# GENE EXPRESSION OF FOXP3, INDOLEAMINE 2,3-DIOXYGENASE (IDO), IL10 AND CSF1 IN THE UTERUS OF COWS THAT RECEIVED AN INTRAUTERINE INFUSION OF MATERNAL AND PATERNAL ANTIGENS

## *EXPRESSÃO GÊNICA DE FOXP3, INDOLEAMINA 2,3-DESOXIGENASE (IDO), IL-10 E CSF1 EM ÚTEROS DE VACAS QUE RECEBERAM INFUSÃO INTRAUTERINA DE ANTÍGENOS PATERNOS E MATERNOS*

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**ABSTRACT:** Sensitization with conceptus antigens has been shown to be useful for improving reproductive performance facilitating maternal acceptance of an allogeneic embryo through the induction of cytokines and immunoregulatory cells in the uterine microenvironment. As FOXP3, IDO, IL10 and CSF1 in the uterus are important on the recognition and development of embryos during early pregnancy, this study aimed to determine whether simultaneous or isolated administration of paternal (semen) and maternal (PBMCs) antigens in the uterus of cow, on the day of estrus, influence the gene expression of these cytokines. Forty crossbred cows were divided into four treatments: T0: Control; T1: Semen; T2: PBMCs (peripheral blood mononuclear cells) from another cow and T3: PBMCs+Semen. Antigens were administered into the uterine body on the estrus day (D0). Uterine biopsies designed for molecular analysis of gene expression were collected *in vivo* seven (D7) and fourteen (D14) days after immunostimulation. Transcripts from FOXP3, IDO, IL-10 and CSF-1 were detected in all RNA samples extracted from uterine biopsies. The semiquantitative analysis showed that none of the treatments caused significant increase in the expression of these genes. Furthermore, on D14 all treatments led to a decline in the number of CSF-1 transcripts; moreover, treatment with PBMCs+Semen also led to a drop in the abundance of IL-10 transcripts. Such results suggest that isolated or simultaneous administration of both antigens would not increase maternal tolerance to embryo alloantigens, nor would it create favorable conditions to its growth and pre-implantation development, at least regarding the effects mediated by these genes on D7 and D14 of the estrous cycle.

**KEYWORDS:** *Bos taurus.* Immunologic induction. Peripheral blood mononuclear cells. Pregnancy. Semen.

## INTRODUCTION

Improving the bovine embryo transfer technique (ET) is strongly encouraged by the breeding industry need to increase genetic development (MERTON et al., 2003). Despite the advantages, pregnancy loss after ET is high, especially during early pregnancy (BERG et al., 2010), and many of the problems related to the recipients (ANDRADE et al., 2012). Most pregnancy losses take place between the 7th and 16th day of gestation (BERG et al., 2010), a period in which the bovine embryo remains free and floating in the uterine lumen, completely dependent on the intrauterine environment to survive and to start its preimplantation growth and susceptible to death from changes in the suitability of this environment during ET (KIMURA; MATSUYAMA, 2014). During this second week, the embryonic mortality after embryo transfer produced *in vitro* or *in vivo* is, on average, almost twice as high compared to the one derived from embryos originating from natural mating or artificial insemination (BERG et al., 2010). Therefore, pregnancy loss in recipients can be attributed to the interactions between the maternal uterus and the pre-implantation embryo (fully allogeneic), leading to failure in maternal recognition of pregnancy and/or mother intolerance of embryo alloantigens (IDETA et al., 2010a, b; MINTEN et al., 2013).

In early pregnancy, interactions between cells and molecules from the immune system of the mother and embryo are crucial for the modulation of the maternal immunoregulatory responses required

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for pregnancy (HANSEN, 2011). Several factors are involved in this immunoregulatory network and including regulatory T cells (Treg), which play key role in antigen-specific immunoregulation and induction of maternal-fetal tolerance (SAITO et al., 2010); indoleamine 2,3-dioxygenase enzyme (IDO), which performs important inhibition role in conventional immunostimulation by tryptophan depletion (DELLÊ; NORONHA, 2009); and Interleukin 10 (IL-10) and colony stimulating factor 1 cytokines (CSF-1), which are important modulators of immune tolerance (OLIVEIRA et al., 2013) and the development of bovine embryos (OSHIMA et al., 2008), respectively.

There is strong evidence for exposure to semen and other sperm components, which causes significant changes in female immune functions before embryo implantation which promotes a state of functional tolerance to paternal alloantigens with direct implications on gestational success (CLARK, 2011). Studies carried out on humans (CRAWFORD et al., 2015), mice (KIM et al., 2015) and pigs (O'LEARY et al., 2004) have demonstrated such semen effect for increasing endometrial receptivity. Moreover, there are studies which promote this immunostimulation with peripheral blood mononuclear cells (PBMCs). Studies conducted on humans (OKITSU et al., 2011) and bovines (IDETA et al., 2010a, b) showed that intrauterine administration of autologous PBMCs prior to ET increased the rate of successful pregnancy. As FOXP3, IDO, IL10 and CSF1 in the uterus are important on the recognition and development of embryos during early pregnancy, this study aimed to determine whether simultaneous or isolated administration of paternal (semen) and maternal (PBMCs) antigens in the uterus of cow, on the day of estrus, influence the gene expression of these cytokines.

## MATERIALS AND METHODS

### Animals

All animal procedures in this study were approved by the Ethics Committee on the Utilization of Animals of the Federal University of Uberlândia. We used non-pregnant crossbred cows with similar ages and the same food and water diet from a commercial farm in the city of Monte Alegre, MG. To determine the effects of antigens on the uterine environment of the recipients, the cows were randomly divided into four treatments: *T0*: control (n=10); *T1*: paternal antigen (Semen, n=10); *T2*: maternal antigen (PBMCs, n=10); and *T3*: paternal and maternal antigens (n=10).

### Collection, preparation and storage of antigens

#### Paternal antigens

The paternal antigens used in this experiment were semen, composed of the seminal plasma and dead spermatozoids. Semen was collected from four donor bulls with the aid of eletroejaculator and/or seminal vesicle massage. This pool of seminal cells was conditioned in microtubes containing 2.0 ml each and stored at -20°C, without diluent or cryoprotectant, which led to sperm viability.

#### Maternal antigens

The maternal antigens used in this experiment were bovine PBMCs. Peripheral blood from a healthy cow was collected and PBMCs were isolated using Ficoll-Paque$^{TM}$ PLUS centrifugation (GE Healthcare, Uppsala, Sweden). After centrifugation, only the leukocyte cloud formed at the interphase between the plasma and the Ficoll was collected and washed three times in PBS. At the end of this process, cells were aliquoted into microtubes at a concentration of (2-4) x $10^7$ cells/ml (IDETA et al., 2010a, b) and stored at -20ºC.

### Intrauterine administration of the antigens

Cows were synchronized to ovulation, and the antigens were inoculated into the uterine body during the peri-ovulatory period with the aid of a pipette of bovine intrauterine infusion. The recipients received equal volume of antigens in accordance with the specific treatment for each animal: *T0*: 4.0 ml of PBS; *T1*: 2.0 ml of semen + 2.0 ml of PBS; *T2*: 1.0 ml of PBMCs [2-4 x $10^7$ cells/ml] + 3.0 ml of PBS; and *T3*: 2.0 ml of semen + 1.0 ml of PBMCs [2-4 x $10^7$ cells/ml] + 1.0 ml of PBS.

### In vivo uterine biopsy

*In vivo* uterine biopsies were collected seven (D7) and fourteen (D14) days after infusion of the antigens (D0 = estrus) to assess the treatment effects on the uterine environment of recipients at the moment which it would occur during the ET procedure and to assess the effects during the period in which the embryo would have already started its preimplantation growth, respectively. Cows were immobilized in a cattle crush cage and anesthetized with 2% xylazine hydrochloride via epidural. A fragment of approximately 3 $mm^3$ of the uterine body was collected from five animals of each treatment and day using forceps for bovine uterine biopsy, which was used for the molecular analysis of gene expression. Every cow, after collection, was

removed from the experiment to avoid interference of inflammatory processes. These biopsies were conditioned in RNase/DNase-free microtubes containing 1:5 wt/vol of RNA stabilization solution (RNAlater of *Ambion*®) and stored at -80ºC.

### *RT-qPCR*

#### RNA extraction and reverse transcription

Total RNA from uterine biopsies was automatically extracted in the Maxwell® 16 Instrument (Promega, Madison, WI, USA) coupled with its tissue RNA extraction kit (Maxwell® 16 LEV simply RNA Tissue Kit) according to the manufacturer instructions and stored at -80°C. For complementary DNA (cDNA) synthesis, total RNA (500 ng) was subjected to reverse transcription using the GoScript™ Reverse Transcription System kit (Promega) according to the manufacturer instructions and stored at -20°C. The purity and concentration of samples were measured with a spectrophotometer NanoDrop® ND1000.

#### Primer design

Primers were designed using the primer design tool from Integrated DNA Technologies®. The chosen parameters were set to qPCR Intercalating Dyes, considering: melting temperature of 60°C, percentage of GC bases of 50%, *primer length* of 18-22pb and amplicon *length* of 80-100pb. The best primer selection was determined by the Basic Local Alignment Search Tool (THORNTON; BASU, 2011). The efficiency curve of the primers was performed with 5 points and the dilution factor 1:4 (initial concentration of 2000 ng of cDNA). Table 1 shows the primers used in this study.

**Table 1.** Sequence of the forward and reverse primers with their references and efficiencies.

| Name | Sequence | IDT Reference | NCBI Reference | Efficiency |
|---|---|---|---|---|
| **FOXP3 F** | TAGGAAAGACAGCACCCTT | 128613172 | NM_001045933.1 | 1,96 |
| **FOXP3 R** | CCTTGAAGACCTTCTCACATC | 128613173 | NM_001045933.1 | |
| **IDO F** | CTACGTGTGGAAGTTGAGAAG | 128613174 | NM_001101866.2 | 1,98 |
| **IDO R** | GTGATGTATCCCAGAACCAAG | 128613175 | NM_001101866.2 | |
| **IL-10 F** | AGCCATGAGTGAGTTTGAC | 128613176 | NM_174088.1 | 2,08 |
| **IL-10 R** | GGAGGTCTTCTTCCCTAGAA | 128613177 | NM_174088.1 | |
| **CSF-1 F** | CTAACATAGCCCTGGAAGAG | 128613180 | NM_174026.1 | 2,06 |
| **CSF-1 R** | CCCAGACTGTAGAAGGAAAG | 128613181 | NM_174026.1 | |
| **SUZ 12 F** | GAAGATGGAGAAGTGGAACA | 121266340 | NM_001205587.1 | 2,12 |
| **SUZ 12 R** | GACGGAGAGGTAAACAAGTATC | 121266341 | NM_001205587.1 | |

F – Forward primer
R – Reverse primer
qPCR

The quantification of gene expression for FOXP3, IDO, IL-10 and CSF-1 was performed in StepOnePlus™ *Real Time PCR Systems* device (Applied Biosystems, Foster, CA, USA) via the comparative method of threshold cycles (Ct). All samples were conducted in duplicate with only one negative control for each gene. We used GoTaq® qPCR Master Mix (Promega) according to the manufacturer's instructions and the standardized amount of cDNA was 300 ng per reaction. Relative gene expression, normalized to SUZ12 (WALKER et al., 2009), was determined based on the mathematical formula of Pfaffl with efficiency corrections (PFAFFL, 2006).

#### Statistical analysis

All analyzes were performed using the software GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA). As defined by the statistical test employed, we used Kolmogorov-Smirnov test of normality, and Kruskal-Wallis test, followed by Dunn's post-test or One Way ANOVA, followed by Tukey's post-test. In all analyses, values of $0.1>p>0.05$ and $p<0.05$ were defined as being trend and statistically significant, respectively (IQBAL et al., 2014).

## RESULTS

Data relating to semiquantitative analysis of relative genic expression for FOXP3, IDO, IL-10 and CSF-1 between the control and treated groups, seven and fourteen days after infusion with antigens, can be seen in Figure 1. All treatments did not promote significant differences in the number of transcripts for FOXP3, IDO and IL-10 at D7 (Figure 1 A, C and E) and D14 (Figure 1 B, D and F) after immunostimulation. However, fourteen days after treatment with both antigens, there was a noticeable tendency for promoted decrease in the number of IL-10 transcripts in relation to the uterus of control cows (Figure 1 F). For CSF-1, all treatments did not promote significant differences in the abundance of these transcripts seven days after immunostimulation (Figure 1 G). However, fourteen days after the treatments with maternal antigens and both antigens led to significantly lower number of CSF-1 transcripts compared with the uterus of control cows (Figure 1 H). Additionally, fourteen days after treatment with paternal antigens, there was a tendency for promoted decrease in the number of CSF-1 transcripts compared with the control (Figure 1 H).

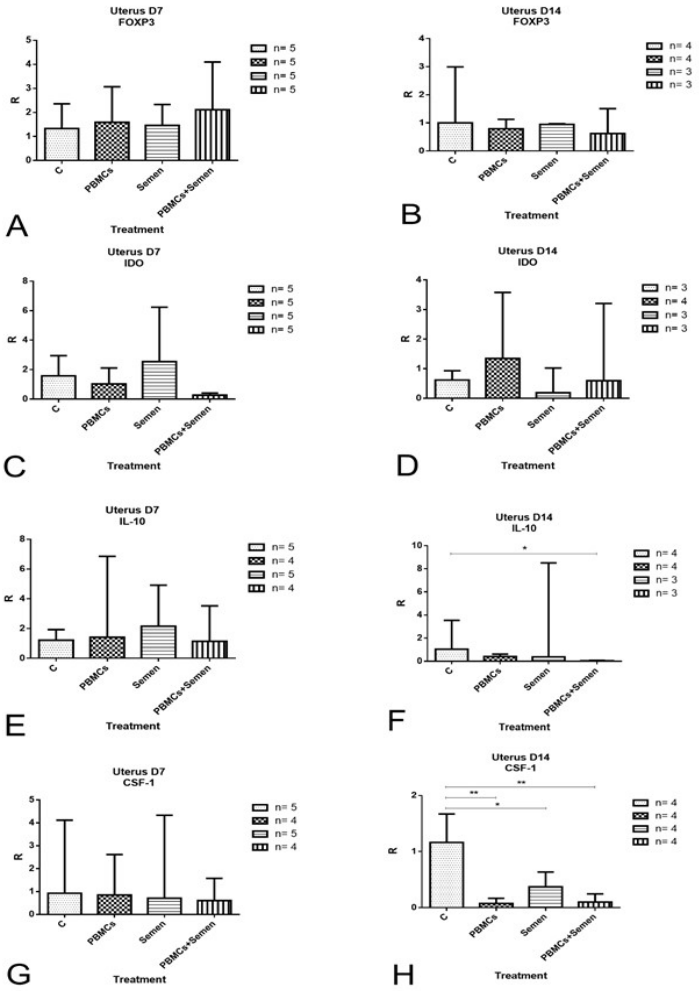


**Figure 1.** Relative genic expression of FOXP3 (**A** and **B**), IDO (**C** and **D**), IL-10 (**E** and **F**) and CSF-1 (**G** and **H**) in the uterus of control and treated cows, 7 (**A**, **C**, **E** and **G**) and 14 (**B**, **D**, **F** and **H**) days after treatment with maternal (PBMCs), paternal (Semen) and both antigens (PBMCs+Semen). Statistical analyses were determined by ANOVA and Tukey's post-test or Kruskal-Wallis and Dunn's post-test. Bars represent the mean ± SD (**A** and **C**) or the median ± *range* (**B**, **D**, **E**, **F**, **G** and **H**). CSF-1 = Colony stimulating factor 1. FOXP3 = *Forkhead Box P3.* IDO = Indoleamine 2,3-dioxygenase. IL-10 = Interleukin 10. R = *Ratio*. C = Control. PBMCs = Bovine peripheral blood mononuclear cells. D7 = Seven days after treatment. D14 = Fourteen days after treatment. n = sample size. * = $p<0.1$. ** = $p<0.05$.

## DISCUSSION

Studies carried out in several species have shown that sensitization with conceptus antigens may be useful in enhancing reproductive performance by facilitating maternal acceptance of an allogeneic embryo and its development through the induction of cytokines and immunoregulatory cells in the uterine microenvironment (O' LEARY et al., 2004, YOSHIOKA et al., 2006, IDETA et al., 2010a, b, OKITSU et al., 2011, CRAWFORD et al., 2015, KIM et al., 2015). Therefore, previous exposure of bovine recipients to embryo antigens, coming from an oocyte donor PBMCs and bull semen used in fertilization, may favor the development of maternal tolerance to embryo alloantigens with direct implications on gestational success after ET.

Uterine gene expression of FOXP3, IDO, IL-10 and CSF-1 during early pregnancy seems to be a consequence of immunomodulatory mechanism adjustments during pregnancy that favor the establishment of appropriate intrauterine environment to tolerate the development of an allogeneic embryo. In this study, transcripts of FOXP3, IDO, IL-10 and CSF-1 were detected in all uterine samples; however, none of the treatments promoted a significant increase in the expression of these genes. Conversely, on D14, all treatments promoted a decrease in the number of CSF-1 transcripts, and the treatment with PBMCs+Semen promoted decrease in the abundance of IL-10 transcripts, showing that this treatment promotes negative effects on the uterine microenvironment.

The absence of significant differences between most of the groups may be assigned to individual variation in expression of each gene within the investigated samples. In addition, this variation may be related to the existing differences of compatibility between the donors of semen and PBMCs and their recipients (MAGALHÃES; BOHLKE; NEUBARTH, 2004), which could have generated the large standard deviations observed in the statistical analyses. The samples that presented high coefficients of variation between samples of the same treatment were considered outliers and, therefore, excluded from the analysis, greatly reducing the amount of analyzed data, and, consequently, causing a sampling bias. This high coefficient of variation can be attributed to existing differences in gene expression between the caruncular and intercaruncular regions of bovine endometrium (MANSOURI-ATTIA et al., 2009).

Regarding the treatment with PBMCs, the absence of significant differences in the number of transcripts for FOXP3 and IL-10 were in accordance with the results from Ideta and collaborators (2010a). Conversely, in the same study, the abundance of CSF-1 transcripts was significantly higher in autologous PBMCs treated groups compared with the control, which may explain why our results observed on D7 and D14 disagreed with Ideta and collaborators (2010a). It is important to note that in our study, we used PBMCs from a bovine female donor, which, among other differences, impedes an accurate comparison with the results observed by other researchers (IDETA et al., 2010a). There is no published work analyzing the effects of intrauterine administration of PBMCs from another individual that is not identical; however, it is well known that such administration increases the exposure of recipients to alloantigens (MAGALHÃES; BOHLKE; NEUBARTH, 2004) and consequently, the immune response may differ from one result from the administration of autologous PBMCs.

Regarding uterine gene expression of CSF-1 during pregnancy in bovine, the results obtained in this study suggest that there may be negative effect from simultaneous or isolated intrauterine administration of semen and PBMCs for the growth and development of the embryo in preimplantation (OSHIMA et al., 2008) because the endometrial expression of CSF-1 was markedly increased between 14 and 17 days of pregnancy (LEE et al., 2003), precisely when the conceptus reaches its rapid growth (IDETA et al., 2010a) and maximum production of INF-τ, promoting the maternal recognition of pregnancy and ideal conditions to implantation (HANSEN, 1997). Moreover, in this experiment, there was no presence of the embryo in the uterus of the recipients, which in fact may have influenced the results because early embryos secrete a wide variety of molecules which modulate the maternal immune system for recognition, establishment and maintenance of pregnancy (HANSEN, 1997, ROBERTS, 2007). For instance, the bovine embryo releases factors which increase the endometrial expression of CSF-1 (YOSHIOKA et al., 2006) and IDO (GROEBNER et al., 2011) during early pregnancy.

## CONCLUSION

In conclusion, this study showed that treatments, isolated or simultaneous of both male and female antigens, do not increase the gene expression of FOXP3, IDO, IL-10 and CSF-1 in bovine uterus and, consequently, do not propitiate increases of maternal-fetal tolerance, at least in

regarding to the effect mediated by these studied genes on D7 and D14 of the estrous cycle. In addition, the effects of immunostimulation after 14 days may depend on the presence of the embryo to find optimal conditions for its growth and preimplantation development. However, the expression of these genes are not the only factors acting for the establishment of endometrial receptivity, and thus, other factors can mediate the increase of pregnancy rates and birth of calves with intrauterine administration of these antigens on the day of estrus. Therefore, more studies must be performed in order to obtain conclusive results about the effects of intrauterine immunostimulation from maternal and paternal antigens on gestational success after bovine ET.

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**RESUMO:** A sensibilização intrauterina com antígenos do concepto tem-se mostrado útil para melhorar o desempenho reprodutivo, facilitando a aceitação materna de um embrião alogênico por meio da indução de citocinas e células imunorreguladoras no microambiente uterino. Como FOXP3, IDO, IL10 e CSF1 no útero são importantes no reconhecimento e desenvolvimento de embriões durante a gestação inicial, este estudo teve como objetivo determinar se a administração simultânea ou isolada de antígenos paterno (sêmen) e materno (PBMCs) no útero de vacas, no dia do estro, influenciam a expressão gênica dessas citocinas. Quarenta vacas mestiças foram divididas em quatro tratamentos: T0: Controle; T1: Sêmen; T2: PBMCs (células mononucleares do sangue periférico) e T3: PBMCs + Sêmen. Os antígenos foram administrados no corpo do útero no dia do estro (D0). Biópsias uterinas projetadas para análise molecular da expressão gênica foram coletadas *in vivo* sete (D7) e catorze (D14) dias após imunoestimulação. Transcritos de FOXP3, IDO, IL-10 e CSF-1 foram detetados em todas as amostras de RNA extraídas de biópsias uterinas. A análise semiquantitativa mostrou que nenhum dos tratamentos causou um aumento significativo na expressão desses genes. Além disso, no D14, todos os tratamentos levaram a um declínio na quantidade de transcritos do CSF-1; Além disso, o tratamento com PBMCs + sêmen também levou a uma queda na abundância de transcritos de IL-10. Estes resultados sugerem que a administração isolada ou simultânea de ambos os antígenos não deve aumentar a tolerância materna aos aloantígenos do embrião, nem deve criar condições favoráveis ao seu crescimento e desenvolvimento pré-implantação, pelo menos em relação aos efeitos mediados por esses genes nos dia sete e catorze do ciclo estral.

**KEYWORDS:** *Bos taurus.* Células mononucleares do sangue periférico. Gestação. Indução imunológica. Sêmen.

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