Full Length Article

EFFECT OF NON-SURGICAL REMOVAL OF CONCEPTUS ON ESTROUS CYCLE LENGTH AND INTERFERON TAU (IFNT) GENE EXPRESSION PROFILE IN WATER BUFFALO *(Bubalus bubalis)*

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ABSTRACT

The physiology of maternal recognition of pregnancy (MRP) in cattle is well explored and a better understanding of phenomenon of MRP is needed in order to improve reproductive efficiency in buffaloes. The present work was conducted to study the effect of non-surgical removal of conceptus and expression profile of interferon tau (IFNT) in the conceptus after post insemination in buffaloes. In the experiment I, 15 female buffaloes in estrus were inseminated using frozen semen of Murrah buffalo and the uterine flushing by non-surgical method was carried out on days 12, 14, 16, 18 and 20 of post insemination for collection of conceptus. Under experiment II, conceptus tissue collected on different days in the experiment I and conceptus tissue collected from slaughter house pregnant reproductive tracts were used for RNA isolation to study real time expression pattern of IFNT on day 12, 14, 16, 18, 20, 30, 40 and 50 of pregnancy by using specific primers (F5'-GCCCTGGTGCTGGTCAGCTA -3' and R5'-CATCTTAGTCAGCGAGAGTC -3'). The estrous cycle length following non- surgical removal of conceptus on day 12, 14, 16, 18 and 20 post-insemination was 23.50±0.96, 36.60±3.35, 41.25±2.67, 42.73 ±2.61 and 45.20±7.03 days, respectively. The mean cycle length increased significantly by 8-47 days following removal of conceptus on day 14, 16, 18 and 20 post-insemination when compared to day 12. The expression of IFNT was found low but at detectable levels as early as on day 12 of gestation. The level of expression increased significantly (P<0.01) on day 14 of pregnancy and remains elevated up to day 16 and

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18. It, thereafter, started reducing significantly (P<0.01) on day 20, 30, 40 and reached undetectable level on day 50 of pregnancy. It may, therefore, be concluded that non-surgical removal of conceptus on day 14, 16, 18 and 20 of pregnancy extended the cycle length up to 8-47 days and strong expression of IFNT during day 14-16 of pregnancy indicating embryonic signal to dam and establishment of Maternal Recognition Pregnancy (MRP) in buffalo.

Keywords: Buffalo, Conceptus, Early pregnancy, Interferon tau, Maternal recognition of pregnancy

INTRODUCTION

Embryonic mortality is one of the most important causes of poor reproductive efficiency in cattle and buffalo. It has been estimated that in bovine about 30-40 % of embryos are lost and most of these losses occur between day 8 and 16 of pregnancy (Sreenan and Diskin, 1986; Pawshe *et al.,* 1994; Binelli *et al.,* 2009). The survivability of embryo during the early period is mostly dependent on the efficiency with which the maternal recognition of pregnancy (MRP) is established (Roberts and Schalue-Francis, 1990). The MRP results from antiluteolytic signaling between the trophoblast of the conceptus and the maternal system. The major conceptus secretory product responsible for initiation of MRP is known as interferon tau (IFNT) secreted by the trophoblast cells between days 13 to 21 and 15 to 24 of gestation in sheep and cattle, respectively (Godkin *et al*., 1982; Bartol *et al.,* 1985).

The primary signal for MRP is IFNT which helps to maintain the structural and functional integrity of the corpus luteum in cattle, sheep and goat (Roberts *et al.,* 1992). IFNT allows progesterone production by the corpus luteum and hence maintains the endometrium in a receptive state for establishment of pregnancy. The elongating conceptus secretes IFNT in large amount which immediately precedes definitive attachment of trophectoderm to the uterine epithelium, i.e. 12–21 days in sheep and 14–25 days in cattle (Helmer *et al.,* 1987; Hansen *et al.,* 1988; Farin *et al.,* 1990). Expression of IFNT has been reported as early as day 7 in sheep and day 12 of pregnancy in cattle. However, the concentrations of oIFN-τ and bIFN-τ mRNA transcripts increased significantly during day 12 to 13 and 15 to 17 in sheep and cattle coinciding with the time of MRP (Farin *et al.,* 1990). Studies on conceptus removal/infusion of conceptus homogenates on days 15 to 17 of cycle in cattle extended the estrous cycle length and lifespan of CL indicating presence of biologically active molecules secreted from the conceptus for its recognition by the mother i.e. period of MRP (Betteridge *et al.,* 1980; Northey and French, 1980). Information as such on period of MRP and expression profile of IFNT mRNA in buffalo is lacking. Therefore, the present study was conducted to ascertain the effect of conceptus removal on cyclic length and expression profile of IFNT during critical period to correlate the period of MRP in buffaloes.

MATERIALS AND METHODS

Experimental animals

The study was conducted on adult female non-descript buffalo (n=15) of 5-8 years of age with good body condition (Score: 4-5), maintained at experimental animal shed of Animal Reproduction Division, IVRI, Izatnagar (UP), India. The animals were housed under intensive system of rearing confined in a large barn having asbestos roof and cemented floor and kept under uniform feeding and managemental conditions. Animals were provided with green fodder, concentrates as per standard and water ad libidum.

Non-surgical removal of conceptus

All the experimental buffaloes were subjected for detection of estrus using a teaser bull followed by rectal examination and day of standing estrus was designated as day 0 of that cycle. Buffaloes detected in estrus were inseminated using frozen semen of Murrah buffalo and flushed by non-surgical method of uterine flushing on days 12, 14, 16, 18 and 20 of post-insemination for collection of conceptus/ remnants as per the procedure described by the Northey and French (1980) with desired modifications. The flushed medium collected in embryo concentrator (0.75µ pore size) and examined under stereozoom microscope for the presence of conceptus parts/remnants. Animals where conceptus/remnants was recovered considered as conceptus positive (n=42) and only included for further study. The conceptus tissues were subjected to RNA isolation using TRIzol (Ambion, USA). Streptopenicillin @ 2.5g was infused in to the uterus following the flushing of uterine horn to prevent endometritis in the experimental buffaloes.

Conceptus tissue collected on different days in the experiment I and the conceptus also collected from morbid genitalia were subjected for RNA isolation. The pregnant (approximately days 20-50) morbid genitalia was brought into laboratory from local slaughter house immediately after slaughter. The genitalia was washed with normal saline (pH 7.0) and the conceptus was removed after dissection of uterus. The age of conceptus was determined by measuring the crown rump length (CRL) (Richardson *et al.,* 1980). Proper care was taken to minimize the effect of ribonuclease (RNAse) activity using 0.1% DEPC treated Milli Q water for preparation of solutions, glass wares and sterile hand gloves etc.

Observation of estrous cycle length

After non-surgical flushing of uterus during different days of post-insemination, the experimental buffaloes were kept under observation to observe onset of estrus. Corpus luteum was palpated through per-rectum throughout experiment for assessment of reproductive status of the experimental buffaloes.

Total RNA isolation and cDNA synthesis

Under experiment II, total RNA was isolated from conceptus/ remnants recovered from experiment I and pregnant morbid genitalia using TRIzol (Ambion, USA) and the integrity of extracted RNA was checked by running agarose gel electrophoresis and visualized typical RNA band pattern. The first strand cDNA was synthesized using

Qiagen RT kit (Germany). Following first strand synthesis, the second strand was PCR amplified using gene specific degenerate primers following manufacture's protocol. The synthesized cDNA was stored at -80°C until use.

Table.1. Details of primer used for real time-PCR

Type of Gene	Primer Sequences	Product Size	Annealing Temperature	Reference
IFNT	Forward: 5'-GCCCTGGTGCTGGTCAGCTA -3' Reverse: 5'-CATCTTAGTCAGCGAGAGTC -3'	564 bp	46 °C	Gutierrez-Adan *et al*. (2004)
GAPDH (House keeping Gene)	Forward: 5'-GTCTTCACTACCATGGAGAAGG-3' Reverse: 5'-TCATGGATGACCTTGGCCAG-3'	197 bp	58°C	Berisha *et al*. (2002)

Real Time RT-PCR

Real time PCR assay was carried out by quantitative Real Time PCR (MX 3000 P System, Stratagene, USA) using specific published primers (Table. 1). Real Time PCR reaction was performed in 96 well plates using Qiagen QPCR Master Mix (Quantitaive Real time polymerase chain reaction (qRT-PCR); Qiagen, USA) following manufacture's protocol. PCR reaction mixture (25 µl) contained: 2.5 µl 10x buffer, 3mM MgCl2, 100 µM of each dNTPs, and 0.2 µM of each primer. In addition, the double-stranded DNA dye, SYBR Green I, (1:3,000 of 10,000 X stock solution) was included in each reaction. The PCR protocol included an initial step of 95°C (10 min), followed by 40 cycles of 95°C (30 sec), 46°C (1.0 min), and 72°C (1.0 min). The real time data was analyzed by delta ct method after normalizing with GAPDH (Livak and Schmittgen, 2001). The arithmetic mean of Ct values (m Ct) was calculated.

Statistical analysis

The statistical analysis was carried out by using computer software (SPSS version 13.0 for windows). The data on the effect of non-surgical removal of conceptus on cycle length and the expression profile of IFNT during early pregnancy was analyzed by independent sample 't' test and paired 't' test, respectively.

RESULTS AND DISCUSSION

Effect of non-surgical removal of conceptus on cycle length

The inter estrous interval or cycle length in conceptus recovered buffaloes were 23.50±0.96, 36.60±3.35, 41.25±2.67, 31.73±2.61 and 45.20±7.03 days after non-surgical flushing of uterus on day 12, 14, 16, 18 and 20 post insemination, respectively (Fig.1). The mean cycle length was significantly higher ($P<0.01$) following non surgical removal of conceptus on day 14, 16, 18 and day 20 of

post-insemination when compared to day 12. The results further revealed that removal of conceptus on day 14, 16, 18 and 20 following insemination resulted prolongation of cycle length by 8 to 47 days.

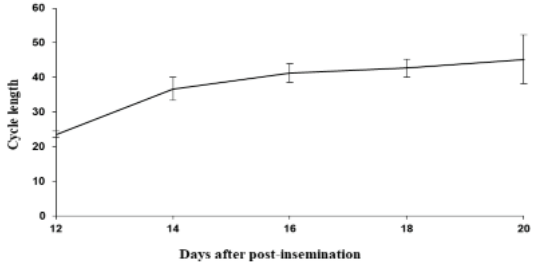

Fig. 1: Effect of non-surgical removal of conceptus on cycle length in buffaloes

In the present study, the mean cycle length was significantly higher on day 14, 16, 18 and 20 post-insemination following non-surgical removal of conceptus when compared to day 12 indicating embryonic signaling to the dam. The findings of the present study, further revealed that removal of conceptus on day 14, 16, 18 and 20 following insemination resulted extension of cycle length by 8 to 47 days signaling presence of IFNT secreted from the conceptus preventing luteolysis thus increases the life span of corpus luteum in buffaloes. However, removal of conceptus on day 12 failed to extend the inter estrus cycle length further indicates the absence of signal from the dam. The results of the present study are in accordance with Northey and French (1980) who also observed extension of cycle length and CL life span in cattle after non-surgical removal of conceptus on day 15 or 17. The extension of cycle length as observed in the present study following removal of conceptus collaborates with the findings in sheep where conceptus removal before day 12 of pregnancy failed to extend inter estrus interval (17 to 18 days) and removal on days 14 and 15 resulted in extension of inter estrus intervals by 24 to 25 days (Moore and Rowson, 1966) and in goat where removal of conceptuses from the uterine lumen between days 13 and 15 did not prolong the lifespan of the CL, but removal on day 17 increased inter-estrus interval by 7-10 days (Gnatek *et al*., 1989) indicating the period of MRP.

Total RNA isolation and cDNA synthesis

On native agarose gel electrophoresis, total RNA was observed as three high intensity ribosomal RNA bands of 28S, 18S and 5S. The concentration of isolated RNA was 1.20 µg per mg of tissue. A_{260}/A_{280} of the obtained RNA was 1.8 indicating the high purity and absence of contamination with protein and DNA. cDNA was synthesized from the extracted RNA using RT-PCR kit (Qiagen, Germany). Amplification of β-actin gene (850bp) was also done every time and considered as a positive control.

Real Time RT-PCR

The relative quantity of IFNT on day 12, 14, 16, 18, 20, 30, 40 and 50 of early pregnancy was 0.334±0.050, 2.398±0.181, 2.699±0.197, 1.538±0.155, 1.094±0.183, 0.788±0.065, 0.656±0.082 and 0.002±0.00 fold changes when compared to the house keeping gene (GAPDH), respectively (Fig.2). The expression of IFN-τ was found low but at detectable levels as early as on day 12 of gestation. The level of expression increased significantly ($P<0.01$) on day 14 of pregnancy and remains elevated up to day 16 and 18.

It, thereafter, started reducing significantly (P<0.01) on day 20, 30, 40 and reached undetectable level on day 50 of pregnancy. The results further revealed a strong expression of IFN-τ on day 14- 16 of pregnancy as compared to day 12 post insemination.

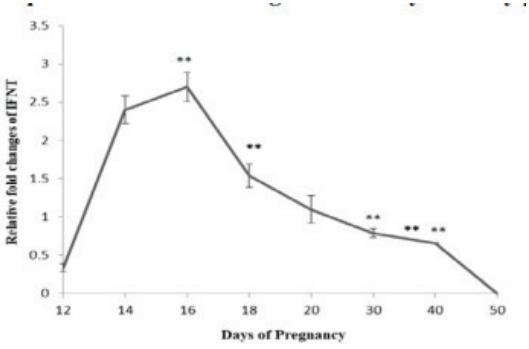

Fig. 2. Relative expression of IFNT during different days of early pregnancy in buffaloes

In ruminants, IFNT is the primary recognition signal secreted by the elongating conceptus that acts on the endometrium to inhibit luteolytic mechanism and ensure the continued production of progesterone thus establishment of pregnancy. The results also support the findings of various authors reported in sheep and cattle for determination of MRP (Northey and French, 1980; Knickerbocker *et al.,* 1986).

The expression of IFNT in the present study was found low but at detectable level on day 12 of gestation. The level of expression increased significantly (P<0.01) on day 14 of pregnancy and remains elevated up to day 16 of post-insemination. It, thereafter, started reducing significantly (P<0.01) on day 20 of pregnancy. The results further revealed a strong expression of IFNT on day 14-16 of pregnancy as compared to day 12 indicates, there may be signal for establishment of maternal recognition of pregnancy during this period in buffaloes. The result of present study is in accordance with other authors who detected expression of IFNT as early as on day 12 but it strongly expressed on day 15/16 of pregnancy and remain static by day 18/19 of pregnancy in cattle (Farin *et al*., 1989; Bertolini *et al*., 2002; Robinson *et al.,* 2006). The mRNA is detectable in low concentration in embryos from day 10 to 12 of pregnancy, increased from day 13 to 15, coincident with the time of maternal recognition of pregnancy and decreases shortly afterwards. The peak production occurs at day 16 in sheep (Godkin *et al.*, 1982) and day 17 in cattle (Bartol *et al.,* 1985) and expression is detectable in the trophectoderm until about day 20 in sheep or day 25 in cattle (Demmers *et al.,* 2001).

Stewart *et al.* (1989) and Farin *et al.* (1990) demonstrated transient expression of oIFN-τ during a limited period of development and localization of its expression to the extra-embryonic trophectoderm of conceptus. The bIFN mRNA is localized exclusively in the trophectoderm of conceptus and not found in either endodermal or yolk sac tissues. However, on the basis of immunocytochemical analysis, bIFN mRNA has been demonstrated in both binucleate and monocucleate cells of trophoblast (Lifsey *et al.,* 1989). A minute expression of IFNT can be detected near the time of blastocyst hatching (Ashworth and Bazer, 1989). The marked increase in the expression of the IFNT gene on day 13 in sheep and day 15 in cattle coincides with the morphological transition of the blastocyst from a spherical to a filamentous form, rather than

strictly correlating with the day of pregnancy (Farin *et al.,* 1990). However, Robinson *et al.* (2006) reported dramatic increase in IFNT protein on day 14-18 accompanied with little changes in the intensity of IFNT expression. Moreover, on the basis of size of embryo, well elongated embryo (> 10 cm) produced more IFNT than smaller embryos but showed similar level of expression. The increase in IFNT mRNA concentrations responsible for the maternal recognition of pregnancy in cattle results from the increase in embryonic size during elongation.

The expression of IFN-τ starts decreasing from day 16 and 25 of pregnancy in sheep and cattle, respectively (Farin *et al.,* 1990). The termination of IFN-τ expression is strictly dependent on implantation since the IFN-τ synthesis ceases as soon as the trophoblast is attached to the maternal caruncles (Guillomot *et al.,* 1990). In the present study, the mild expression of IFN-τ was detected on day 30 and 40 of pregnancy in buffalo confirming the findings of above workers. Therefore, termination of IFNT expression may depend on process of the implantation which starts around day 25 and completes around 50 of pregnancy in buffalo (Campanile *et al.,* 2010). However, the function associated with this extended period of expression as well as secretion of bIFN-τ is unclear (Farin *et al.,* 1990). Therefore, further study is warranted to determine the functional relation of extended period of IFN-τ expression in buffalo.

Expression profile of IFNT in the present study indicated that MRP signal in buffalo from day 14 onwards. The establishment of MRP in cattle has been reported at 16-17 days of pregnancy. In buffalo, occurrence of MRP signal seems to be 1-2 days earlier than cattle. The earlier onset of MRP signal in buffalo may be due to a rapid differentiation and development of embryo in buffalo than the cattle. Growth rate of early embryo in buffalo is faster (4-36 h) as evinced by presence of morula stage in the uterus on 4.5 (108 hrs) and zona hatching occurs on 114-117 h post estrus (Drost and Elsden, 1985; Karaivanov *et al.,* 1987). The early embryonic development in buffalo might be able to signal the mother from day 14 onwards due to its faster growth thus leading to extension of life span of CL and establishment of pregnancy. In the present study, the removal of conceptus on day 14 of pregnancy onwards ensued extension of cycle length and life span of CL indicating embryonic signal and establishment of MRP in buffaloes.

It may, therefore, be concluded that nonsurgical flushing of uterus on day 14, 16, 18 and 20 of pregnancy extended the cycle length up to 8-47 days with extended secretion of progesterone and strong expression of IFNT during day 14-16 of pregnancy supports that the maternal recognition or embryonic signal may have occurred during day 14-16 of pregnancy in buffaloes. This signal is one day ahead in buffaloes, because the early embryonic development is faster compared to cattle.

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