GABRIELA PACHECO MENDES

# Efeito dos tratamentos de estimulação e superovulação usando gonadotrofina coriônica equina (eCG) na expressão de genes relacionados à modelagem celular e angiogênese no corpo lúteo bovino


São Paulo

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Dissertação apresentada ao Programa de Pós-Graduação em Anatomia dos Animais Domésticos e Silvestres da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo para obtenção do Título de Mestre em Ciências

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**Orientador (a):**
Profa. Dra. Paula de Carvalho Papa

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UNIVERSIDADE DE SÃO PAULO

FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA

*Comissão de Ética no uso de animais*

# CERTIFICADO

Certificamos que o Projeto intitulado "Análise das vias de sinalização intracelular no corpo lúteo de bovinos submetidos a tratamentos com eCG", protocolado sob o nº 2715/2012, utilizando 18 (dezoito) vacas, sob a responsabilidade do(a) Profa. Dra. Paula de Carvalho Papa, está de acordo com os princípios éticos de experimentação animal da "Comissão de Ética no uso de animais" da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo e foi aprovado em reunião de 15/8/2012.

We certify that the Research "Analysis of intracellular signaling pathways in a cattle corpus luteum subject to treatment with eCG", protocol number 2715/2012, utilizing 18 (eighteen) cows, under the responsibility Profa. Dra. Paula de Carvalho Papa, agree with Ethical Principles in Animal Research adopted by "Ethic Committee in the use of animals" of the School of Veterinary Medicine and Animal Science of University of São Paulo and was approved in the meeting of day 8/15/2012.

São Paulo, 16 de agosto de 2012.

Denise Tabacchi Fantoni
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Instituição:_____Julgamento:_____

# *DEDICATÓRIA*

*Deus infinitamente bom, que seu nome seja bendito pelos benefícios que me foram concedidos. Eu seria indigno se os atribuísse ao acaso dos acontecimentos ou ao meu próprio mérito.*

*Aos bons espíritos que foram os executores da vontade divina, e principalmente ao meu anjo guardião, o meu agradecimento. Peço-lhes que afastem de mim o sentimento do orgulho e da vaidade, assim como de usá-los de outra forma que não seja para o bem.*

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## RESUMO

MENDES, G. P. **Efeito dos tratamentos de estimulação e superovulação usando gonadotrofina coriônica equina (eCG) na expressão de genes relacionados à modelagem celular e angiogênese no corpo lúteo bovino.** [Effect of superovulatory and stimulatory treatments using equine chorionic gonadotropin (eCG) on the expression of genes related to cellular modeling and angiogenesis in the bovine corpus luteum]. 2014. 40 f. Dissertação (Mestrado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2014.

O uso da gonadotrofina coriônica equina (eCG) tem sido considerado uma potencial ferramenta nos protocolos de estimulação e superovulação para melhorarem o desempenho reprodutivo dos rebanhos. Este fato levou a hipótese de que o tratamento com eCG altera as vias de sinalização intracelular no corpo lúteo (CL) formado. Para testá-la, 18 vacas mestiças de Nelore (*Bos indicus*) foram divididas em três grupos: controle, estimulado e superovulado. O protocolo de sincronização da ovulação com o uso do dispositivo de P4 foi descrito anteriormente (FÁTIMA et al., 2013). O grupo estimulado recebeu 400 UI de eCG no dia da remoção do dispositivo de P4 e o superovulado recebeu 2000 UI de eCG 4 dias antes. Os animais dos grupos controle e estimulado receberam GnRH no dia 10, enquanto os animais superovulados receberam no dia 8. No sétimo dia após  após a administração do GnRH, todos os animais foram abatidos e os CL foram coletados. Foi realizada análise de microarranjo, a partir da qual foram eleitos genes envolvidos na sinalização da esteroidogênese (*ADM, MMP9, NOS2*), da ativação das metaloproteinases da matriz e do receptor ativado de protease que regula a homeostase e inflamação (*PRSS2, PLAU*) e da angiogênese (*ANG e ANGPT1*) e validados por qPCR, western blotting e imuno-histoquímica. A expressão gênica e proteica do PRSS2 e MMP9 foi menor e as células luteínicas grandes e pequenas positivas apresentaram marcação menos intensa nos grupos estimulado e superovulado ($P < 0,05$). A expressão proteica da ANG foi maior no estimulado ($P=0,01$) e superovulado ($P=0,03$) e da proteína ANGPT1 foi maior no estimulado ($P=0,008$). O número de células luteínicas grandes e pequenas positivas para ANG e ANGPT1 foram maiores nos grupos tratados ($P < 0,05$). No estimulado, houve correlação negativa entre os níveis de progesterona e o *MMP9* ($r = -0,66$ e $P = 0,03$) e com a proteína do PRSS2 ($r = -0,63$ e $P = 0,04$). No entanto, houve correlação positiva com a proteína ANG ($r = 0,69$ e $P = 0,03$). No superovulado, houve correlação positiva entre a *ANG e ANGPT1* ($r = 0,96$ e $P = 0,001$). Não houve diferença na expressão de ADM, NOS2 e PLAU nos grupos tratados em relação ao controle. Em resumo, os resultados

obtidos são indicativos de que a eCG altera a expressão relativa de genes e proteínas envolvidas nas vias de sinalização de inflamação e a modelação da membrana celular com uma diminuição na expressão do MMP9 e da PRSS2 e na via da angiogênese com maior expressão de ANG em ambos os tratamentos e ANGPT1 em vacas estimuladas.

Palavras-chave: Corpo lúteo. eCG. Estimulação folicular. Superovulação.

## ABSTRACT

MENDES, G. P. **Effect of superovulatory and stimulatory treatments using equine chorionic gonadotropin (eCG) on the expression of genes related to cellular modeling and angiogenesis in the bovine corpus luteum.** [Efeito dos tratamentos de estimulação e superovulação usando gonadotrofina coriônica equina (eCG) na expressão de genes relacionados à modelagem celular e angiogênese no corpo lúteo bovino]. 2014. 40 f. Dissertação (Mestrado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2014.

The use of equine chorionic gonadotropin (eCG) have been considered a potential tool in stimulation and superovulation protocols to improve the reproductive performance of the herd. This fact led to the hypothesis that eCG treatment alters the intracellular signaling pathways in the formed CL. To test that, 18 crossbred Nellore (*Bos indicus*) cows were divided into three groups: control, stimulated and superovulated. The synchronization protocol with the use of P4 device was previously described (FÁTIMA et al., 2013). The stimulated group received 400 IU eCG at the P4 device removal and superovulated received 2000 IU eCG 4 days before it. Both control and stimulated animals received GnRH on day 10, while superovulated ones received it on day 8. On the seventh day after GnRH administration, CL were collected by slaughter. Microarray analysis was preformed, from which were selected genes involved in the signaling of steroidogenesis (ADM, MMP9, NOS2), activation of matrix metalloproteinases and protease activated receptor that regulates homeostasis and inflammation (PRSS2, PLAU), and angiogenesis (ANG and ANGPT1). They were evaluated by qPCR, western blot and immunohistochemistry. The gene and protein expression of MMP9 and PRSS2 decreased and large and small luteal cells showed weaker staining in stimulated and superovulated groups related to the control group ($P < 0.05$). ANG protein expression was higher on superovulated ($P=0.01$) and stimulated ($P=0.03$) and ANGPT1 protein was higher only in stimulated ($P=0.008$). The number of positive large and small luteal cells for ANG and ANGPT1 were higher in treated groups ($P<0.05$). In stimulated, there were a negative correlation between progesterone levels and *MMP9* ($r = 0.03$ and $P = -0.66$) and the PRSS2 protein expression ($r = 0.04$ and $P = -0.63$). However, there were a positive correlation with ANG protein expression ($r = 0.69$ and $P = 0.03$). In superovulated, there were a positive correlation between *ANG* and *ANGPT1* ($r = 0.96$ and $P = 0.001$) and PRSS2 protein expression was negatively correlated with the ANG protein ($r = 0.04$ and $P = -0.96$). There was no difference in ADM, NOS2 and PLAU expression in treated

groups compared to control. In summary, these findings indicate that eCG alters the relative expression of genes and proteins involved in inflammation and cell modeling signaling pathways by decreasing MMP9 and PRSS2 expression, and on angiogenesis pathway, increasing ANG expression in both stimulated and superovulated animals and ANGPT1 in stimulated cows.

Keywords: Corpus luteum. eCG. Follicle stimulation. superovulation.

**SUMÁRIO**

# 1 EFFECT OF SUPEROVULATORY AND STIMULATORY TREATMENTS USING EQUINE CHORIONIC GONADOTROPIN (ECG) ON THE EXPRESSION OF GENES RELATED TO CELLULAR MODELING AND ANGIOGENESIS IN THE BOVINE CORPUS LUTEUM.

## 1.1 ABSTRACT

The use of equine chorionic gonadotropin (eCG) have been considered a potential tool in stimulation and superovulation protocols to improve the reproductive performance of the herd. This fact led to the hypothesis that eCG treatment alters the intracellular signaling pathways in the formed CL. To test that, 18 crossbred Nellore (*Bos indicus*) cows were divided into three groups: control, stimulated and superovulated. The synchronization protocol with the use of P4 device was previously described (FÁTIMA et al., 2013). The stimulated group received 400 IU eCG at the P4 device removal and superovulated received 2000 IU eCG 4 days before it. Both control and stimulated animals received GnRH on day 10, while superovulated ones received it on day 8. On the seventh day after GnRH administration, CL were collected by slaughter. Microarray analysis was preformed, from which were selected genes involved in the signaling of steroidogenesis (ADM, MMP9, NOS2), activation of matrix metalloproteinases and protease activated receptor that regulates homeostasis and inflammation (PRSS2, PLAU), and angiogenesis (ANG and ANGPT1). They were evaluated by qPCR, western blot and immunohistochemistry. The gene and protein expression of MMP9 and PRSS2 decreased and large and small luteal cells showed weaker staining in stimulated and superovulated groups related to the control group ($P < 0.05$). ANG protein expression was higher on superovulated ($P=0.01$) and stimulated ($P=0.03$) and ANGPT1 protein was higher only in stimulated ($P=0.008$). The number of positive large and small luteal cells for ANG and ANGPT1 were higher in treated groups ($P<0.05$). In stimulated, there were a negative correlation between progesterone levels and *MMP9* ($r = 0.03$ and $P = -0.66$) and the PRSS2 protein expression ($r = 0.04$ and $P = -0.63$). However, there were a positive correlation with ANG protein expression ($r = 0.69$ and $P = 0.03$). In superovulated, there were a positive correlation between *ANG* and *ANGPT1* ($r = 0.96$ and $P = 0.001$) and PRSS2 protein expression was negatively correlated with the ANG protein ($r = 0.04$ and $P = -$

0.96). There was no difference in ADM, NOS2 and PLAU expression in treated groups compared to control. In summary, these findings indicate that eCG alters the relative expression of genes and proteins involved in inflammation and cell modeling signaling pathways by decreasing MMP9 and PRSS2 expression, and on angiogenesis pathway, increasing ANG expression in both stimulated and superovulated animals and ANGPT1 in stimulated cows.

Keywords: Corpus luteum. eCG. Follicle stimulation. Superovulation.

## 1.2 INTRODUCTION

The use of equine chorionic gonadotropin (eCG) has been considered a potential tool for improving plasma progesterone (P4) concentration in the subsequent estrus cycle (BO et al., 1994; SÁ FILHO et al., 2010) and a positive correlation between P4 concentration and pregnancy rates in cattle has been described (SÁ FILHO et al., 2010). In *Bos indicus*, the hormonal treatment with the administration of 300-400 IU eCG before ovulation improved follicular development and increased P4 concentrations on early pregnancy (BARUSELLI et al., 2004). The eCG is a glycoprotein that has the ability to bind to both follicle stimulating hormone (FSH) and luteinizing hormone (LH) receptors in ruminant granulosa or theca cells, which is required for maturation of periovulatory follicles. The eCG half-life is estimated to be 45 h in cows bloodstream allows gonadotropin support before ovulation (MURPHY; MARTINUK, 1991; MURPHY, 2012). Additionally, the use of eCG is important in postpartum anestrus cows with deficiency in LH pulses (ROCHE; MACKEY; DISKIN, 2000).

The establishment of pregnancy in cows requires a cross talk involving the corpus luteum (CL), the uterus and the embryo. Composed by steroidogenic (large and small luteal cells) and nonsteroidogenic cells (endothelial cells, fibroblasts, stroma cells, macrophages and monocytes), the CL undergo an orchestrated growth (MANN; LAMMING, 2001; SKARZYNSKI; OKUDA, 2010; PRING et al., 2012; PROKOPIOU et al., 2013; PUGLIESI et al., 2014). After day 7 of the estrus cycle, the volume of the CL does not increase, its development is due to a hypertrophy of large luteal cells, increase in the number of small luteal cells (SKARZYNSKI et al., 2013; YOSHIOKA et al., 2013) and an increase of P4 production (SKARZYNSKI et al., 2013). Luteal cells produce progesterone (P4), important to promote embryo growth, elongation and the secretion of interferon-tau by trophoectoderm embryonic cells, thus preventing luteolysis (STOCCO; TELLERIA; GIBORI, 2007; FAIR; LONERGAN, 2012; O'HARA et al., 2014).

Data from our research group showed global changes on the expression of genes belonging to pathways related to tissue development and angiogenesis (fibroblast growth factor receptor 2 - FGFR2, transforming growth factor, beta 2 - TGFB2), lipids metabolism (fatty acid binding protein 5 - FABP5, 3-hydroxy-3-methylglutaryl-CoA reductase - HMGCR, peroxisome proliferator-activated receptor gamma - PPARG), and steroidogenesis such as

steroidogenic acute regulatory protein (STAR), binding protein 5 fatty acids (FABP5), cytochrome P450, family 27, subfamily a, polypeptide 1 (CYP271A), acyl-CoA synthetase family member 2 (ACSF2), 3-hydroxy-3 methylglutaryl-CoA reductase (HMGCR) gamma receptor peroxisome proliferator-activated (PPARG), short prolactin receptor (PRLRS) long prolactin (PRLRL) and follistatin (FÁTIMA et al., 2013). In addition, important morphological changes as an increase in the density of mitochondria on stimulated and superovulated animals as well as an increase in the number of large and small luteal cells were observed (RIGOGLIO et al., 2013).

Nevertheless, the molecular effects of these treatments with eCG in luteal cells are strong candidates to explain changes in the production of P4 and morphology. Thereby, starting from a global analysis of differentially expressed genes led to selection of seven genes, which are: matrix metalloproteinase 9 (MMP9) and trypsin 2 (PRSS2) by architecting signaling to tissue destruction and construction of the CL (SORSA et al., 1997; ROSEWELL et al., 2011; PROKOPIOU et al., 2013), Angiogenin (ANG) and Angiopoetin 1 (ANGPT1) involved in the initiation and establishment of angiogenesis (HURLIMAN et al., 2010; SHENG et al., 2014). ANG interacts with endothelial cells and smooth muscle cells to induce responses such as cell migration, invasion, proliferation and formation of tubular structures. For the induction of angiogenesis by ANG is required activity of ribonuclease, degradation of the basement membrane signal transduction and nuclear translocation (GAO; XU, 2008) and has been reported to facilitate luteal vascular growth on CL (BERISHA; MEYER; SCHAMS, 2010). ANGPT-1 have been demonstrated to be involved with perifollicular capillary network changes at the time of the LH surge and may contribute to maintaining and stabilizing the vessel during the final follicular development, ovulation and early CL formation (MIYABAYASHI et al., 2005). The ANGPT1 recruits and interact with periendothelial cells, promoting the stabilization and maintenance of the vessels developed by the vascular endothelial growth factor (VEGF) (TANAKA et al., 2004) serving for vascularization during the follicular development and CL (HAZZARD; CHRISTENSON; STOUFFER, 2000; HAYASHI et al., 2004), and nitric oxide synthase 2, inducible (NOS2) and adrenomodullin (ADM) related to CL steroidogenic function (JABLONKA-SHARIFF; OLSON, 1997; LI; O; TANG, 2011). Sustained nitric oxide (NO) synthesis results in the expression of NOS2 (JAROSZEWSKI et al., 2003) many studies suggest that most effects of NO are performed by binding iron-containing enzymes leading its regulatory role on steroidogenesis can be carried through a direct inhibition of the cytochrome P450 steroidogenic enzyme aromatase (VAN VOORHIS et al., 1995). FSH was unable to stimulate NOS2 mRNA abundance in bovine

granulose cells (ZAMBERLAM et al., 2011). ADM found in human granulosa cells acts as a local factor which increases the production of progesterone as well as ADM is a potent vasodilator and angiogenic factor, it also participates in the formation of CL mainly on the dynamics of vascular structure changes of this organ (MORIYAMA; OTANI; MARUO, 2000). ADM decreased when FSH-stimulated estradiol release in rat follicles as well as eCG-stimulated progesterone release in rat corpora lutea. At estrus, the diminished in ADM would enhance the FSH effect on steroidogenesis, whereas at diestrus, the increase in ADM would attenuate the effect of eCG on progesterone secretion (LI, et al., 2008). In addition, plasminogen activator urokinase (PLAU), which appers to be involved in luteal maintenance (LIU et al., 1997). The remodeling of the connective tissue and tissue degradation is associated with maintenance of the function and luteal regression. The PLAU, as its receptor (PLAUR) allows monocytes, neutrophils and activated T cells migrate to the basement membrane by degrading laminin (PLESNER; BEHRENDT; PLOUG, 1997; KLIEM et al., 2007). In bovine granulosa cells, PLAU increased its expression after FSH and Insulin-like growth factor-1 (IGF-I) stimulation (CAO et al., 2006). However, in rodent granulosa cells PLAU was downregulated by FSH (MACCHIONE et al., 2000).

Therefore, we can infer that the CL requires coordinated action between genes belonging to different pathways for their optimum performance, and hormone treatment-induced changes can modify the balance of the organ. Therefore, the present study hypothesized that treatments, which aimed to stimulate the dominant follicle and increase of the number of ovulated follicles, using eCG alters the expression of genes that participate in signaling pathways related to angiogenesis, the development of CL and, consequently, P4 production.

## 1.3 MATERIALS AND METHODS

### 1.3.1 *Experimental design*

All procedures were carried out in accordance with Committee in Ethics for the Use of Experimental Animals of the Faculty of Veterinary Medicine and Animal Sciences,

University of São Paulo, São Paulo, Brazil (protocol number 2715/2012). Animals were treated as previously described (FÁTIMA et al., 2013). Briefly, 18 Nellore crossbred (*Bos indicus*) cows, aged between 2 and 5 years, were divided into three groups: Control (n=5), stimulated (n=6) and superovulated (n=7). All cows received 2 mg of estradiol benzoate (BE; Estrogin, Farmavet, São Paulo, Brazil) plus 1g of progesterone implant (bovine intravaginal device; progesterone: 1g; Primer, Technopec Brazil) on day 0. On day 8, for the stimulated group, the progesterone device were removed and 0.150 mg of d-cloprostenol (PGF2α, Prolise, Arsa, Buenos Aires, Argentina) was administered plus 400 IU of eCG (Novormon, Syntex, Buenos Aires, Argentina) were administrated (SÁ FILHO et al., 2010; SALES et al., 2011). Ovulation was induced with 0.025 mg of GnRH (Gestran Plus, Arsa, Buenos Aires, Argentina) given 48 hours after the P4 device removal. The superovulatory group received 2000IU eCG on day 4 and 0.150 mg of PGF2α on day 6 (BARUSELLI et al., 2011). The devices were removed and a second dose of PGF2α was administered on day 7. Ovulation was induced with 0.025 mg of GnRH twelve hours after device removal. In the control group, the same treatment as stimulated group was performed, but without the administration of eCG. On day 5 after ovulation, cows underwent ultrasound evaluation and on day 6 after ovulation, all animals were slaughtered. CL were collected, weighed, measured, and frozen in liquid nitrogen or fixed in 4% phosphate-buffered formalin solution for 24 hours for further analysis.

### *1.3.2 Microarray analysis*

Microarray analysis was performed as previously described (FÁTIMA et al., 2013). Firstly, total RNA was isolated using Trizol (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions, and purified using RNAeasy mini kit (Qiagen, Valencia, CA, USA). RNA concentration and purity were assessed by NanoDrop 2000 device (Thermo Scientific, Hudson, USA). Only RNA with integrity number (RIN) greater than or equal to 9 were used. The microarray analyzes were performed at the McGill University and Génome Québec Innovation Centre, Montreal, Canada using the Affymetrix GeneChip Bovine Genome Array (Affymetrix, Santa Clara, USA), containing 23,000 sets of probes and scanned in the GeneChip ® Scanner 3000 (Affimetrix, Santa Clara, CA). The experiments were performed using three samples of each group. The complete microarray data are

deposited in NCBI database bank (GEO http://www.ncbi.nlm.nih.gov/geo), accession number GSE37844. These data generated a list of genes increased (242) or decreased (111) genes in in the stimulated group and increased (111) or decreased (113) in the superovulated group compared to the control group (fold change 1.5 and $P \leq 0.05$). The differently expressed gene list was analyzed in Ingenuity Pathway Analysis 7 software (IPA, Ingenuity Systems, San Francisco, CA; http://www.ingenuity.com/). Therefore, from this analysis, genes related to intracellular signaling pathways were selected to be investigated.

### *1.3.3 Immunohistochemistry for MMP9, PRSS2, ANG, ANGPT1, ADM, NOS2 and PLAU*

Immunoperoxidase method was used to detect MMP-9, Trypsin-2, iNOS, Angiogenin, Angiopoietin-1, AM and uPA proteins in 2 μm tissue sections prepared from on CL per cow using 3 section per CL for each animal per group to assure accuracy of description as already published (MONA E PINTO et al., 2014). The primary antibody (Frame 1) for each protein was diluted in PBS at the following dilutions: MMP-9 1:100; Trypsin-2 1:1000; iNOS 1:200; Angiogenin 1:100; Angiopoietin-1 1:200; AM 1:1000; uPA 1:300 and incubated for overnight at 4 °C. Negative controls were prepared using IgG isotype control antibodies (Normal rabbit IgG for all antibodies; Santa Cruz Biotechnologies, USA). The slides were observed with an Olympus BX 50 microscope equipped with a CCD color video camera (Olympus DP71; Olympus America Inc, Center Valley, PA, USA), and the images were captured using Axio Vision software (Carl Zeiss, Oberkochen, Germany). For quantification of stained positive cells for each protein studied, eight photos in three slides from different regions for each animal were performed and approximately 200 stained positive cells were manually counted.

Frame 1 - Antibodies used for immunohistochemistry and western blotting

| **Antibodies** | **Isotype** | **Immunogen** | **Dilution** | **Supplier (order no.)** |
|---|---|---|---|---|
| MMP-9 | Rabbit Polyclonal IgG | 382-393 Human MMP9 protein | 1:1000 | Biorbyt (orb13583) |
| Trypsin-2 | Rabbit Polyclonal IgG | native trypsinogen from pancreas | 1:1000 | MyBioSource company (MBS622131) |
| iNOS | Rabbit Polyclonal IgG | cytokine-induced murine macrophages | 1:500 | Lifespan Biosciences (LS-11686/37570) |
| Angiogenin | Rabbit Polyclonal IgG | 101-200 Human ANG protein | 1:1000 | Biorbyt (orb101736) |
| Angiopoietin-1 | Rabbit Polyclonal IgG | N-term of Human ANGPTL1 | 1:1000 | Abbiotec (251299) |
| AM | Rabbit Polyclonal IgG | Purified human ADM | 1:500 | Elsasser et al., 2007 |
| uPA | Goat Polyclonal IgG | Purified human PLAU | 1:500 | Acris (AP022255SU-N) |

Source : (MENDES, G. P., 2014).

*1.3.4 Western Blotting*

CL samples were homogenized in buffer containing 50 mM potassium phosphate (pH 7.0), 0.3 M sucrose, 0.5 mM dithiothreitol (DTT), 1 mM ethylenediaminetetraacetic acid (EDTA, pH 8.0), 0.3 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM NaF, and phosphatase inhibitor cocktail (1:100; Sigma-Aldrich). Total protein content was determined using the Bradford method (BRADFORD, 1976) and values were extrapolated against a standard curve of albumin read at 595 nm. Samples containing equal amount (50µg) of proteins were boiled for 5 min in Laemmli solution (0.05 M Tris, pH 6.8, 2% SDS, 15% glycerol, 0.05% bromophenol blue, 9% SDS and 6% 2-mercaptoethanol), and run on a 10% - 15 SDS-PAGE mini gel (Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis) depending on the protein molecular weight, 100 V. After electrophoresis, the proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, CA) under constant current of 120 mA for 2 hours at 4 ° C in Tris HCl 12.5mM, 95 mM glycine, 20% methanol, pH 8.3. Membranes were blocked for 1 h at room temperature with TTBS (0.9% NaCl, 100 mM Tris, 0.1% Tween 20, pH 7.4) containing 5% non-fat dried milk, and incubated overnight, at $4^{o}$C, with primary antibodies (Frame 1). After incubation, the membranes were washed three times with TBS-T and incubated for 1 h with anti-rabbit IgG-peroxidase conjugated (ECL,GE Healthcare, Buckinghamshire, UK) or anti-goat IgG-peroxidase conjugated (Merk Millipore, Darmstadt, Germany) diluted 1:5000 in TTBS. After washing, the proteins were visualized by Enhanced chemiluminescense kit (Amersham Biosciences, Piscataway, NJ, USA). The same blots were stripped and incubated with anti-β-actin monoclonal antibody (MAB 1637; Chemicon International, Inc.) as endogenous control. Images were captured by ChemiDoc MP Image system (Bio-Rad Laboratories) and normalized to actin-beta (ACTB; 42 kDa) using ImageJ Software (Bio-Rad Laboratories).

*1.3.5 Real time PCR for MMP9, PRSS2, ANG, ANGPT1, ADM, NOS2 and PLAU*

A fixed amount of 1 ug RNA was used for cDNA synthesis using Superscript III (Life Technologies, Carlsbad, CA, USA) following manufacturer's instructions. Quantitative real-

time PCR assays were performed as previously described (Fátima et al., 2013) using bovine-specific TaqMan Gene Expression Assays (Frame 2; LifeTechnologies). PCRs for target genes were conducted in duplicates for each sample and the expression was determined by normalizing their signals with GAPDH, tubulin and β-actin signals as housekeeping genes, determined by NormFinder software (ANDERSEN; JENSEN; ØRNTOFT, 2004). The amplification efficiency was analyzed by LinRegPCR program (RAMAKERS et al., 2003), followed by Pfaffl's method (PFAFFL, 2001).

Frame 2 - Primers used for Real Time PCR bovine-specific TaqMan gene expression custom designed assays produced by LifeTechnologies

| **Gene** | **Sequences** | **GenBank nº** |
|---|---|---|
| MMP9 | ID Bt03216000_g1 | AF_135234.1 |
| PRSS2 | ID Bt 03224030_m1 | BC_134794.1 |
| NOS2 | ID Bt03249599_m1 | AF_340236.1 |
| ANG | ID Bt03279285_m1 | BC_111623.1 |
| ANGPT1 | ID Bt03249558_m1 | AF_032923.1 |
| ADM | ID Bt03251626_g1 | AB_055107.1 |
| PLAU | ID Bt03212963_m1 | AF_144761.1 |
| GAPDH | ID Bt03210915_g1 | AB_098979.1 |
| A-Tubulin | ID AIY9YWA | BT_0323101 |
| β-actin | ID Bt03279174_g1 | AB_098930.1 |

Source : (MENDES, G. P., 2014).

### *1.3.6 Statistical Analysis*

The statistical analysis of the microarray data was conducted according to the random variance model of Wright and Simon (2003; significance analysis of microarrays (SAM)). Data from real-time PCR, western blotting and immunohistochemistry were tested for a normal distribution (Anderson–Darling test) and data that did not follow a normal distribution were transformed into logarithms. Differences between control and stimulated group were analyzed by Student's t-test and for the three groups were performed one-way ANOVA followed by the Tukey test. The level of significance was set at $P < 0.05$ for all analyses. The data are presented as the mean ± S.E.M. The statistical analyses were performed using the

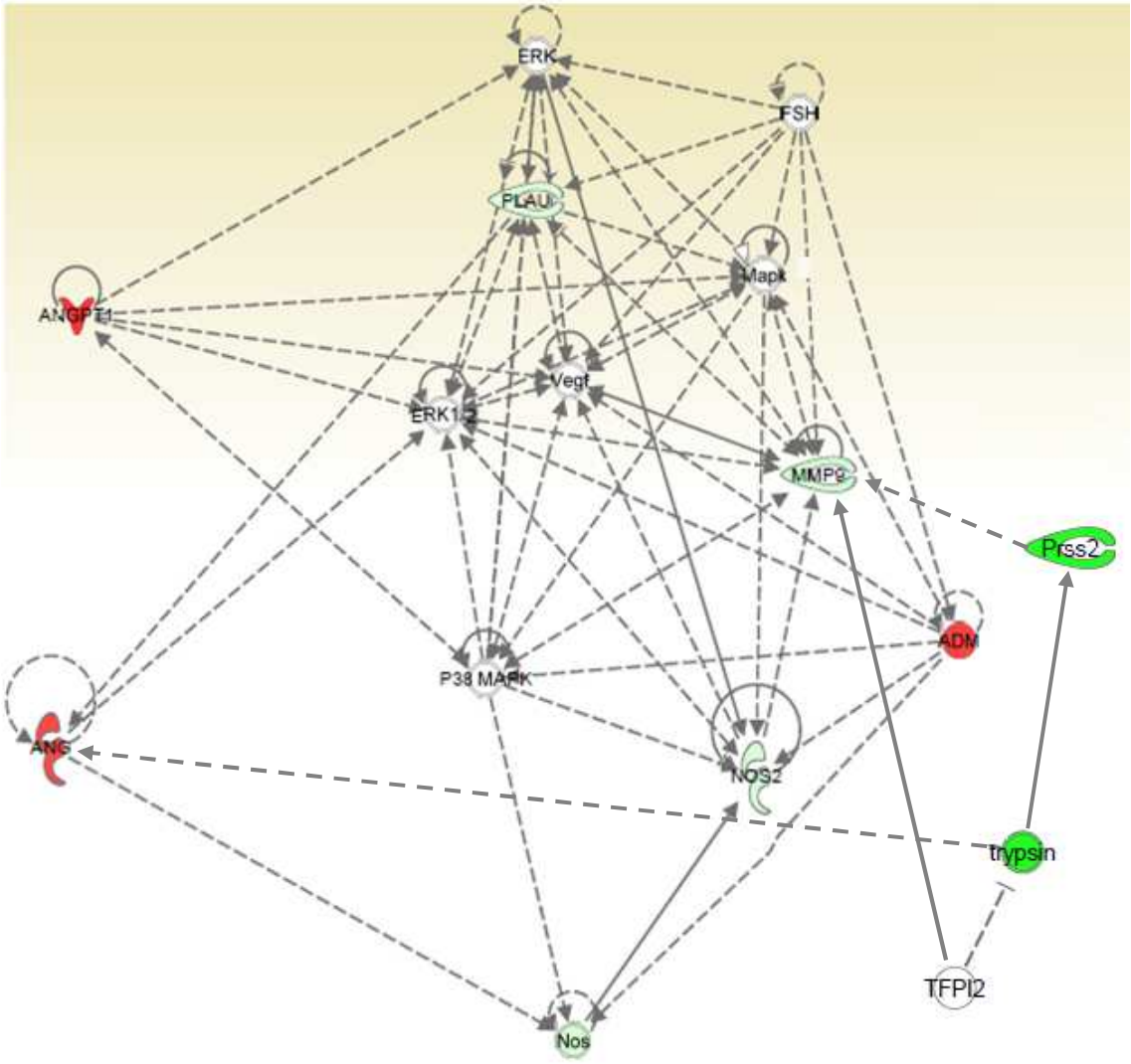
GraphPad Prism software (Version 4.00 for Windows; GraphPad Software, San Diego, CA, USA). Correlations were considered significant at $P \leq 0.05$ using the Pearson correlation test.

## 1.4 RESULTS

### *1.4.1 Microarray*

After submitting the differently expressed gene list to Ingenuity Pathway network functions (Figure 1), seven differently expressed genes that were somehow interconnected were chosen as demonstrated in Table 1.

Figure 1 - Interaction between genes made from the results of the microarray and organized by the IPA program; (red: more intense more increased; blank: not changed; Green: less intense more decreased)




| **Legend** | | | | | |
|---|---|---|---|---|---|
| | Enzyme | | Peptide | | Citoquinase / Growth Factor |
| | Transcriptional regulator | | Complex / Group / Other | | Kinase |
| ——— | Direct interaction | A→B | Acts and inhibits | A⊣B | Inhibits |
| ..... | Indirect interaction | A→B | Acts | | |

Source: (MENDES, G. P., 2014).

Table 1 - Genes differentially expressed in stimulated and superovulated groups with eCG compared to the control group

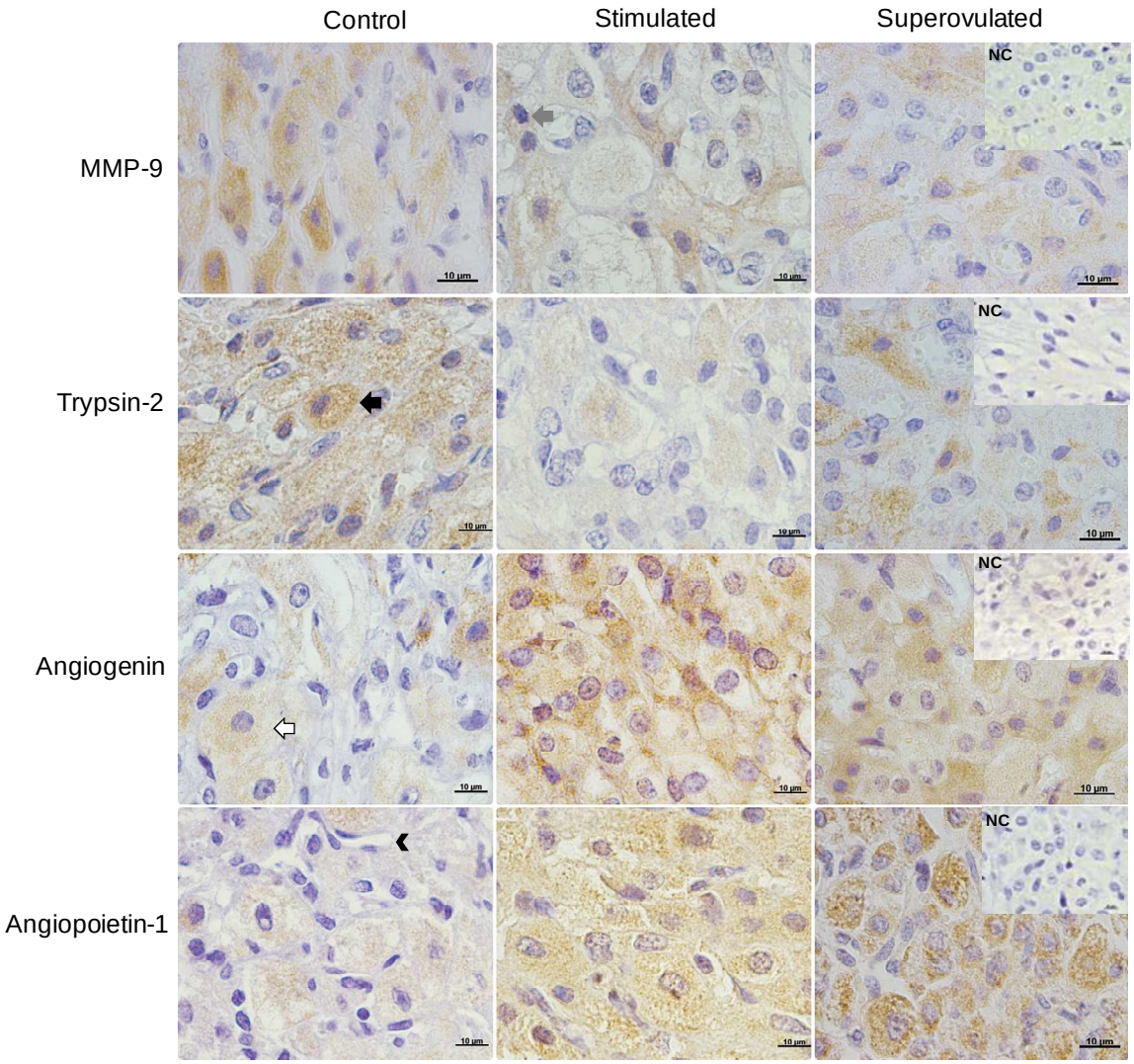
| Gene | Symmetrical raw fold change Stimulated x Control | P value | Symmetrical raw fold change Superovulated x Control | P value |
|---|---|---|---|---|
| MMP-9 | -2,260616* | 3,42E-05* | -2,06436* | 0,004* |
| PRSS2 | -11,60022* | 5,43E-08* | 1,908976 | 0,050 |
| NOS2 | -1,957681* | 0,007* | -1,525* | 0,003* |
| ANG | 1,630326 | 0,012 | | |
| ANGPT1 | 1,830203 | 0,009 | | |
| ADM | 1,692821 | 0,024 | | |
| PLAU | -1,613261* | 0,017* | | |

*Indicates that genes were downregulated (1.5 fold, $P < 0.05$).
Source: (MENDES, G. P., 2014).

### *1.4.2 Immunolocalization of MMP-9, Trypsin-2, AM, iNOS, Angiopoietin-1, Angiogenin and uPA on bovine CL from control, stimulated and superovulated cows*

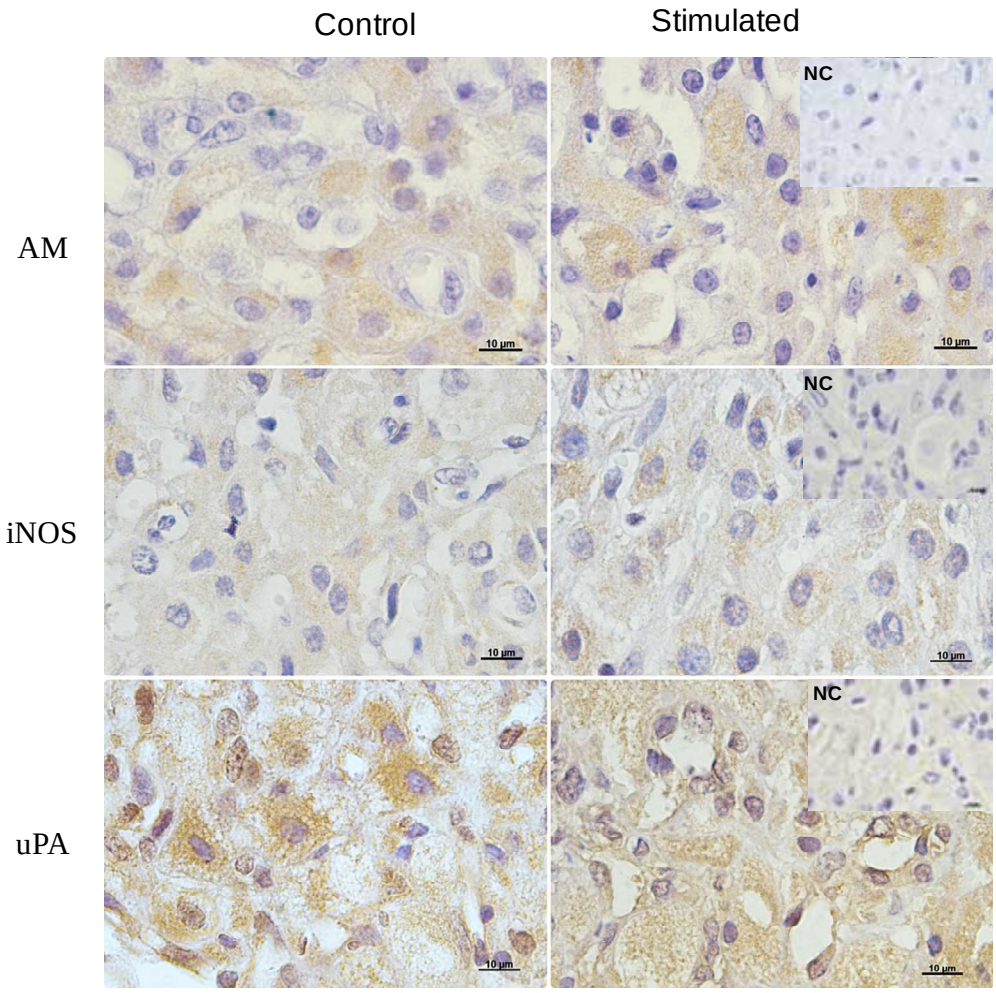
The localization of MMP-9, Trypsin-2, AM, iNOS, Angiopoietin-1, Angiogenin and uPA proteins was verified in the bovine corpus luteum via IHC. Staining was located evenly in the cytoplasm of large and small luteal cells, stroma and endothelial cells on the 3 studied groups without nuclear staining. For MMP-9 protein expression was lower on large (P=0.0002; P=0.002 respectively) and small (P=0.001; P=0.003 respectively) luteal cells on stimulated and on superovulated groups compared with control. Trypsin-2 immunostaining was weaker on large (P=0.0086; P=0.003 respectively) and small (P=0.0001; P=0.01 respectively) luteal cells on stimulated and superovulated groups compared with control (Figure 2). Angiogenin protein expression was higher in small (P= 0.0001; P= 0.005 respectively) and large (P= 0.0001; P= 0.005 respectively) luteal cells in stimulated and superovulated groups. Angiopoietin-2 expression was higher in stimulated and superovulated group on both small (P=0.001; P=0.002 respectively) and large (P=0.063; P=0.03 respectively) luteal cells compared with control (Figure 2). The AM, iNOS and uPA (Figure 3) expressed uniform labelling between both control and stimulated groups showing no differences on total number of positive cells. There was no significant differences on stroma and endothelial total number of positive cells.

Figure 2 – MMP-9, Trypsin-2, Angiogenin and Angiopoietin-1 expression in bovine CL detected by immunohistochemistry. Positive signals can be observed as the orange-brown color in the cytoplasm of large (empty arrow), small (black arrow) luteal cells and stroma (grey arrow) and endothelial (green arrow) cells in the control, stimulated and superovulated animals. NC, negative control. 100x magnification. Bars = 10µm




Source: (MENDES, G. P., 2014).

Figure 3 - AM, iNOS and uPA expression in bovine CL detected by immunohistochemistry. Positive signals can be observed as the orange-brown color in the cytoplasm of large, small luteal cells and stroma and endothelial cells in the control and stimulated animals. NC, negative control. 100x magnification. Bars = 10μm



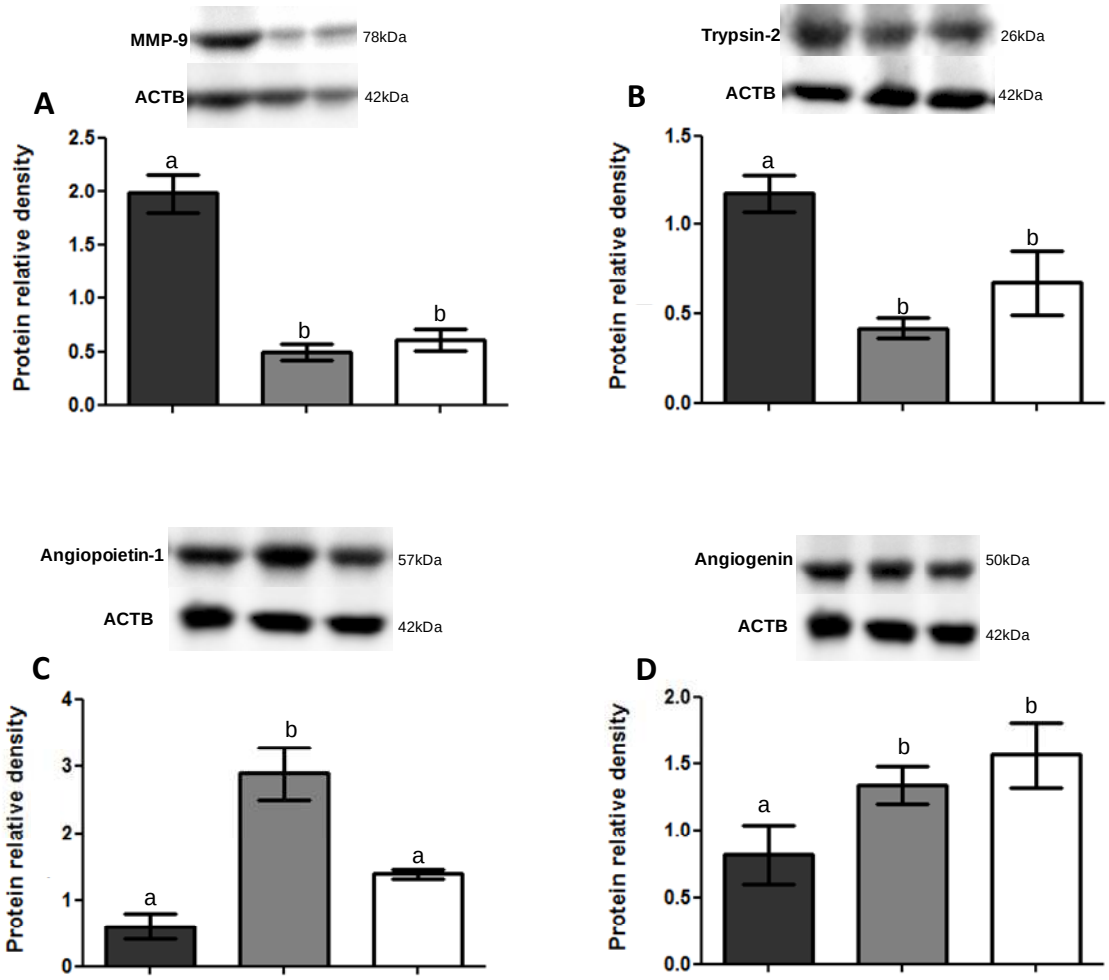

Source: (MENDES, G. P., 2014).

### *1.4.3 Quantitative protein expression of MMP-9, Trypsin-2, AM, iNOS, Angiopoietin-1, Angiogenin and uPA on bovine CL from control, stimulated and superovulated cows*

Western blotting analysis for MMP-9, Trypsin-2, AM, iNOS, Angiopoietin-1, Angiogenin and uPA revealed a single band of the expected sizes (78 kDa, 26 kDa, 20 kDa, 130kDa, 57kDa, 50kDa and 46kDa respectively). When normalized to beta-actin (ACTB, 42 kDa), MMP-9 (P=0.008 and P=0.003 respectively) and Trypsin-2 (P=0.01 and P=0.01 respectively) expression was lower on stimulated and superovulated cows compared with

control. Angiogenin expression was higher on stimulated and superovulated (P=0.01 and P=0.03) and Angiopoietin-1 was higher on the stimulated group (P=0.008) compared with control group (Figure 4). iNOS, AM and uPA showed no significant difference on their expression among the groups (P>0.05) (Figure 5).

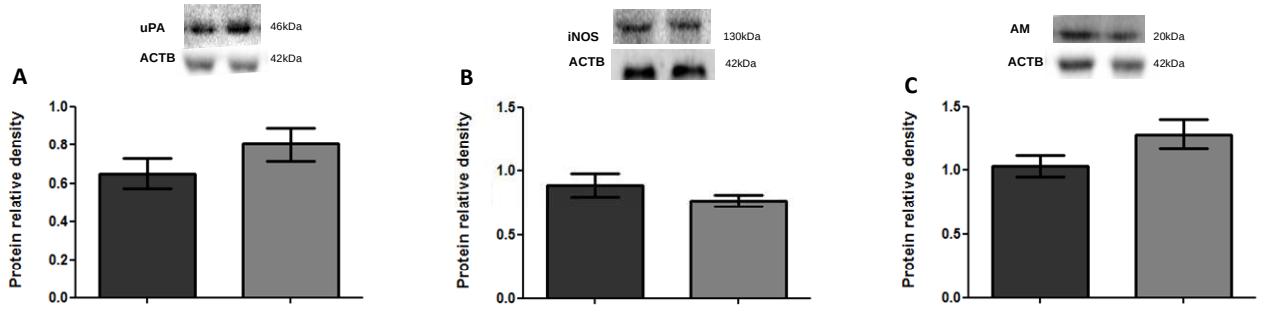
Figure 4 - Protein expression of proteins MMP-9 (A), Trypsin-2 (B), Angiogenin (C) and Angiopoietin-1 (D) in bovine corpus luteum. Dark gray bars represent the control group, light gray the stimulated group and white the superovulated group. Illustrative blots and graphic representing the content expressed by the relative density to β-actin. The bars represent the mean ± standard error (n = 5/control; 6/stimulated; 7/superovulated). Bars with different letters indicate significant differences (P<0.05)




Source: (MENDES, G. P., 2014).

Figure 5 - Protein expression of proteins uPA (A), iNOS (B) and AM (C) in bovine corpus luteum. Dark gray bars represent the control group and light gray the stimulated group. Illustrative blots and graphic

representing the content expressed by the relative density to β-actin. The bars represent the mean ± standard error (n = 5/control; 6/stimulated). Bars with * indicate significant diferences (P<0.05)




Source: (MENDES, G. P. 2014).

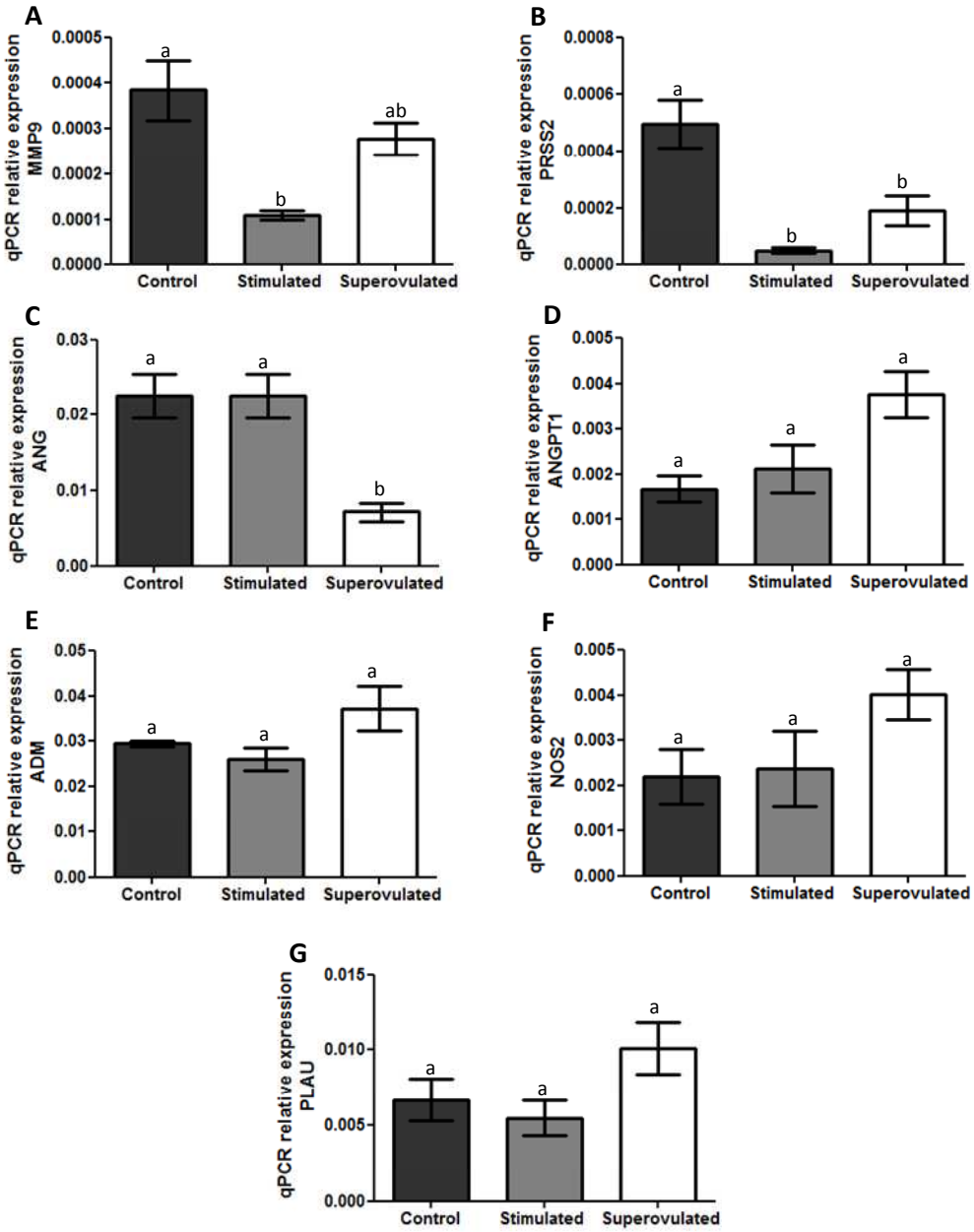
### *1.4.4 Gene expression of MMP9, PRSS2, ADM, NOS2, ANGPT1, ANG and PLAU on bovine CL from control, stimulated and superovulated cows*

The mRNA expression of MMP9, PRSS2, ADM, NOS2, ANGPT1, ANG and PLAU could be detected by real-time PCR (Figure 6). MMP9 and PRSS2 expression was lower (P=0.03 and P=0.01 respectively) in stimulated group and (P=0.04 and P=0.05 respectively) in superovulated groups compared with the control group.

ANG mRNA expression was lower (P=0.01) in superovulated group in relation to control group. ANGPT1 mRNA expression did not differ (P=0.11) in both stimulated and superovulated groups (P=0.55).

Stimulatory treatment with eCG had no effect on ADM, NOS2 and PLAU mRNA expression.

Figure 6 - Gene expression of MMP9 (A), PRSS2 (B), ANG (C), ANGPT1 (D), ADM (E), NOS2 (F) and PLAU (G) in bovine corpus luteum. Data represented as mean ± standard error of relative gene expression (n = 5/control; 6/stimulated; 7/superovulated). Bars with different letters indicate significant differences (P<0.05)




Source: (MENDES, G. P. 2014).

*1.4.5 Correlation between the gene and protein expression of MMP9, PRSS2, ADM, NOS2, ANGPT1, ANG and PLAU and plasma P4*

In stimulated group, progesterone levels were negative correlated with MMP9 (r = -0.66 and P = 0.03) and with PRSS2 protein and Trypsin-2 expression (r = -0,63 and P = 0.04). However, Angiogenin was positively associated with progesterone levels (r = 0,69 and P = 0.03) . In superovulated group ANG and ANGPT1 were positive correlated (r = 0.96 and P = 0.001).

## 1.5 DISCUSSION

In this study, we reported that MMP9 and PRSS2 were downregulated in the CL of cows submitted to stimulation of the dominant follicle and for superovulation using eCG. The ANG protein expression increased after the treatments and mRNA expression decreased in superovulatory treatment. ANGPT1 increased its protein expression after stimulation treatment.

The purpose of this study was to analyze gene and protein expression of genes belonging to formation and degradation of extracellular matrix, angiogenesis, steroidogenesis and luteolysis pathways. In line to our hypothesis that eCG treatment in cows applied on a synchronization protocol with the goal of superovulate or stimulate suppresses mRNA and protein synthesis of MMP9 and PRSS2. It is well known that eight trypsins (1-8) activate proteases as pro-PLAU (KOIVUNEN; HUHTALA; STENMAN, 1989), as well as pro-MMP9 and 2 (SORSA et al., 1997). In addition, we demonstrate in this study that MMP9 followed the decrease of PRSS2 showing a possible association during CL formation. Some studies have shown that for activation of MMPs including MMP2 and MMP9, the human PRSS2 is more potent than other serine proteases (SORSA et al., 1997). However, the signal transduction mechanism by which PRSS2 may suppresses MMP9 in the CL is poorly understood, although in our study their expression followed one another indicating that after ovulation the downregulation is required for CL good formation and perfomance. MMP9 expression is related with hormone action and cell death (PORTELA; VEIGA; PRICE, 2009;

CARBAJAL et al., 2011). Rae et al.,(2009) reported that MMP9 is required for ovulation when remodeling the basement membrane integrity. Metalloproteinase activity is not crucial for steroidogenesis (PELUFFO et al., 2011), however, it is required for tissue reorganization for ovulation and corpus luteum development and was observed predominantly in granulosa cells of the developing follicle as in hamster (SALVERSON et al., 2008), horse (RILEY et al., 2001), pig (RIBEIRO et al., 2006), cow and chicken (ZHU et al., 2014). In human (LIND et al., 2006) and rat (CURRY; SONG; WHEELER, 2001), MMP9 was observed predominantly in primarily theca-interstitial and perifollicular stroma cells, but not in the follicle. LH induces the matrix components disruption, including MMPs, which allows the release of the oocyte and the CL formation by controlling the type of matrix to be remodeled, the specific site location and extent of degradation, being capable of binding LH receptors, the ECG can induce the same mechanism (LAHAV-BARATZ et al., 2003). However, hCG induces MMP2 and MMP9 to support luteinizing of the post ovulated follicle (CURRY; SONG; WHEELER, 2001; LAHAV-BARATZ et al., 2003; ROSEWELL et al., 2011) and is required for the growth and expansion of the follicle, destroying the wall for follicular ovulation, CL formation and dissolution during structural CL regression(SEALS; COURTNEIDGE, 2003).

Especially because eCG most initiates the response in the follicle for the vascular construction on the CL we analyzed the responses in formed CL. Both treatments alter ANG and ANGPT1 expression. ANG mRNA expression decreased in superovulated CLs but protein level increased in superovulated and stimulated cows. The contrast in protein and mRNA expression in superovulated cows may have occurred by ANG mRNA translated, which can increase protein expression under eCG stimulation (CHEN et al., 2002). *In vitro* treatment with hCG and AMPc increased ANG secretion by human granulosa cells (KOGA et al., 2000) and some hormones that are also associated with the production of ANG, as progesterone and factor-α tumor necrosis factor (TNF) (BERISHA; PFAFFL; SCHAMS, 2002) corroborating with our results. However, in our results, ANGPT1 upregulation protein expression in stimulated cows, contrast with Miyabayashi and collaborators (2005) which found that after inducing follicular growth by treatment with eCG in rats. ANGPT-1 showed no changes and ANGPT-2 mRNA increased significantly after 6 and 12 hours of treatment. They suggested that ANGPT-1 may contribute to maintain and stabilize vessels over late follicular development, ovulation and CL formation and early ANGPT-2 can contribute to form vessels in the theca layer in the preovulatory follicle preparing subsequent formation of CL (MIYABAYASHI et al., 2005).

For genes involved in steroidogeneses, our results in microarray analysis in stimulated and superovulated cows compared with control showed downregulation of NOS2, suggesting that the protocol could alter its expression, but on mRNA and protein analysis after validation it did not show any difference among the groups. Gonadotropins regulate NOS2 expression which reaches its maximum expression after 10 days of human chorionic gonadotropin (hCG) injection (JABLONKA-SHARIFF; OLSON, 1997) and play an important role in follicular atresia (ZHANG et al., 2011). Corroborating with our results, Shirasuna et al., (2008) showed no change in NOS2 mRNA expression in bovine CL during the estrus cycle. NO and prostaglandins are angiogenic mediators; at the beginning of CL formation an intensive neovascularization occurs. It is possible that NO modulates COX signaling pathway (PTGS) which is involved in vascular changes in the CL (HURWITZ et al., 2002). Low NO concentrations can negatively regulate $P_4$ synthesis by a cGMP independent pathway in antral granulosa cells of cows (FAES et al., 2009).

On microarray analysis, ADM was upregulated in CL of stimulated cows, which raised the hypothesis that the protocol influenced it and ADM increase expression could be another factor to justified the observed increase in P4. Nevertheless, protein and mRNA results showed no statistical difference after validation. ADM knockout mice decrease fertility and showed a smaller litter size (LI, et al., 2006). A decrease in follicular ADM enhanced estradiol release in rats treated with FSH, and an increase ADM in rats CL treated with eCG can stimulate progesterone release (LI, et al., 2008). However, in human granulosa cells, ADM decreases progesterone (MORIYAMA; OTANI; MARUO, 2000), and rats stimulated with eCG showed an inhibitory effect of ADM over luteal progesterone secretion (LI; TANG; O, 2010). It seems that ADM acts as a local factor that could increase progesterone production in human granulosa luteal cells (MORIYAMA; OTANI; MARUO, 2000). At late pregnancy, ADM expression is higher than in the beginning or in the middle, and may be due to the role of ADM in the inhibition of luteolysis and progesterone production (LI, et al., 2008; LI; O; TANG, 2011). Regarding PLAU expression, no significant difference were observed, even though on microarray analysis PLAU was downregulated in stimulated cows compared to control. It has been reported that this protease is not demanded for luteal function, once PLAU deficient mice did not showed any significant difference in luteal formation and function, ovarian weights, serum progesterone levels and blood vessel densities (LIU et al., 2003). On the other hand, an increase in PLAU and tissue plasminogen activator (tPA) mRNA expression in bovine periovulatory follicles was observed in response to gonadotropin surge, indicating that gonadotropin induces plasminogen activators in the

ovulation (DOW et al., 2002). It has been reported that PLAU is involved in cumulus expansion and subsequent oocyte maturation because PLAU levels in the cumulus were significantly higher at 3 and 6 h in mouse cumulus cells when treated with hCG *in vivo* than *in vitro* (LU et al., 2013).

## 1.6 CONCLUSION

The study of gonadotropin effects on bovine developing CL showed that genes and proteins can be modulated as MMP9 and PRSS2, which decreased their expressions, and genes that participate in the development of new vessels as ANG and ANGPT1, which showed an increase in expression. The use of these protocols suggest that eCG could directly influence expression pathways in the CL leading to improve formation, angiogenesis and function.

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