box 8

PBS - phosphate buffered saline

PDGF - platelet-derived growth factor

PG - prostaglandin

PGE_2 - prostaglandin E_2

PGF_{2a} - prostaglandin F_{2a}

PGR - progesterone receptor

PTGES - prostaglandin E2 synthase

qPCR - quantitative polymerase chain reaction

R - correlation coefficient *i.e.* Pearson coefficient

R^2 - determination coefficient

RIPA - radioimmunoprecipitation assay buffer

RNA - ribonucleic acid

SD - standard deviation

SDHA - succinate dehydrogenase complex flavoprotein subunit A

SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM - standard error of the mean

SMA - smooth muscle actin

SR - sperm reservoir

SSs - systemic sclerosis

ST - standard protein

TGFβ - transforming growth factor-β

TGFβR - transforming growth factor-β receptor

TIMP - tissue inhibitor of metalloproteinases

TNF - tumor necrosis factor-α

TNFRSF - tumor necrosis factor receptor super-family

UTJ - utero-tubal junction

VEGF - vascular endothelial growth factor

VEGFR - vascular endothelial growth factor receptor

vs - versus

WB - western blot analysis

ZP - zona pellucida

α2ap - α2-antiplasmin

CHAPTER I – INTRODUCTION AND OBJECTIVES

1.1. Introduction

Equine reproduction has held an important place from immemorial times as horses were used as means of transport and as war machines, however, gaining greater significance in the world of equine sport especially after the Second World War, when Central Europe assumed a leadership role in breeding a high percentage of horses to compete in the main equestrian disciplines. The equine breeding industry is not only important for the equine sports but it also has a tremendous effect on the economy of some countries and rural regions where agriculture and animal breeding are important and core supports for sustainability of local populations. Apart from Europe, equine breeding is also very important in the western hemisphere, where especially countries like USA, Brazil and Argentina have produced excellent examples of equine athletes.

Mare reproductive function is vital for success in equine breeding programs. Besides the unquestionable importance of the semen quality, mare's reproductive status not only at the time of the insemination, but also during the gestational length, can compromise the success of reproductive activity.

Besides the uterus and the ovaries, the oviduct is another organ which can have a vital role in the reproductive function. Already in the past, several situations were detected in which mares failed to conceive in the absence of any identifiable reproductive tract malfunction (Allen, Wilsher, Crowhurst, Hillyer & Neal, 2006). In particular, the embryo transfer procedure, has provided evidences for the importance of oviductal role, since the uterine flushing is performed at a time, where it is very unlikely that the uterus could have a deleterious effect on the embryo recovery. Historically, the oviduct was seldom ascribed to any significant importance in fertility rates, primarily because of the inability to carry out oviduct clinical evaluation.

In the mare, there are some *post-mortem* reports of pathological changes, such as oviductal cysts (Blue, 1984) and histopathological features, consistent with salpingitis (Saltiel, Paramo, Murcia & Tolosa, 1986).

The equine oviduct is about 20 cm long, and is divided into three main portions: infundibulum, ampulla and isthmus (Yaniz, Lopez-Gatius & Hunter, 2006). Within the oviduct, several events occur, which are ascribed as being of fundamental importance for the reproductive success. Among those events, occur the gamete transport, fertilization and early embryo development. The mare, presents a particular characteristic that is the possibility of the oviduct to retain the unfertilized oocytes and allow only the embryo to reach the uterus, which is attributed to the prostaglandin E_2 released by the blastocyst. For proper oviductal function, hormonal regulation is necessary, as occurs in all female reproductive organs. In cow, oviductal contraction and

secretory activity, may be regulated by ovarian steroids and oxytocin (OXT) (Wijayagunawardane *et al*., 1998). Also, nitric oxide (NO) and cytokines can modulate oviductal functions in cow (Szóstek *et al*., 2011). Prostaglandins (PGs) may also modulate oviductal function through different mechanisms (Wanggren, Stravreus-Evers, Olsson, Andersson & Gemzell-Denielsson, 2008).

Angiogenic growth factors have been associated to several female reproductive functions. Fibroblast growth factors (FGFs) are involved in folliculogenesis, particularly during the final growth of the preovulatory follicle, due to stimulation of angiogenesis and granulosa cell survival and proliferation (Berisha, Sinowatz, & Schams, 2004). Also in gilts, a member of FGF's family was identified in the endometrial epithelium, stroma and myometrium during the estrous cycle and early pregnancy (Katsahambas & Hearu, 1996). In human oviduct, vascular endothelial growth factor (VEGF) mRNA expression was related to the ovulatory period (Lam *et al*., 2003).

The oviductal fluid contains different components. Some of them are also present in serum, while others are characteristic of the oviduct. The oviductal components can vary between proteins, aminoacids, lipids, energy substrates, ions, minerals, steroid hormones, prostaglandins and growth factors (Aguilar & Reyley, 2005). One type of protein, seems to be specific of the oviduct, and has been identified in several animals. It is known as the oviduct specific glycoprotein (OVGP1), but we can find other synonymous such as estrogen dependent oviduct protein, mucin-9, oviductal glycoprotein or oviductin. Since in different species, this glycoprotein has been associated to the sperm cells, the oocyte and the early embryo, some of its hypothetical functions are related to capacitation, fertilization, and early embryo development (Aguilar & Reyley, 2005).

Fibrosis can be considered a normal step of the healing process. Nevertheless, a precise modulation of this process is necessary, otherwise it can result in substantial remodeling of the extra-cellular matrix, leading to scar tissue formation, which in fact consists of an excessive collagen deposition (Wynn, 2007). In many organs, like lung, kidney, liver and heart, fibrosis can result in organ failure. In the mare endometrium, peri-glandular fibrosis is associated to endometrial glands structure disruption and function, leading to infertility (Ferreira-Dias, Nequi, & King, 1994).

The experimental work presented in this thesis is divided into four studies: in the first one, the focus will be on the role of ovarian steroids, OXT and tumor necrosis factor α (TNF) in equine oviduct secretory function modulation (i); in the second study, changes in microvascular density in the equine oviduct will be evaluated and how VEGF, FGF and some of their receptors are expressed in equine oviduct throughout its length and during the estrous cycle (ii); in the third

study, it will be presented how OVGP1 protein and mRNA are expressed in equine oviduct and how it can be related with events that occur in oviduct (iii); in the fourth study, expression of collagen type I (COL1) and type III (COL3) protein and mRNA will be evaluated in equine oviduct as well as *COL1* transcription in the endometrium of the same mares. Also, transcription of some possible players involved in collagen expression will be evaluated, to demonstrate if fibrosis is present in equine oviduct and which pathways could be involved on it (iv).

1.2. Objectives

The major objectives of this work were:

1) To investigate the role of ovarian steroids hormones, oxytocin, and tumor necrosis factor on the modulation of equine oviduct function.
The developed work was published: Pinto-Bravo P., Galvão A., Rebordão M.R., Amaral A., Ramilo D., Silva E., Szóstek-Mioduchowska A., Alexandre-Pires G., Roberto da Costa R., Skarzynski, D.J. & Ferreira-Dias G. (2017). Ovarian steroids, oxytocin and tumor necrosis factor modulate equine oviducto functions. Domestic Animal Endocrinology; 61: 84-99
2) To examine the expression of FGFs and VEGF and their receptors in equine oviduct and relate it with changes in microvascular density and with main oviductal function. In addition, the influence of ovarian steroid hormones, OXT and TNF on VEGF, FGFs and receptors transcription will be addressed.
3) To study how OVGP1 mRNA and protein are expressed in the mare oviduct and possible factors that can modulate OVGP1 production.
4) To investigate collagen expression in equine oviduct, its relationship with collagen expression in endometrium, possible pathways involved in it, and the effect of ovarian steroids, OXT and TNF on COL1 expression.

CHAPTER II – STATE OF THE ART

2.1. Equine oviduct

2.1.1. Anatomy of Oviduct

Anatomically the oviduct is a tubular structure that is present between the ovary and the uterus, and is not in complete contact with the ovary but is in continuation with the latter. In the mare, the oviduct varies in length between 20-30 cm, and is suspended by the mesosalpinx (Hafez & Hafez, 2000a)

In mammal's species, the oviduct is divided into three regions: the infundibulum, the ampulla and the isthmus. Connecting these regions, are described the uterotubal junction (UTJ), and the ampullary-isthmic junction (AIJ), as well as a proximal section connected to the ovarian fimbriae, in the abdominal opening, the ostium (Yaniz, Lopez-Gatius, F. & Hunter, 2006). In the isthmus, the luminal area does not exceed 0.5 mm^2, with no differences shown between phases of the estrous cycle. In the ampulla, multiple epithelial folds are present, frequently quaternary folds. Compared with the isthmus, it presents a larger intraluminal diameter (Mouguelar *et al*., 2015). Ampulla is the middle portion of the oviduct, and represents about half of its length, and it is where oocyte fertilization occurs (Hafez & Hafez, 2000a). The infundibulum is funnel-shaped, with irregular fimbriae present along its margin. Some of them are attached to the cranial pole of the ovary, allowing the rest of the infundibulum to spread over the ventral aspect, covering the ovulation fossa (Kainer, 1993).

2.1.2. Histology of oviduct

Histologically the oviduct presents three concentric layers. According to Muglia & Motta (2001), the serosa is composed of mesothelial and non-striated muscular cells, derived from the uterine broad ligament. The middle layer is a muscular layer (myosalpinx), which is organized in a particular arrangement in different species. The internal layer, is the mucosa, which presents a dense lymphatic and blood vascular network, and folds that are projected internally. The height, width and branching of the mucosa folds are more pronounced in the ampulla and infundibulum. The mucosa presents a simple columnar or pseudostratified epithelium, containing ciliated and secretory cells (Mouguelar *et al*., 2015). Ciliated cells are mainly involved in gamete and embryo transport (Kolle *et al*., 2009), while secretory cells are involved in the synthesis and release of different molecules dissolved in the oviductal fluid, plus a selective transudate of serum (Killian, 2011).

Epithelial cell remodeling, which is regulated by differentiation and/or proliferation of secretory cells, provides the optimal environment for gamete transport, fertilization and early embryonic development (Ito, Kobayashi, Yamamoto, Kimura & Okuda, 2016). Ciliated cells have a slender motile cilium that extends into the lumen. The beat rate of cilia is affected by the levels of ovarian hormones, and is maximal at ovulation and shortly after, when the stroke of the cilia in the infundibulum is closely synchronized and directed toward the ostium. The ciliary beats in the direction of the uterus coupled with oviductal contractions plays an important role keeping oocytes in constant rotation. This is essential to bring oocytes and sperm cells together, and prevent oviductal embryo implantation (Hafez & Hafez, 2000a). In goat oviduct, the epithelium of the infundibulum, ampulla, and AIJ in the follicular phase is extensively ciliated and most cilia extend above the apical processes of the non-ciliated cells. In contrast, in the luteal phase, many ciliated cells are hidden by the bulbous processes of the non-ciliated cells. In the isthmus, and at the utero-tubal junction, the apical surface of the non-ciliated cells are flat or gently rounded at both phases of the estrous cycle (Abe, Onodera, & Sugawara, 1993).

Secretory (non-ciliated) cells contain secretory granules, whose size and number could vary with animal species and estrous cycle phases. The apical surface of these cells is covered with numerous microvilli, and probably secretory granules accumulated in epithelial cells during the follicular phase are released into the lumen after ovulation, causing a reduction in epithelial height (Hafez & Hafez, 2000a). According to Abe (1996), they will be needed for the interaction of oocyte and sperm cells. Several studies have been performed in different species to study the percentage of ciliated and secretory cells. In the cow ampulla, the percentage of FOXJ1-positive cells (a ciliated cell marker) was the highest at the day of ovulation (Day 0) and decreased about 50% by Days 8-12, while in the isthmus it did not change during the estrous cycle (Ito *et al*., 2016). The mitosis rate was the highest at around the time of ovulation in secretory cells (PAX8-positive), in both the ampulla and isthmus (Ito *et al*., 2016). In an early study in cow oviduct, a decrease in ciliated cells and an increase in secretory cells occurred in the infundibulum, during luteal phase (Abe, 1996). The same situation was observed in primates, such as the rhesus monkey (Brenner, 1969). Nevertheless, in other species, such as the golden hamster and the rat, the number of ciliated and secretory cells does not alter during the estrous cycle (Abe, 1994). Apparently, species with a long estrous cycle time span present differences in the percentage of ciliated and secretory cells during the estrous cycle, while the species with short estrous cycle length, do not (Abe, 1996).

Besides changes in the number of the two types of epithelial cells, differences also occur in their appearance during the estrous cycle. In fact, in some species (cows, goats and Chinese Meishan pigs), the height of the ciliated cells in the infundibulum and ampulla falls during the

luteal phase, while in the secretory cells it becomes more prominent. In the isthmus both ciliated and secretory cells remain almost constant throughout the estrous cycle (Abe, 1996).

Eriksen and co-authors (1994) demonstrated in the cow, that number and location of secretory granules in the secretory cells of the oviductal epithelium, show both cyclic and segmental variations. The regional differences in the ultrastructure and in the number of secretory granules in the cells might reflect the fact that the nature of the secretion from oviductal epithelial cells, and their secretory activities differ among the various segments of the oviduct (Abe, 1996).

In bitch, E_2 cause oviduct epithelium cell hypertrophy and differentiation, whereas P_4 induces cell gradual regression, revealing visible morphological changes in the tubal epithelium in the estrous cycle. However, these changes are variable, depending on the oviduct portion (Steinhauer, Boos, & Gunzel-Apel, 2004).

Oviductal fluid from secretory cells, presents differences in its biochemical characteristics throughout the regions. Proteins secreted might have specific roles in the support of embryo as it crosses the oviduct. In cycling pigs, oviduct explants from ampulla and isthmus produce different glycoproteins, soon after fertilization (Buhi, Alvarez, Sudhipong & Dones-Smith, 1990). In sheep, E_2 alone or in addition with P_4, stimulates the production of a glycoprotein by the ampulla, but not by the isthmus (Murray, 1992). Also in the mare, it was reported a higher oviductal secretory activity in ampulla than in isthmus (McDowell, Adams & Williams, 1993).

Some of the oviductal fluid functions may include sperm capacitation, sperm hyperactivation, fertilization and early preimplantation development. The oviductal fluid is composed of a selective transudate of serum and secretory products from the secretory cells. Several protein components are common between oviductal fluid and serum. However, some of them are present in different proportions, others exist in serum but not in oviductal fluid, while others are absent in serum, but exist in oviductal fluid (Hafez & Hafez, 2000a). Some oviductal secretions appear responsive to endocrine estrous cycle influence, while others may be produced at a constant rate (Abe, 1996).

The musculature is mainly formed by a smooth inner circular layer and a thinner external longitudinal layer, continuous with the mesosalpinx (Kainer, 1993, Sisson, 1982). The inner circular muscle increases toward the isthmus, reaching a maximal size in the papilla. Also in the mare, the *tunica muscularis* is almost absent in the infundibulum but become gradually thicker towards the UTJ (Eriksen, Terkelsen, Grondahl, and Bruck, 1994).

Oviductal contractions help to denude the oocyte, promote fertilizations and regulate the transport of the embryo. Unlike intestinal peristalsis, oviductal motility tends to delay slightly the progression of the embryo. The oviduct can present several types of complex contractions such as localized peristalsis-like contractions, segmental contractions and worm-like twisting

of the entire oviduct. Generally, the ampulla is less active than the isthmus. The frequency and amplitude of spontaneous contractions vary with the phase of the estrous cycle. Before ovulation, contractions are gentle in the rate and pattern of contractility. At ovulations, contractions become more vigorous, and the finger-like folds' contract rhythmically and "massage" the ovarian surface (Hafez & Hafez, 2000a).

2.1.3. Biological events that occur in the oviduct

The oviduct is an organ with a vital importance for the reproductive activity, since it is where some important physiological events will occur.

2.1.3.1. Ova Pickup

Based on data from 16 equine oocytes collected from pre-ovulatory follicles, it was concluded that in the horse, as in most other mammals, the ovulating oocyte is normally in metaphase II (King, Bezard, Bousquet, Palmer & Betteridge, 1987).

The viscid mass of cumulus oophorus that contains the oocyte and *corona radiata* cells, adheres to the stigma and remains attached unless it is removed by the action of the cilia in the infundibulum (Hafez & Hafez, 2000b). Studies in hamsters revealed the importance of the cumulus cells and the extracellular matrix of the cumulus-oocyte complex (COC), on the picking up process, and initial adhesion to the infundibulum cilia. Cilia that cover the epithelial cells in infundibulum, beat in the direction of the ostium, and induce a current in the oviductal fluid, that arrests the COC into the oviduct (Talbot, Shur & Myles, 2003).

In humans and mice, the role in P_4 in oocyte transport was suggested. In fact, the presence of P_4 receptors (PGRs) was demonstrated in the lower half of the motile cilia of oviduct epithelial and stromal cells (Teilmann, Clement, Thorup, Byskov, & Christensen, 2006).

2.1.3.2. Sperm at fertilization site

Sperm transport to the oviduct may be completed at 4 h after insemination. Thus, it is worth noting that conception rates are significantly impaired when mare's uterine lavage is performed 0.5 h or 2 h after insemination, but no adverse effect was observed on fertility, if done at 4 h after insemination (Brinsko, Varner and Blanchard, 1991).

A small sub-population of spermatozoa is rapidly transported by myometrial contractions towards the UTJ, colonizing the tubal sperm reservoir (SR). This event is known by the rapid phase of semen transport in the female internal genital tract (Rodriguez-Martinez *et al*., 2001).

Nevertheless, a critical phase of the transport in the uterus is the migration through the UTJ, and a very low percentage of sperm cells can reach it compared to the original population contained in the artificial insemination (AI) dose or ejaculate (Tokuhiro, Ikawa, Benham, & Okabe, 2012).

In cow, most spermatozoa present in the SR in the pre-ovulatory period, remain viable and potentially fertile until they ascend to the upper tubal segments, either shortly before ovulation (Hunter, 1996), or as a continuous stream during the peri-ovulatory period (Larson & Larson, 1985).

In the golden hamster, a change in the sperm surface occurs, which causes the release of spermatozoa, that were attached to the isthmic mucosa, enabling sperm cell migration to the ampulla to fertilize the oocytes (Smith & Yanagimachi, 1991). Pollard and colleagues (1991), have demonstrated that bovine spermatozoa attach to cultured epithelial cells of the oviduct and that spermatozoa were bound by the rostral portion of the intact acrosome to the apical surface of polarized epithelial cells. Moreover, they have shown that the fertilizing capacity was maintained when spermatozoa were incubated with oviductal epithelial cells. Also in the pig, similar interactions between the spermatozoa and the oviductal epithelial cells have been observed (Suarez, Redfern, Raynor, Martin, & Phillips, 1991). As well, boar spermatozoa bound to isthmus explants rather than to oviduct ampulla explants. Also, it was observed that *in vitro* binding of sperm cells was greater in the presence of estrus levels of steroids than in culture medium without steroids. These data indicate the importance of the region and the hormonal status on the SR function, prior to fertilization (Raychoudhury & Suarez, 1991). These studies strongly suggest that the binding of spermatozoa to the oviductal epithelium is influenced by both the region of the oviduct and the hormonal status (Abe, 1996).

When sperm is released from SR, sperm cells need to be guided to reach the fertilization place. Some substances have been identified as potential chemoattractant, such as P_4, present in follicular fluid produced by cumulus cells (Chang & Suarez, 2010). Natriuretic peptide precursor has also been identified as a chemoattractant, which modified the sperm motility pattern and enhance Ca^{++} levels (Bian *et al*., 2012). Temperature can also influence levels of Ca^{++}, and so affect the flagellar bending (Bahat & Eisenbach, 2010). Although not demonstrated *in vivo*, other factors could influence the sperm transport and guidance toward the fertilization site, such as the movement of oviductal fluid, oviductal contractions, oviductal epithelium and even the internal structure of the oviduct (Burkitt, Walker, Romano & Fazeli 2012).

2.1.3.3. Changes in the spermatozoa

2.1.3.1.1. Capacitation

Capacitation includes a delicate reorientation and modification of molecules within the plasma membrane, which enables the sperm cell to bind to the extracellular matrix of the egg (*zona pellucida*; ZP) and the zona then primes the sperm to initiate the acrosome reaction, an exocytotic event required for the sperm to penetrate the zona (Gadella, Rathi, Brouwers, Stout, & Colenbrander, 2001). Hunter and Rodrigues-Martinez (2004) introduced three concepts on the control of capacitation to emphasize the importance of *in vivo* integration: (i) completion of capacitation is a peri-ovulatory event; (ii) suppression of completion of capacitation is an essential storage strategy during a long pre-ovulatory interval; (iii) the process of capacitation comes under the influence of local and systemic ovarian control mechanisms, especially the secretion of P_4 from pre-ovulatory follicles. In several mammal species, the oviduct seems to retard, rather than promote sperm capacitation, and by this way, sustaining their fertilizing capacity (Smith & Nothnick, 1997). Also in humans, proteins secreted from human oviductal tissue are able to inhibit events associated with sperm capacitation (Zumoffen, Caille, Munuce, Cabada, & Ghersevich, 2010). Boar spermatozoa from the isthmus SR, at the period prior and shortly after ovulation do not undergo *in vitro* capacitation, unless they are exposed to the effector bicarbonate (Tienthai, Johannisson & Rodríguez-Martínez, 2004). Besides, co-incubation of boar sperm with SR fluid or its component hyaluronan can also stimulate *in vitro* capacitation. Fluid collected from the ampullary segment, which is rich in bicarbonate, is also able to induce boar sperm capacitation *in vitro* (Rodríguez-Martínez *et al*., 2005). Bicarbonate has a primary early role in carrying about the significant changes in membrane lipid architecture. Early responses to bicarbonate involve protein kinase-A activation, membrane fluidity, and membrane lipid scrambling. These responses only take place in the sperm cell population that has matured appropriately (Gadella & Gestel, 2004).

It has been proposed that a progressive and continuous release of spermatozoa from the SR in the oviducts, occurs before ovulation and that it may be related to the gradual induction of capacitation following exposure to the fluid of the upper tubal segments (Rodriguez-Martinez, 2007). In the presence of elevated pH and extracellular Ca^{++}, a heat-resistant, hydrophilic, <30 kDa component of follicular fluid, can trigger protein tyrosine phosphorylation, to elevate cytoplasmic Ca^{++} and stimulate motility in stallion sperm cells (Leemans *et al*., 2015). A combination of albumin and HCO_3^- markedly induced sperm head-to-head agglutination, which physically prevented stallion sperm to bind to oviduct epithelium (Leemans *et al*., 2016).

2.1.3.1.2. Hypermotility

The condition "hyperactivated motility" was first defined by Yanagimachi (1970), who observed that hamster spermatozoa, after detaching from oviduct epithelial cells, acquire a vigorous motility pattern, with high amplitude and asymmetrical flagellar beating. Later, this term was redefined as the swimming pattern shown by most sperm cells retrieved from the ampulla, at the time of fertilization (Suarez & Ho, 2003). Probably, the increase in intracellular Ca^{++} will trigger this event (Publicover *et al*, 2008), which could be related with the secretion of P_4 (Hunter, 2012). Calcium will enter the sperm cell, through channels in its plasma membrane, known as CATSPER proteins, located in the plasma membrane of the principal piece of the flagellum (Qi *et al*, 2007).

An increase in pH, associated with an increase in HCO_3^- in the oviduct fluid, in combination with an increase of P_4, will activate the CATSPER protein, resulting in an increase of Ca^{++} intra spermatozoa, inducing the hypermotility (Suarez, 2008). In bovine, only sperm incubated with oviduct cells, developed hyperactivated motility (Pollard *et al*., 1991).

In vitro fertilization has remained inexpressible in the horse, presenting very low rates. McPartlin and colleagues (2009) suggested that capacitation and hyperactivation are fundamental for successful IVF in the equine.

2.1.3.4. Fertilization

Fertilization in mammals requires three critical events: (a) sperm migration between cumulus cells (if present); (b) sperm attachment and migration through the *zona pellucida* (ZP); (c) fusion of sperm and ovum plasma membranes. Attachment of the sperm head to the ZP is regulated by specific receptors on the zona surface. One of those specific receptors is the glycoprotein ZP3, to which only sperm with an intact acrosome can bind (Hafez & Hafez, 2000b). The presence of glycosyl transferase, proteinases and glycosidases on plasma membrane covering the sperm head could result in binding to ZP3 through a lock/key mechanism such as that for an enzyme and its substrate (Wassarman, 1990).

Penetration of the ZP by sperm occurs within 5-15 minutes after sperm attachment. Eventually, the acrosome reaction may occur before or after attachment of the sperm head to the glycoprotein receptors on the ZP. Binding of the sperm head to ZP3 allows interactions with several enzymes present in/or attached to the acrosomal membranes, which suggest that a combination of enzymes acts synergistically during penetration. Simultaneously sperm tail propels sperm into the vitelline space (Hafez & Hafez, 2000b).

The fusion between ova and spermatozoa plasma membranes cannot undergo with non-acrosomal activated sperm even though attachment to the membrane surface occurs. Attachment of sperm occurs initially at the equatorial segment of the sperm head and fusion does not involve the plasma membrane over the equatorial segment. Subsequently, the surface of the equatorial region is incorporated into the plasma membrane of the ovum and can be identified in the egg membrane as late as the eight-cell stage (Hafez & Hafez, 2000b).

After penetration of the perivitelline membrane by the spermatozoa, the activated ovum completes meiosis and expels the polar body into the perivitelline space. The remaining maternal haploid chromosomes are then enclosed by a pronucleus, and both pronuclei migrate to the ovum center, for rearrangements in the cytoskeletal framework of the ovum after activation (Hafez & Hafez, 2000b).

Equine oviductal secretions improve IVF rates, probably due to specific secretions of this organ, which is a particular aspect in this mammal, with very low rates for this reproductive technic (Mugnier *et al*., 2009).

2.1.3.5. Embryo development in oviduct

After reaching the two-cell stage about 24h after ovulation, division of the blastomeres continues in a regular manner so that there are 4-6 cells at 24h, and 8-10 cells at 72h. During early cleavage, normal equine embryos have quite large amounts of cellular debris in the perivitelline space and they are normal ellipsoid in shape (Bezard *et al*., 1989). Although the evidence presented leaves little doubt that oviduct secretions have the ability to affect gamete and embryo physiology, the mechanisms by which these effects are implemented are largely undefined (Killian, 2004). In bovine, the use of oviduct epithelial cells (OEC) co-culture systems has improved the in vitro development of embryos (Abe & Hoshi, 1997; Ellington, Carney, Farrell, Simkin, & Foote, 1990). Also, equine embryo co-culture with OEC can support the development of four to eight-cell embryos *in vitro*, and those co-cultured embryos can continue normal development after transfer to recipient mares (Ball & Miller, 1992).

One of the major proteins involved in oviduct embryo development is oviduct specific glycoprotein, which will be further addressed. In 1986, Brown and Cheng identified two major glycoproteins (with 250 and 90 kDa, respectively), which were absent from oocytes collected in the follicular phase, present in the oviductal fluid from estrus, but not from luteal phase. The glycoproteins remained on the zona pellucida of 2-to 4-cell embryos. According to these authors, shortly after ovulation, despite the presence around the egg of cumulus oophorus and corona radiata cells, significant amounts of oviductal glycoproteins can bind firmly to the zona

pellucida. In bovine, oviductal epithelium synthesizes and secretes a class of oviduct-specific glycoproteins (OVGP) that is present in the luminal fluid at the time of fertilization (Boice, Geisert, Blair, & Verhage, 1990). Some glycoproteins secreted by the ampulla and isthmus in explants cultures of oviducts from cycling pigs and from pigs soon after fertilization were identified (Buhi *et al*., 1990). These same authors have shown differences in the production of estrus-associated glycoproteins between the ampulla and isthmus in the pig oviduct (Buhi *et al*., 1992). In sheep, an estrus-associated glycoprotein (90-92kDa) was produced by explants from the ampulla of oviducts of ewes previously treated with E_2 alone, or with E_2 plus P_4, but not from the isthmus (Murray, 1993). In cow, Sendai and co-authors (1994) isolated the amino acid sequence of a portion of an oviductal specific glycoprotein, which was highly homologous (71% identity) to that of a baboon oviduct-specific glycoprotein. They suggested that synthesis and secretion of this glycoprotein can be modulated by ovarian steroids, as previously shown in other species. A year later, Sendai *et al*. (1995) analyzed the amino acid sequence of oviductal glycoproteins (OVGPs) from different species (mouse, cow, baboon and human) and concluded that they presented a high degree of homology between them. O'Day-Bowman *et al*. (1996) conducted an experiment with human oocytes, human OVGP, baboon OVGP and spermatozoa. Their results revealed that *in vivo,* human OVGP bind to fertilized oocytes and probably the association of OVGP with the ZP may play a role in fertilization, possibly through enhancing the binding of sperm to the ZP within the oviduct (O'Day-Bowman *et al*., 1996). Also, this group suggested that a homologous system (*i.e*. gametes and oviductal glycoprotein from the same species) is necessary for the study of oviductal glycoproteins function, since human hemizona assays conducted in the presence of baboon OVGP resulted in a significant decrease ($P < 0.05$) in the number of sperm bound per zona compared with that in culture medium alone despite high homology between human and baboon OVGP.

Exposure of pigs oocytes to OVGP before and during fertilization, reduces the incidence of polyspermy and the number of bound sperm, and increases post cleavage development of blastocyst (Kouba, Abeydeera, Alvarez, Day, & Buhi, 2000). Later, in a very interesting review article (Buhi, 2002) the extensive family of glycoproteins was acknowledged in several animal species, by different authors, such as the oviduct secretory glycoprotein, estrus-associated glycoprotein, oviduct-specific estrus-associated glycoproteins, oviduct glycoprotein, oviductin, MUC-9, glycoprotein GP 125 and oviduct specific glycoprotein, as oviduct-specific oestrogen-dependent glycoproteins (OGPs).

In woman, mRNA of OVGP1 was detected throughout the menstrual cycle, and the highest level was found in the periovulatory period. It continued to be expressed in early pregnancy, but it was absent in the postpartum period and after menopause (Lok, Briton-Jones, Yuen, &

Haines, 2002). This protein was suggested to be related with a supportive role in fertilization and early embryo development. In another study, the role of different hormones was evaluated on human *OVGP1* transcription and E_2 and luteinizing hormone (LH) presented a positive correlation with it, while P_4 exerted a negative effect (Briton-Jones *et al*., 2001). Nevertheless, E_2 failed to maintain oviductin transcription on human oviductal cell culture, while human chorionic gonadotropin (hCG) addition, increased mRNA expression of this glycoprotein (Briton-Jones *et al*., 2003).

In a study with swine it was possible to verify that the oviductal secretory response to spermatozoa was different from its response to oocytes. In fact, the presence of spermatozoa or oocytes in the oviduct altered the secretion of specific proteins. Most of these proteins are known to have an influence on gamete maturation, viability, and function, and these proteins may prepare the oviductal environment for the arrival of the zygote. These authors suggested the presence of a gamete recognition system within the oviduct capable of distinguishing between spermatozoa and oocytes (Georgiou *et al*., 2005)

Osteopontin is another glycoprotein found in many tissues and known to be involved in cell adhesion and cell signaling by binding to integrins. Osteopontin is synthesized by the oviduct epithelium and present in oviduct fluid (Gabler, Chapman & Killian, 2003). Rates of sperm binding, fertilization and embryo development were significantly greater when ova were pre-incubated with oviduct fluid prior to *in vitro* fertilization than if the oviduct fluid used in the pre-incubation contained antibody against osteopontin (Gonçalves, Way & Killian, 2001). It is well known that several unfertilized oocytes, in various stages of degeneration can be found in the mare's oviducts (Onuma & Ohnami, 1975). A group of researchers reported a temporal association between embryonic PGE_2 secretion and the oviductal transport period, which could indicate that embryonic PGE_2 may initiate and be responsible for a selective oviductal transport in the mare (Weber, Vanderwall, Freeman & Woods, 1991a; Freeman, Woods, Vanderwall, & Weber, 1992b).

2.2. THE ROLE OF OVARIAN STEROIDS, OXYTOCIN AND TUMOR NECROSIS FACTOR IN OVIDUCT

The regulation of oviduct muscular and secretory activity for optimal gametes and embryo transport is influenced by ovarian steroids (Wijayagunawardane *et al*., 1998; Wanggren *et al*., 2008; Nelis *et al*., 2015a; Nelis *et al*, 2015b), adrenergic nerves (Killian, 2011; Helm, Owman, Sjoberg, & Walles, 1982), nitric oxide (NO) (Ekerhovd, Brannstrom, Alexandersson

& Norstrom, 1997; Ekerhovd, Brannstrom, Weijdegard & Norstrom,1999; Gawronska, Bodek, & Ziecik, 2000; Szóstek *et al*., 2011), oxytocin (OXT) (Wijayagunawardane *et al*., 1998; Wanggren, Stavreus-Evers, Olsson, Andersson, & Gemzell-Danielsson, 2008; Jankovic, Varjacic, & Protic, 2001) prostaglandins (PGs) (Wijayagunawardane *et al*., 1998; Wanggren *et al*., 2008; Lindblom, Wilhelmsson, Wikland, Hamberger, & Wiqvist, 1983) and cytokine TNFα (TNF) (Szóstek *et al*., 2011), among others. Ovarian steroids also conduct a series of changes through proteomic and non-genomic pathways in the oviduct epithelium, affecting gene expression, proteome and secretion of oviduct fluid (Pérez Martínez *et al*., 2006). In rat oviduct, E_2 induces the expression of some genes, in a different way, before and after mating, and this difference is probably mediated by an E_2 non-genomic signaling pathway operating on gene expression only in unmated rats (Parada-Bustamante *et al.,* 2010*)*. Based on hormonal tissues concentration, it was proposed that estradiol (E_2), progesterone (P_4), oxytocin (OXT), prostaglandin E_2 (PGE_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and endotelin-2, could synergistically control oviductal contraction for optimal embryo transport during the periovulatory period (Wijayagunawardane *et al*., 1998). In woman oviduct, muscular contractions are regulated by prostaglandins (PGs) and P_4, and probably, PG receptors expression is regulated by P_4 (Wånggren, Stavreus-Evers, Olsson, Andersson, & Gemzell-Danielsson, 2008). In the rat oviduct, E_2 increases inositol-1,4,5-trisphosphate (IP_3) by a nongenomic action operated by E_2 receptor I (ESR1) that it involves the activation of calcium–calmodulin protein kinase II (CaMKII) in the smooth muscle cells of rat oviduct (Reuquén *et al*., 2015). This E_2 effect suggests the involvement of IP_3 and CaMKII in the contractile activity, necessary to hasten oviductal egg transport. Recent studies have shown that the equine oviduct is an organ highly responsive to local changes in P_4 and E_2 concentrations affecting oviduct steroidogenic capacities and P_4 receptor expression (Nelis *et al*., 2015a; Nelis *et al*., 2015b). Both E_2 and P_4 exert their actions on the oviduct through their specific receptors. As master regulators of oviduct functions, estradiol receptors (ESRs) and P_4 receptors (PGRs) rule the expression of downstream target genes (Shao *et al*., 2006). In fact, these authors defended a tissue-specific and hormonal regulation of PGR isoform expression in mouse fallopian tube and uterus, where they are potentially involved in regulation of mitochondrial-mediated apoptosis depending on the cellular compartment and a possible interaction between functional PGR protein and growth factor signaling which could present a coordinated role for regulating apoptotic process in both tissues *in vivo*. In mouse fallopian tube and uterus there is a tissue-specific and hormonal regulation of PGR isoform expression, which is potentially involved in mitochondrial-mediated apoptosis depending on the cellular compartment (Shao *et al*., 2006). Also in mouse, ESR1 may suppress the oviductal protease activity, which is paramount to allow normal fertilization and

preimplantation embryo development, since it will control degradation of ZP, avoiding embryo lysis (Winuthayanon *et al*., 2015).

In addition, a possible interaction between functional PGR protein and growth factor signaling could represent a coordinated role for regulating the apoptotic process in both tissues *in vivo*. In cow, gene expression of *ESR2, PGR, OXTR* could be dependent on reproductive tract location, even though it was not influenced by distinct peri-ovulatory steroid environments (Araújo *et al*., 2015). Also in the cow, to investigate the causes of the short estrous cycles that occur frequently in dairy cows after estrus synchronization using $PGF_{2\alpha}$ and gonadotrophin releasing hormone (GnRH), an experiment was designed to evaluate the expression of OXTR, PGR, ESR, cyclo-oxygenase II (COX2) and 20α-hydroxysteroid dehydrogenase. The expression of these proteins was similar between the normal and short-cycle groups. Therefore, the authors concluded that despite evidence from previous studies that short estrous cycles are induced by premature PG release, differences in these receptors or in enzyme expression do not explain the eicosanoids release (Rantala, Mutikainen, Schuler, Katila, & Taponen, 2014). As tubal motility is decreased by the P_4-induced reduction in both beat frequency of cilia and frequency of contractions, this steroid hormone may have an inhibitory action on human tubal activity (Wanggren, Stavreus-Evers, Olsson, Andersson, & Gemzell-Danielsson, 2008; Mahmood, Saridogan, Smutna, Habib, & Djahanbakhch, 1998; Lindblom, Hamberger, & Ljung, 1980). Also, P_4 regulates the expression of endothelin1 and endothelin receptor A in the mouse fallopian tube and by this way, it may control the muscular contraction and eventually gamete transport in the fallopian tube (Bylander, Gunnarsson, Shao, Billig, & Larsson, 2015). It has been shown that *in vivo* P_4 concentrations can be very high in mare oviductal tissue and fluid ipsilateral to the ovulation side (Nelis *et al*., 2015b). Besides, P_4 and E_2 can modulate mare oviduct ciliary activity, cell ultrastructure, transcription of embryotropic genes, as well as oviduct fluid composition, shown by changes in glucose consumption and lactate production (Nelis *et al*., 2015a).

Ovarian steroids themselves could also be involved in $PGF_{2\alpha}$ and PGE_2 production in the rat oviduct (Pérez Martínez *et al*., 2006). Estradiol has been shown to upregulate the expression and activity of prostaglandin synthase 2, an enzyme involved in PGs synthesis. This stimulatory effect may be receptor-mediated (Pérez Martínez *et al*., 2006). In the mare, oviduct treatment with PGE_2 hastens the transport of equine embryos throughout this organ, which suggests a role for embryonic PGE_2 in the initiation of selective oviduct transport (Weber, Freeman, Vandewall, & Woods, 1991; Robinson, Neal, & Allen, 2000). In the presence of sperm cells, bovine oviduct epithelial cells produce $PGF_{2\alpha}$ that might stimulate spermatozoa transport (Kodithuwakku, Miyamoto, & Wijayagunawardane, 2007). The importance of PGE_2 and $PGF_{2\alpha}$

in oviduct, either present in seminal plasma or produced by oviduct cells themselves, may contribute for gametes and embryo transport and for signaling between the embryo and the oviduct, which might be crucial for embryo development (Kodithuwakku, Miyamoto, & Wijayagunawardane, 2007; Kaczmarek *et al*., 2010).

Oxytocin, whose action is mediated by specific OXT receptors (OXTR), also plays a role in oviduct contraction or relaxation, in cyclic and/or pregnant females and in PG synthesis in several species such as cow, woman or bitch (Wanggren, Stavreus-Evers, Olsson, Andersson, & Gemzell-Danielsson, 2008; Kotwica *et al*., 2003; Wijayagunawardane, Gabler, Killian, & Miyamoto, 2003; Derussi *et al*., 2012). In the equine endometrium, OXT increased the secretion of PGE_2 and $PGF_{2\alpha}$ during follicular and mid-luteal phases, while E_2 alone or added with P_4 stimulated $PGF_{2\alpha}$ secretion (Galvão *et al*., 2012). Comparable results were observed in cat endometrium. It was demonstrated that OXT is produced by the early developing *corpus luteum* (CL), and that OXT may regulate PGs secretion especially at the early and mid-luteal phase (Siemieniuch, Mlynarczuk, Skarzynski, & Okuda, 2011). Also in swine, it was proved that LH stimulates $PGF_{2\alpha}$, release and COX2 expression by endometrium stromal cells, while E_2 plus P_4, presented a positive effect on PGE_2 production (Waclawik, Jabbour, Blitek & Ziecik, 2009). In addition, a study on mare endometrium indicated that TNF production is closely related to ovarian steroid actions and the interaction between TNF and PG regulates endometrium physiologic processes (Szóstek, Adamowski, Galvão, Ferreira-Dias, & Skarzynski, 2014).

More recently, it has also been confirmed, that E_2, P_4, OXT, among others, may influence the expression of aquaporins (AQP), either in swine uterus (Skowronski, 2010), or even in swine oviduct (Skowronski, Skowronska, & Nielsen, 2011). Later, this team has also investigated the expression of AQP1 and AQP5 either by the endometrium or by the myometrium. Their results indicate that P_4 upregulated the expression of AQP1/AQP5 mRNAs and proteins in the endometrium and myometrium, E_2 also stimulated the expression of both AQPs, but only in the endometrium, while OXT increased the expression of AQP1/AQP5 mRNAs and proteins in the myometrium (Skowronska, Mlotkowska, Nielsen, & Skowronski, 2015).

2.3. Angiogenic modulation of equine oviduct function

2.3.1. Blood flow supply

The ovarian and the uterine arteries are responsible for the blood supply to the oviduct, but considerable variation exists in the relative contribution of both arteries among species, individuals and hormonal influences. Generally, numerous arterial anastomosis between a

branch of the uterine artery and the uterine branch of the ovarian artery supply the tube (García-Pascual, Labadía, Triguero, & Costa, 1996). In rabbit, the isthmus has a randomly interconnecting subserosal *venous plexus* that surrounds the myosalpinx and a mucosa that is well supplied by arterioles. The ampulla has parallel branching of subserosal arteries and veins and a mucosa poorly supplied by arterioles, that will drain into large veins (Verco, Gannon & Jones, 1983). Although in rats, hamsters, guinea-pigs and monkeys there is a predominant contribution of the uterine artery to the oviductal supply, in cows, sheep and rabbits the ovarian artery appears to contribute to most of the vascular architecture of the oviduct (García-Pascual, Labadía, Triguero, & Costa, 1996).

It has been demonstrated that higher levels of some steroid hormones exist in the arterial blood supplying the uterus and oviduct, than in the systemic blood, because of the transfer of steroids and peptides from ovarian venous and lymphatic effluent to the arterial ovarian blood. This could be ascribed to the close apposition between venous and arterial vessels of the oviduct with extensive areas of contact that allow a countercurrent transfer mechanism (Stefanczyk-Krzymowska, Skipor, Grzegorzewski, Wasowska, & Krzymowski, 1994). Nevertheless, in the mare this countercurrent transfer mechanism was shown to be absent. This was concluded after resection of the oviductal vein that resulted in uterine P_4 concentrations no longer higher in the ipsilateral side, than those on the contralateral side (Weems, Weems, Lee, & Vincent, 1989). However, in the mare, blood is also provided by an accessory ovarian branch from the uterine vein (*ramus uterinus*), which is heavily encircled by a coiled ovarian branch of the ovarian artery that supplies the ovary and also a large part of the oviduct through its cranial branch (Nelis *et al*., 2015a). The uterine branch of the ovarian artery and the middle and caudal tubal branches supply blood to the remainder of the oviduct (Nelis *et al*., 2015a). Besides blood vessels, as described for sheep, there is also an extensive network of lymphatic vessels in the mesovarium that could be involved in transport or diffusion of hormones (Staples *et al*., 1982, Nelis *et al*., 2015a).

The amount of fluid present in the oviduct shows cyclic variations that are parallel to both changes in proliferation rate and secretory activity of epithelial cells, as well as to changes in blood flow to the tube, being maximum at the preovulatory time (Kamwanja & Hansen, 1993). Therefore, the luminal milieu of the oviduct is created and maintained by the transport and permeability properties of the blood-oviductal lumen barrier, together with the secretory activity of the epithelium, both being under ovarian steroid control (Stefanczyk-Krzymowska *et al*., 1994).

2.3.2. Angiogenesis

Angiogenesis is the growth of blood vessels from the existing vasculature. It occurs throughout life in both health and disease, beginning in utero and continuing through old age. Capillaries are needed in all tissues for diffusion exchange of nutrients and metabolites. Changes in metabolic activity leads to proportional changes in angiogenesis and, hence, proportional changes in capillarity and oxygen play a pivotal role in this regulation (Adair & Montani, 2010a). Angiogenesis should not be confused with vasculogenesis, which is defined as the differentiation of endothelial precursor cells, also known as angioblasts, into endothelial cells (ECs) in combination with the formation of a primitive vascular network (Schmidt, Brixius, & Bloch, 2007). Nowadays, it is accepted that both processes are observed during embryonic and adult growth processes (Risau, 1997).

Angiogenesis can occur by two different ways: (i) sprouting, and (ii) intussusception. Sprouting angiogenesis is initiated in poorly perfused tissues when oxygen sensing mechanisms detect a level of hypoxia that demands the formation of new blood vessels to satisfy the metabolic requirements of parenchymal cells. The basic steps of sprouting angiogenesis include enzymatic degradation of capillary basement membrane, EC proliferation, directed migration of ECs, tubulogenesis (EC tube formation), vessel fusion, vessel pruning, and pericyte stabilization (Adair & Montani, 2010a). Intussusceptive angiogenesis is also called "splitting angiogenesis" because the vessel wall extends into the lumen causing a single vessel to split in two. This type of angiogenesis is thought to be fast and efficient compared with sprouting angiogenesis. Intussusceptive angiogenesis mainly causes new capillaries to develop where capillaries already exist (Kurz, Burri & Djonov, 2003).

Many different metabolic fuels are required for cellular metabolism, but oxygen is especially critical because cells have limited stores compared with metabolic substrates such as glucose, fatty acids, and amino acids. This relative inability of tissues to store oxygen can explain why oxygen is a master signal in growth regulation (Adair & Montani, 2010b). Many proangiogenic factors and their receptors can be modulated either directly or indirectly by hypoxia or ischemia in poorly perfused tissues. These include, but are not limited, to the following: vascular endothelial growth factor (VEGF) and its receptors, 1 and 2 (VEGFR1 and VEGFR2), placental growth factor; angiopoietin 1, angiopoietin-2 and their Tie2 receptor; fibroblast growth factor 2 (FGF2), and transforming growth factor-beta (TGFβ) (Adair & Montani, 2010b).

Generally, a low level of O_2 in the tissues causes the release of VEGF, which in turn stimulates angiogenesis. The development of new capillaries increases the supply of O_2 to the tissues, causing VEGF to return to nearly normal levels, thus closing the negative feedback loop (Hang,

Kong, Gu & Adair, 1995). Adenosine is a nucleoside produced in all cells of the body by dephosphorylation of ATP, and is assumed to have a long-term role in maintaining tissue oxygenation by stimulating angiogenesis (Adair, 2005).

Growth factors have been demonstrated to be implicated in several reproductive functions. Among the abundant angiogenic growth factors known, FGF and VEGF will be discussed, due to their relevant role in angiogenesis.

2.3.3. Fibroblast Growth Factor (FGF)

A protein activity identified as fibroblast growth factor (FGF), was identified for the first time in 1973 (Armelin, 1973). The activity of this protein would be studied and referred as basic FGF (or FGF2), due to the overall basic composition of aminoacids and a high isoelectric point (Ornitz & Itoh, 2015). Later, another factor with similar mitogenic activity was identified from the bovine brain. This factor presented a low isoelectric point and was referred as acid FGF, or FGF1 (Thomas, Rios-Candelore & Fitz-Patrick, 1984).

FGF1 and FGF2 belong to the FGF subfamily 1. These FGFs are released from cells by direct translocation across the cell membrane (Prudovsky, Kumar, Sterling & Neivandt, 2013). Extracellular FGF subfamily 1 can pass through the plasma membrane of the target cells, moves through the cytosol, and enters the nucleus (Olsnes, Klingenberg & Wiedlocha, 2003). Potential functions of nuclear FGF subfamily 1 include regulation of the cell cycle, cell differentiation, survival, and apoptosis and it is the only one that can activate all FGF receptors (Bouleau *et al*., 2005).

FGFRs share a high percentage of sequence homology, and consist of three important domains: extracellular ligand-binding domain, single transmembrane domain, and intracellular tyrosine kinase domain (Johnson & Williams, 1993). Despite the general characteristics shared among the family members, an arrangement of isoforms is specific within each family. Structural diversity observed across the isoforms of FGFRs is ascribed to the alternative merging of mRNA sequence (Johnson, Lee, Lu & Williams, 1990). FGFRs signaling is primarily triggered by the binding of the receptors to FGF ligands and the subsequent formation of various complexes to initiate downstream signal transduction (Turner *et al*., 2010).

FGF and its receptors are essential for organogenesis, tissue maturation, homeostasis, response to injury, and cancer development. Some biochemical studies have identified mechanisms that regulate the expression of FGFs, their bioavailability, and their ability to activate cellular responses through interaction with cell surface receptors. Within the cell, signal transduction mechanisms have been identified that reveal interactions with multiple cellular signaling

pathways, complex feedback mechanisms, and regulatory molecules that control FGF signaling, both extracellularly and intracellularly (Ornitz & Itoh, 2015).

Different members of FGF family, mostly FGF1 and FGF2, can induce, under *in vitro* conditions, a complex pro-angiogenic phenotype, including proliferation, migration, protease production, integrin and cadherin receptor expression and intercellular gap-junction communication (Javerzat, Auguste & Bikfalvi, 2002). Besides that, activation of FGFR1 or FGFR2 by FGF1, FGF2 or FGF4, leads to endothelial cell proliferation. FGFR involves the activation of several parallel signaling pathways, because of receptor autophosphorylation (Cross & Claesson-Welsh, 2001). Developmental studies have uncovered redundant functions of FGFs and FGFRs, and interactions with most of the other major signaling pathways, including BMP, WNT, Notch and Hedgehog. The discovery of endocrine FGFs has uncovered new mechanisms that regulate metabolism, lipid, and mineral homeostasis (Ornitz & Itoh, 2015).

FGF family members are present in ovarian follicles of the cow (Berisha, Sinowatz, & Schams, 2004). They may be involved in folliculogenesis and especially during final growth of the preovulatory follicle by stimulation of angiogenesis and granulosa cell survival and proliferation (Berisha *et al*., 2004).

2.3.4. Vascular Endothelial Growth Factor (VEGF)

Expression and transcription of Vascular Endothelial Growth Factor (VEGF) have been evaluated in human oviduct. VEGF is expressed on epithelial cell, smooth muscle fibers and blood vessels. Its transcription was higher during the peri-ovulatory period and in infundibulum and ampulla regions. Also, it presented a positive correlation with FSH and LH serum concentrations (Lam *et al*., 2003). VEGFR signaling is initiated upon binding of a covalently linked ligand dimer to the extracellular receptor domain. This interaction promotes receptor homo- and heterodimerization followed by phosphorylation of specific tyrosine residues located in the intracellular juxta-membrane domain, the kinase insert domain, and the carboxyterminal tail of the receptor. Subsequently, a variety of signaling molecules are recruited to VEGFR dimers giving rise to the assembly of large molecular complexes, so-called signal transduction particles or signalosomes that activate distinct cellular pathways (Stuttfeld & Ballmer-Hofer, 2009).

In swine, several growth factors and their receptors systems were identified in endometrium and oviduct. Epidermal growth factor, VEGF and FGF, as well as their receptors, were detected in porcine oviductal and endometrial tissue during the estrous cycle and at the time of

implantation. Estrous cycle dependent variations in the expression of growth factor systems were associated with specific cell types of the endometrial tissue. These growth factor systems seem to be involved in a supposed paracrine network to successfully establish and maintain pregnancy in pigs (Wollenhaupt, Welter, Einspanier, Manabe, & Brüssow, 2004).

Still in bovine, also VEGF was implicated in oviduct motility and embryo transport (Wijayagunawardane, Kodithuwakku, Yamamoto, & Miyamoto, 2005). These authors suggested that the pre-ovulatory LH-surge, together with increasing E_2 production from the pre-ovulatory follicle associated with basal P_4 levels from the regressing luteal structure, upregulates the oviductal VEGF system (Wijayagunawardane, Kodithuwakku, Yamamoto, & Miyamoto, 2005). Then, VEGF induces the maximum oviductal production of contraction–relaxation-related substances for oviduct contraction and rapid transport of gametes to the fertilization site. In addition, oviductal VEGF elevation caused by the LH-surge, appears to down-regulate the oviductal VEGF system immediately after ovulation, and thereby may contribute to suppress oviductal contraction to ensure embryo slow transport to the uterus at the optimal time.

The relationship between VEGF expression and microvessel density was also evaluated in woman with endometriosis. There appears to be a dysregulation of angiogenic activity in the eutopic endometrium of women with endometriosis and endometriotic lesions with high proliferative activity were accompanied by higher local angiogenic activity and higher levels of VEGF in serum and peritoneal fluid (Bourlev *et al*., 2006).

In swine oviduct, insemination alone as well as ovarian stimulation, affect the mRNA and protein profiles of the VEGF system. Disrupted VEGF system expression may be crucial to many events occurring during the periovulatory period and consequently could lead to deprivation of VEGF-dependent factors that are necessary for proper fertilization, gamete transport, and embryo development (Małysz-Cymborska & Andronowska, 2014).

Establishment and maintenance of CL is a complex event, where cytokines and angiogenic factors could be simultaneously implicated. Galvão and co-authors (2012), suggested a novel auto/paracrine action of cytokines, specifically TNF, on the up-regulation of VEGF for angiogenesis stimulation in equine early CL, while at luteolysis, cytokines down-regulated angiogenesis. Additionally, VEGF stimulated P_4 and PGE_2 production, which may be crucial for CL establishment (Galvao *et al*., 2012).

In human medicine, VEGF family in nowadays under intensive research. The expression of VEGF and VEGFR-2 (i.e. KDR) mRNAs and protein in gestational diabetes mellitus (GDM)-placental tissues, was reduced, suggesting that maternal GDM affects the angiogenic function of placenta (Meng *et al*., 2016). It is also known that cancer cells secrete VEGF to activate

VEGFR-2 pathway in endothelial cells in the vicinity, as part of cancer-related angiogenesis events. Interestingly, activation of KDR signaling is found in breast cancer cells, but its role and regulation are not clear. Ongoing preclinical and clinical studies might prove that pharmaceutically targeting KDR could be an effective therapeutic strategy in treating one of most aggressive type of cancer: the triple-negative breast cancer (Zhu & Zhou, 2015).
Interestingly, an intimate cross-talk may exist among FGF2 and several members of the VEGF family during angiogenesis. Several scientific works indicate that FGF2 can induce neovascularization indirectly by activating the VEGF/VEGFR system (Presta *et al*., 2005).

2.4. Fibrosis in equine oviduct

2.4.1. Fibrosis – the process

Fibrosis is often defined as a wound-healing response that has gone out of control. Repair of damaged tissues is an essential biological process that allows the ordered replacement of dead or damaged cells after injury, a mechanism that is critically important for survival. Damage to tissues can result from various acute or chronic stimuli, including infections, autoimmune reactions, and mechanical injury. Although initially beneficial, the healing process becomes pathogenic if it perpetuates, resulting in substantial remodeling of the extra-cellular matrix (ECM) and formation of permanent scar tissue (Wynn, 2007). When injuries occur, damaged epithelial and/or endothelial cells release inflammatory mediators that initiate an antifibrinolytic coagulation cascade, which triggers formation of both blood clots and a provisional ECM (Kumar, Abbas & Fausto, 2005). Platelets exposure to ECM components is essential for triggering aggregation, clot formation and hemostasis (Esmon, 2005). Consequently, platelet degranulation promotes vasodilation and increased blood vessel permeability, while stimulated myofibroblasts, epithelial and/or endothelial cells produce matrix metalloproteinases (MMPs). Activated platelets also release growth factors such as platelet-derived growth factor (PDGF), a potent chemoattractant for inflammatory cells and transforming growth factor-β1 (TGF-β1), which stimulates ECM synthesis by local fibroblasts (Barrientos, Stojadinovic, Golinko, Brem & Tomic-Canic, 2008). These mechanisms are implicated in the initiation of the fibrotic process (Wynn & Ramalingam, 2012). According to these authors, platelets and damaged epithelial and endothelial cells release a variety of chemotactic factors that recruit inflammatory monocytes and neutrophils to the site of tissue damage. These inflammatory cells in spite of playing an important role removing tissue debris and the killing of invading bacteria, also secrete a variety of toxic mediators, including reactive

oxygen and nitrogen species that are harmful to the surrounding tissues. Consequently, they can further exacerbate the tissue-damaging inflammatory response, leading to scar tissue developing (Wynn & Ramalingam, 2012).

2.4.2. Tumor necrosis factor α

The inflammatory cells will themselves produce several growth factors and cytokines, such as TNF and interleukin 1β (IL1β). Several studies have demonstrated the influence of either TNF or IL1β on lung and liver fibrosis (Miyazaki *et al*., 1995; Kolb, Margetts, Anthony, Pitossi & Gauldie, 2001; Tomita *et al*., 2006). Nevertheless, their effect is not clear and could depend on several factors. TNF is a primary immune and inflammatory regulator which stimulates fibroblast chemotaxis (Postlethwaite & Seyer, 1990), while proliferation is probably mediated by autocrine PDGF (Battegay, Raines, Colbert, & Ross, 1995).

Concerning the collagen production, the effect of TNF is controversial. Previous studies indicated that it inhibits the synthesis of type I collagen in cultured dermal fibroblasts on the transcriptional level, resulting in a dose-dependent reduction of the production of type I collagen. In the same work, a reduction of type III collagen was also reported (Mauviel *et al*., 1988). Furthermore, higher concentrations of TNF reduced the expression of tissue inhibitor of metalloproteinases 1 (TIMP1), thereby promoting the degradation of ECM proteins (Ito, Sato, Iga & Mori, 1990). The effects of TNF on collagen synthesis and on the production of MMPs and TIMPs are not restricted to fibroblasts and are also found in other cell types (Armendariz-Borunda, Katayama & Seyer 1992). In contrast to the results discussed above, a recent study suggested that TNF might promote a profibrotic phenotype in murine intestinal myofibroblasts *in vitro,* where TNF stimulated collagen synthesis, increased expression of TIMP1, and decreased activity of MMP2 (Theiss, Simmons, Jobin & Lund, 2005). Investigators in another *in vitro* study also proposed a profibrotic effect of TNF, indirectly via induction of TGFβ. However, it remains unclear whether the induction of TGFβ is sufficient to overcome the inhibitory effects of TNF on collagen production, since the expression of collagen was not analyzed (Sullivan, Ferris, Pociask & Brody, 2005). The induction of TGFβ upon stimulation with TNF might therefore represent a counterregulatory mechanism to compensate for the inhibitory effects of TNF (Distler, Schett, Gay, & Distler, 2008). As previously referred, the effect of TNF on fibrosis is unclear. Most of *in vitro* studies show antifibrotic effects of TNF, in that it suppresses the production of collagen, reduces the expression of TIMPs, and stimulates the release of MMPs, thereby preventing the accumulation of ECM, while *in vivo* studies demonstrated that inhibition of TNFα impairs the fibrosis process. These differences in the

results between *in vitro* and *in vivo* studies might be explained by the inflammatory component in animal models of experimental fibrosis, which does not exist on *in vitro* experiments. Since TNF exerts a potent inflammatory effect, its direct antifibrotic effects on fibroblasts might be outweighed in experimental models of fibrosis by its important role in driving inflammation (Distler *et al*., 2008).

2.4.3. Transforming Growth Factor β

TGFβ is believed to be the most potent profibrotic cytokines. It is produced by several cells, such as macrophages, lymphocytes, endothelial cells, platelets, and fibroblasts themselves. It is a powerful activator of production of collagen and other extracellular matrix components, often in an autocrine fashion. In addition to its role as a profibrotic cytokine, it can directly induce the differentiation of fibroblasts into collagen-secreting myofibroblasts (Atamas, 2002). TGFβ1 is now widely described as a multifunctional cytokine with broad modulatory activities that affect numerous important biological pathways. These include pathways involved in the regulation of embryogenesis, immunity, carcinogenesis, cell proliferation and migration, wound healing, inflammation and fibrosis, among others (Verrecchia & Mauviel, 2007). The cellular source of TGFβ dictates its activity, with TGFβ derived from macrophages generally showing wound-healing and profibrotic activity and TGFβ secreted from $CD4^+$ T regulatory cells (T_{reg} cells) functioning as an anti-inflammatory and antifibrotic mediator (Kitani *et al*., 2003). TGFβ is functionally implicated with others growth factors. It probably upregulates connective tissue growth factor, which is an autocrine factor associated with collagen and fibronectin production in fibroblasts (Igarashi, Okochi, Bradham, & Grotendorst, 1993; Frazier, Williams, Kothapalli, Klapper & Grotendorst, 1996; Shi-wen *et al*., 2000).).

Fibroblasts are non-hematopoietic, non-epithelial, non-endothelial cells that are widely distributed throughout the mesenchyme where they synthesize ECM proteins which form a structural framework to support tissue architecture and function in steady-state conditions (Ueha, Shand, & Matsushima, 2012). They also play a significant role in tissue repair following multi-factorial tissue damage by forming a provisional ECM, a process preceding re-epithelialization in successful repair. Unfortunately, dysregulated activation, proliferation, and survival of fibroblasts often results in the excessive deposition of ECM proteins and inhibition of re-epithelialization, leading to tissue fibrosis (Gabbiani, 2003). Fibroblasts are immunophenotypically identified as cells negative for hematopoietic, epithelial, and endothelial makers. The lack of specific markers for fibroblasts or possible subpopulations, including

myofibroblasts, complicates the cellular and molecular understanding of these cells *(*Ueha *et al*., 2012).

To repair, regenerate and restore homeostasis after injury, tissue-resident fibroblasts are activated and transformed into myofibroblasts, as previously referred. Myofibroblasts are contractile cells expressing α-smooth muscle (αSMA) actin and myosin bands, and still secrete copious amounts of ECM. They are very important in wound granulation tissue, aiding its contracture and closure towards healing process (Wynn & Ramalingam, 2012). Myofibroblasts could also arise from regenerating epithelial or endothelial cells, or from epithelial stem cells progenitor (via chronic inflammation-induced epithelial–mesenchymal transition), or endothelial-mesenchymal transient cells. Also $CD34^+$ bone marrow-derived progenitor cells, can be recruited, contributing to the myofibroblast pool at the site of wound repair and fibrosis (Wynn & Ramalingam, 2012). The last ones are recognized as fibrocytes. Fibroblasts and myofibroblasts are responsible for the excessive accumulation of COL1, which is responsible for the excessive deposition of ECM during fibrotic process (Ueha *et al*., 2012).

2.4.4. Matrix Metalloproteinases

Other important players in the development of fibrosis, are now considered the matrix metalloproteinases (MMPs). Although MMPs have long been considered to be primarily responsible for turnover and degradation of ECM substrates, they are now recognized as being responsible for mediating crucial functions in a variety of processes, particularly related to immunity and repair, such as cell migration, leukocyte activation, antimicrobial defense and chemokine processing (Gill & Parks, 2008). Common aspects of the MMP family include: 1) the presence of zinc in the active site of the catalytic domain; 2) synthesis of the MMPs as proenzymes that are secreted in an inactive form; 3) activation of the latent zymogen in the extracellular space; 4) recognition and cleavage of the ECM by the catalytic domain of the enzyme; and 5) inhibition of enzyme action by both serum-borne and tissue-derived metalloproteinase inhibitors in the extracellular environment. Currently, the MMP family includes at least 25 related proteolytic enzymes that includes four broad classes: the collagenases, gelatinases, stromelysins, and membrane type enzymes (Curry & Osteen, 2003). Most of these enzymes is secreted by inactive pro-enzymes and will be activated in the extracellular space by numerous factors including MMPs, plasmin, interleukin 1β, TNF and others (Visse & Nagase, 2003). Although some MMPs can degrade matrix, being expected to be under expressed in fibrosis, to resolve the excess of matrix, having indeed an anti-fibrotic action, others can have pro-fibrotic functions (Giannandrea & Parks, 2014). MMPs can

contribute to the degree of initial injury and repair, to the onset and resolution of inflammation, to the activation and de-activation of myofibroblasts, and to the deposition and breakdown of ECM. In other words, MMPs are involved in both augmenting and attenuating many processes that impact fibrosis (Giannandrea & Parks, 2014).

The activity of MMPs depends on its biosynthesis, which could be either over or down-regulated. This indicates that the effectors controlling gene expression may undergo changes, since MMPs might be controlled at the level of transcription; also from the four tissue inhibitors of metalloproteinases (TIMP) – TIMP1, TIMP2, TIMP3 and TIMP4 – which can control MMPs activity (Ra & Parks, 2007).

Researchers concern with the relevance of MMPs in fibrotic conditions, has brought up the use of circulating levels of specific MMPs or possible MMP degradation products as reliable biomarkers of active fibrosis (Leeming *et al*., 2011).

MMP2 mainly influences the degree of collagen deposition during activation of liver hematopoietic stem cells. It is unclear how MMP2 mediates this effect, but a likely mechanism would be proteolysis of a surface protein that results in altered outside-in signaling (Radbill *et al*., 2011). Also in the kidney, Takamiya and co-authors (2013) demonstrated increased collagen deposition and fibroblast activation in diabetic knockout mice for MMP2 gene (Mmp2–/–), supporting the view that MMP2 has an anti-fibrotic role.

In human pulmonary fibrosis *MMP9* expression resulted in less fibrosis in one study (Cabrera *et al*., 2007), whereas in another it was pro-fibrotic (Lee *et al*., 2001), while others concluded it had no role (Kaviratne *et al*., 2004). A significant decrease in αSMA levels in Mmp9–/– mice was observed, suggesting a role for MMP9 in myofibroblast activation or survival (Wang *et al*., 2010).

TIMP1 may promote liver fibrosis by inhibiting the activity of MMPs, such as MMP13 and MMP14, increasing fibrosis in *Timp1*–/– mice (Wang *et al*., 2011). Nevertheless, in pulmonary fibrosis in mice, it was elevated and persisted in bleomycin-injured *Timp3*–/– lungs (Gill *et al*., 2010), suggesting a protective role on this condition. Kidney fibrosis was enhanced in *Timp3*–/– mice subjected to unilateral ureteral obstruction for 2 weeks (Kassiri *et al*., 2009). According to this article, the protective effect of TIMP-3 is due to the suppression of TNFα, which mediates renal fibrosis and regulates expression of several MMPs.

In the mare, ovarian stromal cells also produce important components of the ECM remodeling machinery and therefore, may play a role in the ECM remodeling during follicular growth (Song, Porter, & Coomber, 1999). Also in equine, it was suggested that MMP2 and tissue transglutaminase may play a major role in changes that occur in ECM homeostasis in the case of endometrosis (Walter, Handler, Miller, & Aurich, 2005). By histochemistry techniques the

presence of MMP2, MMP9 and TIMP1 was visualized in equine endometrium, even though with no apparent difference between normal and endometrosis cases (Porto, 2006). Another study verified that the secretion of MMP2 and MMP9 into the uterine lumen is minimal during the normal equine estrous cycle, although increased inhibitory activity was seen by TIMP2 during diestrus. The secretion of MMP2 and MMP9 into the uterine lumen was dramatically increased during endometritis while TIMP2 activity was decreased at the same time. The activity of MMP9 was demonstrated to reside in cytoplasmic granules of endometrial eosinophils, and gelatinase activity was not demonstrated in other cell types using in situ zymography (Oddsdóttir, 2007).

2.4.5. Prostaglandins

The receptors for PGE_2 are present on multiple cell types (Hata & Breyer, 2004) reflecting the ubiquitous functions of PGE_2, which range from nociception and other aspects of neuronal signaling, to hematopoiesis, regulation of blood flow, renal filtration and blood pressure, regulation of mucosal integrity, vascular permeability, and smooth muscle function (Durand & Zon, 2010). The heterogeneous effects of PGE_2 are reflected by the existence of four different PGE_2 receptors, designated EP1, EP2, EP3 and EP4, with an additional level of functional diversity resulting from multiple splice variants of EP3 that exists in at least eight forms in humans and three forms in mice. PGE_2 can be produced by all cell types of the body, with epithelia, fibroblasts, and infiltrating inflammatory cells representing the major sources of PGE_2 during an immune response (Kalinski, 2012).

Studies carried out *in vitro* have suggested that PGE_2 has potentially important bronchoprotective and anti-inflammatory properties, and may elicit bronchodilation when introduced into asthmatic airways *in vivo* (Pavord & Tattersfiel). This eicosanoid also recognized as “epithelium-derived relaxing factor,” may play a key role in regulating airway tone. Airway epithelium removal prevented PGE_2 production and thus increased the contractile response of smooth muscles induced by acetylcholine, histamine, and $PGF_{2\alpha}$ (Aizawa, Miyazaki, Shigematsu & Tomooka, 1988). It was also observed that fibroblast in Idiopathic Pulmonary Fibrosis (F-IPF) have a striking defect in their capacity to synthesize the anti-inflammatory and anti-fibrogenic molecule PGE_2. This reduction in the endogenous capacity of F-IPF to down-regulate their function via PGE_2 may contribute to the inflammatory and fibrogenic response in cases of IPF (Wilborn *et al*., 1995) More recently, Wei and co-authors (2014) confirmed that PGE_2 exerts an essential effect against pulmonary fibrogenesis via EP2-mediated signaling transduction (Wei *et al*., 2014). Also in kidney it was suggested that PGE_2

has an important role in the progression in disease via the EP1/EP3 receptor, whereas EP2 and EP4 receptors are equally important in preserving the progression of chronic kidney failure (Xi, Xu, Chen, Fan, & Wu, 2016).

While PGE_2 has an anti-fibrotic effect, $PGF_{2\alpha}$ is considered to have a pro-fibrotic action. Loss of prostaglandin F receptor (FP) selectively attenuates pulmonary fibrosis while maintaining similar levels of alveolar inflammation and TGFβ stimulation. Deficiency in FP and inhibition of TGFβ signaling additively decrease fibrosis. Furthermore, $PGF_{2\alpha}$ is abundant in bronchoalveolar lavage fluid of subjects with IPF and stimulates proliferation and collagen production of lung fibroblasts via FP, independently of TGF-β (Oga *et al*., 2009). In patients with IPF, the metabolite of $PGF_{2\alpha}$, the 15-keto-dihydro $PGF_{2\alpha}$, was very high and correlated with disease severity and prognosis, which supports a potential pathogenic role for $PGF_{2\alpha}$ in human IPF (Aihara *et al*., 2013). In the mare, endometrial explants exposure to NETs constituents (elastase, myeloperoxidase and cathepsin G), decreased PTGES and increased COL1 transcription (Rebordão *et al*., 2014; Rebordão *et al*., 2018). Besides, long time exposure of luteal phase environment of endometrial explants to some NETs components, resulted in an increase in $PGF_{2\alpha}$ transcription (Rebordão *et al*., 2013).

Also in cardiac tissue, it was proven that $PGF_{2\alpha}$, increased the mRNA and protein levels of collagen I and III, time- and concentration-dependently (Ding *et al*., 2012). In this work, it was also shown that $PGF_{2\alpha}$ induces high FP receptor expression in cardiac fibroblast, and independently of TGFβ. Silencing of FP-receptor gene may exert a protective effect on diabetes cardiomyopathy (DCM) by improving myocardial fibrosis, which could suggest a new therapeutic approach for human DCM (Ding *et al*., 2014).

In a mouse model of bleomycin-induced systemic sclerosis (SSc), plasmin–α2-antiplasmin (α2AP) binding to adipose triglyceride lipase promoted $PGF_{2\alpha}$ synthesis through calcium-independent phospholipase A2 in fibroblasts, and $PGF_{2\alpha}$ synthesis that was promoted by α2AP, induced TGFβ production (Kanno *et al*., 2013). Also in patients with knee osteoarthritis, the infrapatellar fat pad, induced an increase in collagen production, via $PGF_{2\alpha}$ stimulation, with no involvement of TGFβ (Bastiaansen-Jenniskens *et al*., 2013).

CHAPTER III – MATERIALS AND METHODS

In the present section, the different methodologies will be first described. After that, the experimental designs of the various studies performed, will be addressed.

3.1. METHODOLOGIES

3.1.1. Collection of mare internal genitalia and estrous cycle evaluation

From 2010 until 2015, during the period from early April to late September the internal genitalia and blood samples were obtained post mortem at the abattoir, as by-products, from randomly designated cyclic mares according to the different planned laboratory work. After stunning, mares were euthanized, conformed to the Portuguese legislation (DL 98/96, Art. 1º) and European mandates concerning welfare aspects of animal stunning and euthanasia methods (EFSA, AHAW/04-027).

The biological material used was from healthy mares, as determined by *ante-mortem* and *post-mortem* veterinarian examination, and therefore considered appropriate for human consumption. Because the reproductive status of the mares was unknown, the various stages of the estrous cycle (follicular, early and mid-luteal stages) were identified based on follicle size and morphological appearance of the luteal structures (*corpus hemorragicum* - CH, *corpus luteum* - CL, or *corpus albicans* - CA), as described (Roberto da Costa *et al*., 2007; Roberto da Costa *et al*., 2008). Estrous cycle phase was further confirmed by plasma P_4 concentrations in blood samples obtained at the time of exsanguination into heparinized tubes (Monovettes; Ref. 02.265, Sarstedt, Numbrecht, Germany), Briefly, in the follicular phase (FP), the mare ovary had a preovulatory follicle of 35-40 mm in diameter, visible edema of endometrium, plasma P_4 < 1ng/mL and no CL. In the early luteal phase (ELP) a *corpus hemorragicum* (CH) had replaced the ovulatory follicle, large follicles were absent and plasma P_4 > 1ng/mL. Later on, in mid-luteal phase (MLP) a mature CL, was associated with follicles 15 to 20 mm in diameter and plasma P_4 > 6ng/mL, while in the late-luteal phase; the regressing CL was simultaneous with follicles 30–35 mm in diameter and plasma P_4 ranged from 1–2.5 ng/mL (Roberto da Costa *et al*., 2007; Roberto da Costa *et al*., 2008).

Oviducts and uteri from mares with apparent reproductive problems, such as endometritis, were discarded from the study. Thus, oviducts and endometria from the ipsilateral side to the predominant ovarian structure (*i.e.* CH, CL, follicle) from healthy mares were used in this study and based on the criteria above described and grouped, as follows: follicular phase, early-luteal phase, or mid-luteal phase. Endometrial and oviduct segments (infundibulum, ampulla, and isthmus) samples were collected in: (i) RNA later (AM7020, Ambion, Applied Biosystems,

CA, USA) for gene and protein expression quantification; (ii) buffered formaldehyde for histological (hematoxylin-eosin stain; picrosirius red stain) and immunohistochemistry (IHC) studies; or in (iii) 2.5% glutaraldehyde (AppliChem, Germany) in 0.1-M sodium cacodylate buffer for scanning electronic microscopy (SEM). The three segments of the oviduct used for tissue explant culture were immersed in sterile transport medium Hank's balanced salt solution (HBSS; 55021C; Sigma) with 0.1% bovine serum albumin (BSA), 20 µg/mL gentamicin (G1397; Sigma), and 250 g/mL amphotericin (A2942; Sigma). Oviducts for OEC studies were ligated on both edges with surgical clamps and immersed in sterile transport medium. Samples were transported on ice to the laboratory under 2 h.

3.1.2. Histochemical studies

3.1.2.1.Endometrium classification

In a group of randomly assigned mares (n=18), formaldehyde-fixed endometrium histologic sections (4µm) were stained with hematoxylin-eosin, and classified into 3 categories according Kenney (1978). This classification system is based on histopathologic alterations, mainly inflammation, endometrial glands alteration, lymphatic vessels appearance, and fibrosis. Thus, in a healthy endometrium (category I) uterine glands are normal and little to no inflammatory cells are present. Endometria with inflammation and mild to moderate fibrosis are graded as category II. When endometrosis worsens, dilated endometrium glands are surrounded by layers of collagen fibers, which assigns this endometrium to category III that is the most severe score of Kenney classification system (Kenney, 1978). From the same mares, endometria kept in RNAlater were also used for gene expression determination (qPCR). Mare's endometria histopathology according to Kenney's grading system was related to gene expression, estrous cycle phase, and corresponding oviduct gene expression.

3.1.2.2. Picrosirius Red staining

Endometrial and corresponding oviduct 4µm histological sections were stained with Picrosirius Red Stain Kit (connective tissue stain; ref. ab150681), a specific stain for collagen I and III fibers, under polarized light microscopy. A 1% Picrosirius Red solution was prepared with a saturated aqueous solution of picric acid. After de-waxing and hydration of the paraffin sections, cells nuclei were stained with Weigert´s hematoxylin for 8 min, followed by a 10 min. wash in running tap water. The slides were stained in Picrosirius red for 1h, and then were

washed in acidified water (0.5% acetic acid aqueous solution). The excess of water was removed by vigorous shaking of the slides. The slides were dehydrated in three changes of 100% ethanol, cleared in xylene and mounted with mounting medium (Entellan Merck Millipore; ref. 107960).

The sections were observed under polarized light microscopy (Leica Leitz DMRD), photographed (Mag=100x). Collagen type I fibers appeared colored in red, while Collagen type III, stained green. The areas of COL1 and COL3 were measured using a digital image processing system (Leic Qwin V3), and data further subjected to statistical analysis.

3.1.3. Immunohistochemistry

The protein expression of ovarian steroid hormones receptors (ESR1, ESR2, PGR), OXT receptor (OXTR), prostaglandin synthases (AKR1C3, PTGES), and their intensity in specific cells of the infundibulum, ampulla and isthmus, in FP, ELP, and MLP were determined by immunohistochemistry, as described (Ferreira-Dias *et al*., 2007; Rebordão *et al*., 2017). Consecutive 4 µm histological sections were used for identification of each protein under study. Sections were incubated with primary antibody against PGR (mouse monoclonal diluted 1:500, 0.002 mg/mL; MA1-12626, Thermo Scientific), ESR1 (rabbit polyclonal diluted 1:100, 0.01mg/mL, ab16363, Abcam), ESR2 (rabbit polyclonal diluted 1:500, 0.002 mg/mL; ab3577, Abcam), OXTR (rabbit polyclonal diluted 1:500, 0.0004 mg/mL, sc33209, SCBT), AKR1C3 (rabbit polyclonal diluted 1:250, 0.004 mg/mL, ab137546, Abcam), PTGES (rabbit polyclonal diluted 1:250, 0.004 mg/mL¸ PA5-28476, Thermo Scientific). The most adequate primary antibody concentrations were optimized by us. Conditions of incubation with primary antibodies, are summarized in table 1. Immunohistochemistry staining was assessed as the presence of a characteristic brown staining, with a light microscope (Olympus BX51, Tokyo, Japan) equipped with a DP21 Olympus camera (Tokyo, Japan). Negative controls were performed by replacing the primary antibody by mouse IgG (used at the same concentration as primary antibodies, 550878, BD Bioscience) or rabbit polyclonal IgG (used at the same concentration as primary antibodies; ab27478, Abcam) or by 0.1M PBS (pH 7.4). Positive controls were performed with endometrial tissues (Rebordão *et al*., 2017). Oviduct staining area and intensity were assessed on 10 random fields by 3 evaluators blinded to treatment groups. As described by Rebordão and co-authors (2017), intensity of immunolabeling in the oviduct was quantified by a subjective score (1. no staining; 2. weak staining; 3. moderate staining; 4. intense staining). Scores from all evaluators were averaged for each oviduct slide.

Table 1 - Primary antibodies dilution, incubation time and temperature used in immunohistochemistry of equine oviduct.

Primary Antibody	Reference	Dilution	Incubation time	Incubation Temperature
ESR1	Ab16363	1/100	ON	4ºC
ESR2	Ab 3577	1/500	ON	4ºC
PGR	MA1-12626	1/500	ON	4ºC
OXTR	Sc33209	1/500	1 h	RT
PTGES	PA5-28476	1/250	ON	4ºC
AKR1C3	Ab137546	1/250	ON	4ºC

ESR1: estrogen receptor 1; ESR2: estrogen receptor 2; PGR: progesterone receptor; OXTR: oxytocin receptor; AKR1C3: aldo-keto reductase family 1, member 3; PTGES: microsomal prostaglandin E2 synthase 1; ON: overnight; RT: room temperature.

3.1.4. Microvascular density assessment

To evaluate microvascular density in equine oviduct throughout the estrous cycle, 4 µm histological sections were incubated with CD31 Monoclonal Antibody (M0823, Clone JC70A, DAKO-Agilent, Santa Clara, USA), in the dilution of 1: 50. Negative controls were performed by replacing the primary antibody by mouse IgG (used at the same concentration as primary antibody, 550878, BD Bioscience), and by 0.1M PBS (pH 7.4).

For each oviduct portion, number of vessels and microvascular areas were determined on histologic sections, on 10 randomly chosen microscope fields, using a light microscope at a total magnification of 400X, connected to a computerized cell analysis system (CAS, Becton & Dickinson, Erembodegem, Belgium). All blood vessels were evaluated equally without distinguishing their nature (arterioles, venules and capillaries). Vascular area was calculated as the percentage of the area occupied by blood vessels with respect to the total area in each microscopic field on all 10 histologic sections evaluated per each mare. Vessel numbers were also counted on the same histologic sections used for microvascular area determination. Total vascular area and blood vessels number were determined as the mean values for all 10 microscopic fields of the oviduct tissue evaluated for each mare.

3.1.5. Western blot

Protein expression of PGR, ESR1, ESR2, OXTR, AKR1C3 and PTGES, on equine oviduct tissue was assessed by Western Blot (WB), exclusively in the ampulla, in all phases of the estrous cycle studied. Protein expression of FGFR1, FGFR2, KDR, OVGP1 and COL1 was assessed by WB in all portions of the oviduct and in all phases of the estrous cycle. Tissue samples were minced and placed in ice-cold RIPA buffer (50 mM Tris-HCl, pH 7.4, 50 mM

EDTA, 150 mM NaCl and 1% Triton X-100) with protease inhibitor (Complete Mini Protease Inhibitor Cocktail Tablets, 1 tablet per 10 mL of buffer; Roche) and homogenized on ice. After protein extraction, its concentration was determined by Bradford reagent (500-0006; Bio-Rad, Hercules, CA, USA), as referred (Ferreira-Dias *et al*., 2007). To perform the WB analysis of PGR, ESR1, ESR2, OXTR, AKR1C3 and PTGES, 100 μg of protein was separated by SDS-PAGE (12% acrylamide gel, ref. 161-0155; Bio-Rad, Hercules, CA, USA), while for analysis of FGFR1, FGFR2, KDR, OVGP1 and COL1, 40 μg of protein were used, in an 8% acrylamide gel. Proteins were transferred to nitrocellulose membranes (ref: 1620116; Bio-Rad) (Galvão *et al*., 2013). The concentration of each primary antibody was determined according to the minimal dilution at which the specific protein was still expressed in the tissue, and that could guarantee it was not in the saturation range.

3.1.5.1. Evaluation of ESR1, ESR2, PGR, OXTR, PGES, AKR1C3 protein expression

Expression of these proteins was evaluated with the same antibodies used for immunohistochemistry, but diluted at 1:500 for PGR, 1:500 for ESR1, 1:1,000 for ESR2, 1:750 for OXTR, 1:500 for AKR1C3, 1:500 for PTGES, 1:500. Five mares in each estrous cycle phase were used. To normalize the loaded protein, a mouse monoclonal antibody against ß actin (A5441, Sigma, USA) was used at the dilution 1:10,000. The membranes were incubated with the primary antibody overnight at 4ºC, except for OXTR and β actin, which were incubated at room temperature for 1h and 1.5h, respectively. For β actin and PGR, the secondary antibody used was horseradish peroxidase (HRP)-conjugated goat anti-mouse (A2554, Sigma, USA) at 1:10,000. For ESR1, ESR2, OXTR, AKR1C3, conjugated anti-rabbit (*P0448, Dakocytomation*, Carpinteria, CA, USA), at 1:10,000, was used. Conditions of incubation with primary antibodies, are summarized in table 2. Chemiluminescent detection was obtained by incubation of the membrane with SuperSignal® West Pico (34077, ThermoScientific, Waltham, MA, USA) and by its exposure to a photographic film (Kodac BioMax LighFilm; Kodac-Industrie, Chalon-sur-Saone, France). Target proteins expression was normalized dividing the units of arbitrary densitometry by β actin density for each band. After, Image J (http://rsb.info.nih.gov/ij/index.html) was used to evaluate densitometry signals (Miller, 2010).

Table 2 - Primary antibodies dilution, incubation time and temperature used in western blot analysis of equine oviduct.

Primary Antibody	Reference	Dilution	Incubation time	Incubation Temperature
ESR1	Ab16363	1/500	ON	4ºC
ESR2	Ab 3577	1/500	ON	4ºC
PGR	MA1-12626	1/1,000	ON	4ºC
OXTR	Sc33209	1/500	1 h	RT
PTGES	PA5-28476	1/500	ON	4ºC
AKR1C3	Ab137546	1/500	ON	4ºC

ESR1: estrogen receptor 1; ESR2: estrogen receptor 2; PGR: progesterone receptor; OXTR: oxytocin receptor; AKR1C3: aldo-keto reductase family 1, member 3; PTGES: microsomal prostaglandin E2 synthase 1; ON: overnight; RT: room temperature.

3.1.5.2.Evaluation of FGFR1, FGFR2, KDR, OVGP1, COL1 protein expression

Protein expression of FGFR1, FGFR2, KDR, OVGP1, and COL1 was carried out using specific primary antibodies against FGFR1 (Orb 156864, Biorbyt, Cambridge, UK, dilution 1/250), FGFR2 (SC 6930, Santa Cruz Biotechnology, Dallas, USA, dilution 1/250), KDR (Orb 99143, Biorbyt, Cambridge, UK, dilution 1/250), OVGP1 (SC377267, Santa Cruz Biotechnology, Dallas, USA, dilution 1/250), COL1 (20121; Novotec, Lyon, France, dilution 1/1,000). Conditions of incubation with primary antibodies, are summarized in table 3. Five mares in each estrous cycle phase were used, and evaluation was performed on the three portions of oviduct. Explants from ampulla submitted to different treatments, (E_2, P_4, OXT and TNF, as further explained), were also analyzed by WB for COL1 protein expression. Once again, to normalize the loaded protein, a mouse monoclonal antibody against β actin (A5441, Sigma, USA) was used at the dilution 1: 10,000. All the membranes were incubated with the primary antibody overnight at 4ºC, except against β actin, which was incubated for 1.5 h, at room temperature. Membranes first incubated against FGFR2, OVGP1, were further incubated for

Table 3 - Primary antibodies dilution, incubation time and temperature used in western blot analysis of equine oviduct.

Primary Antibody	Reference	Dilution	Incubation time	Incubation Temperature
FGFR1	Orb156864	1/500	ON	4ºC
FGFR2	Sc6930	1/500	ON	4ºC
KDR	Orb99143	1/250	ON	4ºC
OVGP1	Sc377267	1/1,000	ON	4ºC
COL1	20121	1/1,000	ON	4ºC

FGFR1: fibroblast growth factor receptor 1; FGFR2: fibroblast growth factor receptor 2; KDR: kinase insert domain receptor (vascular endothelial growth factor receptor 2); OVGP1: oviduct-specific glycoprotein; COL1: collagen type I.

1.5 h at room temperature with horseradish peroxidase (HRP)-conjugated goat anti-mouse

(A2554, Sigma, USA) at 1:10,000. Membranes incubated against FGFR1, KDR and COL1, were after incubated conjugated anti-rabbit (P0448, Dakocytomation, Carpinteria, CA, USA), at 1:10,000, for 1.5 h, at room temperature. Membranes incubated against βactin, were after incubated for 1h, at room temperature, with (HRP)-conjugated goat anti-mouse (A2554, Sigma, USA) at 1:5,000. Protein expression was visualized using luminol enhanced chemiluminescence (Super Signal West Pico, 34077; Thermo Scientific, Waltham, USA). Chemiluminescence detection and image acquisition was performed by ChemiDoc XRS+ system (Bio-Rad Laboratories, Inc.). Densitometry signal evaluation was assessed using Image Lab^{TM} software. Dividing the units of arbitrary densitometry by standard sample density for each band normalized target protein expression, and values were expressed in terms of Relative Density. Sample Relative Density of each lane was further divided by β-actin loading-control Relative Density for that same lane.

3.1.6. Quantitative Real Time Polymerase Chain Reaction (qPCR) analysis

From mare infundibulum, ampulla and isthmus obtained in the follicular phase, early luteal phase and mid-luteal phase, mRNA was extracted. In addition, from equine endometrium, mRNA was extracted from a group of 18 mares to analyze the expression of some genes, also analyzed in oviduct. mRNA Extraction and Purification Kit (28704; Qiagen, Hilden, Germany), including a DNA-digestion step with an RNase-free DNase Set (50979254; Qiagen), were used according to the manufacturer's instructions. Quantification of RNA was performed using the Nanodrop system (ND 200C; Fisher Scientific, Hamton, PA, USA) and its quality assessed by visualization of 28S and 18S rRNA bands after electrophoresis through a 1.5% agarose gel and red staining (41003; Biotium, Hayward, CA, USA). Reverse transcription was carried out with Reverse Transcriptase Superscript III enzyme (18080093; Invitrogen, GIBCO BRL, Carlsbad, CA, USA) from 400μg total RNA in a 20 μL reaction volume using oligo(dT) primer (27-7858-01; GE Healthcare, Buckinghamshire, UK). Specific primers for progesterone receptor (*PGR*), estradiol receptor 1 (*ESR1*), estradiol receptor 2 (*ESR2*), oxytocin receptor (*OXTR*), prostaglandin E_2 synthase (*PTGES*), prostaglandin F2α synthase (*AKR1C3*), fibroblast growth factor 1 (*FGF1*), fibroblast growth factor 2 (*FGF2*), fibroblast growth factor receptor 1 (*FGFR1*), fibroblast growth factor (*FGFR2*), vascular endothelial growth factor (*VEGF*), vascular endothelial growth factor receptor 1 (*FLT1*), vascular endothelial growth factor receptor 2 (*KDR*), oviduct glycoprotein 1 (*OVGP1*), collagen type 1 (*COL1*), collagen type 3 (*COL3*), matrix metalloproteinase 2 (*MMP2*), matrix metalloproteinase 9 (*MMP9*), tissue inhibitor of metalloproteinase 1 (*TIMP1*), tumor necrosis factor α (*TNF*), tumor necrosis factor

receptor 1 (*TNFRSF1A*), tumor necrosis factor receptor II (*TNFRSF1B*), prostaglandin E synthase (*PTGES*), prostaglandin $F_{2\alpha}$ synthase (*AKR1C3*), prostaglandin E receptor 2 (*EP2*) or prostaglandin $F_{2\alpha}$ receptor (*FP*), as well as reference gene were designed using Primer3 Software and confirmed with Primer Express® (Applied Biosystems, Foster City, CA, USA). To choose the most stable internal control gene under our experimental conditions, four potential reference genes β2-microglobulin (*β2M),* glyceraldehyde 3-phosphate dehydrogenase, succinate dehydrogenase A (*GAPDH*), mitochondrial ribosomal protein L32 (*MRPL32)* and 40β-actin were tested. During the validation process, samples from oviduct (infundibulum, ampulla and isthmus) and endometrium (n=4), from distinct stages of the estrous cycle (FP, ELP and MLP), were run in parallel for the tested genes. The mRNA transcription of *β2M,* for oviduct tissues, and *MRPL32* for the endometrium, were the most stable reference genes and were unaffected by the experimental conditions, with less than a twofold change between stages (Dheda *et al*., 2004). Primers concentrations were optimized to the minimum concentration: lowest cycle threshold ratio. This technique was used in four different experiments:

3.1.6.1. Evaluation of PGR, ESR1, ESR2, OXTR, PTGES, AKR1C3 gene transcription in equine oviduct

Evaluation of the transcription of *PGR, ESR1, ESR2, OXTR, PTGES, AKR1C3* (Table 4) genes throughout the estrous cycle and between infundibulum and ampulla was carried out. This assay was performed in a 7300 Real-Time PCR System (Applied Biosystems, Warrington, UK) using the default thermocycler program for all genes: a 10-min pre-incubation period at 95ºC was followed by 40 cycles of 15s at 95ºC and 1 min at 60ºC, followed by a dissociation step (15 s at 95ºC, 30 s at 60ºC and 15 s at 95ºC). Both the target genes and reference gene were run simultaneously and all reactions were done in duplicate wells on a 96-well optical reaction plate (4306737; Applied Biosystems) in 25 µL reaction volume containing 6.5 µl water, 2 µL forward primer, 2µL reverse primer, 12.5 µL Power SYBER Green Master Mix (4367659; Applied Biosystems) and 2 µL cDNA. All polymerase chain reaction products were run on a 2.5% agarose gel (BIO-41025; Bioline, Luckenwalde, Germany) to confirm specificity. Relative mRNA quantification data were then analyzed with the real-time PCR miner algorithm (Zhao & Fernald, 2005**)**. According to the instructions supplied for the miners algorithm after determination of average cyclic threshold (Ct) and primer efficiency level (E) using the equation $[1/(1+E)^{Ct}]$. Thereafter, the expression of the target genes was normalized against that

of the reference gene and the mRNA expression compared between infundibulum and ampulla at different phases of the estrous cycle.

Table 4 - Primer sequence used for ovarian steroids and oxytocin receptors, and prostaglandin synthases in real time PCR analysis of mare oviductal tissues.

Gene (Acession number)	Sequence 5´- 3	Amplicon (base pairs)
ESR1 (GeneID: 791249)	Forward: ACGATGCCACCAGACCATTT	160
	Reverse: AGCCAGGCACATTCCAGAAG	
ESR2 (GeneID: 100033964)	Forward: CCCTTCACCGAGTCCTCCAT	232
	Reverse: TCCCTGTCCAGAACGAGGTC	
PGR (GeneID: 100033883)	Forward: CCCAGCATGTCGCCTTAGAA	150
	Reverse: AGGGGTTGGCTTTCATTTGG	
OXTR (XM_001491665.2)	Forward: TGGACGCCATTCTTCTTCGT	141
	Reverse: GCCCGTGAACAGCATGTAGA	
AKR1C3 XM_001500921.1	Forward: TGGGTCACTTTCCTTCAACCA	200
	Reverse: CTTCTCCATTGCCTCCCATGT	
PTGES (NM_001081935.1)	Forward: CACGCTGCTGGTCATCAAGA	127
	Reverse: GGTCGTCCCGGTGAAACTG	
β2M (X69083)	Forward: CGGGCTACTCTCCCTGACTG	92
	Reverse: TTGGCTTTCCATTCTCTGCTG	

ESR1: estrogen receptor 1; *ESR2*: estrogen receptor 2; *PGR*: progesterone receptor; *OXTR*: oxytocin receptor; *AKR1C3*: aldo-keto reductase family 1, member 3; *PTGES*: microsomal prostaglandin E2 synthase 1; *β2M*: beta2 microglobulin.

3.1.6.2. Evaluation of FGF1, FGF2, FGFR1, FGFR2, VEGF, FLT1, KDR gene transcription in equine oviduct

Evaluation of the gene transcription of *FGF1, FGF2, FGFR1, FGFR2, VEGF, FLT1, KDR* (Table 5), was performed in the infundibulum, ampulla and isthmus, obtained at different phases of the estrous cycle. Real time PCR was also done on *FGFR1, FGFR2, FLT1* and *KDR* in oviduct explants, previously submitted to different treatments (E_2, P_4, OXT or TNFα). Previous techniques including mRNA extraction and reverse transcription, were performed as described in section 3.1.6. qPCR was carried out by using StepOnePlus™ System (Applied Biosystems, Warrington, UK). Thermocycler program was run for all genes as follows: initial denaturation step (10 min at 95ºC), followed by 40 cycles of denaturation (15 s at 95ºC), and annealing (1 min at 60ºC), followed by a dissociation step (15 s at 95º, 30 s at 60ºC and 15 s at 95º). Also target genes and reference gene were run simultaneously and all reactions were and in duplicate wells on a 96-well optical reaction plate (4306737; Applied Biosystems) in 13 µL reaction volume containing 3.5 µl water, 1 µL forward primer, 1 µL reverse primer, 6.5 µL Power SYBER Green Master Mix (4367659; Applied Biosystems) and 1 µL cDNA. An

Table 5 - Primer sequence used for fibroblast growth factors, vascular endothelial growth factor, and respective receptors, real time PCR analysis of mare oviductal tissues.

Gene (Acession number)	Sequence 5´- 3	Amplicon (base pairs)
FGF1 (XM_005599133)	Forward: GTGGATGGGACAAGGGACAG	187
	Reverse: GGTTTTCCTCCAGCCTTTCC	
FGF2 (NM_001195221)	Forward: GGAGAAGAGCGACCCTCACA	234
	Reverse: ATACTGCCCCGTTCGTTTCA	
FGFR1 (XM_014736560)	Forward: ACCCAACCGTGTGACCAAAG	260
	Reverse: GGTTGTGGCTGGGGTTGTAA	
FGFR2 (XM_014732956)	Forward: CCAGCTCCTCCATGAACTCC	237
	Reverse: TGACTGCTTCCTTGGGCTTC	
VEGF (NM_001081821)	Forward: ATGCGGATCAAACCTCACCA	117
	Reverse: AGGCCCACAGGGATTTTCTT	
FLT1 (NM_001309471)	Forward: AGGCAACGAATTGACCAACG	
	Reverse: GCACCTGCTGTTTTCGGTGT	
KDR (XM_014738773)	Forward: CTTCCAGTGGGCTGATGACC	100
	Reverse: AGCTTCCACCGAAGATTCCA	
β2M (X69083)	Forward: CGGGCTACTCTCCCTGACTG	92
	Reverse: TTGGCTTTCCATTCTCTGCTG	

FGF1: fibroblast growth factor 1; *FGF2*: fibroblast growth factor 2; *FGFR1*: fibroblast growth factor receptor 1; *FGFR2*: fibroblast growth factor receptor 2; *VEGF*: vascular endothelial growth factor; *FLT1*: related tyrosine kinase 1 (vascular endothelial growth factor receptor 1); *KDR*: kinase insert domain receptor (vascular endothelial growth factor receptor 2); *β2M*: beta2 microglobulin.

electrophoresis on 2.5% agarose gel (BIO-41025; Bioline, Luckenwalde, Germany) was run to confirm the specificity of the product. Quantification of relative mRNA data was done by real-time PCR algorithm (Zao & Fernald, 2005), according to the instructions supplied for the miner's algorithm after determination of average cyclic threshold (Ct) and primer efficiency level (E) using the equation $[1/(1+E)^{Ct}]$. The assay concerning the transcripts of *FGFR1, FGFR2, FLT1* and *KDR* in explants was performed in a 7900 HT Fast Real-Time PCR System (ThermoFisher Scientific, Waltham, USA), in the Laboratory of Prof. Dariusz Skarzynski, Department of Reproductive Immunology Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, Olsztyn, Poland, using the default thermocycler program for all genes: a 10-min pre-incubation period at 95ºC was followed by 40 cycles of 15s at 95ºC and 1 min at 60ºC, followed by a dissociation step (15 s at 95ºC, 15 s at 60ºC and 15 s at 95ºC). Also, the specificity of products was confirmed by an electrophoresis run on 2.5% agarose gel.

3.1.6.3. Evaluation of *OVGP1* gene transcription in equine oviduct

Evaluation of mRNA expression of *OVGP1* (table 6), throughout the estrous cycle, between the different portions of the oviduct, and also in explants previously submitted to different treatments (E_2, P_4, OXT or TNF) was carried out. This assay was performed using a StepOnePlus™ System (Applied Biosystems, Warrington, UK), on the same conditions mentioned above. The assay concerning the transcripts of *OVGP1* from explants was performed in a 7900 HT Fast Real-Time PCR System (ThermoFisher Scientific, Waltham, USA), in the Laboratory of Prof. Dariusz Skarzynski, Department of Reproductive Immunology Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, Olsztyn, Poland, using the default thermocycler program for all genes: a 10-min pre-incubation period at 95ºC was followed by 40 cycles of 15 s at 95ºC and 1 min at 60ºC, followed by a dissociation step (15 s at 95ºC, 15 s at 60ºC and 15 s at 95ºC). Also, the specificity of products was confirmed by an electrophoresis run on 2.5% agarose gel.

Table 6 - Primer sequence used for oviduct-specific glycoprotein real time PCR analysis of mare oviductal tissues.

Gene (Acession number)	Sequence 5´- 3	Amplicon (base pairs)
OVGP1 (XM_014740011)	Forward: GCCCTTTCCGCCTTGTCTAT	141
	Reverse: GCCATAGCCTCTTCCCTTGG	
β2M (X69083)	Forward: CGGGCTACTCTCCCTGACTG	92
	Reverse: TTGGCTTTCCATTCTCTGCTG	

OVGP1: oviduct-specific glycoprotein; *β2M:* beta2 microglobulin.

3.1.6.4. Transcription of collagen type I and type III, and collagen putative modulating genes in equine oviduct and endometrium

Evaluation of mRNA expression of *COL1, COL3, MMP2, MMP9, TIMP1, TNF, TNFRSF1A, TNFRSF1B, PTGES, AKR1C3, EP2*, and *FP* (Table 7), in the three portions of oviduct and endometrium, of the same group of mares was carried out. This assay was also performed in a StepOnePlus™ PCR System (Applied Biosystems, Warrington, UK) using the same default thermocycler program for all genes, as well as all the same procedures were equally run. Thereafter, the expression of the target genes was normalized against that of the reference gene and the mRNA expression compared at different phases of the cycle regarding *COL1* and *COL3*. The levels of these transcripts were correlated to those in the endometrium. Even though estrous cycle phase was not considered, the transcription of *MMP2, MMP9, TIMP1, TNF, TNFRSF1A, TNFRSF1B, PTGES, AKR1C3, EP2*, and *FP* was correlated between oviduct and endometrium.

Table 7 - Primer sequence used for collagen and putative modulating genes, real time PCR analysis of mare oviductal and endometrial tissues.

Gene (Acession number)	Sequence 5´- 3	Amplicon (base pairs)
COL1A2 (XM_001492939.3)	Forward: CAAGGGCATTAGGGGACACA	196
	Reverse: ACCCACACTTCCATCGCTTC	
COL3A1 (AF117954.1)	Forward: CAAAGGAGAGCCAGGAGCAC	98
	Reverse: CTCCAGGCGAACCATCTTTG	
MMP2 (XM_001493281.2)	Forward: TCCCACTTTGATGACGACGA	115
	Reverse: TTGCCGTTGAAGAGGAAAGG	
MMP9 (NM_001111302.1)	Forward: GCGGTAAGGTGCTGCTGTTC	177
	Reverse: GAAGCGGTCCTGGGAGAAGT	
TIMP (NM_001082515.1)	Forward: CAAGTTCGTGGGGACCTCAG	141
	Reverse: CTCTCCATAGCGGGGGTGTA	
PTGES (NM_001081935.1)	Forward: CACGCTGCTGGTCATCAAGA	127
	Reverse: GGTCGTCCCGGTGAAACTG	
AKR1C3 (XM_001500286)	Forward: TGGGTTCCGCCATATTGATT	151
	Reverse: CAACTCGGGTCGAAGGAAAG	
EP2 (NM_001127352.1)	Forward: TGACCATCACCTTCGCCG	179
	Reverse: GACCGCAGCACTCTTAGCACA	
FP (NC_009148.2)	Forward: GTGCAATGCCATCACAGGAA	225
	Reverse: GCCATTCGGAGAGCAAACAG	
TNF (AB035735)	Forward: ACCGAATGCCTTCCAGTCAA	143
	Reverse: CATTTGCACGCCCACTCA	
TNFRSF1A GU166822.1	Forward: TCAACGGCACAGTGCATCT	98
	Reverse: CAGGACATGCTCTCTT	
TNFRSF1B (XM_014737672.1)	Forward: TGCATACTTCCAAGGCAGGAG	108
	Reverse: GCACACCACGTTTGATGTCG	
ALK5 (XM_014735928.1)	Forward: CACCATCGAGTGCCAAATGA	210
	Reverse: CTCCTCTCCACTTCCCTCGC	
TGFBR2 **(**NM_001301147.1)	Forward: TGCTGCCTGTGTGACTTTGG	107
	Reverse: TCTGGGGCCATGTATCTTGC	
β2M (X69083)	Forward: CGGGCTACTCTCCCTGACTG	92
	Reverse: TTGGCTTTCCATTCTCTGCTG	
MRPL32 (XM_001492042)	Forward: AGCCATCTACTCGGCGTCA	144
	Reverse: GTCAATGCCTCTGGGTTTCC	

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3.1.7. Enzyme Immunoassays

3.1.7.1. Hormone determinations

Concentrations of PGE_2 and $PGF_{2\alpha}$ in oviductal conditioned media and P_4 in plasma were determined by direct enzyme immunoassay (EIA) as described (Galvão *et al*., 2010). The standard curve for P_4 ranged from 0.39 to 100 ng/mL. Concentration of P_4 at 50% binding (ED50) was 4.2 ng/mL. The intra-assay coefficient of variation (CV) was 5.5%. The PGE_2 standard curve ranged from 0.38 ng/mL to 100 ng/mL and the concentration at 50% binding (ED50) was 6.24 ng/mL. The intra- assay CV was 1.5%. The $PGF_{2\alpha}$ standard curve ranged from 0.07 to 20 ng/mL, and the ID50 was 1.84 ng/mL. The intra-assay CV was 7.3%. Hormones concentration in culture media was normalized for the number of live cells (OEC) after cell viability assessment or for explants weight (base line corresponded to control level).

3.1.7.2.OVGP1 determination

Concentrations of OVGP1 oviductal conditioned media was determined by direct enzyme immunoassay (EIA) (#LS-F 12237-1, LifeSpan BioSciences, Inc, Seattle, USA). The OVGP1 standard curve ranged from 78 pg/mL to 5,000 pg/mL and the intra- assay CV was <10%. OVGP1 concentration in culture media was normalized for explants weight (base line corresponded to control level).

3.1.8. *In vitro* studies

3.1.8.1. Oviduct epithelial cells (OEC) isolation

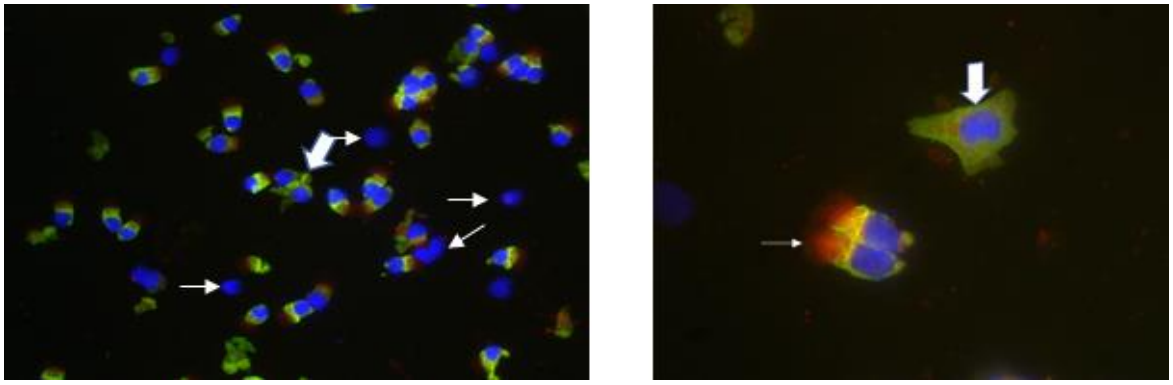
At the laboratory, oviducts previously ligated on both edges with surgical clamps, were first dissected from the surrounding tissue, rinsed in an ethanol solution (70%) and further immersed in culture medium Dulbecco's modified eagle's medium (DMEM) and F-12 Ham medium (D/F medium; 1:1 [v/v], D-8900; Sigma) with 20 µg/mL gentamicin and 0.1% bovine serum albumin (BSA; 735078; Roche Diagnostics GmbH Mannheim, Germany) added. For OEC preparation, only the ampulla was used. It was cut open longitudinally and the mucosa layer gently scraped with a scalpel blade. Cells were filtered through a metal wire mesh (100 µm) with a cell dissociation Sieve – Tissue Grinder Kit (cd1-1kt, Sigma), to remove the undissociated tissue fragments. The filtrate was washed twice by centrifugation (120 g/10 min) with D/F with 0.1%

BSA and 20 µg/mL gentamicin added. Supernatant was discarded and erythrocyte lysis was accomplished after treating the pellet with Red blood lyses buffer (R7757, Sigma). Another washing step was performed. Cells were resuspended in DMEM and F-12 Ham medium (D/F medium; 1:1 [v/v], D-8900; Sigma) containing 10% fetal bovine serum (FBS; 26140-079, Gibco, USA), 20 µg/mL gentamicin and amphotericin [250 µg/mL]. Cell viability, was higher than 88% as determined by trypan blue dye (T8154, Sigma).

3.1.8.2. Oviduct cell assessment

In order to identify the epithelial (ciliated or secretory) nature of oviduct isolated cells, 100µL of oviduct cell suspension at a concentration of $5x10^5$ cell/mL were submitted to a cytospin (Cytospin 2, Shandon) for 8 min at 370 g. Oviductal cells present in the pellet were then fixed with 4% paraformaldehyde (30 min at 4ºC), washed in PBS (10 min), Triton 0.25% (10 min), and PBS (10 min). Blocking [PBS with 2.5% w/v bovine serum albumin and (A7906; Sigma-Aldrich, Inc.) tween (0.05%; Sigma-Aldrich)] was performed at room temperature, for 1 h. For double-staining, primary antibodies (mouse anti-cytokeratin, 1:100, MSK019, Zytomed; goat anti-RAB 27B, 1:50, 0.004 mg/mL, SC 22993, Santa Cruz) were diluted in blocking solution and incubated overnight at 4°C. After washing (4 times with PBS), slides were incubated with the secondary antibodies (donkey anti-mouse Alexa 488, 1:300, Invitrogen; chicken anti-goat Alexa 594, 1:300, Invitrogen), for 30 min at room temperature. Slides were then washed in PBS (10 min) and stained with Hoeschst 33258 (Sigma, 20 µL stock solution/mL PBS) for 15 min. After, slides were washed twice with PBS for 10 min, and coverslips mounting was performed with Mowiol (Sigma). All these procedures were carried out at room temperature. Cells were observed under the fluorescence microscope and representative images were acquired (x400 or x1,000 magnification) (Figure 1). Cell count and identification was performed on 300 cells, at random (Mag 400x). Hoescht stained blue all cells nuclei, regardless of cell types, while cytokeratin stained green the cytoplasm of epithelial cells (ciliated and secretory cells), and RAB 27B marked secretory granules in red, in epithelial secretory cells.

Figure 1 - Oviductal epithelial cells obtained after a 24 h incubation and submitted to cytospin.



Nuclei were stained in blue by Hoeschst 33258 and cytoplasm of epithelial cells stained bright green with anti-cytokeratin antibody (thick arrows). Secretory cells depict red granules of secretion stained by RAB27 (thin arrows). In non-epithelial cells cytoplasm was not visible and only nuclei stained blue (dashed arrows). Left photo: Mag = 400X; Right photo: Mag = 1,000X.

3.1.8.3.Oviductal explants preparation

As referred for OEC preparation, once in the laboratory, the oviducts were carefully dissected from the surrounding tissue, rinsed in an ethanol solution (70%) and further immersed in culture medium D/F medium; 1:1 [v/v], D-8900; Sigma) with 20 µg/mL gentamicin and 0.1% BSA. Afterwards, each of its three regions - infundibulum, ampulla and isthmus - was fragmented into small pieces (2-4 mm) and placed in a petri dish. Three washing steps were performed with D/F with 20 µg/mL gentamicin and 0.1% BSA. Oviduct explants (about 25 mg/mL) were cultured in D/F medium (1:1 [v/v]) containing 0.1% BSA, 20µg/mL gentamicin and 250 µg/mL amphotericin, in 24-well culture plates (142475, Nunc, Kamestrupvej, Denmark), at 37ºC in a humidified atmosphere (5% CO_2, 95% air) in an incubator chamber (Heraus, Hera Cell 150).

3.1.8.4.Ovarian steroid hormones, OXT and TNF effect on *in vitro* prostaglandins and OVGP1 secretion

3.1.8.4.1. Oviductal epithelial cells

After cell viability assessment, OEC collected from the ampulla in the follicular or mid-luteal phases (n=5 mares/stage) were incubated in a 24-well culture plate (#142475, Nunc, Kamestrupvej, Denmark), at a concentration of $5x10^5$ cell/mL for 24 h with: (i) no exogenous treatment (Control); (ii) P_4 (10^{-7}M; P0130, Sigma); (iii) E_2 (10^{-9}M; E8875, Sigma); (iv) OXT (10^{-7}M; O3252, Sigma) or (v) TNF (10 ng/mL; T6674, Sigma). Conditioned media from negative control and treatment groups were stored with 5µL EDTA and 1% acetylsalicylic acid

(v/v) (Sigma Aldrich, #A2093) (Szóstek, Galvão, Ferreira-Dias & Skarynski, 2014), and stored at -80°C until PGE_2 and $PGF_{2\alpha}$ concentration assessment by direct enzyme immunoassay (EIA), as described below (*Cf* **3.1.7.1**).

3.1.8.4.2. Oviduct explants

Oviduct explants from follicular phase, early-luteal phase and mid-luteal phase (n=5/stage) were incubated for 24 h as described above for OEC culture. After, conditioned media with acetylsalicylic acid and EDTA added (as referred above) were kept at -80°C until PGE_2 and $PGF_{2\alpha}$ concentration assessment, or without any supplement, and also stored at -80ºC until OVGP1 contraction assessment, as previously referred (*Cf* **3.1.7.1**). Oviduct explants collected after incubation, were kept in RNAlater, at -80ºC, until RNA extraction was carried out, and qPCR was completed, in order to evaluate the transcription of *FGFR1*, *FGFR2*, *FLT1*, *KDR*, (*Cf* **3.1.6.2**), transcription of *OVGP1* (*Cf* **3.1.6.3**), and transcription of *COL1* (*Cf* **3.1.6.4**), as well as COL1 protein expression by WB (*Cf* **3.1.5.2**).

3.1.8.5. Sperm cells effect on *in vitro* OVGP1 secretion

Stallion semen was collected with an artificial vagina (Model Hannover). After it was diluted in Equipro®, and placed inside a syringe, in anaerobic conditions, at the final concentration of 200 x 10^6 SPZ/mL. Before use, semen was centrifuged at 400g, for 10 min, and resuspended in culture medium at a final concentration of 100 x 10^6 SPZ/mL. Oviduct explants from follicular (n=3 mares) and early-luteal phases (n=3 mares), were cultured in triplicate in a 6 well-plate (Nunclon, #140675, NUNC, Roskilde, Danmark) in 3mL of culture medium alone (DMEF/F12, # 11039, Gibco) - (i) Control; or with (ii) medium + SPZ, separated from explants by an insert (Transwell, #3414, Corning, NY, USA); or (iii) medium + SPZ + explants in direct contact. After incubation, conditioned medium was maintained at -80ºC, until OVGP1 determination by EIA (*Cf.* **3.1.7.2**).

3.1.9. Statistical analysis

Data concerning the various studies were submitted to different statistical analysis, as follows:

1. One-way analysis of variance by Bonferroni Compare all Pairs of Columns Test (ANOVA; GraphPAD PRISM, Version 5.00, GraphPad Software, San Diego, CA, USA). Significance was defined as $P < 0.05$:

- Relative quantification of gene transcription by real-time polymerase chain reaction in equine oviduct of *ESR1, ESR2, PGR, OXTR, PTGES, AKR1C3, FGF1, FGF2, FGFR1, FGFR2, VEGF, VEGFR-1, KDR, OVGP1, COL1, COL3,* between portions, throughout the estrous cycle, and between different mares ages in the case of *COL1, COL3.*
- Relative quantification of ESR1, ESR2, PGR, OXTR, PTGES, AKR1C3 protein expression by WB analysis in the ampulla of equine oviduct, throughout the estrous cycle.
- Comparison of vascular areas and number of vascular structures in equine oviduct between portions throughout the estrous cycle.
- Relative quantification of FGFR1, FGFR2, VEGFR2, OVGP1 protein expression by WB analysis between portions and throughout the estrous cycle.
- Relative quantification of COL1 protein expression by WB analysis between portions and throughout the estrous cycle.

2. T-test (GraphPAD PRISM, Version 5.00, GraphPad Software, San Diego, CA, USA). Significance was defined as $P < 0.05$:
    - Analysis of differences between the transcription of ovarian steroid hormones, OXTR and PG synthases between infundibulum and ampulla.
    - Analysis of differences in the transcription of *COL1* and *COL3* between mares with different degree of endometrial fibrosis.
    - Area of fibrosis, referred as area of COL1 and COL3 deposition in endometrium and oviduct, evaluated by PSR.

3. Correlation test and whenever Pearson coefficient was significant at $P < 0.05$), Bi-variable Linear Correlation was performed in order to investigate the existence of a dependent and independent variables (IBM SPSS Statistic Analysis Version 24, IBM SPSS Software, Armonk, NY, USA). Significance level was $P < 0.05$:
    - Between oviduct *COL1* and endometrium *COL1*;
    - Between oviduct *COL3* and endometrium *COL3*;
    - Between oviduct *COL1* and *MMP2, MMP9, TIMP1, TNF, TNFRSF1A, TNFRSF1B, PTGES, AKR1C3, EP2, FP, ALK5 and TGFβRII,* in oviduct
    - Between *COL3* oviduct and *MMP2, MMP9, TIMP1, TNF, TNFRSF1A, TNFRSF1B, PTGES, AKR1C3, EP2, FP, ALK5 and TGFβRII,* in oviduct

- Between oviduct *COL1* and *MMP2, MMP9, TIMP1, TNF, TNFRSF1A, TNFRSF1B, PTGES, AKR1C3, EP2, FP, ALK5 and TGFβRII* in endometrium
- Between oviduct *COL3* and *MMP2, MMP9, TIMP1, TNF, TNFRSF1A, TNFRSF1B, PTGES, AKR1C3, EP2, FP, ALK5 and TGFβRII* in endometrium

4. One-way analysis of variance by Dunnett's Multiple Comparison Test (ANOVA; GraphPAD PRISM, Version 5.00, GraphPad Software, San Diego, CA, USA). Significance was defined as $P < 0.05$.
   - For the analysis of differences between (i) the transcription of infundibulum and ampulla; and (ii) ovarian steroid hormones and OXTR and PG synthases, data were analyzed using t-test (T-test; GraphPAD PRISM, Version 5.00, GraphPad Software, San Diego, CA, USA).

3.2. EXPERIMENTAL DESIGN

Study I - The role of ovarian steroids, oxytocin and tumor necrosis factor in the modulation of equine oviduct function

Experiment I: Ovarian steroid hormones, oxytocin receptors and PG synthases mRNA transcription and protein expression in oviduct

Transcription of ovarian steroid hormones and oxytocin receptors, and PGs synthases was analyzed by qPCR in infundibulum and ampulla of mare oviduct in follicular (n=5), early (n=5) and mid-luteal (n=5) phases. Protein expression was assessed by western blotting only in the ampulla and by immunohistochemistry in all regions of the oviduct in the follicular, early and mid-luteal phases, as described in (*Cf.* **3.1.3**, **3.1.5.1** and **3.1.6.1**).

Experiment II: Ovarian steroid hormones, OXT and TNF effect on in vitro prostaglandins secretion

a) Oviduct epithelial cells (OEC) - PGE_2 and $PGF_{2\alpha}$ production

After cell viability assessment (*Cf.* **3.1.8.2**), OEC collected from the ampulla in the follicular or mid-luteal phases (n=5 mares/stage) were incubated in a 24-well culture plate (#142475, Nunc, Kamestrupvej, Denmark), at a concentration of $5x10^5$ cell/mL for 24 h with: (i) no

exogenous treatment (Control); (ii) P_4 (10^{-7}M; P0130, Sigma); (iii) E_2 (10^{-9}M; E8875, Sigma); (iv) OXT (10^{-7}M; O3252, Sigma) or (v) TNF (10 ng/mL; T6674, Sigma) (*Cf.* **3.1.8.4**). Conditioned media from negative control and treatment groups were stored with 5µL EDTA and 1% acetylsalicylic acid (v/v) (Sigma Aldrich, #A2093) (Szóstek *et al*., 2014**)**, and stored at -80°C until PGE_2 and $PGF_{2\alpha}$ concentration assessment by direct enzyme immunoassay (EIA), as described above (*Cf.* **3.1.7.1**).

b) Oviduct explants - PGE_2 and $PGF_{2\alpha}$ production

Oviduct explants from the ampulla, from follicular phase, early-luteal phase and mid-luteal phase (n=5/stage) were incubated (*Cf.* **3.1.8.3**) for 24 h as described above for OEC culture. After, conditioned media with acetylsalicylic acid and EDTA added (as referred above) were kept at -80°C until PGE_2 and $PGF_{2\alpha}$ concentration assessment (*Cf.* **3.1.7.1**).

Experiment III: Ultrastructure of OEC and oviduct

Additional OEC were placed in separate wells, under the same culture conditions of temperature and atmosphere for 72 h, on a round gelatinized coverslip for scanning electron microscopy (SEM) evaluation. Coverslips for OEC assessment by SEM were fixed in 2.5% glutaraldehyde (AppliChem, Germany) in 0.1 M sodium cacodylate buffer for 24 h. The OEC samples were subsequently dehydrated in a graded ethanol series. Oviduct tissue was also fixed as OEC, for further SEM processing. Samples were dried using the critical point drying method and sputter coated with gold palladium, mounted on stubs, observed under a SEM (JEOL5200-LV) and photographed.

Study II – Microvascular density and the expression of FGFs, VEGF, and receptors in equine oviduct

Experiment I: Vascular structures of oviduct were observed and analyzed using CD31 marker and the area compared among diverse groups

Formaldehyde-fixed oviduct histologic sections (4 µm) of infundibulum, ampulla and isthmus were obtained from mares in follicular (n=6), early luteal phase (n=6) and mid luteal phase (n=6), and CD31 immunohistostaining blood vessels were marked. Vascular areas and number vascular structures presents in oviduct were as described previously (*Cf.* **3.1.4**), and compared among groups.

Experiment II: Angiogenic growth factors transcription in equine oviduct

Transcription of angiogenic growth factors *FGF1*, *FGF2* and *VEGF*, and their receptors *FGFR1*, *FGFR2*, and *KDR* transcription were analyzed by qPCR in infundibulum, ampulla and isthmus of oviducts from mares in distinct phases of estrous cycle (n=5 mares/each phase) as previously referred. Also, transcription of FGFR1, FGFR2 and KDR was evaluated from explants submitted to culture for 24 h, as mentioned above (*Cf.* **3.1.6.2**).

Experiment III: Angiogenic growth factors receptors protein expression in equine oviduct

Protein expression of FGFR1, FGFR2 and KDR was analyzed by WB. Data between the various segments of the oviduct were compared, also considering the distinct phases of the estrous cycle. The oviducts used in this experiment were the same ones used for qPCR (*Cf.* **3.1.5.2**).

Experiment IV: Ovarian steroid hormones, OXT and TNF effect on gene transcription of *FGFR1*, *FGFR2*, *FLT1* and *KDR* in equine explants

Ampulla oviduct explants from follicular phase, early luteal phase and mid-luteal phase (n=5/stage) were incubated for 24 h as described above. Explants were submitted to qPCR, to evaluate the effect of E_2, P_4, OXT and TNF on *FGFR1*, *FGFR2*, *FLT1* and *KDR* transcription (*Cf.* **3.1.6.2**).

Study III – Oviduct specific glycoprotein expression in equine oviduct

Experiment I: OVGP1 transcription in equine oviduct

OVGP1 transcription was analyzed by qPCR in infundibulum, ampulla and isthmus of oviduct from mares in distinct phases of estrous cycle (n=5 mares/each phase) as previously referred (*Cf.* **3.1.6.3**).

Experiment II: OVGP1 protein expression in equine oviduct

Protein expression of OVGP1 was also evaluated by WB. Data between groups of mares in distinct phases of estrous cycle, and also between portions of oviduct were compared (*Cf.* **3.1.5.2**). The oviducts used in this experiment were the same ones used in experiment I.

Experiment III: Ovarian steroid hormones, OXT and TNF effect on *in vitro* mRNA expression of *OVGP1* in equine explants and on OVGP1 secretion

Ampulla oviduct explants from follicular phase, early-luteal phase and mid-luteal phase (n=5/stage) were incubated for 24 h as described above. After, tissues were kept in RNAlater, at -80ºC, until RNA extraction was done and qPCR was completed, in order to evaluate the transcription of *OVGP1 ((Cf* **3.1.6.3**). Media was also kept at -80°C until OVGP1 concentration assessment, by EIA (*Cf* **3.1.7.2**).

Experiment IV: Spermatozoa effect on *in vitro* OVGP1 secretion

The effect of sperm cells (spz) on equine OVGP1 production by equine oviduct portions in follicular phase explants (infundibulum, ampulla, isthmus) was investigated. Media collected after explants tissues culture (*Cf* **3.1.8.4**), were used for OVGP1 concentration assessment, as described before (*Cf* **3.1.7.2**).

Study IV - Collagen in equine oviduct: possible relationship with endometrial fibrosis and pathways involved

This study was designed to assess collagen presence in mare oviduct (infundibulum, ampulla, isthmus), with respect to endometrium and putative factors involved on its deposition. Endometria and oviducts were collected *post-mortem* from cyclic mares (n=18) in follicular, early and mid-luteal phases, aged from 4 to 20 years old, and endometria with different Kenney´s classifications.

Experiment I: *COL1* and *COL3* transcription in equine oviduct

A quantitative evaluation of *COL1* and *COL3* gene transcription in oviduct was performed. Data were analyzed according to (i) the oviduct portion, (ii) the age of the mares, and (iii) the phase of the estrous cycle (n=18) (C*f* **3.1.6.4**).

Experiment II: COL1 protein expression in equine oviduct

The same mares used in the previous experiment, were also used to evaluate the protein expression of COL1. Again, data were analyzed with respect to (i) the oviduct portion, (ii) the age of the mares, and (iii) the phase of the estrous cycle (n=18) (C*f* **3.1.5.2**).

Experiment III: COL1 *vs* COL3 protein in equine oviduct and endometrium

This experiment was carried out to determine the relationship between COL1 and COL3 protein in oviduct and endometrium collected from the same mares (n=18), and of known endometrium Kenney´s classification (*Cf.* **3.1.2.1**).
Endometrium and the three portions of oviduct, of each mare, were stained by PSR and slides were observed and evaluated as referred (C*f* **3.1.2.2**).

Experiment IV: Possible association between COL1 and COL3 transcripts in oviduct and endometrium

In the same group of mares (n=18), oviduct collagen type I and III transcripts were evaluated, as so in the corresponding endometria. The purpose of this evaluation, was to investigate the possible relation in *COL1* and *COL3* transcription between endometrium and oviduct (C*f* **3.1.6.4**).

Experiment V: Putative pathways involved in collagen transcription in equine oviduct

To assess the putative pathways involved in collagen deposition in the mare oviduct *MMP2, MMP9, TIMP, PTGES, AKR1C3, EP2, FP, TNF, TNFSFR1A, TNFSFR1B, ALK5 and TGFBR2*, gene transcription was carried out by qPCR, from the same group of mares (n=18) as previously mentioned (C*f* **3.1.6.4**). Gene transcription was performed in all portions of equine oviduct and correlated with COL1 and COL3 transcription. In cases of a significant correlation coefficient, linear regression was performed, between *COL1* or *COL3* (dependent variable), and the putative gene (independent variable).

Experiment VI: Possible link between endometrial collagen pathways and oviduct collagen transcription

Since oviduct and endometrium are functionally and physically related, some pathways involved in endometrium collagen deposition were analyzed in order to understand if they could be related with oviduct fibrosis (n=18).
Endometrial transcription of *MMP2, MMP9, TIMP, PTGES, AKR1C3, EP2, FP, TNF, TNFSFR1A, TNFSFR1B, ALK5 and TGFBR2,* genes was evaluated (C*f* **3.1.6.4**). Data were analyzed by correlation method between *COL1* and *COL3*, and the gene implicated in endometrial fibrosis. If results were significant, a linear regression test was also performed.

Experiment VII: Ovarian steroid hormones, OXT and TNF effect on *in vitro* gene transcription of *COL1* in oviduct explants

Ampulla explants from follicular phase, early-luteal phase and mid-luteal phase (n=5/stage) were incubated for 24 h as described above (*Cf.* **3.1.8.3**). Explants were subjected to qPCR, to evaluate the effect of E_2, P_4, OXT and TNF on *COL1* transcription (C*f* **3.1.6.4**).

Experiment VIII: Ovarian steroid hormones, OXT and TNF effect on *in vitro* protein expression of *COL1* in oviduct explants

Ampulla oviduct explants from follicular phase, early-luteal phase and mid-luteal phase (n=5/stage) were incubated for 24 h as described above (*Cf.* **3.1.8.3**). Explants were processed for WB, to evaluate the effect of E_2, P_4, OXT and TNF on COL1 expression (C*f* **3.1.5.2**).

CHAPTER IV – RESULTS

4.1. The role of ovarian steroids, oxytocin and tumor necrosis factor in the modulation of equine oviduct function

4.1.1. Ovarian steroid hormones and oxytocin receptors and PG synthases mRNA transcription and protein expression in oviduct

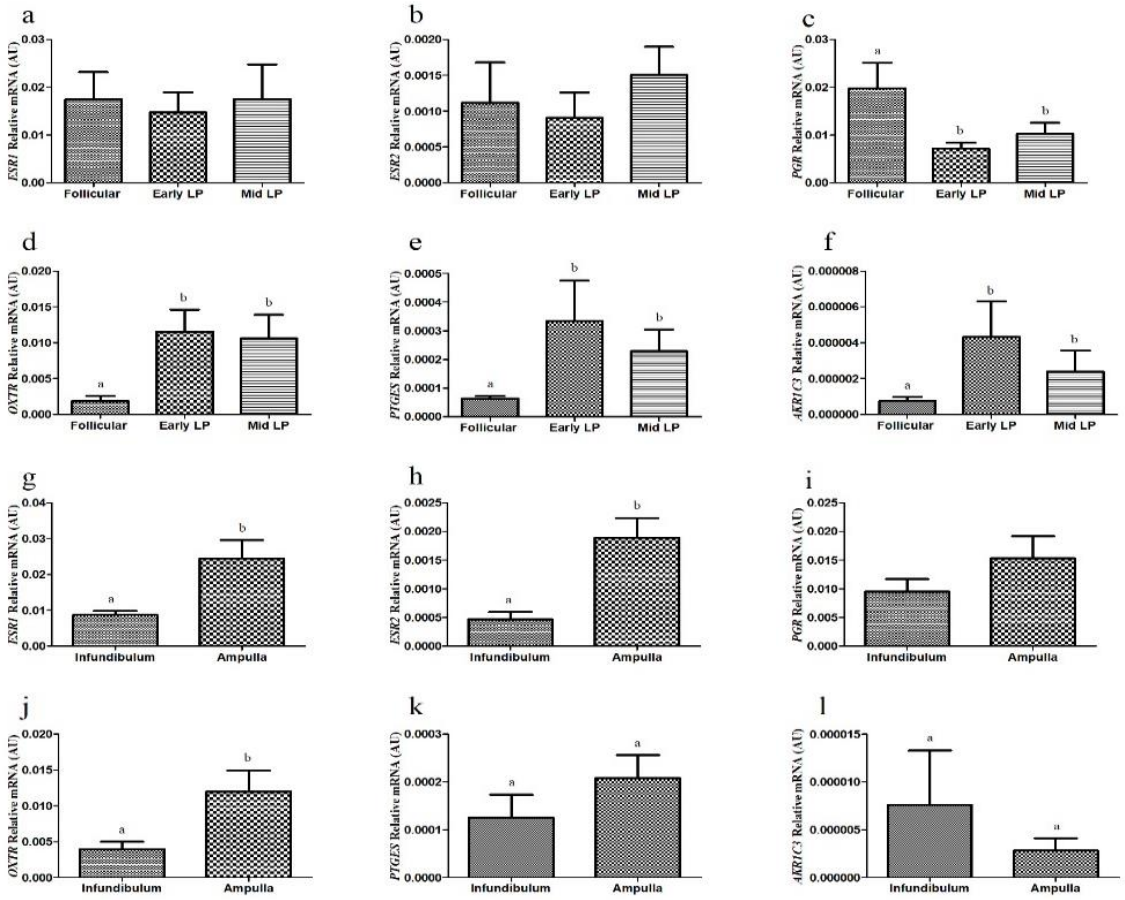
Transcription of ovarian steroid hormones and oxytocin receptors, and PGs synthases was analyzed by qPCR in infundibulum and ampulla of mare oviduct in follicular, early and mid-luteal phases. Protein expression was assessed by western blotting only in the ampulla and by immunohistochemistry in all regions of the oviduct in the follicular, early and mid-luteal phases.

Estradiol receptors *ESR1* and *ESR2* transcription in mare´s oviduct did not change throughout the estrous cycle (Fig. 2a, 2b). In contrast, *PGR* mRNA levels were increased in the follicular phase ($P < 0.05$; Fig. 2c). When the portion of the oviduct was taken into consideration, the highest transcription of *ESR1* and *ESR2* was present in the ampulla ($P < 0.05$; Fig. 2g, 2h). However, no difference in *PGR* transcription was noted between those regions of the oviduct (Fig. 2i). Unlike E_2 receptors, *AKR1C3* transcription in mare´s oviduct was up-regulated in ELP ($P < 0.05$; Fig. 2f). In the follicular phase, *OXTR* and *PTGES* mRNA levels were decreased ($P < 0.05$; Fig. 2d, 2e). Even though the highest transcription of *OXTR* was present in the ampulla ($P < 0.05$; Fig. 2j), no difference in *PTGES* or *AKR1C3* mRNA levels was noted between infundibulum and ampulla (Fig. 2k, 2l).

Since for almost all genes, transcription was the highest in the ampulla, protein expression analysis by western blot was performed solely on that portion of mare oviduct. Protein expression of ESR1 was the largest in follicular phase when compared to early and mid-luteal phases ($P < 0.05$; Fig. 3a; Fig. 4a); ESR2 was the highest in follicular phase when compared to mid-luteal phase ($P < 0.05$; Fig. 3b; Fig. 4b); and PGR increased in follicular and early-luteal phases when related to mid-luteal phase ($P < 0.05$; Fig. 3c; Fig. 4c). Also, OXTR protein expression was the strongest in follicular phase oviducts when compared to early and mid-luteal phases ($P < 0.05$; Fig. 3d; Fig. 4d). Nevertheless, the enzymes involved in the synthesis of PGE_2 (PTGES) or $PGF_{2\alpha}$ (AKR1C3) did not show any differences in protein expression between the phases of the estrous cycle studied ($P > 0.05$; Fig. 3e, 3f; Fig. 4e, 4f).

Immunohistochemistry study demonstrated the presence of ESR1, ESR2, PGR, AKR1C3, and PTGES protein in mare oviduct epithelial cells (Fig. 5a-e), while OXTR was only expressed in

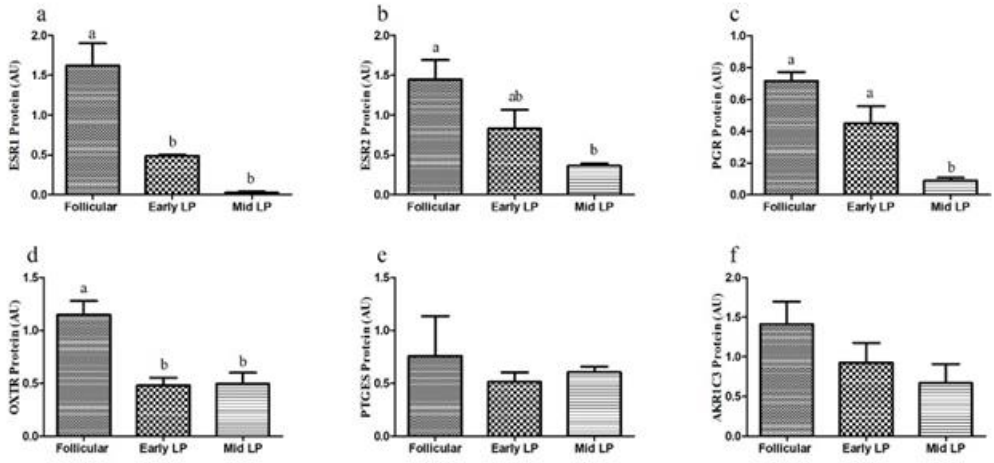
Figure 2 - Relative quantification of gene transcription by real-time polymerase chain reaction in equine oviduct of *ESR1*, *ESR2*, *PGR*, *OXTR*, *PTGES* and *AKR1C3*.



n=5 samples for each estrous cycle phase; n=5 for each portion of oviduct analyzed of *ESR1* (a) and (g), *ESR2* (b) and (h), *PGR* (c) and (i), *OXTR* (d) and (j), *PTGES* (e) and (k), *AKR1C3* (f) and (l). Transcription of target genes was normalized against that of the reference gene (*β2M*). Bars represent mean ± SEM. AU: arbitrary units. Different letters indicate significant differences ($P < 0.05$).

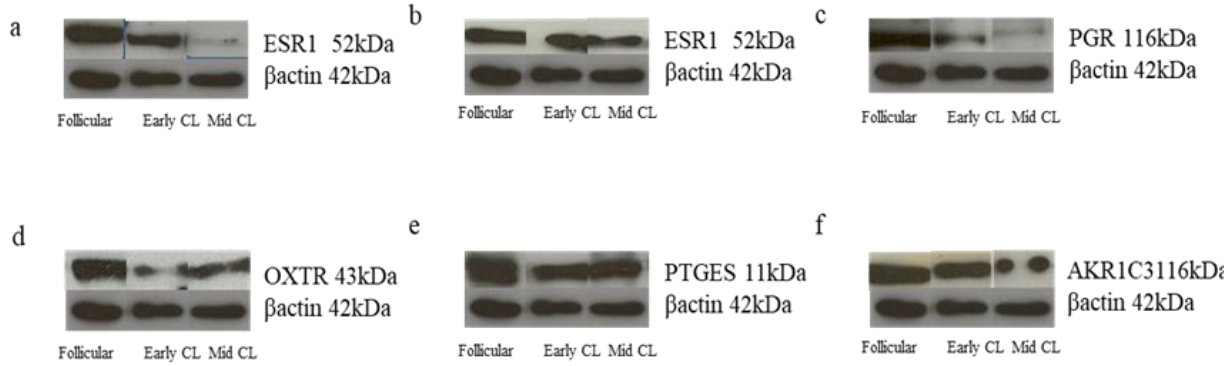
the stroma of the mucosa folds (Fig. 5f), without any significant changes between oviduct portions or estrous cycle phases ($P > 0.05$). While for PGR, ESR1, AKR1C3 and PTGES oviduct cell nuclei stained deep brown and cytoplasm stained in a lighter brown, ESR2 staining was mainly present in the cytoplasm of epithelial cells while the nucleus appeared blue (Fig. 5b).

Figure 3 - Relative quantification of ESR1, ESR2, PGR, OXTR, PTGES and AKR1C3 protein expression by western blot analysis in the ampulla of equine oviduct.



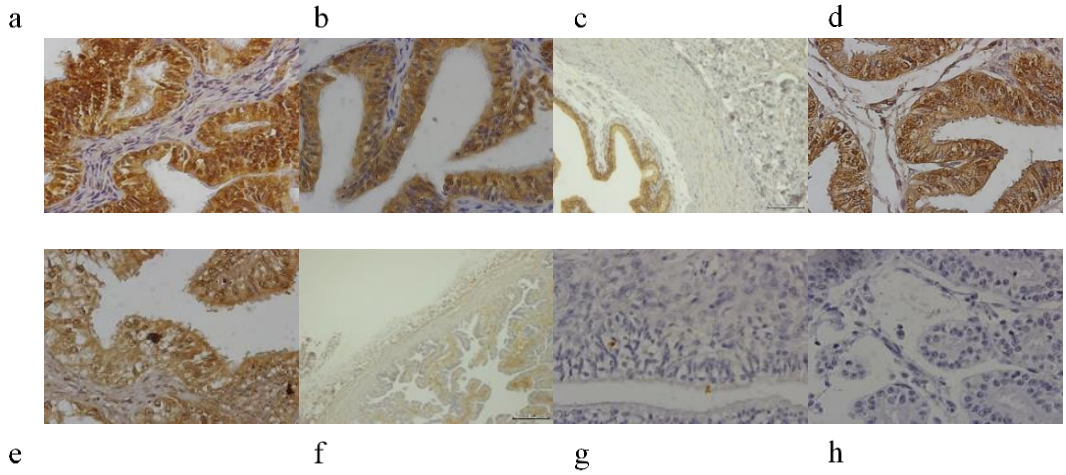
Relative quantification of ESR1, ESR2, PGR, OXTR, PTGES and AKR1C3 protein expression by western blot analysis in the ampulla of equine oviduct (n=3 for each phase). Bars represent mean ± SEM. AU: arbitrary units. Different letters indicate significant differences ($P < 0.05$).

Figure 4: Panels representative of protein expression in equine ampulla, evaluated by WB, of ESR1, ESR2, PGR, OXTR, PTGES and AKR1C3



(a) ESR1; (b) ESR2; (c) PGR; (d) OXTR; (e) PTGES; (F) ARK1C3. Data were normalized against β-actin density values

Figure 5 - Representative images of equine oviduct immunostained for the presence of ESR1, ESR2, PGR, OXTR, PTGES and AKR1C3.



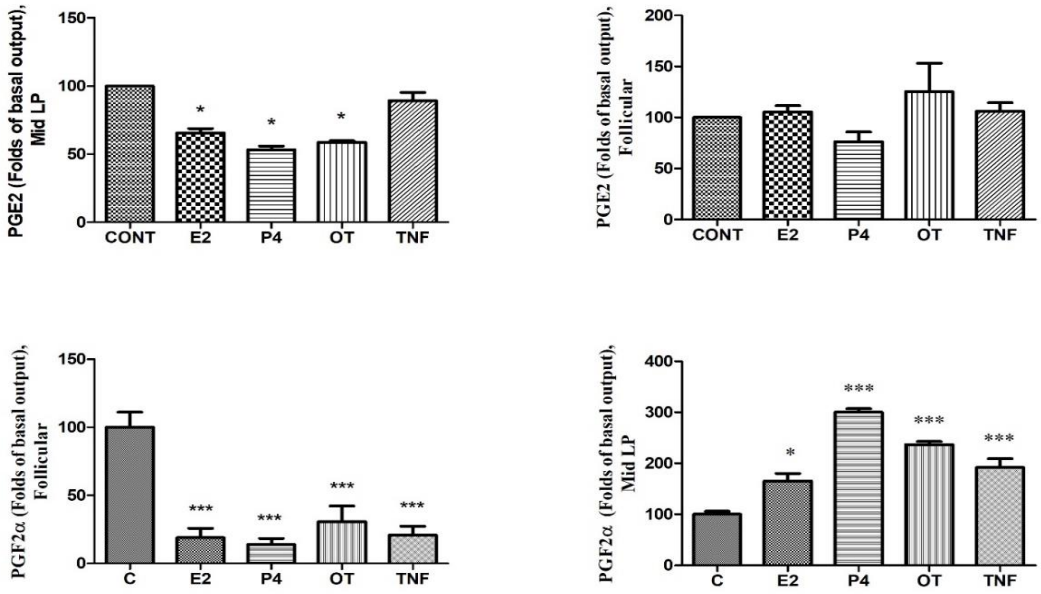
(a) ESR1 in the follicular phase (FP); (b) ESR2 in early luteal phase (ELP); (c) PGR in the mid-luteal phase (MLP) depicting tunica muscularis where staining was less evident than in OEC; (d) AKR1C3 in the FP; (e) PTGES in ELP; (f) OXTR in the MLP depicting tunica muscularis where staining was less evident than in connective tissue of mucosa folds. Negative controls: (g) primary antibody replaced by rabbit IgG; (h) primary antibody receptor replaced by mouse IgG. Positive staining is shown in brown. Because all hormone receptors and/or synthases stained equally throughout the estrous cycle, random images are show from each phase. Immunostaining was performed in 4-µm oviduct histologic sections.

4.1.2. Ovarian steroid hormones, oxytocin and TNF effect on prostaglandins secretion

4.1.2.1. Oviduct epithelial cells (OEC) - PGE2 and PGF2α production

Among all oviduct isolated cells, 90.2% were epithelial cells and 65% of them were secretory in nature. The secretory capacity of mare OEC in culture was shown by the production of prostanoids (PGE_2 and $PGF_{2\alpha}$). Even though P_4, E_2 or OXT decreased PGE_2 production by OEC collected from mares in the follicular phase, when compared both to control and TNF groups ($P < 0.05$; Fig. 6a), none of the treatments tested affected PGE_2 production in mid- luteal phase (Fig. 6b). A down-regulation of oviduct $PGF_{2\alpha}$ production in the follicular phase was also caused by all treatments (P_4, E_2, OXT, TNF) used ($P < 0.05$; Fig. 6c). In contrast, mid-luteal phase OEC were stimulated for the production of this eicosanoid ($P < 0.05$; Fig. 6d).

Figure 6 - Effects of E_2, P_4, OXT and TNF on PGE_2 and $PGF_{2\alpha}$ production by equine ampulla oviductal epithelial cells.

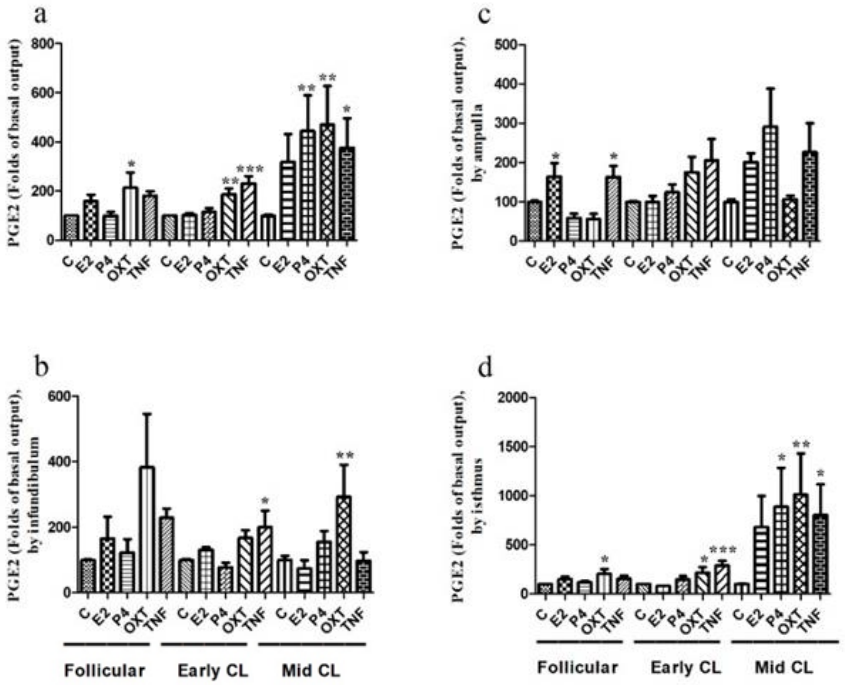


Effects of E_2, P_4, OXT and TNF on PGE_2 (a, b) and $PGF_{2\alpha}$ production (c, d) by equine oviductal epithelial cells (OEC) from follicular (a, c) or mid-luteal (b, d) phases. Data show the mean ± SEM percentage changes compared with basal (control: C) output. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

4.1.2.2.Oviduct explants - PGE_2 and $PGF_{2\alpha}$ production

When the results were analyzed regarding the phase of estrous cycle, oviduct explants from follicular phase mares showed an increase in PGE_2 production, when treated with OXT ($P < 0.05$; Fig. 7a). In the early luteal phase, this hormone production was up-regulated when tissues were treated with OXT or TNF ($P < 0.05$; Fig. 7a), whereas in the mid-luteal phase a stimulatory effect was noted after a 24 h treatment with P_4, OXT or TNF ($P < 0.05$; Fig. 7a). Only for explants from the infundibulum, PGE_2 production was stimulated by TNF in early luteal phase ($P < 0.05$; Fig. 7b), and by OXT in mid-luteal phase ($P < 0.05$; Fig. 7b). However, no effect was observed for any treatment in the follicular phase. In explants from the ampulla, an increase in PGE_2 in conditioned culture medium was detected only in tissues from mares in the follicular phase treated with E_2 or TNF ($P < 0.05$; Fig. 7c). No effect of the treatments was noted during early and mid-luteal phases. Also, PGE_2 production from isthmus explants was stimulated by OXT in the follicular phase ($P < 0.05$; Fig. 7d), by OXT or TNF in the early luteal phase ($P < 0.05$), and by P_4, OXT or TNF in the mid-luteal phase ($P < 0.05$; Fig. 7d).

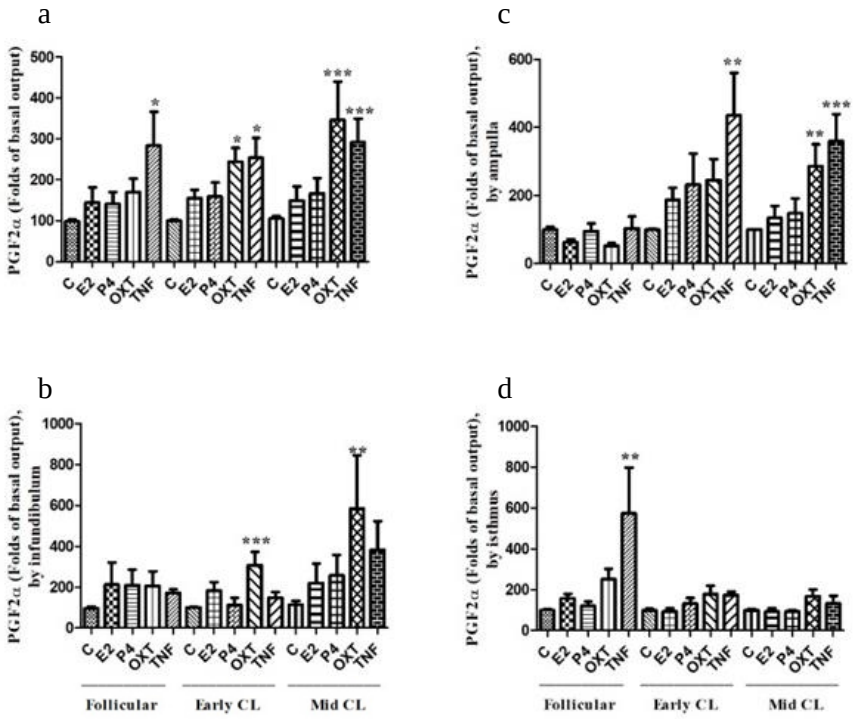
Figure 7 - Effects of E_2, P_4, OXT and TNF on PGE_2 production by equine oviduct explants.



Effects of E_2, P_4, OXT and TNF on PGE_2 production by equine oviduct explants from follicular phase, early-luteal phase and mid-luteal phase. Analysis was performed considering explants from all portions of oviduct (a), and from each portion of oviduct: infundibulum (b), ampulla (c) and isthmus (d). Data show the mean ± SEM percentage changes compared with basal (control: C) output. * $P < 0.05$, *, $P < 0.01$, *** $P < 0.001$.

Whenever the oviduct explants were considered as a whole, taken into consideration the estrous cycle phase alone, an increase in $PGF_{2\alpha}$ production was observed for tissues treated with TNF in follicular phase ($P < 0.05$), or with OXT or TNF in early and mid-luteal phases ($P < 0.05$; Fig. 8a). However, when the different portions of the oviduct were considered separately, different results were found. Infundibulum explants exposed to OXT showed an up-regulation in $PGF_{2\alpha}$ production in early and mid-luteal phases ($P < 0.05$; Fig. 8b). Nevertheless, in ampulla explants, TNF was capable of stimulating $PGF_{2\alpha}$ production in early and mid-luteal phases ($P < 0.05$). In addition, treatment of the ampulla with OXT in mid-luteal phase up-regulated $PGF_{2\alpha}$ production ($P < 0.05$; Fig. 8c). In the follicular phase, isthmus tissue treated with TNF was the only one to show an increase in $PGF_{2\alpha}$ production compared to other treatments ($P < 0.05$; Fig. 8d).

Figure 8 - Effects of E_2, P_4, OXT and TNF on $PGF_{2\alpha}$ production by equine oviduct explants



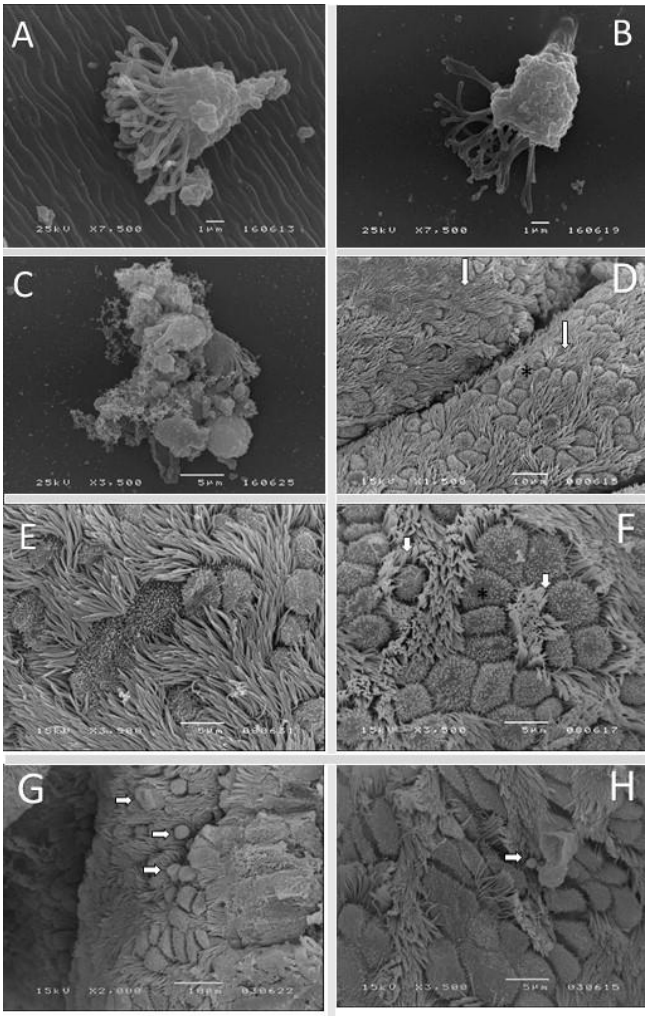
Effects of E_2, P_4, OXT and TNF on $PGF_{2\alpha}$ production by equine oviduct explants from follicular phase, early-luteal phase and mid-luteal phase. Analysis was performed considering explants from all portions of oviduct (a), and to each portion of oviduct: infundibulum (b), ampulla (c) and isthmus (d). Data show the mean ± SEM percentage changes compared with basal (control: C) output. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

4.1.3. Ultrastructure of OEC and oviduct

Oviduct cell assessment by SEM showed cells with a ciliated like appearance, as well as secretory cells, even after a 72 h culture, compatible with a viable cell culture (Fig. 9A-C). Portions of equine oviduct depicted ciliated cells, as well as secretory cells covered by microvilli (Fig. 9). Ultrastructure images of OEC showed a mix pattern of prismatic ciliated cells and a greater amount of non-ciliated cells that present a rounded surface with microvilli. The height of the cilia and ciliated cells density vary and are more evident in the ampulla during the follicular phase (Fig. 9D, E), concealing to some extent the borders of secretory cells (non-ciliated aspect). This contrasts with the presence of shorter cilia and less density of ciliated cells in the infundibulum at the same reproductive phase (Fig. 9F).

Secretory activity with the appearance of protruding buds of secretion can be observed in OEC secretory cells of the endometrium mainly in the ampulla, in the luteal phase (Fig. 9G, H).

Figure 9 - Scanning electronic microscope images of oviductal epithelial cells and oviduct explants



Mare oviductal epithelial cells (OEC) observed in culture (a, b and c) and intact oviduct tissue (d, e, f, g and h). OEC culture presents a ciliated cell (A) and a non ciliated cell, or a cell starting to lose its cilia prior to adherence (B). Bar = 1 µm. C – Cluster of OEC depicting prismatic ciliated and secretory cells. Many debris of secretion are present. Bar = 5µm. D and E - secretory (*) and ciliated cells (arrows) of the ampulla during the follicular phase. Note that the cilia (arrows) are quite high and the densely ciliated epithelium conceals to some extent the borders of the secretory cells (*). This contrasts to shorter cilia and less density of ciliated cells (arrow) in the infundibulum area presented in f. F -Secretory cells present a rounded surface with microvilli. D – Bar = 10µm, e and f= Bar= 5µm. g and h - Protruding buds of secretion (small arrows) from secretory cells in the ampulla in the luteal phase. G= 10 µm and H= 5 µm.

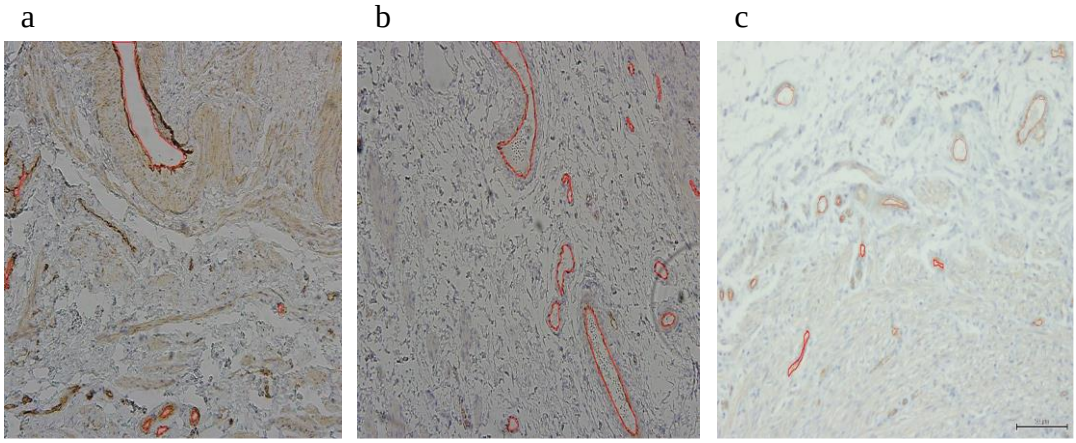
4.2. Microvascular density and the expression of FGFs, VEGF, and receptors in equine oviduct

4.2.1. Microvascular density in equine oviduct

Microvascular structures of oviduct were assessed on histologic sections immunostained with an antibody against CD31 (Fig. 10 a-c) and vascular areas compared among groups. When all

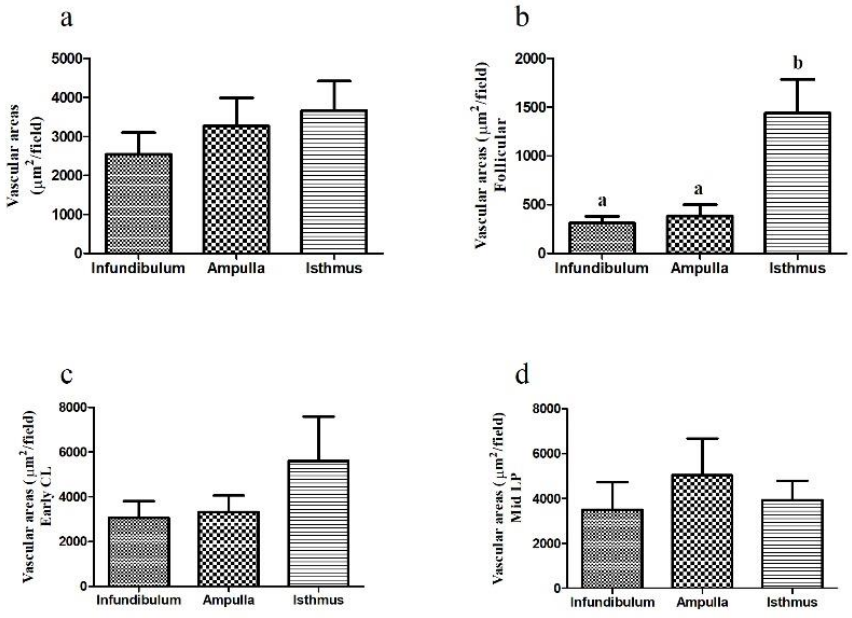
the mares were taken together, and compared between different regions of the oviduct (infundibulum, ampulla and isthmus) were compared, no statistical difference was found in vascular areas ($P > 0.05$; Fig. 11a), while the number of vascular structures was higher in the isthmus, compared with the ampulla ($P < 0.05$; Fig 12a).

Figure 10 - Representative images of immunostaining with CD31.



Images of immunostaining with CD31, allowing to visualize vascular structures in equine oviduct. a: infundibulum; b: ampulla; c: isthmus.

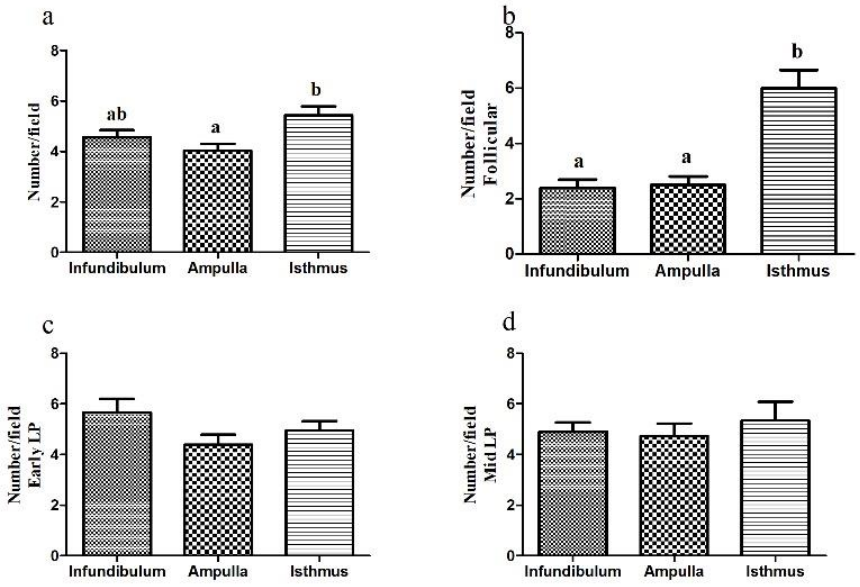
Figure 11 - Vascular areas present in the oviduct.



Total area of microvascular structures present in 10 randomly microscopic field. Analysis was performed considering samples from all estrous phase (a), and from each estrous cycle phase: follicular (b), early (c) and mid luteal phases (d). Bars represent mean ± SEM. Different letters indicate significant differences ($P < 0.05$).

When mares were separated according to their estrous cycle phase (follicular phase, early luteal phase and mid luteal phase), it was noted that in the follicular phase, the isthmus presented the largest vascular area and the highest number of vascular structures ($P < 0.05$; Fig 11b and Fig 12b), which was not noted in either early or in the mid luteal phase (Fig 11c and 11d; Fig 12c and 12d). These results show that the oviduct, particularly the isthmus, undergo vascular changes during the estrous cycle, mainly increasing its vascular bed in the follicular phase.

Figure 12 – Number of vascular structures present in the oviduct



Total structures present in 10 randomly microscopic field. Analysis was performed considering samples from all estrous phase (a), and from each estrous phase: follicular (b), early (c) and mid luteal phase (d). Bars represent mean ± SEM. Different letters indicate significant differences ($P < 0.05$).

4.2.2. Angiogenic growth factors transcription in equine oviduct

Some angiogenic growth factors (*FGF1*, *FGF2* and *VEGF*) and receptors (*FGFR1*, *FGFR2*, and *KDR*) transcription was analyzed by qPCR in infundibulum, ampulla and isthmus of oviduct from mares in distinct phases of estrous cycle.

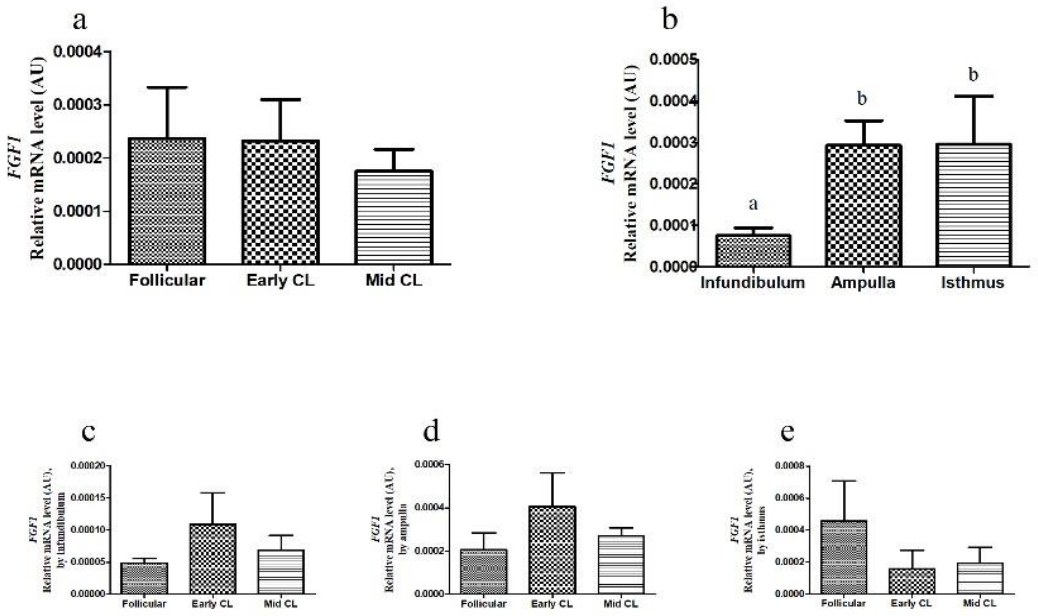
FGF1 only presented differences when results were analyzed without distinction between the estrous cycle phases, when both the ampulla and the isthmus showed the highest transcription ($P < 0.05$; Fig. 13b). Nevertheless, when the results were evaluated throughout the estrous cycle, and of each oviduct segment throughout the estrous cycle, there was no difference ($P > 0.05$; Fig. 13a, c-e).

FGF2 presented a similar pattern of transcription to *FGF1*, except that the ampulla did not present such higher transcription as the isthmus ($P < 0.05$; Fig. 14b).

Regarding *VEGF* no difference occurred on its transcription throughout the estrous cycle either between the portions of oviduct, or even when each individual portion was considered separately throughout the estrous cycle (Fig. 15a-e).

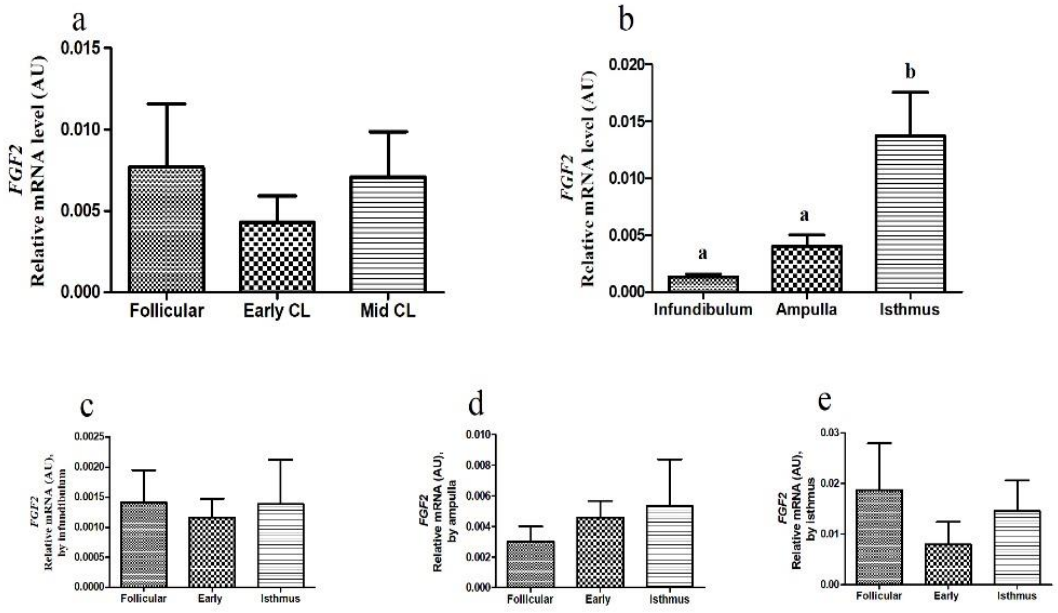
FGFR1 transcripts did not differ along the estrous cycle (Fig. 16a), while the isthmus presented a higher transcription when compared with the infundibulum ($P < 0.05$) (Fig. 16b). When the three portions were considered separately throughout the estrous cycle, only the infundibulum presented differences between the follicular phase and the mid luteal phase ($P < 0.05$) (Fig. 16c). Neither the ampulla nor the isthmus presented any difference during the estrous cycle (Fig. 16d, e). With respect to *FGFR2* no difference on its transcription was detected either during the estrous cycle, among oviduct portions, or when its portions were considered individually throughout the estrous cycle (Fig. 17a-e).

Figure 13 - Relative quantification by mRNA transcription of *FGF1*



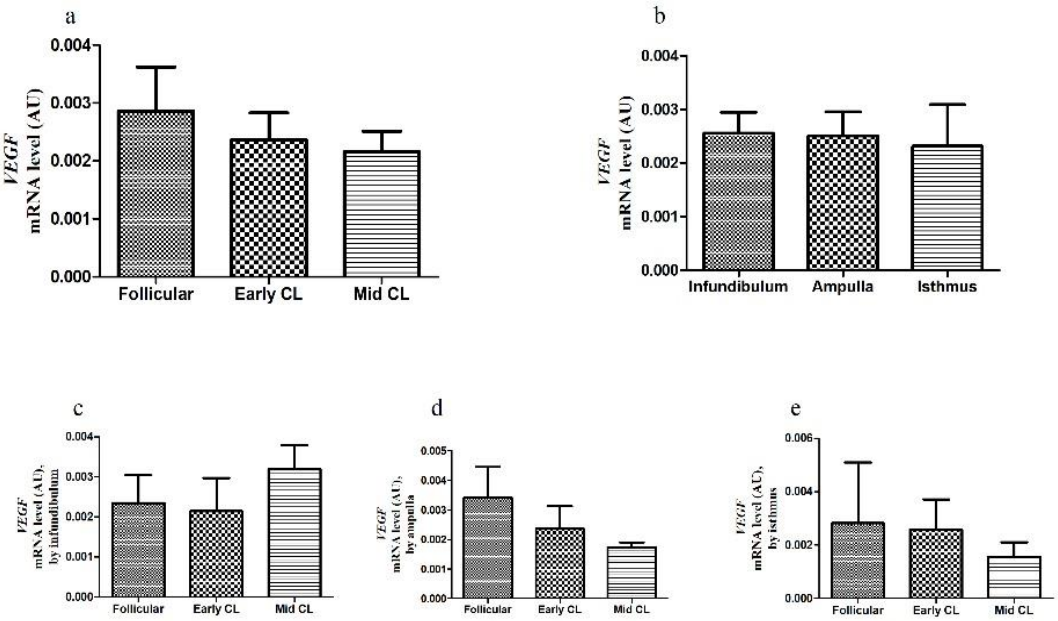
Relative quantification of mRNA transcription by qPCR in equine oviduct (n=5 samples for each estrous cycle phase; n=5 for each portion of oviduct analyzed) of *FGF1*. Transcription of target gene was normalized against that of the reference gene (*β2M*). Bars represent mean ± SEM. AU: arbitrary units. Different letters indicate significant differences ($P < 0.05$).

Figure 14 - Relative quantification by mRNA transcription of *FGF2*



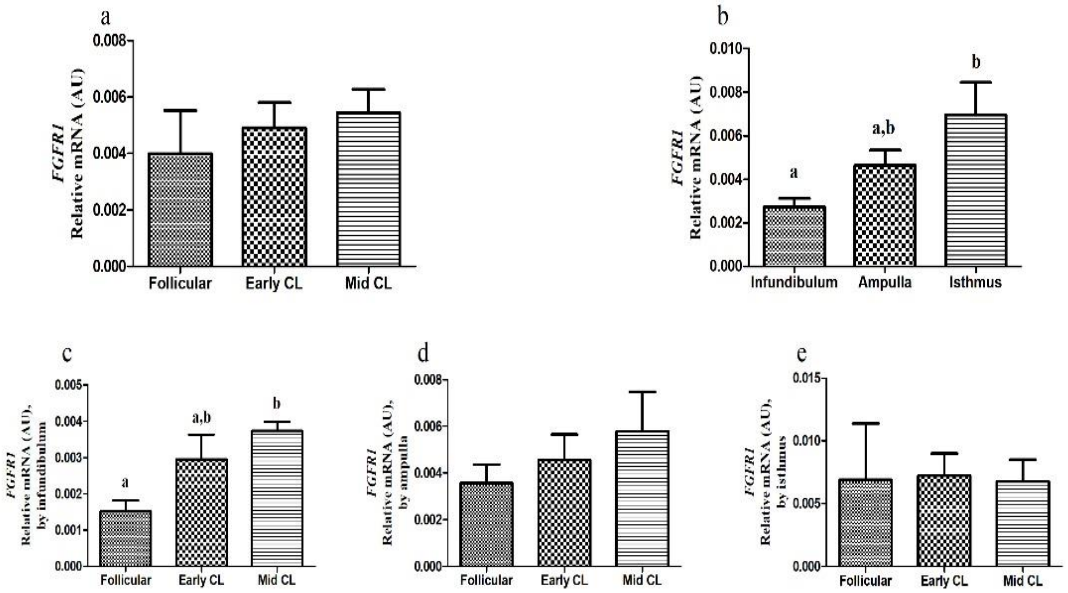
Relative quantification of mRNA transcription by qPCR in equine oviduct (n=5 samples for each estrous cycle phase; n=5 for each portion of oviduct analyzed) of *FGF2*. Transcription of target gene was normalized against that of the reference gene (*β2M*). Bars represent mean ± SEM. AU: arbitrary units. Different letters indicate significant differences ($P < 0.05$).

Figure 15: Relative quantification by mRNA transcription of *VEGF*



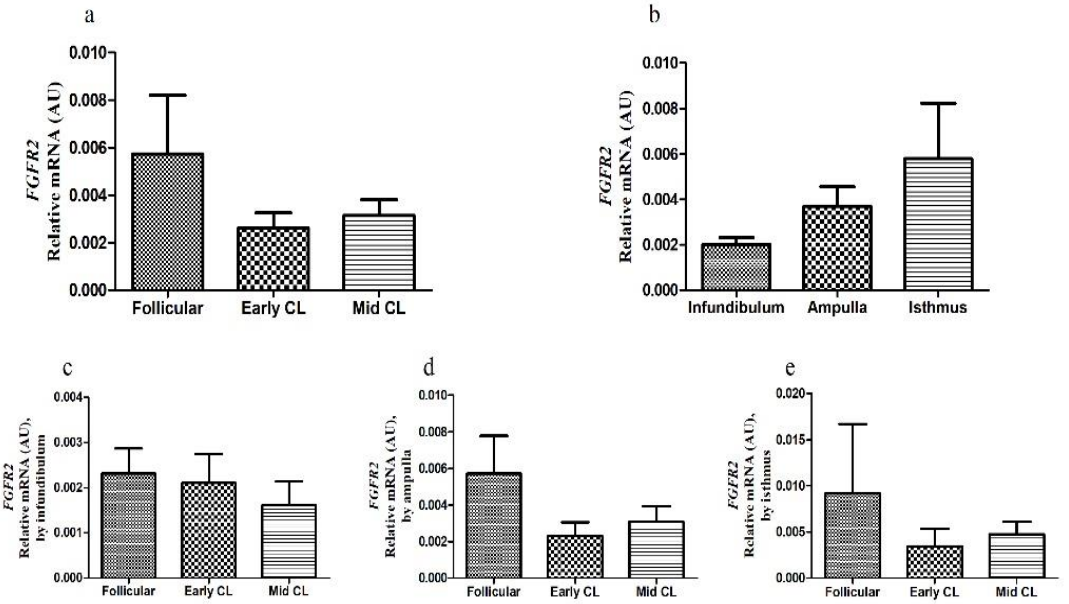
Relative quantification of mRNA transcription by qPCR in equine oviduct (n=5 samples for each estrous cycle phase; n=5 for each portion of oviduct analyzed) of *VEGF*. Transcription of target gene was normalized against that of the reference gene (*β2M*). Bars represent mean ± SEM. AU: arbitrary units. Different letters indicate significant differences ($P < 0.05$).

Figure 16 - Relative quantification by mRNA transcription of *FGFR1*



Relative quantification of gene transcription by qPCR in equine oviduct (n=5 samples for each estrous cycle phase; n=5 for each portion of oviduct analyzed) of *FGFR1*. Transcription of target gene was normalized against that of the reference gene (*β2M*). Bars represent mean ± SEM. AU: arbitrary units. Different letters indicate significant differences ($P < 0.05$).

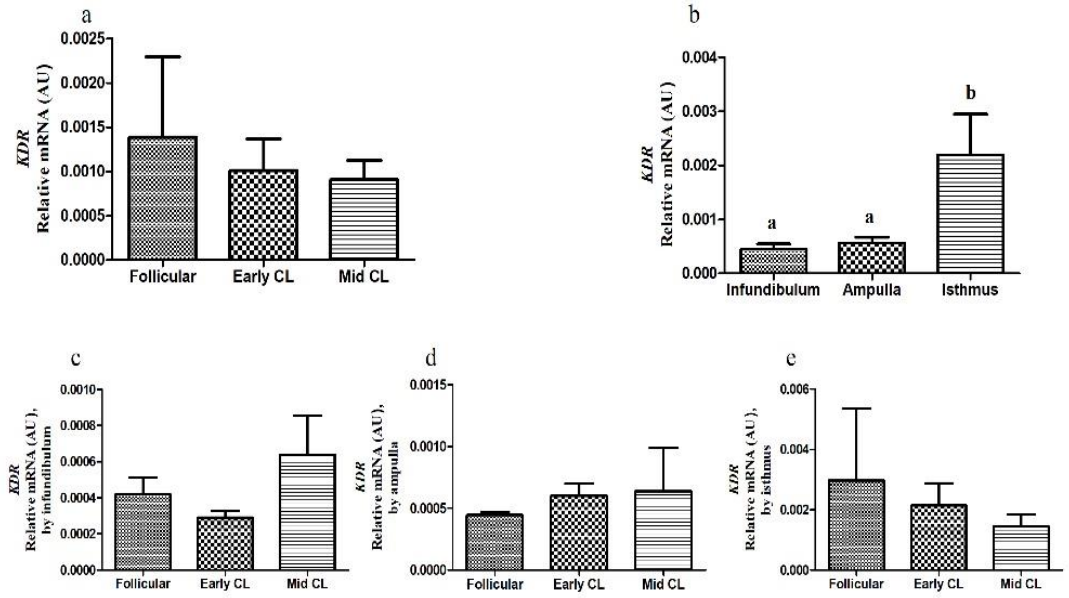
Figure 17 - Relative quantification by mRNA transcription of *FGFR2*



Relative quantification of gene transcription by qPCR in equine oviduct (n=5 samples for each estrous cycle phase; n=5 for each portion of oviduct analyzed) of *FGFR2*. Transcription of target gene was normalized against that of the reference gene (*β2M*). Bars represent mean ± SEM. AU: arbitrary units. Different letters indicate significant differences ($P < 0.05$).

When the estrous cycle influence was disregarded, VEGFR-2 (*KDR*) presented the highest transcription in the isthmus, when compared with the infundibulum and the ampulla ($P < 0.05$; Fig. 18b). Also, it did not present any difference in its mRNA levels either throughout the estrous cycle, or in separated portions of the oviduct along the estrous cycle (Fig. 18a, c-e).

Figure 18 - Relative quantification by mRNA transcription of *KDR*



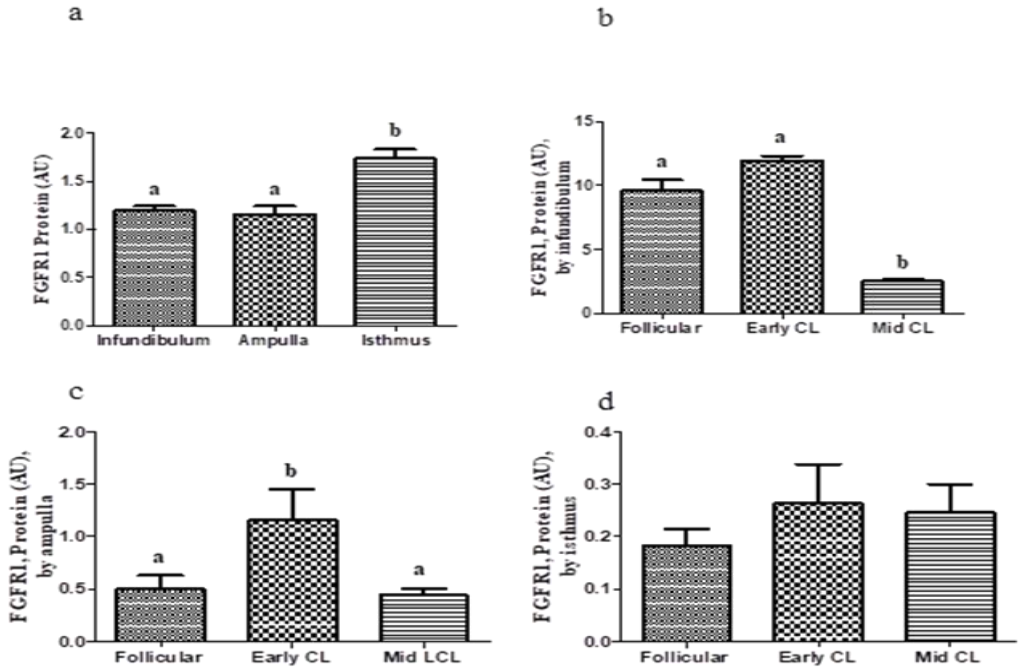
Relative quantification of gene transcription by qPCR in equine oviduct (n=5 samples for each estrous cycle phase; n=5 for each portion of oviduct analyzed) of *KDR*. Transcription of target gene was normalized against that of the reference gene (*β2M*). Bars represent mean ± SEM. AU: arbitrary units. Different letters indicate significant differences ($P < 0.05$).

4.2.3. Angiogenic growth factors receptors expression in equine oviduct

The protein expression of FGFR1, FGFR2 and VEGFR2 (KDR) was assessed by WB analysis, and data between groups of mares in distinct phases of estrous cycle, and between portions of oviduct were compared.

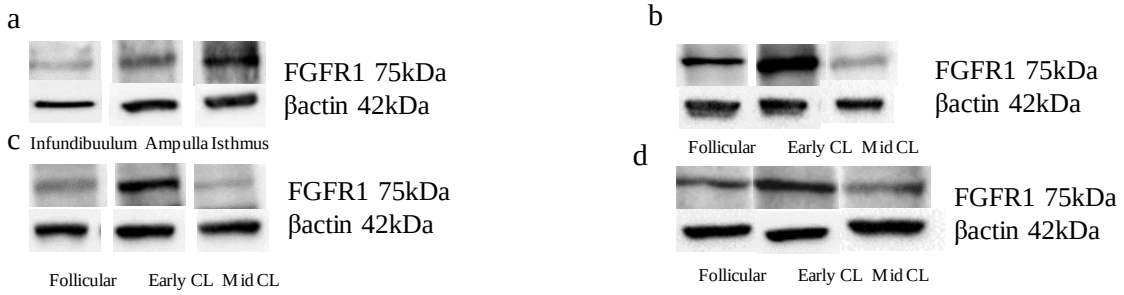
FGFR1 presented a higher protein expression on isthmus, than in infundibulum or ampulla ($P < 0.05$, Fig. 19a, 20a). When the expression of each oviduct portion was evaluated, in infundibulum, protein expression was the highest in follicular and early luteal phases ($P < 0.05$; Fig. 19b, 20b). Also in ampulla, expression was increased in early-luteal phase ($P < 0.05$; Fig. 19c, 20c), while in isthmus there was no difference throughout the estrous cycle ($P > 0.05$, Fig. 19d, 20d).

Figure 19 - Quantification of FGFR1 protein expression by WB.



Quantification of FGFR1 protein expression by WB in the equine oviduct (n=3 for each phase or portion). Bars represent mean ± SEM. AU: arbitrary units. Different letters indicate significant differences ($P < 0.05$).

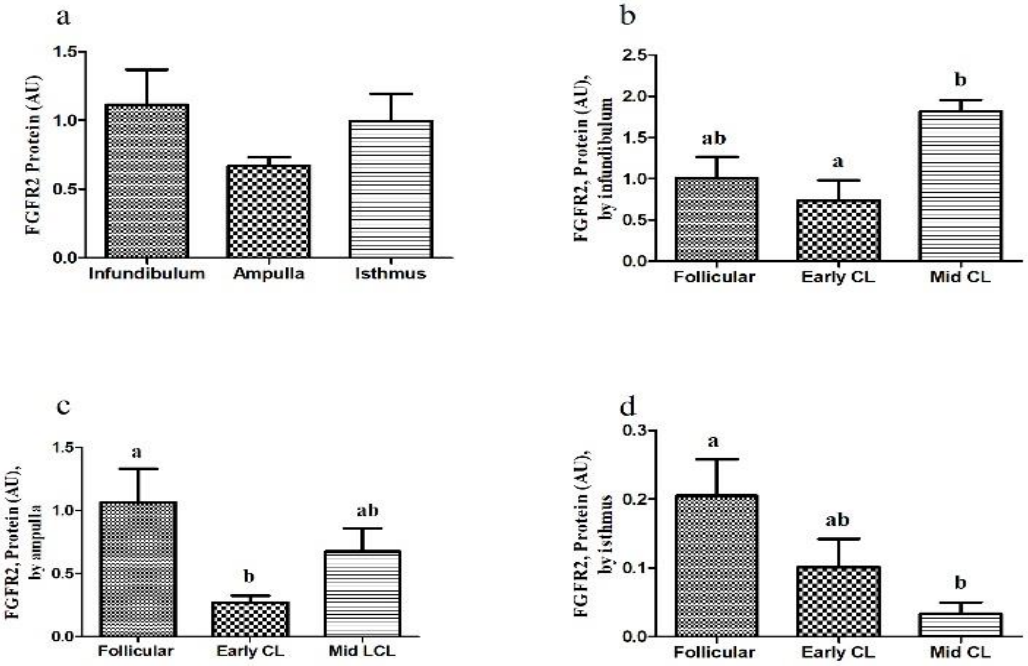
Figure 20 - Panels representative of protein expression of FGFR1, evaluated by WB.



Panels representative of protein expression of FGFR1, evaluated by WB, in equine oviduct. a: Differences between oviduct portion; b: Differences in infundibulum throughout the estrous cycle; c: Differences in ampulla throughout the estrous cycle; d: Differences in isthmus throughout the estrous cycle. Data were normalized against β-actin density values.

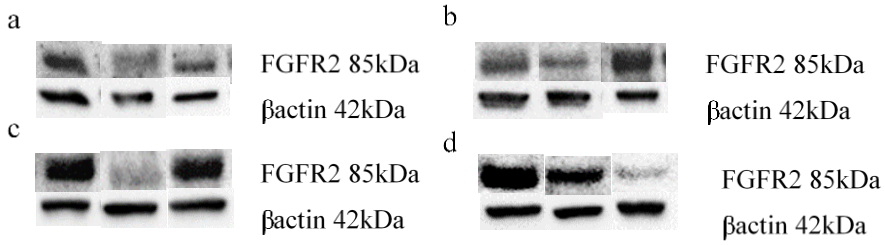
The protein expression of FGFR2 between the three portions of the oviduct was similar (Fig. 21a, 22a). Nevertheless, in the infundibulum itself, an increase in protein expression occurred in the mid luteal phase, with respect to the early-luteal phase ($P < 0.05$; Fig. 21b, 22b). In the ampulla, the highest expression was observed in the follicular phase, compared with the early-luteal phase ($P < 0.05$; Fig. 21c, 22c). In the isthmus, the expression was also greater in follicular phase, and the lowest in mid-luteal phase ($P < 0.05$; Fig. 21d, 22d).

Figure 21 - Quantification of FGFR2 protein expression by WB.



Quantification of FGFR2 protein expression by WB in the equine oviduct (n=3 for each phase or portion). Bars represent mean ± SEM. AU: arbitrary units. Different letters indicate significant differences ($P < 0.05$).

Figure 22 - Panels representative of protein expression of FGFR2, evaluated by WB.

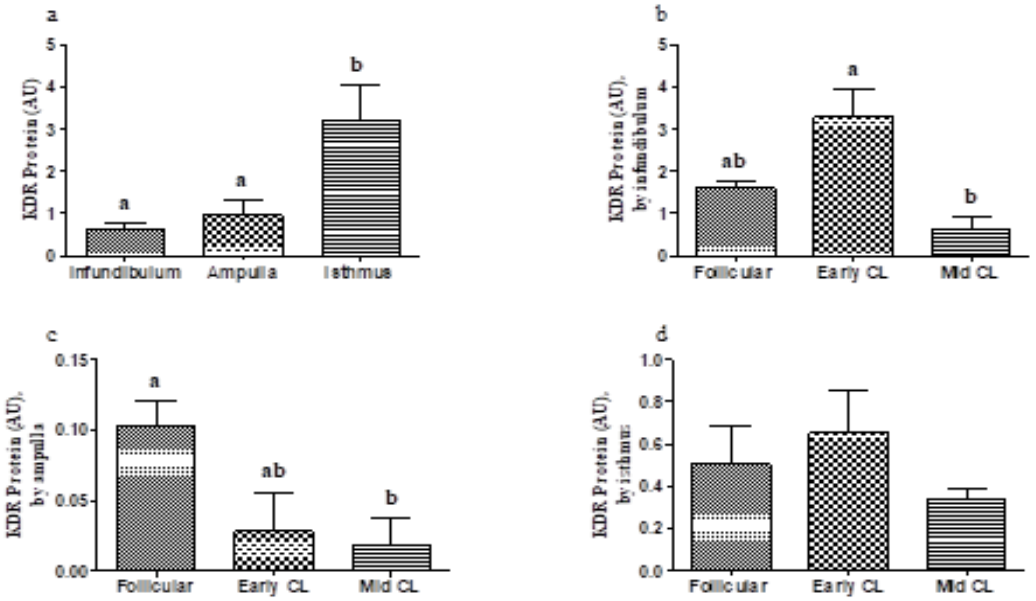


Panels representative of protein expression of FGFR2, evaluated by WB, in equine oviduct. a: Differences between oviduct portion; b: Differences in infundibulum throughout the estrous cycle; c: Differences in ampulla throughout the estrous cycle; d: Differences in isthmus throughout the estrous cycle. Data were normalized against β-actin density values.

KDR protein expression in the isthmus was greater than in infundibulum or ampulla ($P < 0.05$; Fig. 23a, 24a). When the three portions were compared during the estrous cycle, the infundibulum presented the highest expression on the early-luteal phase ($P < 0.05$; Fig. 23b, 24b).

In the ampulla, KDR protein expression presented its highest expression in the follicular phase ($P < 0.05$; Fig. 23c, 24c), while in the isthmus the estrous cycle did not influence its expression (Fig. 23d, 24d).

Figure 23 - Quantification of KDR protein expression by WB.



Quantification of KDR protein expression by WB in the equine oviduct (n=3 for each phase or portion). Bars represent mean ± SEM. AU: arbitrary units. Different letters indicate significant differences ($P < 0.05$).

Figure 24 - Panels representative of protein expression of KDR, evaluated by WB.

Panels representative of protein expression of KDR, evaluated by WB, in equine oviduct. a: Differences between oviduct portion; b: Differences in infundibulum throughout the estrous cycle; c: Differences in ampulla throughout the estrous cycle; d: Differences in isthmus throughout the estrous cycle. Data were normalized against β-actin density values.

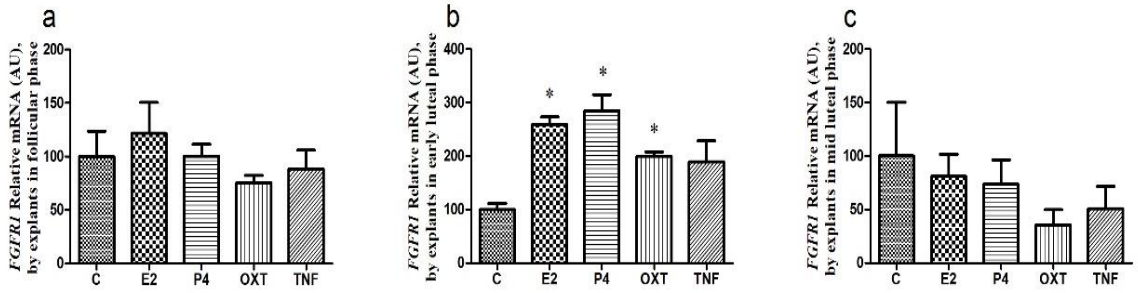
4.2.4. Ovarian steroid hormones, oxytocin and TNF effect in transcription of FGFR1, FGFR2, FLT1 and KDR in equine explants

Transcription of *FGFR1*, *FGFR2*, *VEGFR-1* and *KDR* was carried out on equine oviductal explants treated with E_2, P_4, OXT and TNF. Results were compared with the control group (explant in culture medium alone).

Regarding *FGFR1*, in early-luteal phase, the ovarian hormones E_2, P_4 and OXT upregulated its transcription ($P < 0.05$, Fig. 25b), while neither the follicular nor the mid-luteal phases altered it (Fig.25a, c).

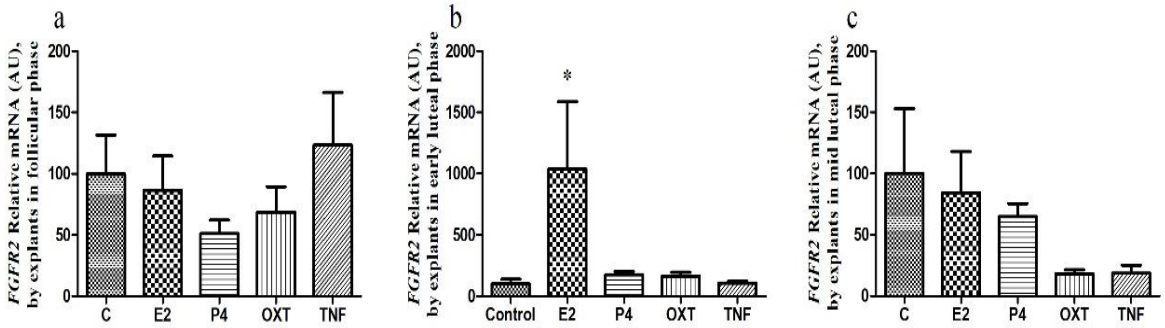
With respect to *FGFR2*, only E_2 upregulated its transcription in early-luteal phase ($P < 0.05$; Fig. 26b), and no difference in follicular and mid-luteal phases was observed (Fig. 26a, c).

Figure 26 - Effects of E_2, P_4, OXT and TNF on *FGFR1* transcription in equine ampulla oviduct explants



Effects of E_2, P_4, OXT and TNF on *FGFR1* transcription in mare ampulla explants from follicular phase, early-luteal phase and mid-luteal phase analyzed by qPCR. Transcription of target gene was normalized against that of the reference gene *(β2M)*. Data show the mean ± SEM percentage changes compared with basal (control: C) output. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 25 - Effects of E_2, P_4, OXT and TNF on *FGFR2* transcription by equine ampulla oviduct explants

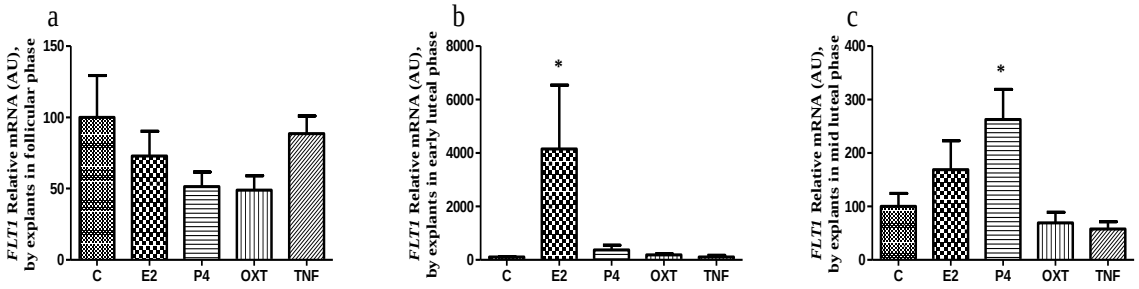


Effects of E_2, P_4, OXT and TNF on *FGFR2* transcription by equine oviduct ampulla explants from follicular phase, early-luteal phase and mid-luteal phase analyzed by qPCR. Transcription of target gene was normalized against that of the reference gene *(β2M)*. Data show the mean ± SEM percentage changes compared with basal (control: C) output. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

While in early-luteal phase E_2 up-regulated *FLT1* transcription ($P < 0.05$; Fig. 27b), while in mid-luteal phase P_4 had a positive effect on its transcription ($P < 0.05$; Fig. 27c). Also in early-

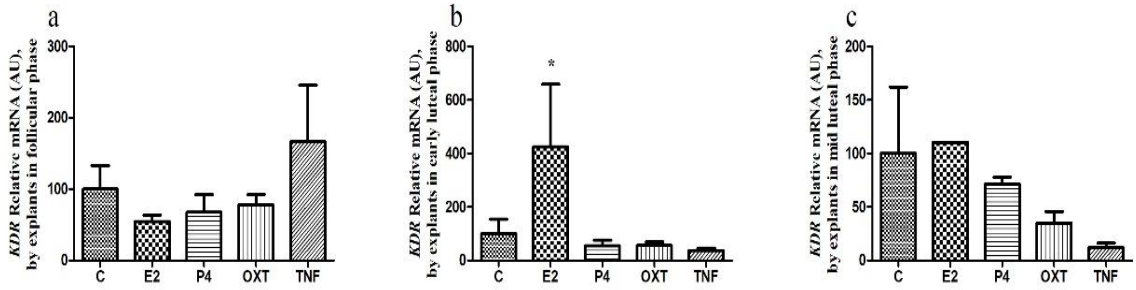
luteal phase, E_2 also upregulated *KDR* ($P < 0.05$; Fig. 28b) but no difference in follicular and mid-luteal phases was noted (Fig. 28a-c).

Figure 27 – Effects of E2, P4, OXT and TNF on *FLT1* transcription by equine ampulla oviduct explants



Effects of E_2, P_4, OXT and TNF on *FLT1* transcription in equine oviduct ampulla explants from follicular phase, early-luteal phase and mid-luteal phase analyzed by qPCR. Transcription of target gene was normalized against that of the reference gene (*β2M*). Data show the mean ± SEM percentage changes compared with basal (control: C) output. * $P < 0.05$.

Figure 28 - Effects of E_2, P_4, OXT and TNF on *KDR* transcription by equine ampulla oviduct explants



Effects of E_2, P_4, OXT and TNF on *KDR* transcription in equine ampulla explants from follicular phase, early-luteal phase and mid-luteal phase analyzed by qPCR. Transcription of target gene was normalized against that of the reference gene (*β2M*). Data show the mean ± SEM percentage changes compared with basal (control: C) output. * $P < 0.05$.

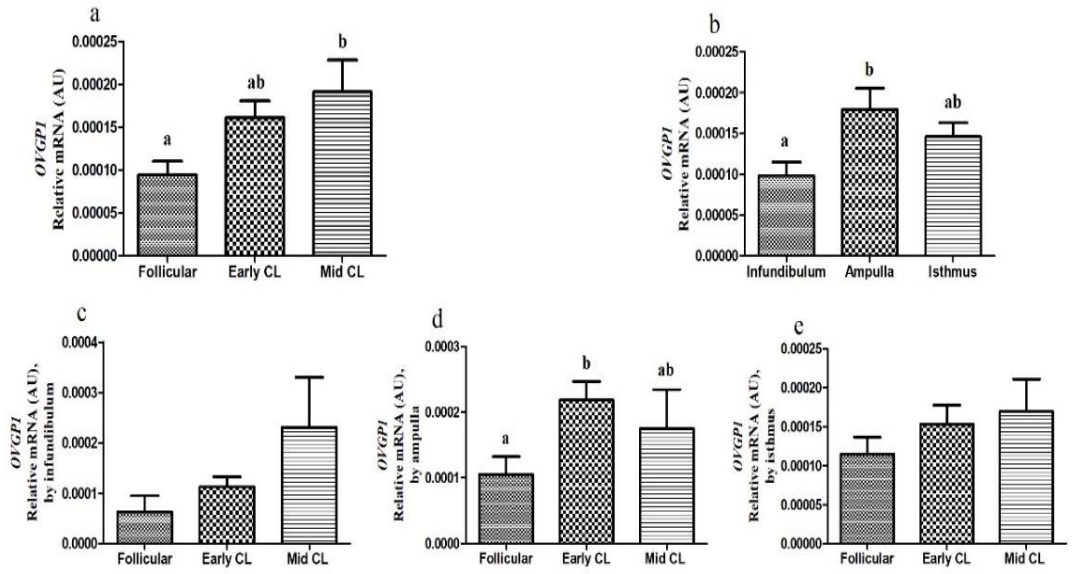
4.3. Oviduct specific glycoprotein expression in equine oviduct

4.3.1. Oviduct specific glycoprotein transcription in equine oviduct

Oviduct specific glycoprotein (*OVGP1*) gene was analyzed in mares' oviduct in distinct phases of the estrous cycle, and in its three portions, by qPCR. When all samples were evaluated together, *OVGP1* presented the highest transcription in mid luteal phase, compared with follicular phase, but like early-luteal phase ($P < 0.05$; Fig. 29a). Also, it was in the ampulla and

the isthmus where *OVGP1* presented a largest mRNA levels ($P < 0.05$; Fig. 29b). When the three oviduct portions were considered separately, it was in the ampulla where there was an increase in transcription in early and mid-luteal phases, compared with follicular phase ($P < 0.05$; Fig. 29d). Neither the infundibulum nor the isthmus presented any differences on *OVGP1* mRNA levels (Fig. 29c, e).

Figure 29 - Relative quantification by mRNA transcription of *OVGP1*



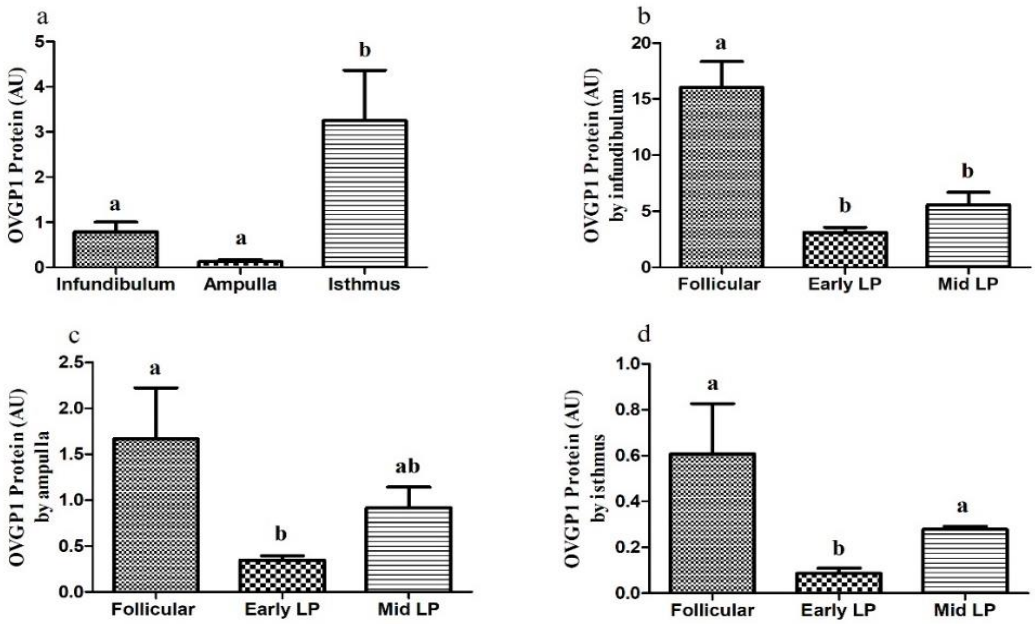
Relative quantification of gene transcription by qPCR in equine oviduct (n=5 samples for each estrous cycle phase; n=5 for each portion of oviduct analyzed) of *OVGP1*. Transcription of target gene was normalized against that of the reference gene (*β2M*). Bars represent mean ± SEM. AU: arbitrary units. Different letters indicate significant differences ($P < 0.05$).

4.3.2. OVGP1 expression in equine oviduct

OVGP1 expression was also evaluated by WB analysis in all portions of the oviduct and in each individual segment, throughout the estrous cycle. Comparison between portions of oviduct, regardless of the phase of the estrous cycle, demonstrated a higher OVGP1 protein expression in isthmus, compared with infundibulum or ampulla ($P < 0.05$; Fig. 30a, 31a).

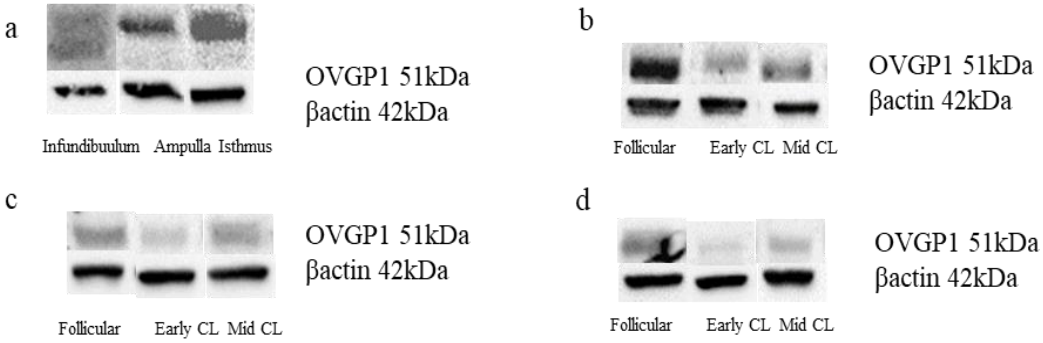
When the expression of each oviduct portion throughout the estrous cycle was considered, in all portions, a higher OVGP1 expression was observed in the follicular phase ($P < 0.05$) (Fig. 30b-d, Fig. 31b-d).

Figure 30 - Quantification of OVGP1 protein expression by WB.



Quantification of OVGP1 protein expression by WB in the equine oviduct (n=3 for each phase or portion). Bars represent mean ± SEM. AU: arbitrary units. Different letters indicate significant differences ($P < 0.05$).

Figure 31 - Panels representative of protein expression of OVGP1, evaluated by WB



Panels representative of protein expression of OVGP1, evaluated by WB, in equine oviduct. a: Differences between oviduct portion; b: Differences in infundibulum throughout the estrous cycle; c: Differences in ampulla throughout the estrous cycle; d: Differences in isthmus throughout the estrous cycle. Data were normalized against β-actin density values.

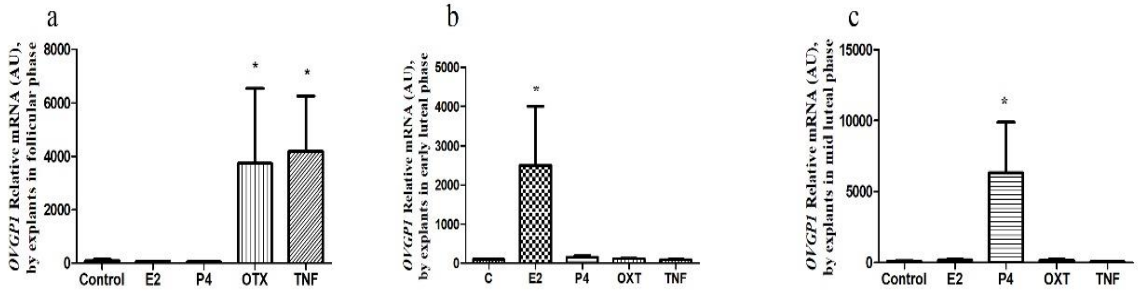
4.3.3. Ovarian steroid hormones, oxytocin and TNF *in vitro* effect on in vitro transcription of OVGP1 in oviduct explants, and on OVGP1 secretion

4.3.3.1. Transcription of *OVGP1* in oviduct explants

Transcription of *OVGP1* was assessed on oviduct explants treated *in vitro* with E_2, P_4, OXT and TNF. Results were compared with the control group, which consisted of oviduct explants incubated in culture medium alone.

In the follicular phase, OXT and TNF up-regulated *OVGP1* transcription ($P < 0.05$; Fig. 32a); in the early luteal phase, E_2 had a stimulatory effect ($P < 0.05$; Fig. 32b), but in mid-luteal phase, it was P_4 that stimulated transcription ($P < 0.05$; Fig. 32c).

Figure 32 - Effects of *in vitro* E_2, P_4, OXT and TNF on *OVGP1* transcription by equine ampulla oviduct explants



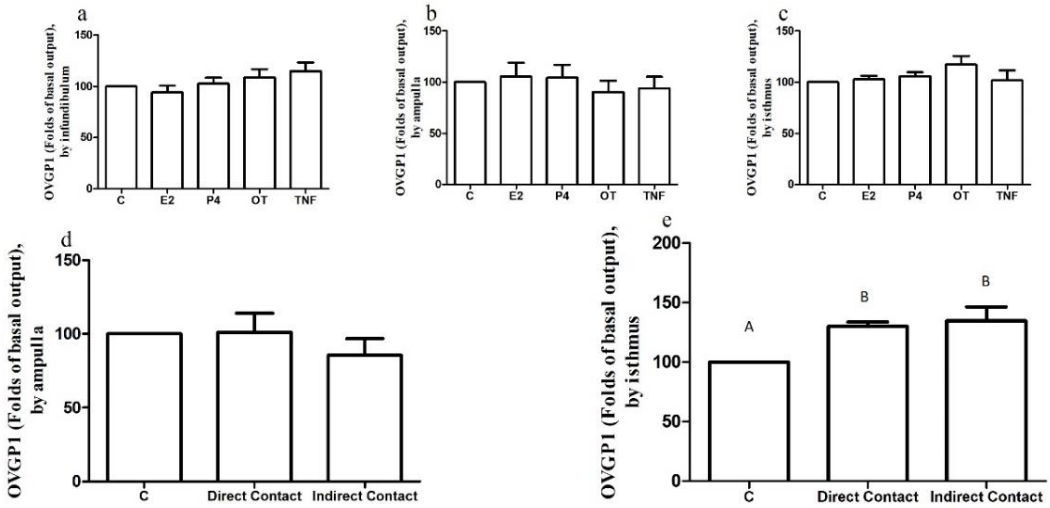
Effects of E_2, P_4, OXT and TNF on *OVGP1* transcription by equine oviduct ampulla explants from follicular phase, early-luteal phase and mid-luteal phase analyzed by qPCR. Transcription of target gene was normalized against that of the reference gene (*β2M*). Data show the mean ± SEM percentage changes compared with basal (control: C) output. * $P < 0.05$.

4.3.3.2. OVGP1 production by explants

OVGP1 was measured by EIA in the culture medium conditioned by oviduct explants that had been submitted to E_2, P_4, OXT and TNF treatments, and under direct or indirect contact with spermatozoa, only for tissues from ampulla and isthmus from early-luteal phase. All results were compared with a control group, which was not subjected to any specific treatment.

No portion of the oviduct presented any difference on OVGP1 production under E_2, P_4, OXT or TNF treatment (Fig. 33 a-c). Explants from ampulla did not show any difference under direct or indirect contact with spermatozoa (Fig. 33 d), but in the isthmus the secretion of OVGP1 was upregulated under either direct or indirect contact with spermatozoa ($P < 0.05$; Fig. 33 e).

Figure 33 - Effects of E_2, P_4, OXT, TNF and spermatozoa, on OVGP1 production



Effects of E_2, P_4, OXT and TNF on OVGP1 production by equine oviduct explants. Analysis was performed from each portion of oviduct: infundibulum (a), ampulla (b) and isthmus (c), and after direct contact (d) and indirect contact (e) with spermatozoa. Data show the mean ± SEM percentage changes compared with basal (control: C) output. * $P < 0.05$.

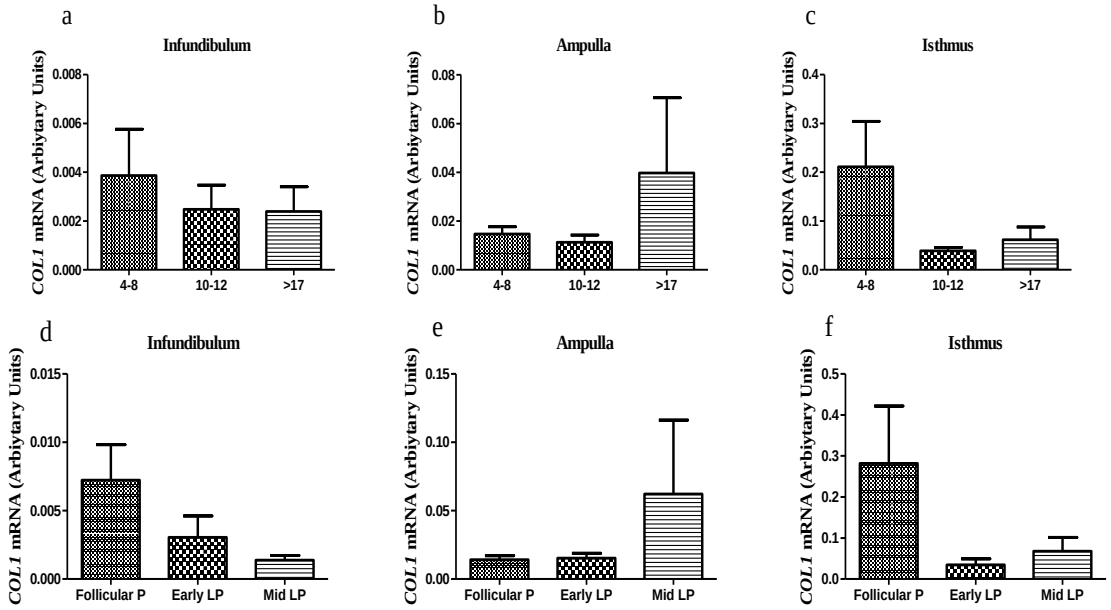
4.4. Collagen in equine oviduct: possible relationship with endometrial fibrosis and pathways involved

4.4.1. Collagen type I (COL1) and collagen type III (COL3) mRNA transcription in equine oviduct

COL1 and *COL3* genes were determined in mares' oviducts in distinct phases of the estrous cycle, and on its three portions, by qPCR. The analysis performed, besides considering the portion of the oviduct, the phase of estrous cycle, and age of mares, also considered Kenney's endometrial classification, with special emphasis on the presence of fibrosis.

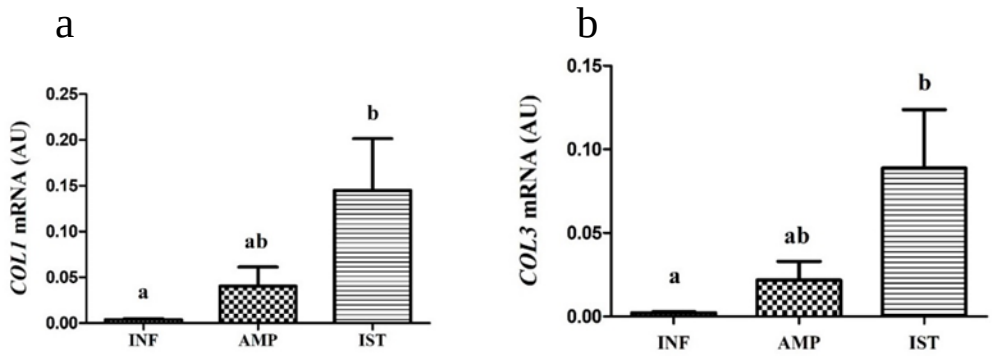
Neither the estrous cycle phase, nor the age of mares had any effect on *COL1* mRNA levels (Fig. 34 a-f). However, when the various portions of the oviduct were considered, the isthmus presented the highest *COL1* transcription level ($P < 0.05$; Fig. 35a).

Figure 34 - Relative quantification by mRNA transcription of *COL1* in oviduct throughout estrous cycle and between different mares' ages.



Relative quantification of gene transcription by real-time polymerase chain reaction in equine oviduct throughout the estrous cycle and between different mares' ages (n=6 samples for each estrous cycle phase; n=6 for each group of ages) of *COL1*. Transcription of target gene was normalized against that of the reference gene (*β2M*). Bars represent mean ± SEM. AU: arbitrary units. Different letters indicate significant differences ($P < 0.05$).

Figure 35 - Relative quantification by mRNA transcription of *COL1* and *COL3* through the oviduct



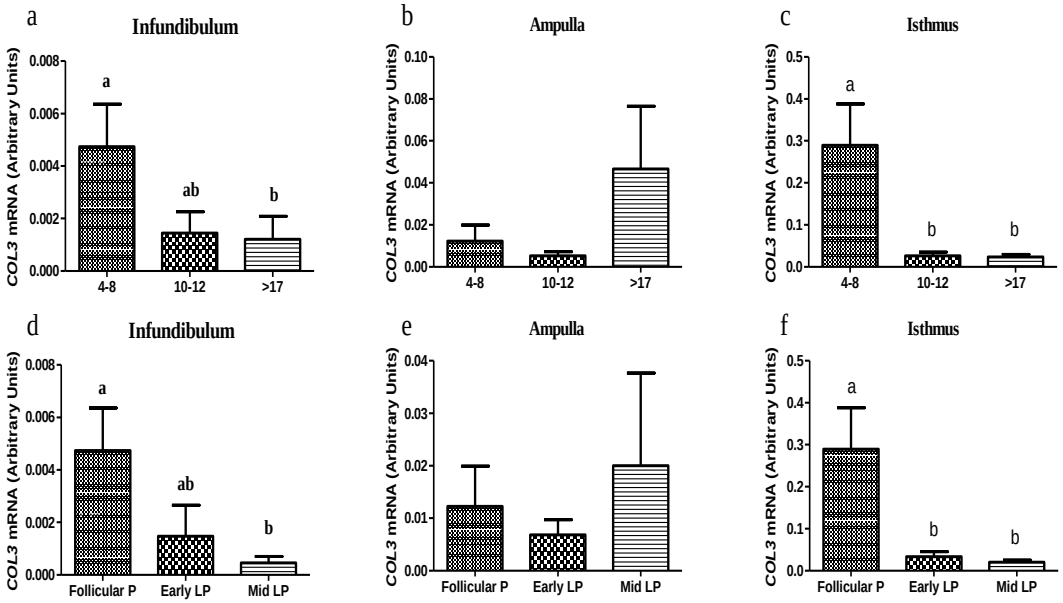
Relative quantification of gene transcription by qPCR in equine oviduct through portions (n=18 samples for each portion) of *COL1* (a) *and COL3* (b). Transcription of target gene was normalized against that of the reference gene (*β2M*). Bars represent mean ± SEM. AU: arbitrary units. Different letters indicate significant differences ($P < 0.05$). INF- infundibulum; AMP- ampulla; IST- isthmus.

Likewise, *COL3* also present the highest RNA transcription in the isthmus ($P < 0.05$, Fig 35b). When mares' age was considered, the youngest mares (4-6 years old) presented the highest transcription of *COL3* in the isthmus but also in the infundibulum ($P < 0.05$; Fig. 36a, c). This

increase in transcription in those specific anatomic portions of the oviduct was observed in the follicular phase ($P < 0.05$; Fig. 36d, f) In the ampulla, neither the estrous cycle phase, nor the age of the mares had any effect on *COL3* mRNA levels (Fig. 36b, e).

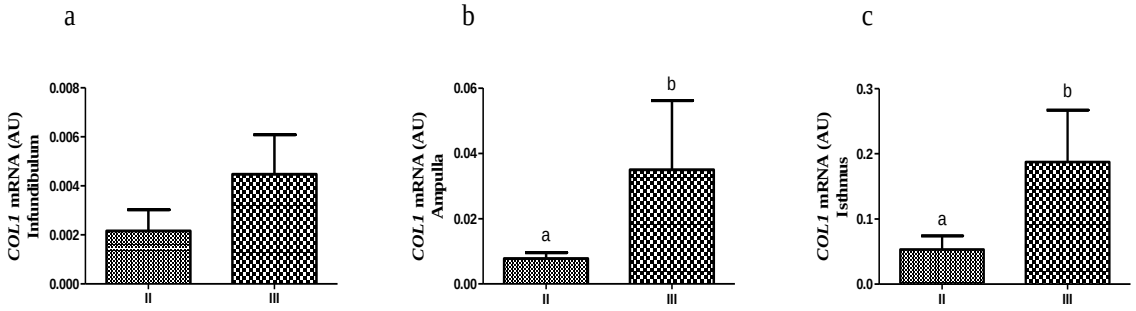
Interestingly, *COL1* transcription was higher in mares classified in category III (Kenney, 1978), than in mares classified in category II, in ampulla and isthmus ($P < 0.05$; Fig. 37b, c), but not in infundibulum (Fig. 37a). Nevertheless, *COL3* did not present the same differences, since transcription did not presented differences between these two groups of mares, in neither portion (Fig.38a-c).

Figure 36 - Relative quantification by mRNA transcription of *COL3* in oviduct throughout estrous cycle and between different ages



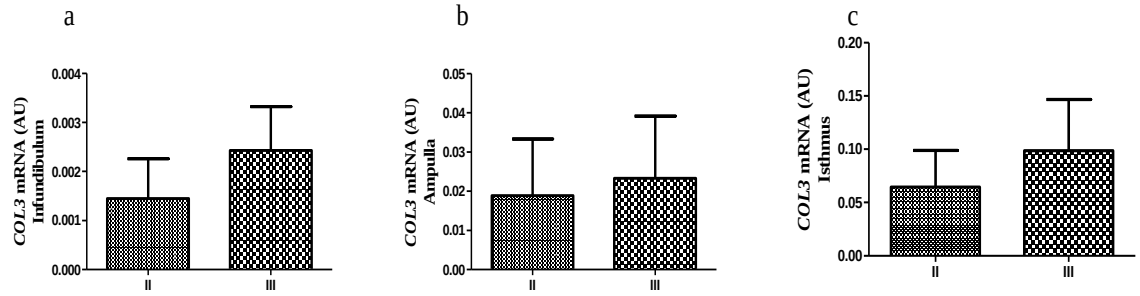
Relative quantification of gene transcription by real-time polymerase chain reaction in equine oviduct throughout the estrous cycle and between different mares' ages (n=6 samples for each estrous cycle phase; n=6 for each group of ages) of *COL3*. Transcription of target gene was normalized against that of the reference gene (*β2M*). Bars represent mean ± SEM. AU: arbitrary units. Different letters indicate significant differences ($P < 0.05$).

Figure 37 - Relative quantification by mRNA transcription of *COL1* in oviducts of mares with different Kenney's endometrial classification



Relative quantification of *COL1* gene transcription by real-time polymerase chain reaction in equine oviduct between mares with different degrees of endometrial fibrosis (n=9 samples for each endometrial classification). Transcription of target gene was normalized against that of the reference gene (*β2M*). Bars represent mean ± SEM. AU: arbitrary units. Different letters indicate significant differences ($P < 0.05$).

Figure 38 - Relative quantification by mRNA transcription of *COL3* in oviducts of mares with different Kenney's endometrial classification

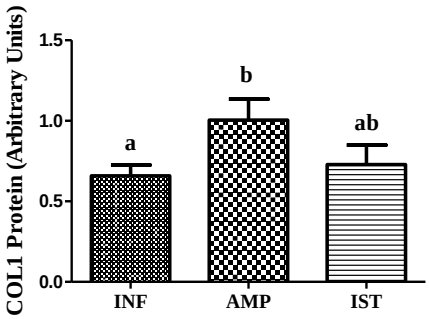


Relative quantification of *COL3* gene transcription by real-time polymerase chain reaction in equine oviduct between mares with different degrees of endometrial fibrosis (n=9 samples for each endometrial classification). Transcription of target gene was normalized against that of the reference gene (*β2M*). Bars represent mean ± SEM. AU: arbitrary units. Different letters indicate significant differences ($P < 0.05$).

4.4.2. COL1 protein expression in equine oviduct

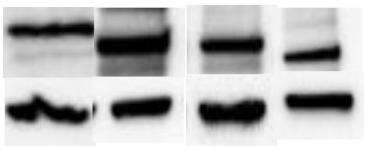
Expression of COL1 protein was evaluated by western blot analysis in the same oviduct samples used for transcription analysis. COL1 presented a higher protein expression in the ampulla, than the infundibulum ($P < 0.05$; Fig. 39, 40).

Figure 39 - Quantification of COL1 protein expression by WB, through the oviduct



Quantification of oviduct COL1 protein expression by WB in the equine oviduct (n=6 for each phase; n=6 for each portion). Bars represent mean ± SEM. AU: arbitrary units. Different letters indicate significant differences ($P < 0.05$). INF- infundibulum; AMP- ampulla; IST- isthmus.

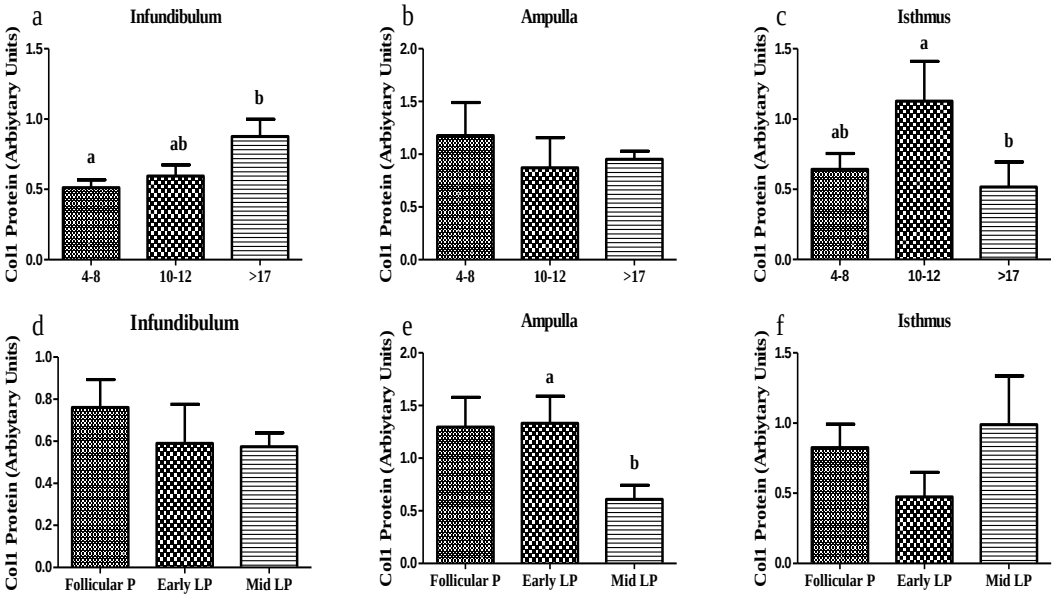
Figure 40 - Panel representative of protein expression of COL1 in mare oviduct, evaluated by WB



Panels representative of protein expression of COL1, evaluated by WB, in equine oviduct through different portions. Data were normalized against β-actin density values.

The age of mares (4-8 years old; 10-12 years old; >17 years old) from whom the oviducts were retrieved was also considered for COL1 gene expression analysis. In the infundibulum, a higher COL1 protein expression was present in the oldest mares (>17 years old) ($P < 0.05$; Fig. 41a, 42a); while in the ampulla, age had no effect (Fig. 41b, 42b). The isthmus of mares from 10 to 12 years old, showed a higher protein expression, than older mares ($P < 0.05$; Fig. 41c, 42c).

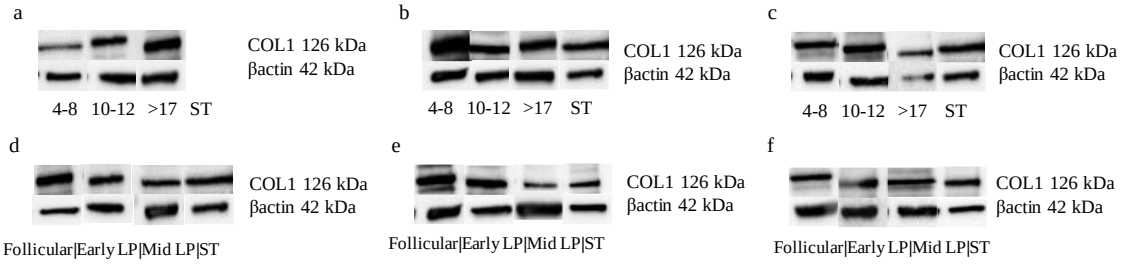
Figure 41 - Quantification of oviduct COL1 protein expression by WB throughout estrous cycle and between different ages



Quantification of oviduct COL1 protein expression by WB analysis in the equine oviduct (n=6 for each phase; n=6 for each group of ages). Bars represent mean ± SEM. AU: arbitrary units. Different letters indicate significant differences ($P < 0.05$).

The phase of the estrous cycle did not affect COL1 protein expression either in the infundibulum, or in the isthmus (Fig. 41d, f; 42d, f), while in the ampulla, its expression fell in mid luteal phase ($P < 0.05$; Fig. 41e, 42e).

Figure 42 - Panels representative of protein expression of COL1, evaluated by WB

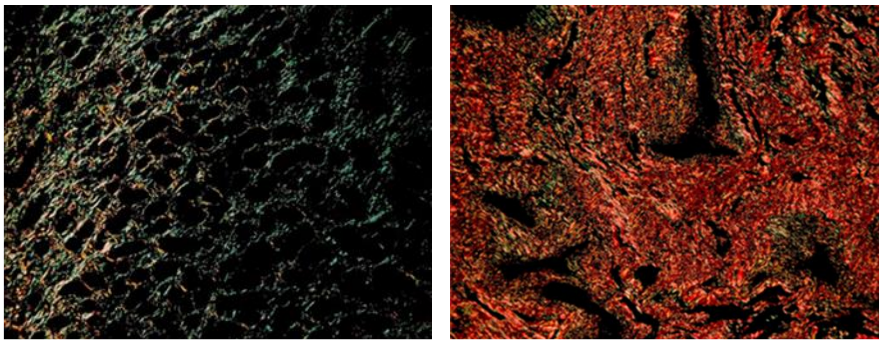


Panels representative of protein expression of COL1, evaluated by WB, in equine oviduct, throughout the estrous cycle and between different mares' ages. a and d: infundibulum; b and e: ampulla; c and f: isthmus. Data were normalized against β-actin density values.

4.4.3. Quantification of COL1 *vs* COL3 protein expression in oviduct and endometrium

COL1 and COL3 protein expression were analyzed by the histochemical stain Picrosirius Red (PSR), which under a polarized light microscope, allows to distinguish between COL1 and COL3. COL1 will stain red, while COL3 will stain green (Fig. 43).

Figure 43 - Mare endometrium stained with PSR



Mare endometrium stained with PSR, observed and photographed under polarized light microscopy (Leica Leitz DMRD; Mag=100X). On the left panel is depicted a healthy endometrium with mostly COL3 fibers (stained in green), while on the right panel most collagen fibers are COL1 (stained in red) in severe endometrosis.

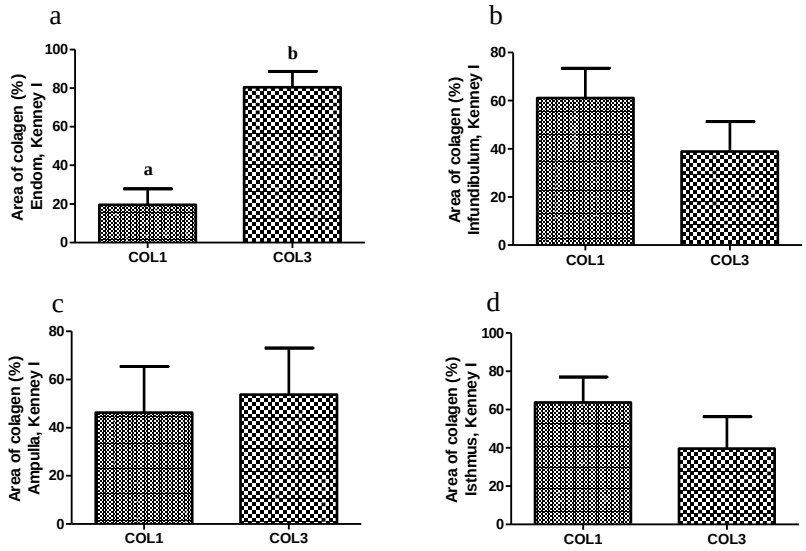
In all mares, endometrium was analyzed, as well as each portion of oviduct: infundibulum, ampulla and isthmus. Mares were assigned to different groups according their endometrium Kenney's histopathological category: I, II or III. Only the percentage of each collagen type present in the whole photographed area was considered, and it was related to the other collagen type. This way, all the other cellular components were deducted, so the real proportion of collagen deposition and the relationship between both types of collagen was determined.

For the mares in the category I, an increase in the area with COL3 fibers with respect to COL1 was depicted in endometrium ($P < 0.05$; Fig. 44a;). In all the portions of the oviduct, there was no difference between their expression ($P > 0.05$; Fig. 44b, c, d;).

In the case of mares in category II, there was no difference in collagen type I or type III fibers distribution, in the endometrium (Fig. 45a). Nevertheless, all oviduct portions, showed a higher transcription of *COL1* than *COL3* ($P < 0.05$; Fig. 45b-d).

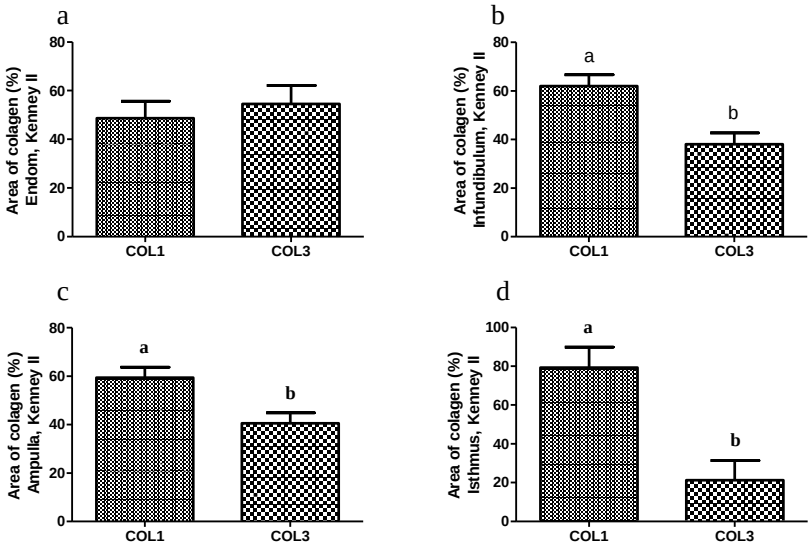
Interestingly, in the case of mares classified in Kenney III, while no difference was noted in infundibulum and ampulla (Fig. 46b, c;), endometrium and isthmus presented a higher percentage of fibers of COL1 *vs* COL3 ($P < 0.05$; Fig.46 a, d).

Figure 44 - Relative proportion of COL1 and COL3 in mares' oviduct and Kenney I endometria



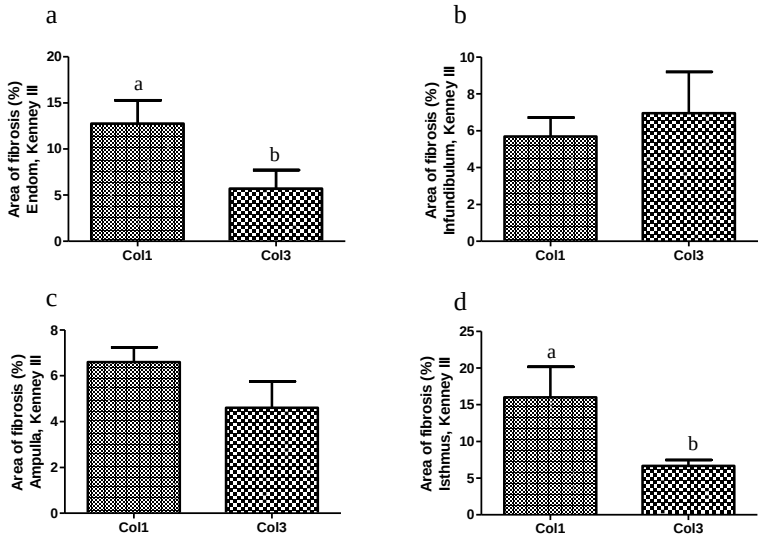
Relative proportion of COL1 and COL3 in equine oviduct and endometrium, from mares with endometrium previously classified as **Kenney I**. Histologic sections were stained with picrosirius red, photographed under polarized light microscopy and measured using a digital image processing system (Leic Qwin V3). Bars represent mean ± SEM. AU: arbitrary units. Different letters indicate significant differences ($P < 0.05$).

Figure 45 - Relative proportion of COL1 and COL3 in mares' oviduct and Kenney II endometria



Relative proportion of COL1 and COL3 in equine oviduct and endometrium, from mares with endometrium previously classified as **Kenney II**. Histologic sections were stained with picrosirius red, photographed under polarized light microscopy and measured using a digital image processing system (Leic Qwin V3). Bars represent mean ± SEM. AU: arbitrary units. Different letters indicate significant differences ($P < 0.05$).

Figure 46 - Relative proportion of COL1 and COL3 in mares' oviduct and Kenney III endometria



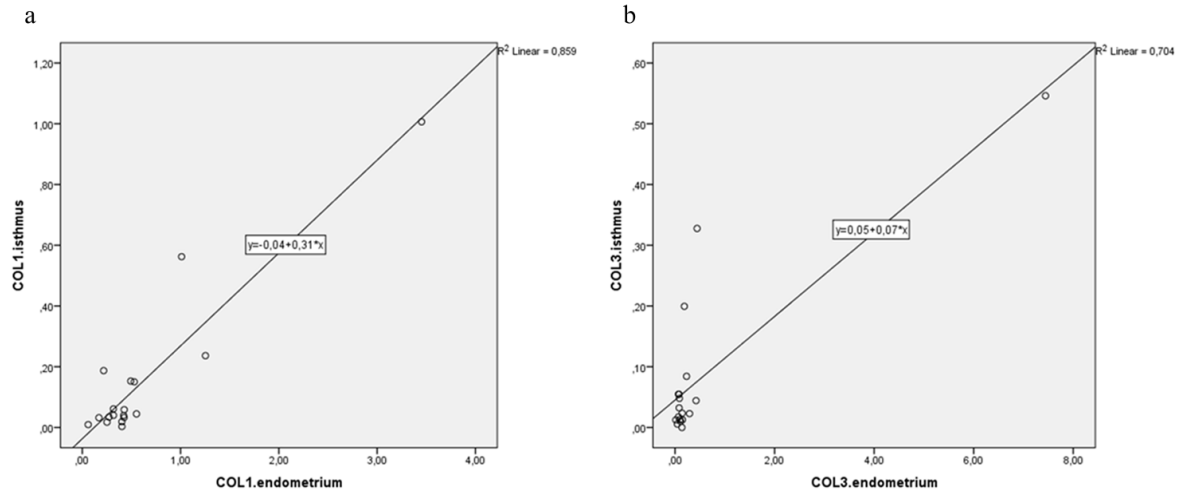
Relative proportion of COL1 and COL3 in equine oviduct and endometrium, from mares previously classified as **Kenney III**. Histologic sections were stained with picrosirius red, photographed under polarized light microscopy and measured using a digital image processing system (Leic Qwin V3). Bars represent mean ± SEM. AU: arbitrary units. Different letters indicate significant differences ($P < 0.05$).

4.4.4. Possible linkage between fibrosis in endometrium and oviduct

Transcription of *COL1* and *COL3* in endometrium and oviduct of the same mares was analyzed by linear regression, to investigate if the transcription in endometrium could influence the transcription in oviduct. *COL1* in infundibulum and in ampulla, presented a lower correlation coefficient (R) (0.443 and 0.129, respectively), but *COL1* in isthmus showed a significant correlation and determination coefficient (R^2) values (R: 0.927; R^2: 0.859; $P < 0.05$). The linear regression in this case was given by the following equation: *COL1* isthmus = - 0.037 + 0.306 *COL1* endometrium (Fig 47a).

In infundibulum and ampulla, *COL3* also presented a low correlation coefficient value (R= 0.282 and R= 0.041 respectively), but again *COL3* in isthmus showed a significant correlation and determination coefficient values (R: 0.839; R^2: 0.704; $P < 0.05$) and the linear regression between the two variables was given by the following equation: *COL3* isthmus = 0.045 + 0.069 *COL3* endometrium ($P < 0.05$; Fig. 47b).

Figure 47 - *COL1* (a) and *COL3* (b) linear regression between isthmus and endometrium



Linear regression between *COL1* (a) and *COL3* (b) gene transcription in isthmus and *COL1* and *COL3* transcription in endometrium. R^2 represents the determination coeficient ($P < 0.05$).

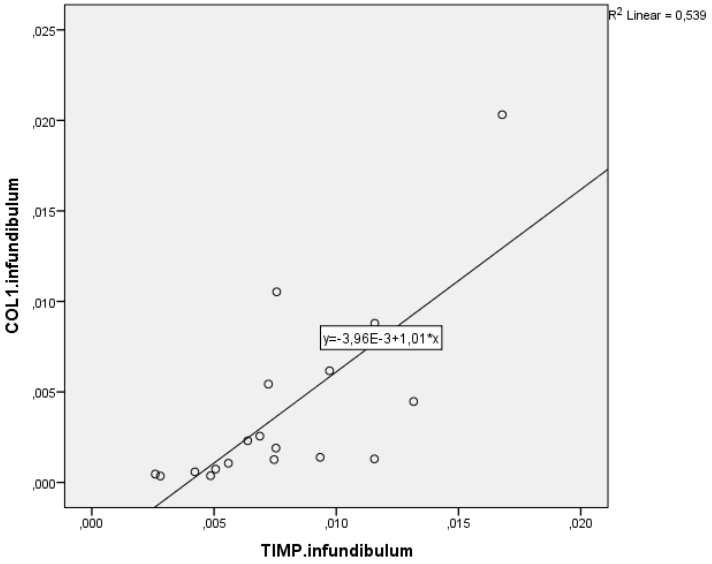
4.4.5. Putative mechanisms involved in COL1 and COL3 transcription in oviduct

In order to investigate the putative mechanism that could be involved in *COL1* transcription, on each oviduct portion, the correlation between *COL1* (infundibulum, ampulla and isthmus) and *MMP2*, *MMP9*, *TIMP*, *PTGES*, *EP2*, *AKR1C3*, *FP*, *TNF*, *TNFRFS1A*, *TNFRSF21B*, *ALK5 and TGFβRII* transcripts was evaluated

Infundibulum

Correlation coefficient between *COL1* and *TIMP* in infundibulum, was 0.734 ($P < 0.01$).
The linear regression between these two variables was represented by the following equation: *COL1* infundibulum = -0.004 + 1.01 *TIMP* infundibulum ($P < 0.05$; Fig. 48). The determination coefficient was 0.539 ($P < 0.05$). With respect to *COL3* in the infundibulum, none of the genes evaluated presented a significant correlation with it.

Figure 48 – Linear regression between COL1 and *TIMP1* in mare infundibulum



Linear regression between *COL1* and *TIMP1* gene transcription in infundibulum in oviduct. R^2 represents the determination coeficient ($P < 0.05$).

Ampulla

In the ampulla, none of the genes studied presented a significant correlation with *COLI* transcription. The transcription of *COL3* showed a significant correlation ($P < 0.05$) with: *MMP2* (R=0.939), *MMP9* (R=0.586), *TIMP* (R=0.628), *PTGES* (R=0.893), *FP* (R=0565), *TNFRSF1A* (R=0.691) and *TNFRSF1B* (R=0.877) ($P < 0.05$). Linear regression was significant for *MMP2*, *MMP9*, *PTGES*, *AKR1C3* and *TNFRSF1A* (R^2=0.950; Fig. 33) and the respective equation was *COL3* ampulla = -0.02 + 0.583 *MMP2* + 112.24 *MMP9* – 15.718 *PTGES* + 27.303 *ARK1C3* – 1.515 *TNFRSF1A.*

Isthmus

In isthmus, *COL1* presented a significative correlation value ($P < 0.05$) with *AKR1C3*, *ALK5* and *TGFβRII* (R= 0.802, R= 0.844 and R= 0.666, respectively). The linear regression equation was *COL1* isthmus = -0.186 + 13.302*AKR1C3* – 0.465*TGFβRII* + 120.030*ALK5*, with a significative determination coefficient (R^2: 0.847; $P < 0.05$).

Also, *COL3* presented a significative correlation value with *AKR1C3*, *ALK5* and *TGFβRII* (R= 0.749, 0.785 and 0.578, respectively) and the liner regression equation was *COL3* isthmus = -0.113 + 9.866*AKR1C3* – 0.268*TGFβRII* + 43.880*ALK5*, with a significant determination coefficient (R^2: 0.741; $P < 0.05$).

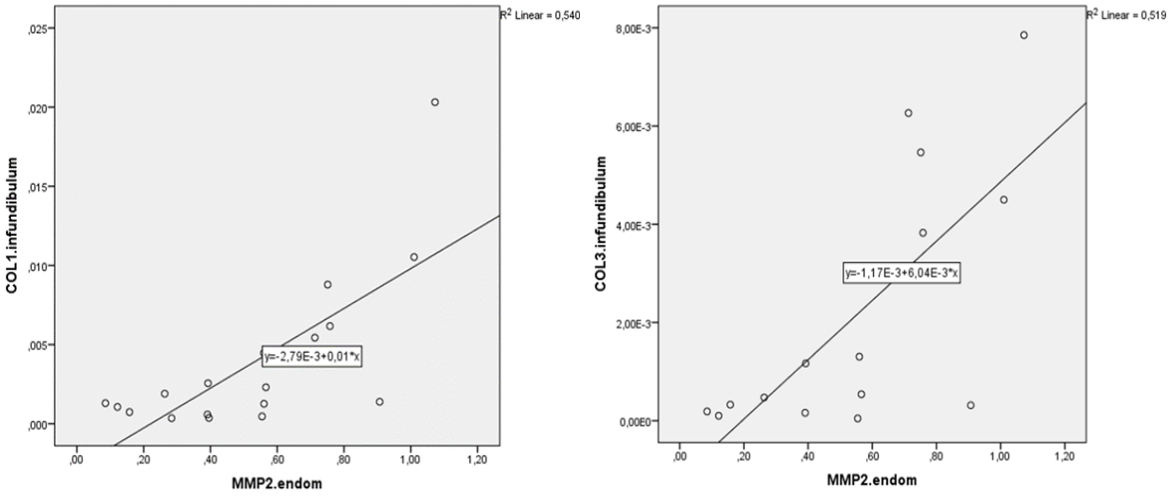
4.4.6. Possible linkage between endometrial collagen pathways and oviduct collagen transcription

Since collagen transcription in oviduct was correlated with collagen transcription in endometrium, in this section possible linkage between oviduct collagen transcription and endometrial collagen pathways was investigated, and presented the following results.

Infundibulum

In infundibulum, correlation coefficient was statistically significant either for *COL1* and *COL3*, with respect to *MMP2* (R= 0.735 and R= 0.72, respectively). The corresponding determination coefficient was R^2= 0.54 and R^2= 0.519 (Fig. 49a, b).

Figure 49 - Linear regression between collagen transcription in infundibulum and *MMP2* in endometrium

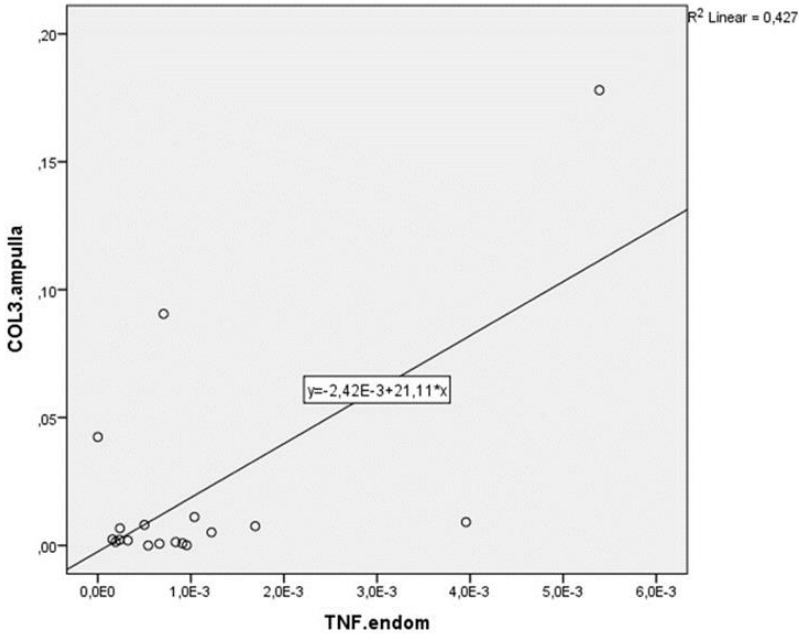


Linear regression between oviduct collagen (I and III) transcription in oviduct and *MMP2* transcription in endometrium. R^2 represents the determination coeficient ($P < 0.05$).

Ampulla

In ampulla, only *COL3* presented a significant correlation value with *TNF* mRNA in endometrium: R= 0.654; R^2= 0.427 (Fig. 50).

Figure 50 - Linear regression between transcription of *COL3* in ampulla and of *TNF* in endometrium



Linear regression between *COL3* (ampulla) transcription and *TNF* (endometrium). R^2 represents the determination coeficient ($P < 0.05$).

Isthmus

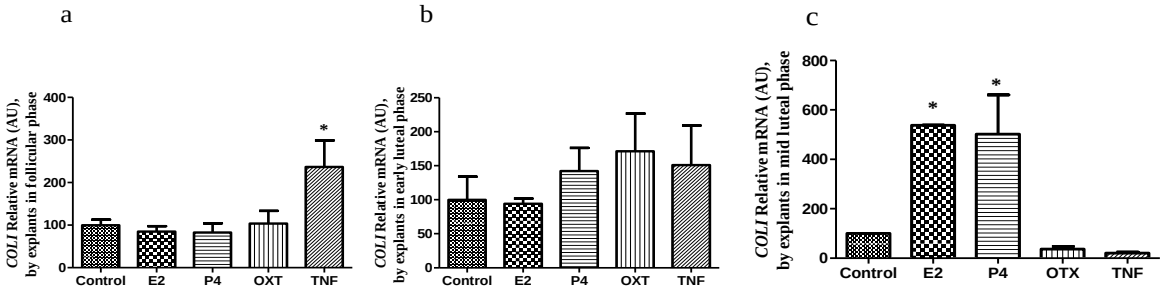
In isthmus, *COL1* showed a significant correlation with *MMP2* and *MMP9* (R=0.525, 0.569, respectively). Linear regression between *COL1* (dependent variable) and *MMP2* + *MMP9* (independent variables) was given by the following equation *COL1* = - 0.176 + 0.289*MMP2* + 8.553*MMP9* (R^2: 0.416, $P < 0.05$). *COL3* also present significant correlations values with *MMP2* and *MMP9* (R= 0.497, 0.540, respectively). Linear regression equation was the following: *COL3* = - 0.089 + 0.153*MMP2* + 4.561*MMP9* (R^2= 0.375, $P < 0.05$).

4.4.7. Ovarian steroid hormones, OXT and TNF on *in vitro* transcription of *COL1* in oviduct explants

Transcription of *COL1* was analyzed in ampulla oviduct explants treated with E_2, P_4, OXT and TNF, and results were compared with a control group, which was subjected to culture medium alone.

In follicular phase, TNF up-regulated *COL1* transcription ($P < 0.05$; Fig. 51a), while in early-luteal phase, no treatment had any effect on *COL1* transcription ($P > 0.05$; Fig. 51b). In mid luteal phase, either E_2 and P_4, stimulated *COL1* transcription ($P < 0.05$; Fig. 51c).

Figure 51 - Effects of E_2, P_4, OXT and TNF on *COL1* transcription in equine ampulla oviduct explants



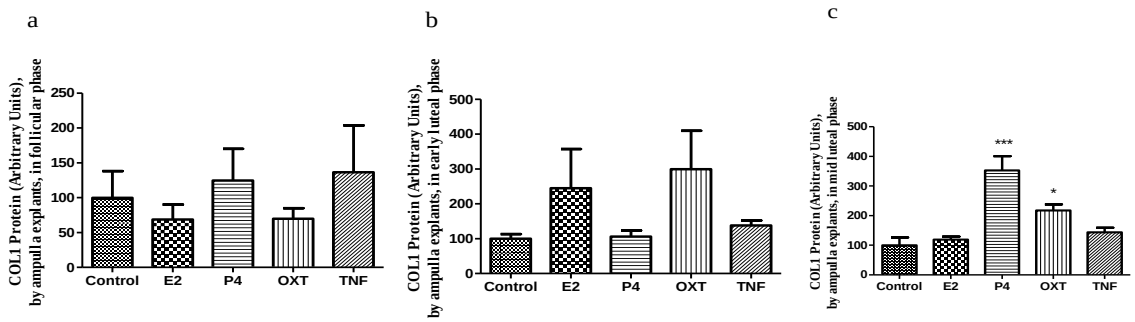
Effects of E_2, P_4, OXT and TNF on *COL1* transcription in equine oviduct ampulla explants from follicular phase, early-luteal phase and mid-luteal phase analyzed by qPCR. Transcription of target gene was normalized against that of the reference gene (*β2M*). Data show the mean ± SEM percentage changes compared with basal (control: C) output. * $P < 0.05$.

4.4.8. Ovarian steroid hormones, OXT and TNF on *in vitro* protein expression of COL1 by oviduct explants

Expression of COL1 was analyzed on the same ampulla oviduct explants treated with E_2, P_4, OXT and TNF, previously used for mRNA levels assessment, and results were compared with a control group (explants incubated in culture medium alone).

In contrast to mRNA transcription, COL1 protein did not show any significant difference, with any treatment in explants from the ampulla region obtained in the follicular phase ($P > 0.005$; Fig 52a). In early-luteal phase, also no treatment induced any difference in COL1 protein expression ($P > 0.005$; Fig 52b). Nevertheless, in mid-luteal phase P_4 and OXT, up-regulated COL1 expression ($P < 0.05$; Fig. 52c).

Figure 52 - Effects of E_2, P_4, OXT and TNF on COL1 expression in equine ampulla oviduct explants



Effects of E_2, P_4, OXT and TNF on COL1 expression in equine ampulla oviduct explants from follicular phase, early-luteal phase and mid-luteal phase analyzed by WB; n=6 for each phase. Data show the mean ± SEM percentage changes compared with basal (control: C) output. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

CHAPTER V – DISCUSSION

5.1. The role of ovarian steroids, oxytocin and tumor necrosis factor in the modulation of equine oviduct function

Ultrastructure images of the OEC obtained from the infundibulum ipsilateral to the dominant ovarian structure showed a mixed pattern of prismatic ciliated cells and a greater amount of non-ciliated cells that present a rounded surface with microvilli assuring an intimate cell-to-cell contact determinant in endometrium health and movement of the ovum towards the uterus (Henry-Suchet, 2000; Urzua, Stambaugh, Flickinger & Mastroianni 1970). Several of the observed aspects and patterns of ciliated and secretory cells have been reported in different portions of cow, pig, mare and goat oviduct, as well as in mare endometrium (Stalheim, Gallagher & Deyoe, 1975; Ferreira-Dias, Nequin & King, 1994; Gandolfi, Brevini, & Moor, 1994). Protruding buds of secretion found in the ampulla in the luteal phase might suggest the importance of this area in the production of nutrients for the first stages of embryonic development (Gandolfi, Brevini, & Moor, 1994).

In the present study, in the follicular phase, under the physiological action of E_2, *PGR* mRNA levels increased in mare oviduct. These findings regarding PGR expression agree with previous reports on steroid hormones and receptors (Graham & Clarke, 1997; Rottmayer *et al*, 2006). Also in the cow, either exogenous E_2 treatment of oviductal cells (Rottmayer *et al*, 2006), or endogenous physiologic E_2 action in the follicular phase increased *PGR* mRNA expression in the ampulla (Kenngott, Vermehren, Sauer, Ebach & Sinowatz, 2011). In contrast, P_4 stimulation resulted in a reduction of *PGR* transcripts or protein expression in cow, sheep and rabbit oviducts (Hyde, Blaustein, & Black, 1989; Ulbrich, Kettler & Einspanier, 2003; Garcia-Palencia *et al*, 2007). Classically, gene expression of *PGR* in target tissues is up-regulated by E_2, but downregulated by P_4 (Graham JD & Clarke, 1997). With declining peripheral P_4 concentrations during luteolysis, this inhibition diminishes causing a strong upregulation of PGR expression (Kenngott *et al*, 2011), while E_2 stimulates PGR in the oviduct (Hai, Logeat, Warembourg & Milgrom, 1977). This agrees with our results, where PGR gene transcription and protein expression in mare ampulla were the highest in the follicular phase and depicted in epithelial cells. These findings may suggest that circulating estrogens in the follicular phase could have a stimulatory effect on both PGR transcription and translation processes. In another study on mare oviduct, immune-localization of PGR was stronger in the ampulla than in the isthmus, present in epithelial nuclei, but in contrast with our findings, it was also depicted in the smooth muscle cell nuclei of the *lamina muscularis propria* (Nelis *et al*, 2015b). Also in cow oviduct, PGR were immuno-localized in epithelial cells nuclei, stroma and muscle layer, even though muscle layer only stained for PGR in the early luteal phase and not in others

(Ulbrich *et al*, 2003; Saint-Dizier, Sandra, Ployart, Chebrout & Constant, 2012). Nevertheless, another work showed intense staining for PGR in secretory epithelium cells and muscle cells in the bovine ampulla in the follicular phase, while ciliated cells only showed a week nuclear staining (Kenngott *et al*, 2011). These discrepancies between our results and the aforementioned studies in mare and cow oviduct regarding PGR protein localization may be ascribed to species difference between cow and mare, different days of oviduct sample collection, or different techniques and antibodies used. As in other species, the physiologic role of P_4 mediated by genomic *PGR* in mare oviduct may be responsible for decreasing cilia activity and oviduct contractility in the luteal phase (Saint-Dizier *et al*, 2012). Besides, cellular events leading to cell survival or apoptosis are also mediated by PGR expression modulation in different cell types in the mouse oviduct, and may be determined by a putative interdependence between this steroid receptors and growth factors (Shao *et al*, 2007).

Even though transcription of *ESR1* and *ESR2* did not change in mare oviduct throughout the estrous cycle, protein expression of both receptors was higher in the follicular phase, as for PGR. This lack of agreement between mRNA transcription of ESR1 and ESR2 and protein expression in mare oviduct may be explained by the fact that gene expression is regulated at multiple levels (*e.g.*, transcriptional and post-transcriptional) to maintain oviduct function under physiologic conditions (Vogel & Marcotte, 2013; Dahan, Gingold & Pilpel, 2011). Protein abundance reflects a dynamic balance among the processes of spanning the transcription, processing and damage of mRNAs, translation, localization, modification and destruction of the resulting proteins (Vogel & Marcotte, 2013). These processes are often extensively linked and may recurrently regulate each other through feedback loops, as revised by Dahan *et al* (2011). Estrogen dominance in the follicular phase might have a positive effect on the translation of these genes, resulting on a higher level of protein, which will be necessary to prepare the oviduct for the fertilization process.

When infundibulum and ampulla of the mare oviduct were considered, there was an *ESR1* and *ESR2* mRNA up-regulation in the ampulla, in contrast to *PGR*. The expression of *ESR1* mRNA by laser-assisted microdissection (LAM) and *in situ* hybridization in cow oviduct epithelium was higher in the follicular phase than in the mid-luteal phase (Kenngott *et al*., 2011). Also, a distinct upregulation of *Esr1* mRNA in the stromal cells of bovine ampulla was seen in the follicular phase (Kenngott *et al*., 2011). In cow oviduct, *ESR1* mRNA, *in situ* hybridization revealed transcripts in epithelial cells and in stromal cells mainly in the follicular phase, while hybridization signals appeared weaker in the mid-luteal phase (Kenngott *et al*., 2011). Murine oviduct epithelium has also a tissue-specific set of target genes for ESR that respond to E_2 stimulation by regulating a tissue specific selective E_2 receptor modulators response (Moyle-

Heyrman, Schipma, Dean, Davis, & Burdette, 2016). In mare oviduct, the ampulla, which has the largest luminal perimeter, appears to be the most active secretory region, providing a large contact surface and nutrients to support biological events, such as early embryo cleavage (Mougelar *et al*., 2015). Thus, assessment of protein expression of the genes under study was only performed in the ampulla. These differences in steroid receptors gene expression are probably related with the process of gamete preparation, fertilization and for an adequate early embryo development (Mougelar *et al*., 2015; López-Úbeda *et al*., 2015). In fact, suppression of oviductal protease activity mediated by E_2-epithelial ESR1 signaling is required for fertilization, preimplantation and embryo development (Winuthayanon *et al*., 2015).

In the follicular phase, even though *OXTR* mRNA was down-regulated in mare oviduct, with respect to early and mid-luteal phases, its protein expression was up-regulated under endogenous estrogen influence. and mostly expressed in the ampulla. This could be explained by a translational delay, also observed in the cow oviduct in respect to *ESR1* (Ulbrich *et al*, 2003). The presence of OXTR on cow oviduct and OXT stimulatory effect on oviduct motility were also evident in the follicular-phase (Kotwica *et al*., 2003). In sheep oviduct, OXTR were up-regulated at estrus when compared to the luteal phase (Ayad, Guldenaar & Wathes, 1991). In both the ampullary and isthmic regions of the sheep oviduct OXTR binding sites were confined to smooth muscle (Ayad *et al*., 1991). Regarding OXT action on cow oviduct function, OXT completely blocked *in vitro* oviductal contraction in the follicular phase (Wijayagunawardane *et al*., 2008). In addition, in woman Fallopian tube, OXT administration first resulted in a short contractile response followed by a pronounced inhibition of muscular activity or relaxation through a specific effect on OXTR (Wanggren *et al*., 2008; Jankovic *et al*., 2001). In disagreement with those findings, *in vivo* oviductal musculature of the ewe reached a peak in sensitivity to physiological concentrations of OXT at estrus, in the ampulla and utero-tubal junction, increasing oviduct contractility (Shao *et al*., 2007). As reported in humans, OXT may contribute for a fast transport of sperm to the oviduct under E_2 influence (Kunz, Beil, Huppert, & Leyendecker 2007). Once in this work, *OXTR* mRNA was the highest in the ampulla and its OXTR protein was expressed in the connective tissue of mare oviduct mucosa folds, we may suggest it is possibly related to contractility/motility and/or relaxation. Nevertheless, since the precise mechanism of mare oviduct cyclic contractility mediated by OXT is unknown, one is unable to present a definite role on contractility *vs.* relaxation events.

It is long known that prostaglandins (PGs) are involved in reproductive functions, such as ovulation, fertilization, implantation and parturition. As previously referred for the gilt and cow (Kaczmarek *et al*., 2010; Wijayagunawardane *et al*., 2003; Siemieniuch, Woclawek-Potocka, Deptula, Okuda & Skarzynski, 2009), we have shown the mare oviduct is capable of producing

PGE_2 and $PGF_{2\alpha}$ *in vitro*. Production of PG in mare oviduct depended on estrous cycle stage, oviduct regions, and cell types. These are highly coordinated mechanisms regarding interactions of autocrine/ paracrine factors. They may be necessary for oviduct contractility, for sperm cells and oocyte transport, and early embryo development and transport (Kodithuwakku *et al*., 2007; Kaczmarek *et al*., 2010). Oviduct contractions have been reported to be modulated by PGs, through effects of PGE_2 on relaxation of smooth muscle, and PGF2α on contraction (Kunz *et al*., 2007; Spilman & Harper, 1975). In our study, *PTGES* and *AKR1C3* transcription in mare´s oviduct was down-regulated in the follicular phase, but with no change in gene expression. While *PTGES* mRNA levels were up-regulated in the ampulla, *AKR1C3* transcription was increased in the infundibulum. Also in the cow, PTGES was mostly expressed in the ampulla and to a lesser extent in the infundibulum and isthmus of the oviduct (Marey *et al*., 2014). In the rat oviduct, the stimulatory effect of E_2 on the enzyme prostaglandin-endoperoxide synthase 2 (PTGS-2; COX-2) expression and activity may be receptor-mediated (Pérez-Martínez *et al*., 2006).

While ovarian steroids treatment down-regulated PGE_2 and $PGF_{2\alpha}$ production by equine OEC in the follicular phase, PGF2α release increased in mid-luteal phase, in disagreement with cow OEC (Szóstek *et al*., 2011). In contrast, in the present study, when oviduct explants were cultured, PGE_2 production was up-regulated by E_2 in the ampulla in the follicular phase, by P_4 in the isthmus explants in mid-luteal phase, but no effects of ovarian steroids on PGE_2 were recorded in the infundibulum. Treatment of equine oviduct explants with OXT resulted in increased production of PGE_2 by the isthmus (follicular, early and mid-luteal phases) and by infundibulum (mid-luteal phase); and release of $PGF_{2\alpha}$ by the infundibulum (early and mid-luteal phases) and by the ampulla (mid-luteal phase). When TNF was tested *in vitro*, PGE_2 was increased in the infundibulum in the early-luteal phase; in the ampulla in the follicular phase; and in the isthmus in the early and mid-luteal phases. Regarding *in vitro* TNF effect on mare oviduct, $PGF_{2\alpha}$ was increased in the isthmus in the follicular phase and in the ampulla in early and mid-luteal phases. In an *in vitro* microdialysis study, infusion of LH alone or in combination with P_4 or E_2 stimulated a pronounced release of PGE_2 and $PGF_{2\alpha}$ in the oviducts from cows in the follicular and postovulatory phases (Wijayagunawardane *et al*., 2001). It is worth noting that PGE_2 rich microenvironment in the oviduct might have an anti-inflammatory action and might prevent sperm cells phagocytosis by neutrophils, as in the cow (Marey *et al*., 2014). This suppression of immune-response has been ascribed to the action of E_2 that binds to ESR1, and plays a vital role in down-regulating some of the immune responses in the oviduct to provide a supportive environment for fertilization and embryo development (Wijayagunawardane & Miyamoto, 2004). We might speculate a concerted action of $PGF_{2\alpha}$ and PGE_2 on oviduct

contraction and relaxation, respectively, as in the cow oviduct (Siemieniuch *et al*., 2009). These differences on prostanoids production could be ascribed to different types of cells involved, such as OEC from the ampulla or different cell types included in the oviduct explants from the 3 different anatomical regions of the oviduct. This explant culture system allows cell-to-cell integrity and cell-to-cell communication to be maintained. As in the cow, TNF and OXT stimulated oviduct PGE_2 and $PGF_{2\alpha}$ production (Siemieniuch *et al*., 2009; Wijayagunawardane & Miyamoto, 2004). In the cow oviduct, this stimulation by TNF occurred in the follicular phase, when the oviduct was considered as a whole, or by both the ampulla and the isthmus (Wijayagunawardane *et al*., 2003; Siemieniuch *et al*., 2009). In contrast, in the luteal phase, no stimulatory effect on eicosanoids production was seen in the entire oviduct, but only in the cow ampulla (Wijayagunawardane *et al*., 2003; Siemieniuch *et al*., 2009). In early-luteal phase, TNF did not influence the contractility in either fragment of cow oviduct (Siemieniuch *et al*., 2009). When taken together, TNF seems to play some role as a modulator of $PGF_{2\alpha}$ and PGE_2 production and for transferring the embryo from the oviduct to the uterus (Siemieniuch *et al*., 2009). Both $PGF_{2\alpha}$ and OXT are involved in ovum capture in rabbit by the oviduct ampulla by stimulating contractility, thus altering intraductal pressures (Wijayagunawardane & Miyamoto, 2004). TNF system may optimize the release of contraction-related substances and modulate local contraction to regulate the oviductal transport of the gametes and embryo (Wijayagunawardane *et al*., 2003). As it was suggested for the cow (Wijayagunawardane *et al*., 2001), also in the mare, the increasing E_2 concentration from the Graafian follicle, simultaneous with a basal P_4 plasma level from the regressing corpus luteum, stimulates oviductal production of PG resulting in oviductal contraction for a rapid transport of gametes.

In conclusion, this work has shown that *ESR1*, *ESR2*, *OXTR*, *PTGES* and *AKR1C3* gene transcription and/or translation is estrous cycle dependent and varies with oviduct portion (infundibulum *vs* ampulla) and cell type. Ovarian steroids, OXT and TNF stimulation of $PGF_{2\alpha}$ and/or PGE_2 production is also estrous cycle dependent and changes in the different portions of mare oviduct. It has been shown that steady-state transcript abundance only partially predicts protein abundances (Dahan *et al*., 2011). Differential transcription level and protein localization in various portions of the oviduct throughout the estrous cycle, as well as PG production, suggest coordinated physiologic actions and mechanisms of ovarian steroid hormones, OXT and TNF in the equine oviduct. They may synergistically control oviductal contraction for optimal embryo transport during the periovulatory period, and provide further evidence for the local delivery of ovarian steroids to the adjacent reproductive tract (Wijayagunawardane *et al*., 1998).

5.2. Microvascular density and the expression of FGFs, VEGF, and receptors in equine oviduct

In mare internal genitalia, the reproductive organs, such as the ovary and endometrium, do not present the same microvascular changes throughout the estrous cycle. Specifically, the endometrium has not shown any differences in microvascular density (Roberto da Costa *et al*., 2007). In contrast, the corpus luteum (CL) has shown differences on its microvascular density, according to P_4 production (Ferreira-Dias *et al*., 2005). In the present work, in the oviduct, the highest microvascular density and number of blood vessels per area were depicted in the isthmus, during the follicular phase, under estrogens influence. Also in the rabbit, by the time that the oocyte is at AIJ, it was observed a dilatation of the isthmic subserosal venous plexus (Verco, Gannon & Jones, 1984b). Still in the rabbit, during pregnancy, an increase in microvasculature was observed, probably due to increased levels of circulating placental hormones (Verco, Gannon e Jones, 1984a) Thus, as an attempt to understand the observed changes in microvascular density in equine oviduct, and due to the importance of angiogenesis in reproductive organs, the expression of the most important angiogenic factors were evaluated in mare oviduct. Transcription of *VEGF* in equine oviduct did not change between portions and throughout the estrous cycle, in agreement with what was observed in the cow (Gabler, Einspanier, Schams & Einspanier, 1999), but in opposition to women, who presented a higher transcription in infundibulum and ampulla and a higher transcription during the peri-ovulatory period (Lam *et al.*, 2003). In swine oviduct, different results from ours were recently observed by Albors and co-authors (2017), since *VEGF* showed a higher transcription in ampulla, with respect to isthmus. In the present work, the transcription of VEGF receptor (KDR) increased in the isthmus, when the estrous cycle phase was not considered. Nevertheless, in spite of no estrous cycle effect in *KDR* mRNA transcription in the mare oviduct, in the sow oviduct, the transcription of *KDR*, either in ampulla or isthmus, raised in early and late-luteal phases (Albors *et al*., 2017). Nevertheless, besides the highest KDR protein expression in mare isthmus, when the three portions were analyzed as separate entities, we got a higher expression in the infundibulum in early-luteal phase, while in the ampulla, the highest expression occurred in follicular phase. In swine oviduct, transcription of *KDR*, either in ampulla and isthmus, increased in early and late luteal phases (Albors *et al*., 2017). In general, KDR is a protein associated with vasculogenesis, angiogenesis, cell proliferation and vascular permeability (Ferrara & Davis-Smyth, 1997). In mare oviduct, as previously referred, KDR (either as protein or mRNA) was mostly expressed in the isthmus, which agreed with the highest microvascular density in the oviduct. In agreement with our results, several experimental works confirm the

relationship between VEGF with angiogenesis, in different organs and species. For instance, in spinal cord damage of the rat, VEGF was associated with vascular changes following different types of injury in the central nervous system (Bartholdi, Rubin & Schwab, 1997). In tracheal capillaries of mouse, after VEGF signaling was blocked with a VEGF-receptor tyrosine kinase inhibitor, a decrease in capillaries around 30% was observed 21 days after (Baffert *et al*., 2006). In equine CL, Galvão and co-authors (2012), suggested a positive effect of TNF on the regulation of VEGF for angiogenesis stimulation during the early luteal phase, while at luteolysis, it is down-regulated. Also in human cancer, neutralizing monoclonal antibodies against VEGF and small molecule tyrosine kinase inhibitors targeting VEGFRs have been shown to block its angiogenic activity, resulting in tumor vascular regression, anti-tumor effects and improvements in patient survival (Sia, Alsinet, Newell & Villanueva, 2014).

In the cow endometrium, *VEGF* presented a higher transcription during early and mid-luteal phases, while the protein presented a higher expression only during early luteal phase. Both receptors (*FLT1* and *KDR*), presented a higher transcription during early and mid-luteal phases, but also some differences concerning the protein expression were shown (Tasaki *et al*., 2010). This group also demonstrated that VEGF could stimulate $PGF_{2\alpha}$ production. In equine oviduct, E_2 up-regulated *FLT1* and *KDR* transcription in ampulla explants, during early luteal phase, which is agreement with what was referred in the cow (Wijayagunawardane *et al*., 2005). Nevertheless, in equine oviduct we did not observe the positive effect of TNF in *KDR* transcription, as it was noted in equine CL (Galvão *et al*, 2012). These findings might suggest that VEGF system regulation in oviduct is mediated by different pathways.

It seems that VEGF system presents differences in the oviduct among species, on its transcription and expression, between portions and estrous cycle phases. These differences suggest various roles on oviduct function, either related with angiogenesis, or related with secretion of oviductal fluid or even related with oviduct contractibility, as it has been suggested for swine oviduct (Albors *et al*., 2017).

Like other angiogenic/growth factors FGF's family is involved in several processes including cell growth, proliferation, differentiation and cell survival (Thisse & Thisse, 2005). In bovine ovarian follicle, FGF's family members are involved in folliculogenesis, especially during the final stage of the follicular phase by stimulation of angiogenesis and granulosa cell survival and proliferation (Berisha *et al*., 2004). In gilts, FGF2 has also been identified in endometrial epithelium, stroma and myometrium during the estrous cycle and early pregnancy, but without any differences on its expression (Katsahambas & Hearn, 1996). Nevertheless, another research group, using immunolocalization techniques in swine, reported an increase in FGF2 in endometrium luminal epithelium and stroma on days 12 and 14 of gestation (Gupta, Bazer &

Jaeger, 1997). In equine oviduct, there were also no differences in mRNA *FGF2* and *FGF1* levels throughout the estrous cycle, but a different transcription on its portions was noticed. While *FGF1* presented a higher transcription in isthmus and ampulla, *FGF2* mRNA levels were more expressed in isthmus. In addition, *FGFR1* and *FGFR2* transcription was not influenced by the estrous cycle, and only *FGFR1* showed a higher transcription in isthmus. These results, propose different roles of both ligands and receptors studied. As observed for *VEGF*, *FGFR1* also had a higher transcription in the isthmus, which is the portion with the highest microvascular density. These findings might suggest a role of the angiogenic factors VEGF and FGF, mediated by their specific receptors, on isthmus angiogenesis and/or microvascularization. In fact, when comparing protein expression of all oviduct portions, also FGFR1 had the highest protein level in the isthmus, which could reinforce the role of this receptor, modulating the action of these growth factors in blood vessel development. FGFR2 protein expression presented interesting differences. Either in ampulla and in isthmus, the highest protein expression occurred in the follicular phase, while in the infundibulum it depicted the highest expression in mid-luteal phase. The increased expression of this receptor could be ascribed to the largest microvascular density in the isthmus, in the follicular phase (Gabler *et al*. 2004). As well, these data might reinforce the importance that FGF system likely represents in creating the proper conditions for oviduct physiological events in the follicular phase (Archibong, Petters & Johnson, 1989; White *et al*., 1989). In fact, also in other reproductive endocrine organs, such as the CL, FGF has been associated to luteal cell proliferation (Grazul-Bilska *et al*, 1995), and to luteal P_4 production (Grazul-Bilska *et al*., 2001).

5.3. Oviduct specific glycoprotein expression in equine oviduct

In this work, mRNA *OVGP* transcription was augmented in ampulla and isthmus, compared with infundibulum. In ampulla, *OVGP* transcription was the highest in early and mid-luteal phase. Nevertheless, OVGP protein expression was increased in isthmus, and when each oviduct segment was analyzed separately, follicular phase was the one that presented the largest expression. Differences between mRNA and protein expression, may be explained by the fact that gene expression is regulated at multiple levels (e.g., transcriptional and post-transcriptional) to maintain oviduct function under physiologic conditions (Vogel & Marcotte, 2013). Besides, proteins are more stable than mRNAs, with a maximum half-life of approximately 46 h, compared to 7 h for mRNAs (Vogel & Marcotte, 2013). Protein abundance reflects a dynamic balance among the processes of spanning the transcription, processing and

damage of mRNAs, translation, localization, modification and destruction of the resulting proteins.

As already demonstrated in this work, isthmus had the highest expression of other proteins, such as FGFR1 and KDR. Caudal isthmus of the mouse oviduct acts as a reservoir for spermatozoa during the estrus (Suarez, 1987). This is probably one main reason for these proteins to exhibit a higher expression in this oviduct portion. Concerning OVGP1, its expression has shown the same pattern in all equine oviduct portions, with the highest expression in follicular phase. This confirms that also in the mare, this protein has a core importance in the process that occurs in oviduct. In fact, in 1990, Boice claimed that the bovine oviduct secretes a class of a specific glycoprotein that is present in the luminal fluid at the time of fertilization. Later on, it was concluded that the number of sperm that bound *in vitro* to human oocytes in the presence of OVGP, was superior to when only control medium was used (O'Day-Bowman *et al*., 1996). Also in the sow, the addition of OVGP increased the cleavage of blastocysts, and reduced the incidence of polyspermy in oocytes (Kouba *et al.*, 2000). In woman, a positive correlation between serum E_2 and LH, and mRNA *OVGP* transcription, besides a negative correlation with P4 have been reported (Briton-Jones and co-authors (2001). Indeed, it was in the peri-ovulatory period in woman that *OVGP* transcription was the highest (Lok *et al*., 2002). These results agree with the results reported in this work, substantiating the highest OVGP expression in mare oviduct, in the follicular phase, when both gametes meet in the oviduct and when fertilization will occur.

The hormones OXT, ovarian steroids, and cytokine TNF showed a somewhat different modulatory role on *OVGP* mRNA levels. Transcription of *OVGP1* in follicular phase, was stimulated by OXT and TNF; by E_2 in early-luteal phase, and P_4 in mid-luteal phase. It has already been demonstrated that OXT and TNF can stimulate PGs productions, either in cow (Szóstek *et al*., 2011) or as demonstrated by us, in the mare (Pinto-Bravo *et al*., 2017). Although the relationship between PGs and OVGP1, in mare oviduct has not yet been defined, OXT and TNF also upregulated *OVGP1* transcription during follicular phase, which could suggest their involvement in oviduct proteomic profile in this phase of the estrous cycle. In addition, E_2 up-regulation of *OVGP* mRNA transcription during early luteal phase could be the result of a higher level of this hormone in the follicular phase, which could prime the oviductal tissue for a further E_2 positive effect.

Progesterone, the main steroid hormone in the luteal phase, has exhibit its stimulatory effect on *OVGP1* transcription during this phase, on equine oviduct explants. This observation could be ascribed as a process for preparation for a higher expression of the OVGP protein, during the

follicular phase, due to gene expression regulation at multiple levels, and different half-lives of transcripts and proteins (Vogel & Marcotte, 2013).

When considering OVGP secretion, in ampulla explants no treatment tested exerted any effect, when compared to control. These results are in disagreement with OVGP protein expression and transcripts. This could be explained by the time of tissue incubation (24 h), or the doses of the factors tested that could not have been the most adequate ones to trigger differences on OVGP production. Other possible reasons, could be related with post-transcriptional and/or translation regulatory mechanisms (Vogel & Marcotte, 2013).

This work, also demonstrated that sperm cells can up-regulate the secretion of OVGP by equine isthmus, which agrees with other works. Georgiou and co-authors (2005), demonstrated in swine, that the presence of spermatozoa or oocytes in the oviduct alter the secretion of specific proteins by oviduct. In fact, in rabbit, several oviductal proteins are altered 2 h after male gametes exposure (Artemenko *et al*., 2015), and in rabbit, semen increases OVGP secretion (Steinberger *et al*., 2017). Nevertheless, the increase in OVGP secretion, in the presence of sperm, was only observed in the isthmus, but not in the ampulla. Since the isthmus has a paramount importance as a sperm reservoir and in preparation of spermatozoa for fertilization, the up-regulation of OVGP in this portion of the oviduct by the influence of sperm reinforces the importance of this glycoprotein.

Our data suggest that direct contact between spermatozoa and oviduct epithelium is not essential, since there was no difference when isthmus explants were incubated directly with sperm cells, or without direct contact with sperm. We may suggest that spermatozoa themselves will secrete substances, which will induce the epithelial cells to secrete OVGP, creating the adequate oviductal milieu for fertilization and early embryo development. Nevertheless, our data are slightly in disagreement with previous works in rabbit, which stated the absolute need for a direct contact between oviduct epithelial cells and sperm cells, to enable the oviduct to secrete specific proteins, in response to insemination (Artemenko *et al*., 2015).

Therefore, more research is necessary to clarify the mechanisms involved in the cross-talk between oviduct epithelial cells, spermatozoa and even oocytes and embryos.

5.4. Collagen in equine oviduct: possible relationship with endometrial fibrosis and pathways involved

In order to search for fibrosis in mare oviduct, collagen presence was assessed and its presence correlated to endometrium fibrosis, mare´s age and Kenney´s classification (1978). To the best

of our knowledge, this is the first time that endometrial fibrosis is related to oviduct structural collagen changes in the equine species. The present study showed differences in mRNA transcription of *COL1* and *COL3*, particularly amongst the three anatomical segments of the oviduct. In fact, *COLI* mRNA transcripts were increased in isthmus, while no influence of estrous cycle or mare´s age was found. Regarding *COL3*, although it also presented a higher transcription in isthmus, it had some differences in infundibulum and isthmus regarding the estrous cycle phase and the mare's age. When we compared the expression of COL1 by WB analysis, among the three portions of oviduct, in different age mares and throughout the estrous cycle, we concluded that the ampulla presented a higher COL1 expression than the infundibulum. In the ampulla COL1 expression was increased in follicular phase and early-luteal phase with respect to mid-luteal phase. The infundibulum presented a higher expression in older mares, while the ampulla did not presented any difference between different ages. Also, in equine endometrium it was reported that the distribution of type I collagen was dependent on the estrous phase cycle (Walter, Handler, Reifinger & Aurich, 2001). In equine oviduct there was a clear relationship between age and collagen expression, since COL1 protein was increased in the infundibulum of older mares; in the ampulla, COL1 expression did not show any difference, but in the isthmus, middle age mares presented COL1 highest expression. Regarding the development of equine chronic endometrial disease (based on Kenney's classification) and its possible relation with age, it was concluded that mares up to 9 years old should not present any sings of it and only mares with only 17 years old, or more, are likely to have severe signs of chronic endometrial disease (Ricketts & Alonso, 1991). More recently, another study also confirmed a positive relation between animal age and disease severity, since they proved that endometrosis in older mares (> 12 years old), was more severe than in young mares (2 ≅ 4 years old) (Aresu *et al*., 2012). In conclusion, there seems to occur differences in *COL1* and *COL3* transcription, either due to the segment of the oviduct considered, either to the phase of the estrous cycle and even due to the age of the mare. Also, the expression of COL1 protein can be influenced by the same factors on different scales.

Among the group of mares in this study, *COL1* mRNA, in ampulla and isthmus, was higher in mares previously classified as Kenney III, than those mares classified as Kenney II. Nevertheless, these changes were not verified in *COL3* transcription. These facts tempt us to consider, even though with caution, that COL1 may be more involved in the fibrotic process in oviduct, than COL3.

The PSR stain is actually a technique involved in some controversy. Traditionally it has been considered as a reliable method to evaluate and differentiate type I from type III collagen, under a polarized light microscope (Rittie, 2017). However, some authors have reported that polarized

colors only reflect fiber thickness and packing (Lattouf *et al*., 2014). Nevertheless, even though PSR data should be analyzed with caution, in our opinion, this technique revealed interestingly results in the present study. The proportion of COL1 expression *vs* COL3 was analyzed, both in endometrium and oviduct by PSR stain. In the group of mares classified as having a Kenney category I endometrium, there was no difference concerning the relation of both types of collagen in the oviduct, interestingly, in the endometrium of these mares, COL3 presented a higher expression than COL1, which agrees with the fact that COL3, might have no deleterious effect on fibrosis (Masseno, 2009). In mares classified as Kenney II, there was no difference between COL3 expression and COL1. Nevertheless, COL1 expression was superior than COL3 in all portions of the oviduct. In Kenney's III group, in endometrium COL1 was more expressed than COL3, but in oviduct, the same pattern was observed only in isthmus. These results are partially in agreement with others, in which COL3 was more expressed in mares classified in category I (as in our study), but also in category II (Lunelli, Cirio, Leite, Camargo & Kozicki, 2013). Nevertheless, this group did not consider mares in category III, so we cannot compare it clearly with our results. However, the present work is fully in covenant with what was reported by Masseno (2009), who acknowledged that COL3, is predominant in healthy endometrium and will be gradually replaced by COL1, as fibrosis advances.

Taken in together, oviduct *COL1* and *COL3* transcription and/or protein expression could be influenced by different factors, like mares' age, and Kenney's endometrium classification. Since oviduct COL1 transcription in ampulla and isthmus showed differences in mares presenting different degree of periglandular fibrosis, as well as differences in percentage of COL1 and COL3 deposition in the oviduct, in a dissimilar fashion to the endometrium, claim the need for a specific classification grid about the degree of oviductal fibrosis. Indeed, this could clarify the presence of pathological changes in the equine endometrium, but also in the oviduct and possible relationship with infertility and reproductive failure. It is important to remember that mares with Kenney's category III endometrium have more degenerative structures and fewer cellular organelles, lack cilia in the lumen of the glands, and despite extensive fibrotic tissue in the lamina propria and inflammatory cells in most tissue layers (Ferreira-Dias, Nequin & King, 1994; Ferreira-Dias, Nequin & King, 1999). As far as we know, no knowledge is available about the relationship between equine oviduct structural arrangement of its components and layers, and the normal function of this organ, as it exist in endometrosis. Transcription of either *COL1* and *COL3* in isthmus was highly correlated with collagen transcription in the endometrium. These aspects suggest that the fibrotic process occurring in endometrium, can have repercussions on the *neighbor* oviduct, particularly in the isthmus. Using the rat as a model, it was also suggested that cases of endometriosis, present structural

and functional abnormalities of the oviduct, such as attenuated intercellular signaling, oviduct contractility, impaired immunoregulation, stem cell-mediated tissue repair and tissue fibrosis (Yang, Yang, Lin, Yang & Shen, 2015).

In the present work, TNF showed a questionable relationship with collagen transcription in equine oviduct, although there is linkage between TNF transcription in endometrium and *COL3* transcription in ampulla. The cytokine TNF may have a controversial effect on fibrosis in different organs and species. Distler (2008), in a very interesting review, presented several examples where TNF can either exhibit a profibrotic effect, or an antifibrotic action. In fact, as previously referred, TNF inhibits both the transcription of collagen in cultured dermal fibroblasts, and the synthesis of COL3 and fibronectin (Mauviel *et al*., 1988). Also, TNF is involved in the resolution of established pulmonary fibrosis through reducing and programming the status of profibrotic macrophages (Redent *et al*., 2014). Moreover, TNF has been referred as a profibrotic cytokine by many other authors. Theiss and co-authors (2005) defended that TNF promotes a profibrotic phenotype of intestinal myofibroblast. Very recently, Hon and co-authors (2018), explained how TNF can up-regulate the expression of nuclear factor kappa B, which can induce the differentiation of lung resident mesenchymal stem cell into myofibroblast, and so exacerbates pulmonary fibrosis. In human endometriosis, TNF can act indirectly, by stimulation of MMPs expression (Sillem, Prifti, Monga, Arslic & Runnebaum, 1999). In the equine oviduct, TNF up-regulated *COL1* transcription just during the follicular phase. No difference was observed either during early or mid-luteal phase. Nevertheless, COL1 did not change its protein expression, under the influence of TNF, throughout the estrous cycle. In conclusion, in equine oviduct, TNF does not exhibit a clear effect on collagen type I deposition. Since TIMP1 inhibits collagenases and other enzymes, it may not induce liver fibrosis by itself, but can significantly exacerbate hepatic fibrosis (Yoshiji *et al*., 2000). In liver fibrosis, coincident with an increase in collagen deposition, an increase in the expression and release of TIMP1 occurs (Robert *et al*., 2016). In a rat model, where pulmonary fibrosis was induced by paraquat, not only MMP2 and MMP9, but also TIMP1 was upregulated, 21 days after the initial treatment (Wang *et al*., 2011). Furthermore, TIMP1 can modulate the expression of genes related to liver fibrosis, and so, TIMP1 pathway could be considered a potential target for therapeutic intervention of fibrotic diseases. In oviduct tissues there are some reports of TIMP1 expression (Buhi *et al*., 1997; Zampini, Argañaraz, Miceli & Apichela, 2014; Peng *et al*., 2015). In goat oviduct, TIMP1 was upregulated by E_2 and presented a positive effect on viability of cultured oviductal epithelial cells. For that, it was associated to fertilization process and early embryonic development (Peng *et al*., 2015). In equine endometritis some bacteria can induce the release of neutrophil extracellular traps (NETs), which can be implicated in in collagen

deposition in endometrium, namely by changing TIMP and TGFβ transcription (Rebordão *et al*., 2014). The results of our work, point out to some influence of TIMP1 on *COL1* transcription, at least in the infundibulum, since both variables presented a significant Pearson coefficient, and linear regression indicated that 53.9% of *COLI* transcription in infundibulum, could be ascribed to *TIMP1* transcription. Nevertheless, this pathway does not seem to have any particular effect either in *COL1* or *COL3* transcription in ampulla or isthmus.

In equine oviduct, none of the MMPs evaluated showed a significant correlation with collagen type transcription. However, *MMP2* transcription in endometrium presented a significant correlation with both types of collagen in infundibulum, and isthmus, while MMP9 was only correlated with *COL1* and *COL3* transcripts in isthmus. Concerning MMP9, it is usually elevated in lung tissues and bronchoalveolar lavage fluids obtained from mice lungs where fibrosis was induced after intratracheal administration (Cabrera *et al*., 2007). Also, MMP9 can activate TGFβ contributing to enhance the pool of this cytokine (Yu & Stamenkovic, 2000). In equine endometrium MMP9 was found in stromal and glandular epithelial cells, but with no significant differences in expression between young and old mares. Also, the expression of this enzyme did not significantly differ between mares with endometrosis and the control group (Aresu *et al*., 2012). MMP9 is mainly produced by leucocytes, and in horse, is more associated with acute inflammation, than with chronic conditions (Clutterbuck, Harris, Allaway & Mobasheri, 2010).

In the kidney, in an experimental study conducted in rats, it was concluded that Mmp2, was related with hydronephrosis and renal fibrosis, during unilateral ureteral obstruction (Tveitarås *et al*., 2015). In ocular lens, MMP2 plays a role in matrix contraction via TGFβ, which reinforces the relationship between MMPs and TGFβ (Eldred *et al*., 2012). In a group of ectopic tubal implantation cases, it was evaluated the expression of several MMPs and TIMPs, and related with hCG levels and the depth of invasion. It was concluded that the expression was higher in cases of deeper implantation of trophoblast (Qiu, Xie, Chen & Gemzell-Danielsson, 2011). According to these authors, an imbalance between MMPs/TIMPs expression at the ectopic implantation, may lead to extensive destructive degradation of the extracellular matrix. Matrix metalloproteinases are also present in the ovary, where they can be involved in diseases like polycystic ovarian syndrome (Light & Hammes, 2015). These results were lately confirmed by another research group, who also found a positive relation between the expression of MMP2 and MMP9, with the incidence of polycystic ovarian syndrome (Ranjbaran *et al*., 2016). Expression of MMP3 and TIMP2 were evaluated in serosal tissues from intraperitoneal organs and adhesions. It was observed that differences on their expression may predispose an organ to develop more adhesions than other (Chegini *et al*., 2002). Also, this same group argued that

serum levels of TIMP2 may be useful to identify individuals with an increased risk to develop adhesions. In equine endometrium, MMP2 is present in dilated and fibrotic endometrial glands, and in significant amounts in regions presenting peri-glandular fibrosis, which can indicate an active role in the development of chronic equine endometrial degenerative fibrosis/endometrosis (Walter, Handler, Miller, & Aurich, 2005). Still in equine endometrium, another group detected MMP2 in stromal cells by immunohistochemistry, and like MMP9, no difference was found between old and young mares (Aresu *et al*., 2012). In bovine oviduct, MMP2 among other proteins of MMPs, was evaluated during the estrous cycle. It was observed that MMP2 concentration was increased around time of ovulation, compared with luteal phase (Gabler, Killian & Einspanier, 2001). These authors concluded that MMPs, which were regulated distinctly in the oviduct, could be implicated in fertilization and early embryo development, as it was previously referred about the TIMP1, in the goat. Despite several experimental works have demonstrated the role of MMPs and even TIMPs in the development of fibrotic diseases, and others point out to their importance in reproductive functions in oviduct, there are not many, explaining their possible involvement in fibrogenesis in oviduct of mammal species. In mice, in which neutropenia was previously induced, hydrosalpinx was achieved by *Chlamydia muridarum* urogenital infection. Mice submitted to neutropenia, showed a significant reduction in MMP9 production, and after resolution of infection, a significant reduction in hydrosalpinx, providing evidences that neutrophils produce MMP9, which play a significant role in the development of fibrosis in oviduct (Lee, Schripsema, Sigar, Murray, Lacy & Ramsey, 2010). In conclusion, since MMPs are mostly released by leucocytes, and MMPs are generally involved in fibrotic processes in different organs, including some reproductive organs, and the endometrium is an organ with a very dynamic leucocyte population, it may justify the fact that *MMP9 and MMP2* transcription in the endometrium, may influence collagen transcription in oviduct.

In this experimental work, it was possible to conclude that PGs transcription was correlated with collagen transcription, particularly in ampulla and isthmus. In ampulla, *PTGES* seems to be inversely related with *COL3* transcription, while *AKR1C3* was positively associated with either *COL3* transcription in ampulla, or with *COL1* and *COL3* transcription in isthmus. Several studies have referred the anti-fibrotic role of PGE_2, and the pro-fibrotic action of $PGF_{2\alpha}$. For instance, in intestinal disease, PGE_2 significantly decreased intestinal inflammation and collagen deposition in a murine model (Baird, Lloyd, Lawrance, 2015). In hepatic fibrosis induced in rats, PGE_2 could mediate a reduction in the fibrotic process (Zakaria & El-Sisi, 2016). Also in cases of pulmonary fibrosis induced by bleomycin, $mPGE_2$ exerted an essential effect against pulmonary fibrogenesis via EP2 mediating signaling transduction (Wei *et al*.,

2014). PGE_2 can not only prevent differentiation of fibroblasts into myofibroblasts, but can even reverse the established myofibroblast differentiation, as it was demonstrated in a study carried out with human fibroblast obtained from patients with IPF (Garrison *et al*., 2013). In addition, $PGF_{2\alpha}$, has been implicated in the development of IPF and stimulates collagen production of lung fibroblast via FP (Oga *et al*., 2009; Olman, 2009; Aihara *et al*., 2013). In equine endometritis, prostaglandins may be related to endometrial fibrosis pathogenesis by stimulating collagen deposition (Rebordão *et al*., 2014). Also in skin, $PGF_{2\alpha}$ is implicated in fibrosis development (Kanno *et al*., 2013). In osteoarticular diseases, isoforms of $PGF_{2\alpha}$ were found in patients suffering from osteoarthritis. Collagen production by fibroblast-like synoviocytes was positively associated with $PGF_{2\alpha}$, which suggest a profibrotic role of this prostanoid, also in articular tissue (Remst, Blaney Davidson & van der Kraan, 2015). Based on the examples mentioned, and once that both enzymes related with PGs synthesis (PTGES and AKR1C3) were correlated with collagen transcription, we may conclude that these hormones are implicated in collagen deposition in equine oviduct, particularly in isthmus.

In equine oviduct, although TGFβ mRNA was not evaluated, its receptors ALK5 and TGFβRII, were assessed. Intestingly both receptors were significantly correlated with either COL1 or COL3 transcription in isthmus. In human medicine, there is a tremendous interest about the role of TGFβ in the development of fibrotic disease, in several organs. For instance, in chronic kidney disease (CKD), TGFβ is considered the "master regulator of fibrosis" (Meng, Nikolic-Paterson, & Lan, 2016). Several articles have demonstrated the influence of TGFβ in CKD, either about its origin (mainly from macrophages), either about its influence on the expression of secondary molecules/proteins, also involved in the process (Meng, Chung & Lan, 2013; Shen, Liu, Fan & Qiu, 2014; Muñoz-Félix, González-Núñez, Martínez-Salgado & López-Novoa, 2015). Besides that, and based on the influence that TGFβ has on CKD, the demand for new molecules which could represent a possible treatment for this disease has been pursued (Wu, Shi, Lu, Ma & Cheng, 2015; Park *et al*., 2013). Several studies have addressed the role of TGFβ in liver fibrosis, as well as possible modalities of treatments (Tu *et al*., 2014; Roy *et al*., 2015; Crosas-Molist, Bertran, & Fabregat, 2015). Also in cardiac fibrosis, several mediators may be implicated in the process, including TGFβ (Kong, Christia & Frangogiannis, 2014). In equine endometrium, it was proved that prolonged exposure to NETs, up-regulated TGFβ transcription, also confirming its influence in the development of equine endometrosis (Rebordão *et al*., 2014).

Nodal is a member of TGFβ superfamily, that has been under study in the reproductive tract. It has already been identified in bovine uterus and oviduct (Argañaraz *et al*., 2013), and in mare endometrium (Morazzo *et al*., 2017). Nevertheless, its functional importance has not been

established, but it was related either with pregnancy maintenance and/or fertility process in the cow (Argañaraz *et al*., 2013). In contrast, in the mare, Nodal might be involved in endometrium fibrosis (Morazzo *et al*., 2017). Based on the evidences that TGFβ is implicated in fibrogenic processes in several organs, our results suggest that this cytokine is also involved in the collagen deposition in equine oviduct, in particular in the isthmus.

Since fibrosis is a very complicated and multi-factorial process, and it may be implicated in several organ functions, including the oviduct, therefore, further studies in this area is recommended for a better understanding of its involvement in equine fertility.

CHAPTER VI – CONCLUSIONS

The results reached with this work allow for a better understanding of some mechanisms implicated in oviduct regulation. These data put forth and strengthen the hypothesis that exacerbated collagen deposition in equine oviduct may be responsible for infertility and point us possible players in this pathological process.

Regarding the results from these four studies, it is possible to draw the following conclusions:

1. Ovarian steroid hormones, oxytocin, and PGs modulate oviductal function in a coordinated mode, providing proper conditions for regular oviductal function.
2. Angiogenic factors FGF1, FGF2, VEGF and respective receptors expression in equine oviduct, agree with microvascular density changes.
3. A higher expression of OVGP was detected in isthmus and in the follicular phase.
4. Sperm cells will increase OVGP secretion, providing an adequate milieu for fertilization and early embryo development.
5. These data suggest that OVGP is important for sperm cells final preparation and/or capacitation, since it is in the isthmus, and during follicular phase, that male gametes will be present in this oviduct segment
6. Mare oviduct can suffer fibrosis process since COL1 and COL3 present differences on their expression.
7. Transcription of *COL1* and *COL3* in equine oviduct is correlated with transcription of the same genes in endometrium
8. Mares endometrium classified in category III presents the same relationship between COL1/COL3 as the isthmus. Therefore, we might conclude that in these cases, endometrium can reflect what is occurring in the isthmus.
9. Transcription of *COL1* and *COL3* is correlated and dependent of several pathways in oviduct.
10. Some genes in endometrium, which can be implicated in collagen expression in this organ, are also linked to *COL1* and *COL3* transcription in oviduct, confirming a linkage of fibrosis between these two organs.
11. Collagen expression can be differentially up-regulated by ovarian steroids and oxytocin, since they stimulated mRNA or protein expression in different estrous cycle phases.
12. In equine oviduct, in contrast to other organs in other animal species, TNFα does not influence collagen expression.

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