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Effect of Exogenous Progesterone Treatment on Quantitative Expression of IFN-tau in Caprine Skin Fibroblast Cells Model.

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Abstract

In ruminants, there is a strong positive correlation exists between the time that increase in plasma progesterone concentrations and embryonic IFN tau production rate and it's survival. In the present study IFN- tau EGFP gene construct was nucleotransfected in adult caprine skin fibroblast cells and these nucleotransfected cells were treated with exogenously supplemented progesterone at various doses (2.5 – 20 ng/ml). q-RT PCR results from the progesterone treated samples reports that there is no significant amplification in the expression of IFN-tau in goat fibroblast cell culture in comparison to standards on external supplementation of progesterone even at higher concentration over and above normal physiological range.

Keywords: Progesterone, IFN- tau, Caprine skin fibroblast, Nucleofection, q-RT PCR.

Institution where research was conducted

Dairy Cattle Physiology Division, National Dairy Research Institute (NDRI), Karnal, Haryana, India.

Introduction

A strong association has been observed among the *in vivo* growth of conceptuses, their production of IFN-tau, and the level of serum progesterone in ruminants (Mann

and Lamming, 2001). IFN-tau is a major secretory protein produced by the trophectoderm cells of ruminant conceptuses at about Day 15-17 and plays a very important role in establishment of pregnancy by its antiluteolytic effect (Bazer et al.1994). The preimplantation blastocyst depends for nutrients on endometrial secretions, and the secretory endometrium is, in turn, dependent on progesterone. This relationship leads to a positive feedback loop at the time of IFN-tau synthesis, whereby IFN-tau maintains luteal progesterone secretion, and progesterone supports the blastocyst development. Progesterone stimulates and maintains endometrial functions necessary for conceptus growth, implantation, placentation, and development to term (Spencer *et al*. 2004). In cattle, concentrations of progesterone in early pregnancy clearly affect embryonic survival during later pregnancy (Mann and Lamming 1999). Heifers and ewes with lower concentrations of progesterone in the early luteal phase had retarded conceptus development with less IFN-tau secretion. (Mann and Lamming, 2001). Increasing serum concentrations of progesterone from days 2- 5 or days 5- 9, enhanced conceptus development and size on day 14 in heifers (Garrett *et al*. 1988) and day 16 in cows (Mann *et al*. 2006). Wang et al. (2002) demonstrated that IFN-tau can induce apoptosis in bovine uterine epithelial cells and that this effect is modulated by progesterone supplemented at a concentration of 10 ng/ml. IFN-tau and progesterone co-stimulate expression of number of genes, particularly in the endometrial epithelium like Cystatin C, Cathepsin L, Galectin 15, Hypoxia Inducible Factors (HIF),Gastrin Releasing Peptide (GRP),which are important for blastocyst attachment and elongation (Song et al. 2008). The present study is a novel attempt to study the effect of exogenous progesterone treatment on quantitative expression of IFN-tau in Caprine skin fibroblast model.

Materials and Methods

Preparation of Goat fibroblast cells

Skin biopsy was taken aseptically from the ear pinna of adult goat and primary culture of fibroblast cells were prepared from these tissue explants as per standard protocol. The somatic cell culture lines were established, by sub culturing known number of trypsinised cells in DMEM supplemented with 20%FBS.

Nucleotransfection of Goat fibroblast cells

Nucleo-transfection was carried out using IFN-τ EGFP gene construct (fig.1) (EGFP-Enhanced Green Fluorescent Protein helps in tracking the gene construct) which was procured from the Chromous biotech, Bangalore, India. Aseptically harvested later passage cells ($4x10^5$cells) were used for nucleofection as per manufacturer's protocol (Amaxa biosystem, Germany) using suitable nucleofector program (U- 23) with 2µg IFN-τ EGFP gene construct.

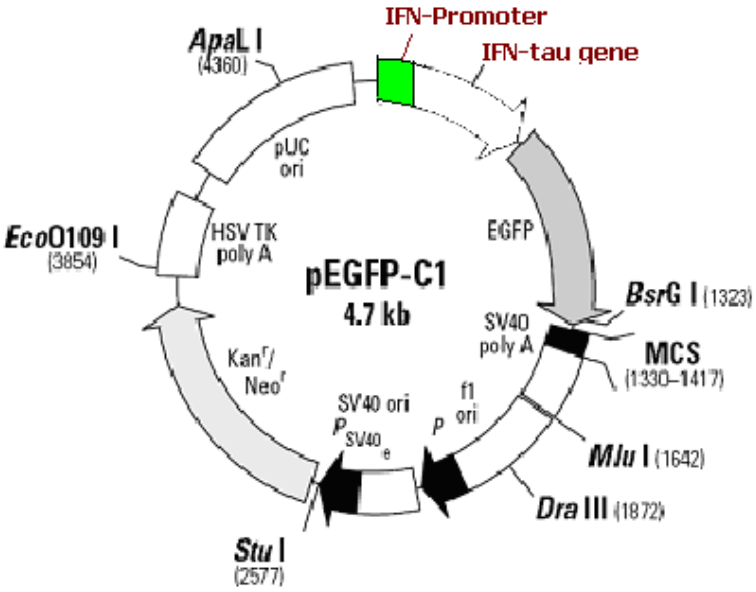


Figure 1: IFN τ gene construct using pEGFP-C1(4.7 kb) vector.

Exogenous Progesterone treatment on transected cells

Nucleo-transfected goat fibroblast cells at later passages were selected for progesterone treatment. Equal number of cells (1.6 x 10^5) were seeded in four tissue culture flasks (75cm^2). Once the cells got attached, (after24hrs) supplemented the progesterone with doses ranging from 2.5ng/ml-20ng/ml (2.5, 5, 10 and 20ng/ml). Cultures were treated with hormone for 48h. At the end of the time period, the cells were harvested after trypsinisation. Resuspended the cells in 1ml of the growth medium (DMEM containing 20%FBS) and observed under inverted microscope using specific attachment for GFP.

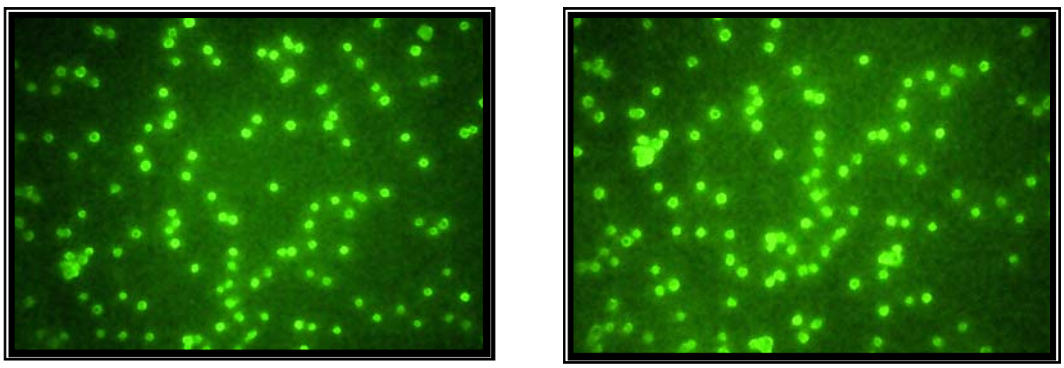
Quantification using q-RT PCR

Total RNA was isolated from nucleofected fibroblast cells (Norgens RNA,DNA,Protein extraction kit) which were treated with progesterone.and was subjected to q-RT PCR for accurate quantification of mRNA levels from various samples with known quantity of IFN tau as internal control.SYBR green qRT-PCR was performed on a Real Time PCR (Bio RAD) using IFN-tau specific primers.

Result and Discussion

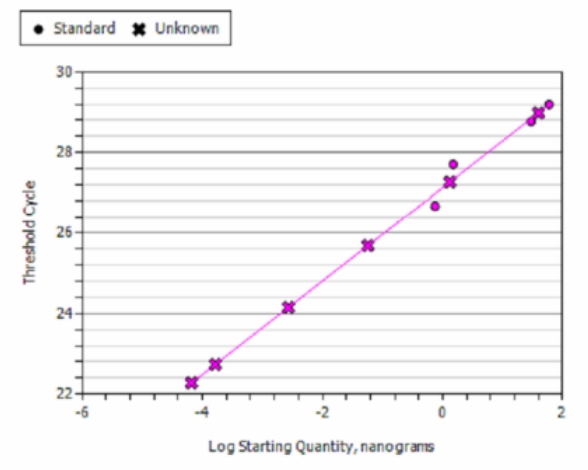
Direct evidence for an effect of exogenous progesterone concentration on IFN-tau production comes from cows supplemented with progesterone. In a study conducted by Mann et al. (1999) in cows reported that with low plasma progesterone concentration on day 5 after insemination, the IFN-tau concentration in uterine flushings on day 16 was found to be low and thereby affected fetus development adversely. Exogenous progesterone supplementation improved blastocyst

development, increased uterine IFN-tau concentrations and ultimately the pregnancy got maintained to term. In the present study IFN-tau transfected Goat fibroblast cells after adherence to the flask, were treated with progesterone at different concentrations. ranging from 2.5-20 ng/ml. After 48 hrs of incubation the cells were started to detach from the flask bottom and observed to float in the medium. Trypsinised cells were observed under fluorescent microscope and based on the number of cells exhibiting the fluorescence it was observed that there is no significant increase in the number of fluorescent cells after progesterone treatment (Fig-2). From the q-RT-PCR standard curve chart it was observed that even though Ct (threshold cycle)values are found to be decreasing for progesterone treated samples in a dose dependent manner, the ▲Ct was found to be insignificant when compared with the standards(Fig.3). It is inferred that there was no significant amplification in the expression of IFN-tau in goat fibroblast cell culture on external supplementation of progesterone even at higher concentration over and above normal physiological range (1-5ng/ml depending upon breeding status in goats) (Flemming et al.,1990). In another study conducted by Pavelic et al. (1983) demonstrated that administration of progesterone stimulated the growth of aplastic carcinoma of the breast *in vivo*. But in *in vitro* conditions when progesterone was supplemented into cell culture of the same tumour, did not show any effect on the growth of fibro sarcoma, melanoma, Ehrlich tumour, myeloid leukemia. Similarly in our studies even though there is a strong positive correlation exist between the concentration of serum progesterone and trophectoderm derived IFN-tau protein in ruminants as reviewed above but failed to recreate the scenario in in *vitro derived* goat fibroblast cell culture model system. This may be due to the lack of some unknown autocrine and paracrine factors involved in the activation of serum responsive element (SRE) in fibroblast cell model in *invitro* conditions as reported by Wilhelm et al. (1998)



a b

Figure 2: IFN-tau-EGFP gene expression as observed under fluorescence microscope post Nucleofection of goat fibroblast cells using U-23 program before (a) and after (b) progesterone treatment. The percentage of fluorescent cells ranged between 60 – 70% in both the cases.



Standard Curve Spreadsheet Data

Fluor	Well	Type	Ident.	Rep	Ct	Log SQ	SQ	SQ Mean	SQ SD	Ct Mean	Ct SD	Set Point
SYBR	G05	Std	std4	4	26.66	-0.125	7.50E-01	7.50E-01	0.00E+00	26.66	N/A	N/A
SYBR	G04	Std	std3	3	27.71	0.176	1.50E+00	1.50E+00	0.00E+00	27.71	N/A	N/A
SYBR	G03	Std	std2	2	28.77	1.477	3.00E+01	3.00E+01	0.00E+00	28.77	N/A	N/A
SYBR	G02	Std	std1	1	29.19	1.778	6.00E+01	6.00E+01	0.00E+00	29.19	N/A	N/A
SYBR	G07		20ng	6	22.28	-3.786	1.64E-04	1.64E-04	0.00E+00	22.74	N/A	N/A
SYBR	G08		10ng	5	22.74	-4.179	6.63E-05	6.63E-05	0.00E+00	22.28	N/A	N/A
SYBR	G09		5ng	4	24.15	-2.566	2.72E-03	2.72E-03	0.00E+00	24.15	N/A	N/A
SYBR	G10		2.5ng	3	26.55	-0.500	3.16E-01	3.16E-01	0.00E+00	26.55	N/A	N/A

Figure 3: q-RT-PCR standard curve data for Progesterone treated cells at different Concentrations in comparison to standard.

Conclusions

In the present study IFN- tau EGFP gene construct was nucleotransfected in adult caprine skin fibroblast cells and these nucleotransfected cells were treated with exogenously supplemented progesterone at various doses (2.5 – 20 ng/ml). q-RT PCR results from the progesterone treated samples reports that there is no significant amplification in the expression of IFN-tau in goat fibroblast cell culture in comparison to standards on external supplementation of progesterone even at higher concentration over and above normal physiological range. This study with further improvisation can be used in future to see the change in the quantitative expression of IFN tau gene in Goat fibroblast cell culture medium upon exogenous progesterone supplementation.

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