APPLICATION OF RECOMBINANT BOVINE INTERFERON ALPHA (rbIFNα) FOR *CORPUS LUTEUM* MAINTENANCE IN CYCLING BUFFALOES

THESIS SUBMITTED TO THE
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FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY
IN
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(ANIMAL PHYSIOLOGY)

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2002

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To

Dr. B.S. Prakash

.... For he gave me the impetus

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This is to certify that the thesis entitled, **"APPLICATION OF RECOMBINANT BOVINE INTERFERON ALPHA (rbIFN$_\alpha$) FOR *CORPUS LUTEUM* MAINTENANCE IN CYCLING BUFFALOES"** submitted by **Mr. D.P. MISHRA** towards the partial fulfilment of the award of the degree of **DOCTOR OF PHILOSOPHY** in **ANIMAL PHYSIOLOGY** of the **NATIONAL DAIRY RESEARCH INSTITUTE (DEEMED UNIVERSITY)**, Karnal (Haryana), India, is a bonafide research work carried out by him under my supervision, and no part of the thesis has been submitted for any other degree or diploma.

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LIST OF ABBREVIATIONS

amol	=	Attomol
BSA	=	Bovine serum albumin
CPM	=	Counts per minute
DPM	=	Disintegration per minute
EIA	=	Enzyme immunoassay
i.m.	=	Intramuscular
IFN	=	Interferon
IgG	=	Immunoglobulin G
kDa	=	Kilo dalton
LH	=	Luteinizing hormone
ng	=	Nanogram
PBS	=	Phosphate buffer saline
pg	=	Picogram
$PGF_{2\alpha}$	=	Prostaglanding $F_{2\alpha}$
PGFM	=	13,14-dihydro-15-keto-$PGF_{2\alpha}$
pmol	=	Picomol
$rbIFN_{\alpha}$	=	Recombinant bovine interferon alpha
RIA	=	Radioimmunoassay
$roIFN_{\alpha}$	=	Recombinant ovine interferon alpha
rpm	=	Revolutions per minute
µg	=	Microgram
µl	=	Microlitre

ABSTRACT

A study was undertaken to investigate the efficacy of recombinant bovine interferon alpha (rbIFN$_\alpha$) for *corpus luteum* maintenance in cycling Murrah buffaloes. Fifteen non-lactating cycling buffaloes free of any physiological anatomical and infectious disorders were selected from the National Dairy Research Institute's herd. The animals were maintained under standard feeding and managemental conditions in a separate paddock throughout the experimental period. Plasma progesterone analysis was used for estrous cycle monitoring. Heat detection was carried out by visual observations, vasectomized bull parading and plasma progesterone analysis. As an essential pre-requisite for carrying out the study a sensitive enzyme immunoassay was standardized for 13,14-dihydro-15-keto PGF$_{2\alpha}$ (PGFM) in buffalo plasma and biologically validated in peri-estrus, cyclic, early pregnancy, reproductive infections and ovarian cystic conditions in buffaloes. The buffaloes were categorized into groups I, II and III, consisting of 5 buffaloes each. Each of the buffaloes in groups I, II and III were administered 4, 8 and 16 mg/day of rbIFN$_\alpha$ through the intramuscular route on days 14 through 16 post-estrus. In case of each animal, blood samples were collected two hours before and 8 hours after the first injection of rbIFN$_\alpha$ at 15 minutes intervals along with rectal temperature measurements at hourly intervals, twelve hours before and eighty hours after the first injection of rbIFN$_\alpha$. Thereafter, blood samples were collected daily till estrus in each animal. The rbIFN$_\alpha$ treated cycles were compared with two cycles pre-treatment and two cycles post-treatment. The 16 mg/day dose of rbIFN$_\alpha$ significantly ($P<0.01$) extended estrous cycle lengths and was selected to be used in the oxytocin mediated PGFM release experiment. The blood samples collected during the estrous cycle length study, were analyzed for LH and progesterone through sensitive enzyme immunoassay and radioimmunoassay, respectively. The influence of rbIFN$_\alpha$ on oxytocin mediated PGFM release was studied in six buffaloes. The buffaloes were administered an oxytocin challenge (50 IU) on day 17 of their control cycles. In the subsequent cycles, rbIFN$_\alpha$ (16 mg/day) was administered on days 14 through 16 with the oxytocin administration in a similar manner. Blood samples were collected 2 hours before and 6 hours after the oxytocin administration at 15 minutes interval in both the cases.

The following conclusions were drawn from the present study. The PGFM enzymeimmunoassay standardized and validated in the present study is sufficiently reliable and specific for estimation of PGFM levels that can help in reproductive health monitoring in buffaloes. rbIFN$_\alpha$ administration can extend *corpus luteum* function. Administration rbIFN$_\alpha$ produced a transient rise in rectal temperature associated with a decrease in progesterone profiles for a brief period. However, rbIFN$_\alpha$ did not suppress peripheral LH levels. At the same time, it significantly ($P<0.01$) decreased oxytocin mediated PGFM release. Therefore, it can be concluded from the present investigation that rbIFN$_\alpha$ is a cytokine that can extend *corpus luteum* function through anti-luteolytic mechanisms in buffaloes. The results suggest a possibility of exploring rbIFN$_\alpha$ administration post-insemination in buffaloes as a practical tool for fertility improvement in this species.

सारांश

मदचक्रीय मुर्रा भैंसों में कार्पसल्यूटियम के अनुरक्षण में पुर्नयोगज गौ इन्टरफेरोन अल्फा (rbIFNα) की क्षमता की जांच हेतु अध्ययन किया गया। इस हेतु राष्ट्रीय डेरी अनुसंधान संस्थान की पशुशाला से 15 दूध न देने वाली मदचक्रीय भैंसों को चुना गया जो कि किसी भी प्रकार के शरीर कार्यिकी एवं संक्रमणीय विकारों से मुक्त थी। सभी पशुओं को प्रयोग के दौरान मानक पोषण एवं प्रबन्धन के अर्न्तगत अलग से एक वाड़े में रखा गया। मदचक्र के अनुश्रवण हेतु प्लाज्मा प्रोजेस्ट्रोन विश्लेषण का प्रयोग किया गया। मद जाँच का अध्ययन दृष्टिप्रेक्षण, नसबन्धित साँड एवं प्लाज्मा प्रोजेस्ट्रोन स्तर के माध्यम से किया गया। इस अध्ययन हेतु आवश्यक संवेदनशील एन्जाइमइम्यूनोऐसे (EIA) को भैंसों के प्लाज्मा में 13,14–डाइहाईड्रो–15–कीटो पी.जी.एफ.$_2$अल्फा (PGFM) के लिए मानकीकृत किया गया और भैंसों के पेरीमद, मदचक्रीय, प्रारम्भिक गर्भावस्था, जननीय संक्रमण तथा पित्ताशय अण्डाशय हेतु जैविक रूप से मानकीकृत किया गया। भैंसों को 5, 5 के तीन समूहों में बांटा गया। तीनों समूहों I,II एवं III की भैंसों में क्रमशः 4,8,16 मिग्रा. rbIFNα के प्रतिदिन अन्तः पेशीय मार्ग से मदचक्र के 14वें से 16वें दिन तक दिया गया। सभी पशुओं के रक्त नमूने,rbIFNα के प्रथम टीके के दो घंटे पूर्व तथा 8 घंटे पश्चात् 15 मिनट के अंतराल में लिए गए। प्रथम टीके से 12 घंटे पूर्व तथा 80 घंटे पश्चात 1 घंटे के अन्तराल पर गुदातापलिया गया। तत्पश्चात मदचक्र आने तक प्रतिदिन प्रत्येक पशु का रक्त नमूना लिया गया। rbIFNα उपचारित मदचक्र की तुलना उपचार से पूर्व के 2 मदचक्रों तथा उपचारोपरान्त 2 मदचक्रों से की गई। rbIFNα की 16 मिग्रा. की प्रतिदिन मात्रा ने मदचक्र की अवधि को सार्थक रूप से बढ़ाया तथा इसे आक्सीटोसिन मध्यस्थ पी.जी.एफ.एम. स्राव प्रयोग के लिए चुना गया। मदचक्र अवधि अध्ययन हेतु संग्रहित किये गये रक्त नमूनो का विश्लेषण ल्यूटिलाइज हार्मोन तथा प्रोजेस्ट्रोन हेतु संवेदनशील एंजाइमइम्यूनोऐसे (EIA) तथा रेडियोइम्यूनोऐसे (RIA) से क्रमशः किया गया। आक्सीटोसिन मध्यस्थ पी.जी.एफ.एम. स्राव पर rbIFN α के प्रभाव का अध्ययन 6 भैंसों पर किया गया। भैंसों में नियंत्रित मदचक्र के 17वें दिन पर 50 अर्न्तराष्ट्रीय इकाई आक्सीटोसिन दिया गया। आगे के मदचक्रों में rbIFNα की 16 मिग्रा. प्रतिदिन की मात्रा 14वें से 16वें दिन तक आक्सीटोसिन के समान दी गई। दोनों अवस्थाओं में आक्सीटोसिन देने से 2 घंटे पूर्व तथा 6 घंटे पश्चात 15 मिनट के अन्तराल पर रक्त के नमूनें एकत्रित किये गये।

किये गये अध्ययन से इस प्रकार के निष्कर्ष प्राप्त हुए, इस अध्ययन द्वारा मानकीकृत पी.जी.एफ.एम. इन्जाइमइम्यूनोऐसे (EIA) को पी.जी.एफ.एम. के स्तरों के आकलन में पर्याप्त रूप से विश्वसनीय एवं विशिष्ट पाया गया जो कि भैंसों के जनन स्वास्थय के अनुश्रवण में सहायक हो सकता है। rbIFNα का प्रयोग कार्पसल्यूटियम की कार्यक्षमता को बढ़ाता है। rbIFNα के प्रयोग से अल्प समय हेतु गुदातापमें आशिंक बढ़ोत्तरी एवं साथ–साथ प्रोजेस्ट्रोन स्तर में कमी पाई गई। जबकि इसने परिधीय ल्यूटिलाइज हार्मोन के स्तर को कम नहीं किया। ठीक इसी समय, rbIFN αने आक्सीटोसिन मध्यस्थ पी.जी.एफ.एम. स्राव को सार्थक रूप से कम किया । अतः उपस्थित अध्ययन से ये निष्कर्ष निकलता है कि rbIFNα एक ऐसा साइटोकाइन है जो कि भैंसों में प्रतिल्यूटोलिटिक क्रियाविधि द्वारा कार्पसल्यूटियम की कार्यक्षमता को बढ़ा सकता है। परिणाम बताते हैं कि भैंसो में वीर्यसंचनोपरान्त प्रजनन उर्वरता के सुधार हेतु rbIFNα का प्रयोग एक व्यवहारोपयुक्त विधि साबित हो सकती है।

CHAPTER - 1

Introduction

1. INTRODUCTION

Dairying constitutes an integral part of the agrarian economy of India. India's status as the largest milk producing country in the world is attributed to its large bovine population comprising of 194.7 million cattle and 79.5 million buffaloes (Dairy India, 1997). Buffaloes in particular are multipurpose animals contributing more than half of the total milk produced annually in the country despite being less than 40 percent of the total bovine population. Good reproductive performance is an essential pre-requisite for high milk production. This entails the requirement of the ability to mate and conceive in quick time, and deliver the viable youngone at the end of normal gestation period. In this regard, the buffalo is found lacking, due to certain reproductive problems, which include poor conception rates, embryonic mortality and repeat breeding, that makes it a sluggish breeder. As reproductive efficiency is an essential consideration of economic buffalo production, high incidences of embryonic mortality leading to repeat breeding disturbs the reproductive rhythm and renders buffalo production less profitable. Embryonic mortality leading to repeat breeding is one of the most serious and frustrating problems concerning buffalo reproduction; serious due to economic losses and frustrating due to the reason that the problem is well concealed and is difficult to correct.

Clinical cause of embryonic mortality and repeat breeding is known to be luteal insufficiency, post-breeding (Arthur *et al.*, 1989). The *corpus luteum* is essential for progesterone secretion and indispensable for establishment of successful pregnancy. Implantation allows the conceptus and uterine endometrium to achieve intimate contact for nutrient exchange and endocrine communication. At the appropriate time, the conceptus must produce hormones or protein signals to signal its presence to the maternal system.

This signal is necessary for *corpus luteum* maintenance, production of progesterone and continued endometrial development and secretory activity. This critical period when the conceptus must signal its presence to allow pregnancy to be established is called maternal recognition of pregnancy (Short, 1969). The conceptus from several mammalian species prior to implantation secrete proteins, that belong to the family of interferons (Imakawa *et al.*, 1987). In ruminants, the blastocyst in the pre-implantation stage secretes trophoblastic proteins, which have structural homology to the alpha group of interferons (IFN_{α}). The secretion of these proteins are considered as the first signals for the maternal recognition of pregnancy. In these species, maternal recognition of pregnancy is brought about by these proteins, acting on the endometrium and inhibiting the release of prostaglandin F_2 alpha ($PGF_{2\alpha}$) thus maintaining the *corpus luteum* (Bazer *et al.*, 1991). These proteins are expressed prior to the time at which actual luteolysis would occur in cycling cows (Thatcher *et al.*, 1997), and sheep (Farin *et al.*, 1990) post-ovulation. It is established that the insufficient production of these proteins causes premature luteolysis of the *corpus luteum* (Bazer *et al.*, 1994), and hence cessation of progesterone production which results in embryonic mortality and repeat breeding in the cow and sheep. An emerging biotechnique which has been attempted for *corpus luteum* maintenance and sustenance of progesterone production during early pregnancy in European and American livestock, especially sheep, goat and cattle, is the exogenous administration of recombinantly derived interferons post-insemination.

Uterine infusion or intramuscular administration of recombinant bovine interferon alpha ($rbIFN_{\alpha}$) to non-pregnant sheep or cattle has been shown to prolong the luteal phase, bring down embryonic mortality and significantly enhance pregnancy rates (Plante *et al.*, 1988; Nephew *et al.*, 1990; Schalue-Francis *et al.*, 1991; Flint *et al.*, 1991; Martinod *et al.* (1991).

The application of recombinant interferons post-insemination has shown to alter the balance favourably for *corpus luteum* maintenance through their antiluteolytic mode of action in cattle and sheep (Plante *et al.*, 1989; Nephew *et al.*, 1990; Newton *et al.*, 1990). This has further strengthened the possibility of these interferons being used as fertility enhancing agents in other ruminant species as well.

In the Indian context, such a biotechnique can be particularly useful for addressing the problem of repeat breeding in buffaloes as investigations conducted so far also suggest that embryonic mortality is higher in buffaloes in comparison to cattle (Singh and Gangwar, 1976; Chatterjee *et al.*, 1985; Tomar and Verma, 1987; Kaul and Prakash, 1994). The current available literature points out to a complete lack of information regarding the role of interferons particularly in relation to pregnancy in case of buffaloes. No studies have been conducted till date on the possible application of recombinant interferons for *corpus luteum* maintenance or improvement of pregnancy rates in any of the Indian livestock species.

Establishment of successful pregnancy is a complex interplay of endocrine factors involving major hormones like luteinizing hormone, prostaglandins and progesterone. The influence of administration of recombinant interferon alpha on circulatory profiles of luteinizing hormone, prostaglandins and progesterone in any of the Indian livestock species is also yet to be determined. Further, the applicability of this new biotechnique for improving fertility in buffaloes can be gauged only after initially determining its role on *corpus luteum* maintenance and changes in endocrine and physiological responses brought about by its application.

For this, basic investigations are needed with a systematic approach involving studies on (a) the efficacy of recombinant interferon alpha (rbIFN$_\alpha$) in extending luteal function, and (b) endocrine changes associated with recombinant interferon application in buffaloes.

These studies will then form the basis to explore the subsequent feasibility of practical application of recombinant bovine interferon for augmentation of fertility. With this backdrop, the present investigation has been taken up in buffaloes with the following specific objectives :

1) To study the influence of rbIFN$_\alpha$ dose response on estrous cycle length.

2) To study the endocrine changes (PGFM, progesterone and luteinizing hormone) associated with rbIFN$_\alpha$ administration.

CHAPTER - 2

Review of Literature

2. REVIEW OF LITERATURE

2.1 THE SCOPE

The process of fertilization of an ovum by a spermatozoon initiates a complex series of developmental, metabolic and endocrine events which results in the establishment of pregnancy. The establishment of pregnancy induces a blockade of *corpus luteum* (CL) regression resulting in maintenance of CL. In ruminants, CL maintenance is of fundamental importance during the estrous cycle of a cyclic animal beyond its life span for successful establishment of pregnancy (Martal *et al.*, 1997). The primary function of the *corpus luteum* is the secretion of the hormone progesterone, an essential requirement for maintenance of normal pregnancy (Niswender *et al.*, 2000). CL life span is influenced and can be manipulated by mechanisms and their interactions associated with secretions from the pituitary, ovary, uterus and significantly during early pregnancy by the conceptus (Garverick *et al.*, 1992). The ruminant conceptus during the pre-implantation stage secretes trophoblastic proteins, which have structural homology to the alpha group of interferons (IFNα), serving as the first signals for maternal recognition of pregnancy (Bazer *et al.*, 1991). The insufficient production of these signalling proteins result in the demise of a CL, lack of progesterone production and embryonic mortality in ruminants (Martal *et al.*, 1987). Extensive research efforts have been carried out in the last decade with the objectives of reducing incidences of embryonic mortality through *corpus luteum* maintenance and improving pregnancy rate in these species by application of recombinant interferons and conceptus secretory proteins, structurally related to IFNα group of molecules (Godkin *et al.*, 1984; Homeida *et al.*, 1986; Helmer *et al.*, 1987; Gnatek *et al.*, 1989; Nephew *et al.*, 1990; Martinod *et al.*, 1991; Mirando *et al.*, 1990; Plante *et al.*, 1991; Meyer *et al.*, 1995; Spencer *et al.*, 1995; Martal *et al.*, 1997).

In order to comprehensively identify the research investigations undertaken by different workers with regards to the application of recombinant interferons for CL maintenance in ruminants, information dealing with, endocrine regulation of CL maintenance, embryonic signal mediated alteration of luteolysis, maternal recognition of pregnancy, luteal insufficiency due to failure of adequate maternal recognition of pregnancy resulting in embryonic mortality and application of recombinant interferons for CL maintenance in ruminants have been reviewed separately. While, all the information regarding application of recombinant interferons for CL maintenance thereby reducing embryonic mortality or improving pregnancy rates pertains to European and American livestock, there are no reports regarding the application of recombinant interferons and the physiological and endocrinological changes brought about by them in the treated animals, available for any of the Indian livestock species or their crosses.

2.2 *CORPUS LUTEUM* : THE TRANSIENT ENDOCRINE GLAND

During the estrous cycle of ruminants there are continuous phases of growth and regression of intraovarian components. Initiation of estrous cycle in ruminants involves the growth of several follicles of which only one is destined to dominate, mature and finally ovulate. Soon after ovulation the flattened and soft ovary indicates a small depression at the site of ovulation, where the ruptured follicles get transformed in to a transitory endocrine structure, known as the *corpus luteum* (CL) .It is basically composed of the granulosa and theca cells. Granulosa cell layers form infoldings filling up the cavity left by the ova. The central core of the stromal tissue and large blood vessels present in these folds develop and distend respectively. Hypertrophy and luteinization of the granulosa cells soon after ovulation leads to formation of the luteal tissue. The luteal cells can be classified in to three groups: large luteal cells (>25 µm in diameter), small luteal cells (10-20 µm in diameter) and smaller non-steroidal cells (<10 µm in diameter). The small luteal cells have large number of receptors for luteinizing hormone (LH), while the large luteal cells have majority of $PGF_{2\alpha}$ receptors. The small luteal cells are known to be

six times more sensitive than the large luteal cells. As the cycle progresses the small luteal cells get transformed into large luteal cells. The CL has a definite pattern of development during its lifespan. In ruminants 3-4 days after ovulation, the CL develops at the site of ovulation .By 7-8 days it is fully formed measuring 2.0-2.5 cm in diameter in cattle. In cattle the CL is a firm, non yielding structure on the surface of the ovary which becomes firmer and harder with age. Blood flow is high in the ovary containing CL, which makes available the extraovarian factors essential for CL function. On day 17 of the cycle a marked reduction in size of the CL is noticeable followed by a rapid regression around 24-48 hours before the onset of estrus. The transient nature of the *corpus luteum* (CL) is the most intriguing feature of this gland of internal secretion (Pate, 1994). The major secretion of the CL is the hormone progesterone, known to have its biologic actions on the reproductive tract and the hypothalamo-hypophyseal axis (Niswender and Nett, 1988). It is of evolutionary significance that the simplest steroidogenic pathway is for progesterone, which plays a key role in successful pregnancy establishment (Niswender *et al.*, 1994). In the CL, cholesterol is utilized as the substrate for progesterone synthesis. Usually, this cholesterol is obtained from high density lipoproteins (HDL) and low density lipoproteins (LDL), rather than being synthesized *de novo* from acetate (Niswender *et al* 2000). Uptake of LDL involves receptor-mediated endocytosis, whereas uptake of HDL involves binding to specific membrane binding sites and shuffle of cholesterol into the cell by an unknown mechanism. Cholesterol from various sources in the CL can be incorporated into cholesterol esters by acyl CoA cholesterol acyl transferase (ACAT), and stored as lipid droplets. Lipoprotein binding sites increase proportionately in relation to increased rate of progesterone secretion on the CL (Rajkumar *et al.*, 1985; Brannian *et al.*, 1991).

Release of cholesterol from cholesterol esters is dependent on (a neutral cholesterol esterase) hormone sensitive lipase. Activity of this enzyme is regulated by phosphorylation of two serine residues, brought about by cyclic AMP dependent protein kinase A (PKA) and Ca^{++}/calmodulin dependent protein kinase resulting activation and deactivation, respectively.

The rate-limiting step in progesterone biosynthesis is the cleavage of the side chain of cholesterol. It involves transport of cholesterol from cytoplasm to the mitochondria and from the outer to the inner mitochondrial membrane, for side chain cleavage, with the aid of cytoskeleton. The cholesterol transport involves mediation by steroidogenesis activator peptide, sterol carrier protein 2, endozepines / benzodiazepenes and lipoxygenase metabolites. At the inner mitochondrial membrane, the cholesterol side chain cleaving enzyme ($P450_{SCC}$) is localized. Three proteins are involved in conversion of cholesterol to pregnenolone : adrenodoxin, adrenodokin reductase and cytochrome $P450_{SCC}$. Messenger mRNAs for these proteins are regulated similarly and thus mRNA for $P450_{SCC}$ is often used to monitor transcription of genes encoding enzymes in this complex (Rodgers *et al.*, 1987). Conversion of pregnenolone to progesterone is catalyzed by 3β-hydroxy steroid dehydrogenase, Δ^5, Δ^4 isomerase (3-βHSD). CL secretes progesterone which is essential for normal pregnancy in ruminants (Wiltbank, 1994). In turn, the CL secreted progesterone regulates the length of estrous cycle. Not only length of estrous cycle is an index of CL maintenance, but the progesterone levels are indicative of luteal activity and CL life span (Smith *et al.*, 1994). The CL life span is regulated by the uterine luteolysin $PGF_{2\alpha}$ (McCracken *et al.*1972). The bovine CL has receptors for $PGF_{2\alpha}$ (Powell *et al.*, 1975), and the binding affinity of the receptors increases 203 fold from day 13 to 20 of the estrous cycle (Rao *et al.*, 1979). The binding of $PGF_{2\alpha}$ is low in the early luteal phase in cattle, although receptors are present (Rao *et al.*, 1979), due to shunting of blood flow away from the ovary accounting for the refractoriness of the CL to the $PGF_{2\alpha}$ (Inskeep, 1973; Henricks *et al.,* 1974). But in late luteal phase $PGF_{2\alpha}$ causes the lysis of the CL, known as luteolysis. The ephemeral nature of the CL is of importance for providing repeated opportunities for fertilization, as a functional CL suppresses final stages of follicular development and ovulation (Pate, 1994). So luteolysis must therefore occur at the appropriate time, however, for successful establishment of pregnancy

to occur embryonic or conceptus mediated signals are vital for prevention of luteolysis (Bazer *et al.*, 1991; Plante *et al.*, 1991; Meyer *et al.*, 1995; Spencer *et al.*, 1995; Martal *et al.*, 1997) in ruminants.

2.3 ENDOCRINE REGULATION OF CL MAINTENANCE

Corpus luteum (CL) maintenance and the endocrine regulation of CL functions are vital for the successful maintenance of pregnancy. The major endocrine regulators of CL life span are luteinizing hormone, prostaglandins ($PGF_{2\alpha}$), progesterone and the embryonic signals (IFNs). The various actions of each of these with regards to the CL lifespan and function are detailed in the following sections.

2.3.1 Luteinizing Hormone (LH)

The *corpus luteum* is formed by the action of a surge of luteinizing hormone on the pre ovulatory follicle. The luteal cells undergo a series of structural and functional changes known as luteinization which results in a shift from a structure which secretes predominantly oestradiol to one that secretes progesterone (Baird, 1992).

2.3.1.1 Luteinizing hormone (LH) during estrous cycle

Cow

It is well established that CL lifespan requires pituitary support (Denamur *et al.*, 1966). LH plays a critical role in the maintenance of luteal structure and in cows there is evidence of LH being the prime luteotropic factor (Hoffmann *et al.*, 1974). LH interacts with the receptors on the CL, the concentration and number of which increases from ovulation to reach a maximum in the mid luteal phase of the cycle (Fritz *et al.*, 1982). *In vitro* LH stimulates the production of progesterone in luteal cells. In sheep and cow only small luteal cells responds to LH with an increase in cAMP and progesterone production. The basal production of progesterone by the large luteal cells greatly exceeds that of small luteal cells but is unresponsive to the action of exogenous LH. It has been established that CL function is relatively

independent of pituitary luteotropic support early in the cycle (Days 2-5) but requires it in mid cycle (Days 10). Also in the absence of luteolytic influences from the uterus, the CL requires pituitary luteotropic support (Baird, 1992). The gonadotrophin support requirement for *corpus luteum* maintenance differs among species (Nalbandov, 1973; Nicoll, 1974). Support for the obligatory role of LH is well established in sheep as continuous intravenous infusion of ovine LH into intact normal ewes on day 12 of cycle prolongs luteal phase and is known to sustain progesterone secretion and maintain the CL (Fuller and Hansel, 1970; McCracken *et al.*, 1971; Karsch *et al.*, 1971). In early cycle of ruminants the CL is resistant to the withdrawal of LH support but during the mid or late luteal phase of the cycle withdrawal of LH support results in rapid cessation of progesterone secretion (Baird, 1992). Therefore, LH is well known to be the prime luteotrophic factor (Kaltenbach *et al.*, 1968; McNeilly and Land, 1979) which influences progesterone secretion positively, but continuous exposure reduces secretory levels (Collett *et al.*, 1973). During luteal phase, LH is secreted in a pulsatile manner, with the pulse frequency decreasing from 1per hour on day 1 to 1 per every 4 to 6 hours by day 12 (Baird, 1978). Garverick *et al.* (1970) reported that neither the blood plasma LH nor the progesterone differed significantly between cows and heifers nor there was a significant interrelation between groups and days. However the plasma LH and progesterone varied during the days of estrous cycle. LH increased 3 folds (0.35 to 1.4 ng/ml) during the last 5 days preceding estrus as compared to the basal levels throughout the cycle. The correlation between plasma LH and progesterone was insignificant during the luteolytic phase but positively correlated during the luteal phase of the cycle (Garverick *et al.*, 1970). After the peak LH levels prior to estrus, the LH levels declined to basal levels and remained with little variation at the base line of 0.35 -0.5 to 1.4-1.7 ng/ml during the remainder of cycle till the next preovulatory peak in cycling cows (Carr, 1971; Ingalls *et al.*, 1973, Arije *et al.*, 1974) and a basal level of 0.5-3 ng/ml was reported in cyclic cattle (Peterson *et al.*, 1975). During the estrus cycle LH increased from 1.4ng/ml on day 7 to 5.9 ng/ml on a day before estrus and reached 11.8 ng/ml on the day of estrus in Holstein-

Fresian cattle (Swanson *et al.,* 1972). In cattle the pattern of LH release in systemic circulation during 3 phases of estrous cycle (day 3- early luteal, day 10 or 11- midluteal and day 18 or 19 late luteal phase) showed that LH fluctuated in a pulsatile manner. During the early luteal phase pulses were classified as low amplitude (0.3-1.8 ng/ml) and high frequency (20-30 pulses/24 hrs) with individual cattle exhibiting an inherent rhythmic pattern. During the midluteal period pulses were classified as high amplitude (1.2-7.0 ng/ml) and low frequency (6-8 pulses/24 hrs) without individual animals exhibiting any inherent rhythmic pattern. During late luteal phase on day 18 or 19 exhibited preovulatory surge of LH with a frequency more like the early luteal than midluteal period with LH levels exhibiting a pulsatile pattern of release. Amplitude of the pulses was greater during the ascending than the descending portion of the surge. This suggested the pulsatile fluctuation of LH was dependent on the period of estrous cycle and probably mediated by ovarian steroids (Rahe *et al.,* 1980).

Physiological levels of estradiol and progesterone are known to suppress pulses of LH characteristic of tonic LH secretion. Estradiol decreases LH pulse amplitude but not frequency and also inhibits the height of LH peak resulting from exogenous gonadotropin-releasing hormone stimulation. However progesterone decreases the frequency of LH pulses without reducing their amplitude or response to exogenous gonadotrophin releasing hormone. This is suggestive of the fact that progesterone suppresses tonic LH secretion by acting on the hypothalamus to decrease the frequency of gonadotropin-releasing hormone pulses, while estradiol may suppress the response of the hypophysis to gonadotropin-releasing hormone and thereby decrease LH pulse amplitude in sheep and cattle.(Goodman and Karsch ,1980).During the midluteal phase of the cow LH pulses paralleled FSH pulses with occasional additional pulses of FSH suggesting that the frequency of LH secretion may be modulated to a greater extent by ovarian steroids than by FSH pulse frequency . LH pulses were also found to mediate pulses of estradiol and both LH and FSH synergistically acting to result in pulses of progesterone (Walters *et al.,* 1984). During the periovulatory phase

of estrous cycle in cattle the LH surge is the result of an increase in frequency and amplitude of LH pulses (Walters and Schallenberger, 1984). During luteal regression the reduction of progesterone concentration below a certain threshold level in the presence of low estradiol concentrations probably eliminated the negative feedback effect on gonadotropin secretion thereby allowing frequency and amplitude of pulses to increase, the increase in LH pulse frequencies probably stimulated estradiol secretion. (Schallenberger *et al.*, 1984).

Buffalo

LH levels in cyclic buffaloes (Bubalus bubalis) at the onset of estrus varied from 20.8±3.43ng/ml in hotter months (June to August) and 21.2 ± 0.98 ng/ml) during cooler months (October-December). The peak levels of LH persisted for about 4 hrs. During the rest of the estrous cycle the LH levels fluctuate between 1-2 ng/ml in this species there was decreased LH level and LH peak during the hotter months of the year. (Kaker *et al.*, 1980). Rao and Pandey (1983) did not observe any difference in the basal LH levels throughout the estrous cycle during different seasons in buffaloes. The values of LH on the day of estrus in cold, warm, hot dry and hot humid, seasons decreased progressively during estrous cycle of buffaloes. In swamp buffaloes basal LH levels decreased toward the midluteal phase and progressively increased during the follicular phase. (Kanai and Shimuzu, 1984). Heranjal *et al.* (1976) reported the LH levels from 14.03-38.50ng/ml on the day of estrus. Basal levels of LH ranged from 5.4-9.8 ng/ml and fluctuated on different days during the course of estrus cycle in Murrah buffaloes. Sheth *et al.* (1978) observed LH levels of 23.9, 11.9, 19.1 and 19.2 ng/ml at the beginning of estrus at the end of estrus or during periovulatory period, on day 9 and day 15 of the cycle respectively in Surti buffaloes. Heranjal *et al.* (1979a,b) found low levels of LH in anestrus buffaloes fluctuating between 6.95-9.37 ng/ml as compared to the normal estrus cycle levels of 8.26-9.57 ng/ml in buffaloes during normal estrous cycle.

Janakiraman *et al.* (1980) in anestrus surti buffaloes observed LH levels at the beginning, end of estrus, on day 9 , and day 15 of the cycle during different seasons of July-October, November to December and March to June were 18.16, 17.97, 19.13, 19.20; 16.21, 15.37, 15.19, 14.81, and 19.56, 14.36, 17 and 16.56 ng/ml, respectively. Galhotra *et al.* (1981) investigated the basal levels of LH in different groups of buffaloes, pre and post pubertal pregnant heifers lactating nonpregnant, lactating pregnant and pregnant non lactating and found mean levels fluctuating between 0.6 to 1.4 ng/ml but the differences observed were not significant. Arora and Pandey (1982) studied LH levels in Murrah buffalo heifers having estrous cycle length of 19-25 days, LH levels were highest on the day of insemination (22.9 ng/ml) which declined to basal levels day after estrus (2.51 ng/ml) on the rest of the days levels ranged from 1.26-3.23 ng/ml. The decrease in circulating LH during the luteal phase was inversely related to high progesterone levels, which accompanied the development of *corpus luteum*. Avenell *et al.* (1985) in Swamp buffaloes reported that LH concentrations increased from basal levels of 5.0 ng/ml (1-8 ng/ml) to a peak of 35 ng/ml (20-52 ng/ml) over a period of 4 hrs and returned to basal levels within 8 hrs. Peak LH concentrations recorded 6.5±1.1 hrs after the onset of estrus.

Aboul Ela and Barakawi (1988) reported that LH pulse frequency higher in winter (3.2/8 hr period) than in summer (2.1/8 hr period) during the follicular phase frequency and amplitude of LH pulse were significantly higher in winter (3.6/8 hr period and 3.78 ng/ml, respectively) than in summer (2.8/8 hr period and 2.46 ng/ml).

2.3.1.2 Luteinizing hormone (LH) during early pregnancy

Cow

The LH levels were found to be significantly lower in pregnant cows as compared with the nonpregnant cyclic cows, possibly due to the negative feedback effect of elevated progesterone levels associated with early pregnancy (Henricks *et al.*, 1971). In zebu cows LH peaks of 200ng/ml and 5500ng/ml were reported around 120 and 210 days of pregnancy, rest of the

levels within this period fluctuated between 1-15 ng/ml. After 210 days of pregnancy the levels fell to 1-4ng/ml till two weeks prior to parturition, when levels dropped to undetectable levels (Carr, 1971). Schams *et al.* (1972) reported LH levels varying between 1-1.4 ng/ml with irregular peaks in the first 110 days of gestation in cows. Wettemann and Hafs (1973) reported a lower level of LH in pregnant (8.7±1.6 ng/ml) than nonpregnant (12.0±3.2 ng/ml) cows, between days 18-75 of pregnancy.

Buffalo

Arora and pandey (1982) reported that the LH levels were highest on the day of insemination .The levels did not vary significantly till days 256-258 of pregnancy and fluctuated around the basal levels. Heranjal *et al.* (1976) reported basal levels of LH ranging from 5.4 to 9.8 ng/ml, fluctuating on different days of the estrous cycle. No sharp peaks were observed during the equivalent days of estrous cycle like the cyclic animals. Jain (1982) reported highest levels on the day of insemination (Day 0). The LH levels on this day were significantly ($P<0.05$) higher from days 1, 7, 9, 13, 15, 17, 18, 19, 21 and 22 in early pregnant buffaloes.

2.3.1.3 LH assay procedures

The assay procedures for LH, in bovine plasma are summarized with their salient features as following. The available information presents a need for a suitable assay procedure, which is simple, reliable and specific for estimation of LH in buffalo plasma.

Summary of LH assay procedures in bovine plasma

Author	Method	Species	Salient feature of the assay
Niswender *et al.*, 1969	RIA	Cattle	Sample size = 200 µl Duration of assay = 96 – 120 hours Sensitivity = 0.125 pg/ tube
Heranjal *et.al.*, 1976	RIA	Buffalo	Sample size = 200 µl Duration of assay = 96 – 120 hours Sensitivity = 0.125 pg/ tube

Sheth *et al.*, 1978	RIA	Buffalo	Sample size = 100-300 µl Duration of assay = 96 hours Sensitivity = 0.125 ng/ml
Arora, 1980	RIA	Buffalo	Sample size = 250 µl Duration of assay = 96-120 hours Sensitivity = 28.18 pg/tube
Janakiraman *et.al.*, 1980	RIA	Buffalo	Sample size = 100-300 µl Duration of assay = 96 hours Sensitivity = 0.125 ng/ml
Kaker *et al.*, 1980	RIA	Buffalo	Sample size = 100-300 µl Duration of assay = 96 hours Sensitivity = 0.125 ng/ml
Rahe *et.al.*, 1980	RIA	Cattle	Sample size = 200 µl Duration of assay = 96 – 120 hours Sensitivity = pg/ml
Batra, 1981	RIA	Buffalo	Sample size = 200 µl Duration of assay = 96 – 120 hours Sensitivity = 35.32 ± 1.09 pg/ml
Galhotra *et.al*, 1981	RIA	Buffalo	Sample size = 200 µl Duration of assay = 96 – 120 hours Sensitivity = pg/tube
Jain, 1982	RIA	Buffalo	Sample size = 200 µl Duration of assay = 96 hours Sensitivity = 31.2 pg/tube
Rao, 1982	RIA	Buffalo	Sample size = 200 µl Duration of assay = 96 – 120 hours Sensitivity = 31.62 pg/tube
Suri, 1983	RIA	Buffalo	Sample size = 200 µl Duration of assay = 96 – 120 hours Sensitivity = 200 pg/ml
Avnell *et.al.*, 1985	RIA	Swamp Buffalo	Sample size = 200 µl Duration of assay = 96 – 120 hours Sensitivity = 0.04 ng/tube

Palta, 1985	RIA	Buffalo	Sample size = 100 µl Duration of assay = 96 – 120 hours Sensitivity = 40 pg/tube
Aboul-Ela and Barakawi, 1988	RIA	Swamp Buffalo	Sample size = 200 µl Duration of assay = 96 – 120 hours Sensitivity = 0.04 ng/tube
Goodman and Karsch, 1990	RIA	Cattle	Sample size = 200 µl Duration of assay = 96 – 120 hours Sensitivity = pg/ml
Mutayoba *et.al.*, 1990	EIA	Cattle	Sample size = 20 µl Duration of assay = 48 hours Sensitivity = 8 pg/well
Singh, 1990	RIA	Buffalo	Sample size = 200 µl Duration of assay = 96 – 120 hours Sensitivity = 19 pg/tube
Singh, 1998	RIA	Buffalo	Sample size = 100 µl Duration of assay = 38 hours Sensitivity = 9 pg/tube

2.3.2 PROSTAGLANDIN $F_{2\alpha}$ ($PGF_{2\alpha}$)

The prostaglandin $F_{2\alpha}$ is known to be the most important hormone concerned with regulation of CL function and luteolysis (Niswender and Nett, 1988).

2.3.2.1 $PGF_{2\alpha}$during estrous cycle

Sheep

In sheep mean concentration of PGF was found to be 8.8 ng/ml in uterine venous blood on day14-16 of the oestrous cycle while the levels on day 13 was less than 2 to 3 ng/ml (Bland *et al.,* 1971). A higher level of 20-40 ng/ml was estimated two days prior to estrus (McCracken *et al.,* 1972). A fall in progesterone was observed after $PGF_{2\alpha}$ administration (Thorburn and Nicoll, 1971). Thorburn *et al.* (1972) reported PGF (22.3 ng/ml) spikes lasting for 1-3 hours. PGF levels were found to be at basal levels but both magnitude

and frequency till 4 days prior to estrus, reaching peak levels 24-36 before estrus at the time of luteolysis inform of spikes, during the estrous cycle of nonpregnant cyclic ewes but this was not observed in pregnant ewes (Fitzpatrick and Sharma, 1973; Caldwell *et al.*, 1972; Singh, 1975; Peterson *et al.*, 1976). No difference was observed between pregnant and nonpregnant sheep on day 15 post conception (Pexton *et al,* 1975a; Land *et al.,* 1976; Nett *et al*, 1976). However the quantity of PGF apparently being transported to ovarian arterial plasma was higher in pregnant than in nonpregnant ewes, suggesting the alteration of luteolytic process during pregnancy. The concentration of PGF in ovarian venous plasma was positively correlated with the concentration in the uterine vein (Pexton *et al.,* 1975b). It was evident that initial priming action of progesterone for 7-10 days are required for uterine endometrium to release $PGF_{2\alpha}$ affecting luteolysis (Baird *et al.,* 1976a; Lewis *et al.*, 1976; Louis *et al.*, 1977). High levels of PGFM were associated with inhibition of luteal activity, with small spikes PGFM observed between days 13-17 with luteolytic peak day 17 (Kindahl *et al.*, 1976c). Kindahl *et al.* (1976a) reported a basal level of 50-80 pg/ml with 5 peaks 3 days prior to estrus with maximum levels of 500 pg/ml. However, the parent compound $PGF_{2\alpha}$ did not show any variation. During luteolysis PGFM increased in pulsatile manner lasting for 1-5 hours (Kindahl *et al.*, 1976b). Baird *et al.* (1976b) reported that the functional regression of the CL was indicated by a drop in plasma progesterone profiles, 4 days before the onset of estrus (On day 12-13 of the cycle). The rise $PGF_{2\alpha}$ occured on days 12-14, that coincided with the time of decline of progesterone levels, and reached highest levels in spurts on the day before estrus, while very little $PGF_{2\alpha}$ from the uterus before day 12 of the cycle with levels remaining at basal levels .Cox *et al.* (1974) observed the first peak of PGF on day 13 of the oestrous cycle and inferred the rise of $PGF_{2\alpha}$ on day 13 was due to the increase in estradiol 17β on day 11-12 of cycle in nonpregnant cyclic sheep. Release of PGFM continued till progesterone levels were elevated over critical a threshold level. Release of $PGF_{2\alpha}$was found to be stimulated by falling levels of progesterone (Kindahl *et al.,* 1979). Scaramuzzi *et al.* (1974) reported the first significant increase in the release of

$PGF_{2\alpha}$ on day 12 of the cycle which was followed by a fall in progesterone levels on day 13.The maximal release of $PGF_{2\alpha}$ occured on day 16, was probably induced by the preovulatory release of estradiol. The fall in the progesterone levels may also believe to induce release of PGF from the uterus (Liggins *et al.,* 1972). In sheep endometrial $PGF_{2\alpha}$ levels were reported to be higher on day 14 than on days 3, 5 or 11 of cycle (Wilson *et al.*, 1972). The simultaneous increase of $PGF_{2\alpha}$ in the uterus coincided with the elevated levels in the uterine venous blood preceding estrus points to its role as the luteolysin in this species. Mean $PGF_{2\alpha}$ levels in peripheral blood serum in mature crossbred ewes on days 1, 4, 8, 12, 14 and 16 of the oestrous cycle were 0.466, 0.416, 0.413, 1.656, 1.232 and 0.742 ng/ml, respectively (Singh, 1975). The highest levels of $PGF_{2\alpha}$ were observed on day 12 (1.656 ng/ml) and on day 14 (1.232 ng/ml) of the cycle coinciding with the time of luteal regression. During the proestrus period blood samples collected at the interval of 4 hours (day 12 through 15 of the cycle). Peaks of $PGF_{2\alpha}$ 1.998 ng/ml, 1.6000 ng/ml, 1.228 ng/ml were observed on days 13, 14, 15 of the estrous cycle.

Cow

$PGF_{2\alpha}$ in utero ovarian venous plasma showed spikes of reaching levels up to 45.4 ng/ml, two days prior to estrus with simultaneous decrease of progesterone levels (Nancarrow *et al.*, 1973). Exogenous administration of $PGF_{2\alpha}$ influences endogenous levels for 10 minutes in peripheral plasma and for 15 minutes in the utero ovarian plasma in jersey cows (Lammond *et al.*, 1973). Minor fluctuations of PGFM (100-300 pg/ml) levels in the peripheral circulation during late luteal phase, while two major peaks of PGFM (0.8-1.2 ng/ml) were found to be associated with falling levels of progesterone (Fairclough and Payne, 1975). In cows low levels of PGF were observed in the uterine venous plasma on days 1-14(.162±0.44), which showed an increasing trend from day15 onwards (between 1.5-3.0ng/ml), while the peripheral plasma progesterone levels were still high. Though the PGF levels showed these fluctuations no such changes were observed in the ovarian

artery or the jugular vein or the endometrial tissue from the uterine horns adjacent or opposite to the functional CL (Shemesh and Hansel, 1975).. A similar pattern in the PGF and progesterone levels in jugular plasma, uterine vein, ovarian artery was observed in the luteal phase on days 10-17 in cattle (Hansel *et al.*, 1976). Peterson *et al.* (1975) reported a decline in the peripheral plasma progesterone levels prior to the proestrus rise in estradiol and the major $PGF_{2\alpha}$ activity coincided with the declining progesterone levels at the active stage of estradiol secretion in dairy cows during estrus. Kindahl *et al.* (1976) determined progesterone and the major metabolite of $PGF_{2\alpha}$, PGFM (13,14- dihydro-15-keto $PGF_{2\alpha}$) at hourly intervals in the peripheral circulation in two heifers during luteolysis. PGFM rise was found to occur in 2-3 days in rapid pulses with 1-5 hour duration prior to and during luteolysis, which coincided with the declining levels of progesterone. In pregnant heifers neither any decrease in progesterone nor any increase in the PGFM levels were observed. Shemesh *et al.* (1979) reported the inhibitory effect of the midcycle bovine follicular fluid on prostaglandin synthatase as well as luteinization, but this inhibition disappeared in the preovulatory follicles.

Buffalo

$PGF_{2\alpha}$ is known to be luteolytic during oestrous cycle of buffaloes (Bachlaus *et al.*, 1979; Kamonapatna *et al.*, 1979). Batra and Pandey (1983) reported that the mean levels ranging from 200 to 250 pg/ml throughout the oestrous cycle with peak levels of 600 to 900 pg/ml during luteolysis.

2.3.2.2 $PGF_{2\alpha}$ during early pregnancy

Sheep

The PGF pattern on days 13-14 of pregnancy varies widely ewes (Cox *et al.*, 1974). However, the surges of $PGF_{2\alpha}$ during the day 12-16 of the estrous cycle were abolished or remained much reduced in early pregnant animals (Thorburn *et al.*, 1973 Barcikowski *et al.*, 1974). This was confirmed by the estimation of (the primary metabolite of $PGF_{2\alpha}$) PGFM in the peripheral circulation of sheep. Although the pulsatile pattern of the PGFM was

suppressed in the early pregnancy, but there is an elevation of he basal levels during this period (Zarco *et al.*, 1988). During the early pregnancy of sheep the reduction of $PGF_{2\alpha}$ or PGFM pulses were suggested to be the result of suppression of endometrial oxytocin receptors mediating the $PGF_{2\alpha}$ pulsatile release (McCracken, 1980; Roberts and McCracken, 1976). This was confirmed by a marked reduction in the oxytocin receptor concentrations in the sheep endometrium during early pregnancy (McCracken *et al.*, 1984; Sheldrick and Flint, 1985). Webb *et al.* (1981) reported a decrease in PGFM pulse frequency and amplitude as well as absence of large peaks on days 13-14 post breeding in early pregnant ewes.

Cow

In cattle the survival of embryo during early pregnancy is dependent on sustained progesterone secretion, by means of alteration of the luteolytic process and associated maintenance of CL (Thatcher *et al.*, 1984, McCracken *et al.*, 1999). An oestradiol induced increase in plasma PGFM levels were found to be significantly reduced in pregnant cows at day 18 and 20 compared to day 18 cyclic cows. Ovarian artery concentration of $PGF_{2\alpha}$ was considerably reduced in early pregnant cows. Tissues from uterine endometrium, ovarian vein and ovarian artery of day 17 pregnant cows accumulated less of $PGF_{2\alpha}$ *in vitro*, than day 17 cyclic cows (Thatcher *et al.*, 1984). The amplitude of PGFM pulses were significantly reduced in case of early pregnant cows on days 14-16 post breeding (Parkinson and Lamming, 1990). Early pregnancy in cows significantly reduced oxytocin induced PGFM release along with suppression of endometrial oxytocin receptors, but did not influence the expression of the transcriptionally regulated estrogen receptors on day 16 of pregnancy (Robinson *et al.*, 1999).

Buffalo

A negative correlation between $PGF_{2\alpha}$ and progesterone was obtained during day 15-18 in pregnant Murrah buffalo heifers. However a positive correlation was evident during day 9-13 of pregnancy in these

animals (Jain, 1982). Peripheral levels of PGFM did not show significant variations during early pregnancy. There was also no significant difference in PGFM levels between primiparous and multiparous buffaloes (Batra and Pandey, 1983)

2.3.2.3 $PGF_{2\alpha}$ assay procedures

The available information on $PGF_{2\alpha}$ presents the need of a simple, specific and direct assay procedure, which could be carried out with high accuracy and reliability in plasma samples without extraction procedures.

Summary of $PGF_{2\alpha}$ assay procedures in bovine plasma

Author	Method	Species	Salient feature of the assay
Fairclough and Payne, 1975	RIA	Cattle	Sample size = 200 µl Duration of assay = 24 hours Sensitivity = 20 pg/ tube
Kindahl *et.al.*, 1976	RIA	Cattle	Sample size = 200 µl Duration of assay =24 hours Sensitivity = 50 pg/ ml
Batra, 1981	RIA	Buffalo	Sample size = 100 µl Duration of assay = 24 hours Sensitivity = 2.83 pg/ml
Eley *et al.*, 1981	RIA	Cattle	Sample size = 200 µl Duration of assay = 24-36 hours Sensitivity = 40.1 pg/ml
Jain, 1982	RIA	Buffalo	Sample size = 300 µl Duration of assay = 24-36 hours Sensitivity = 2 pg/ml
Prakash, 1983	RIA	Buffalo	Sample size = 100µl Duration of assay = 24hours Sensitivity = 50pg/tube
Guilbault *et al.*, 1984	RIA	Cattle	Sample size = 200 µl Duration of assay = 24 hours Sensitivity =10 pg/ml

Kaker *et al.*,1984	RIA	Cattle	Sample size = 200 µl Duration of assay = 24 – 36 hours Sensitivity = 30.3 pg/ml
Alam and Dobson, 1986	RIA	Cattle	Sample size = 200 µl Duration of assay = 24 hours Sensitivity = 12.2 pg/ml
Meyer *et al.*,1989	EIA	Cattle	Sample size = 2 µl Duration of assay = 20 hours Sensitivity = 160 a mol/well
Parkinson and Lamming, 1990	RIA	Cattle	Sample size = 500 µl Duration of assay = 24 – 36 hours Sensitivity = 53.3 pg/ml
Robinson *et al.*, 1999	RIA	Cattle	Sample size = 100 µl Duration of assay = 24 hours Sensitivity = 52 pg/ml

2.3.3 Progesterone During Estrous Cycle

Progesterone is the prime hormone produced by the CL. The levels of progesterone are indicative of CL function (Niswender *et al.*, 2000).

2.3.3.1 Progesterone during estrous cycle

Cow

Pope *et al.* (1969) reported the plasma progesterone level in 8 normal cows and two ovariectomized cows. Progesterone levels near the time of ovulation were below 2 ng/ml. Mean peak levels for twenty-two cycles were 9.0 ng/ml and occurred on an average of 13 days after ovulation. The time of most rapid fall in progesterone levels was on an average 4 days before ovulation. Mean levels during first 14 days after estrus in normal cycles were not different from those during the first 14 days after insemination in early pregnancies; they then declined in the cycle but not during the early

pregnancies. Mean progesterone levels in the plasma of ovariectomized cows up to 200 days after operation were below 2 ng/ml; levels showed a slow but significant rise during this period.

Stabenfeldt *et al.* (1969) determined progesterone daily in peripheral plasma of 6 cows for a total of seven complete estrous cycles. Progesterone levels ranged from less than 0.5 ng/ml plasma during follicular phase to 6.6 ng/ml plasma (6.1 to 10.2 ng/ml) at peak luteal phase. Progesterone levels in cows with 21 days cycles increased rapidly from day 3 to day 8 with a much slower rate of increase from day 9 to 17. These cows showed a decrease in progesterone of more than 50 percent from the previous day on day 18 (two cows), 19 (one cow) and 21 (two cows). Two other cows with cycles of 22 and 23 days duration both had a similar decline on day 20. A variable time interval of 1 to 5 days was observed between decline and the occurrence of estrus.

Donaldson *et al.* (1970) studied the progesterone concentrations in peripheral plasma of cows and reported mean concentration was lowest at estrus (0.44 ng/ml) and then increased to a maximum of 6.8 ng/ml about day 14 of the 21 day cycle. The concentration decreased rapidly during the last 4 days of cycle, reaching low levels on the day before estrus.

Adeyemo and Heath (1980) studied the progesterone concentration in Brown Swiss, Holstein and White Fulani cattle during two consecutive cycles in dry and wet seasons in a tropical climate. The concentration fluctuated throughout the estrous cycle and were lowest (less than 1 ng/ml) from a day before to 2 or 3 days after estrus. Increases become pronounced from day 4 of the cycle and peak values ranging between 3.6 and 7.6 ng/ml occurred between day 7 and 15 of the cycle. The mean concentration of progesterone at peak secretion (days 9 to 12) was significantly higher in the dry season (5.07 ± 0.08 ng/ml) than in wet season (4.83 ± 0.08 ng/ml), but the difference was not statistically significant ($P>0.05$). The analysis of variance showed that breed effect was significant ($P<0.05$) during luteal phase, the mean level being 5.0 ± 0.17, 5.2 ± 0.14 and 4.5 ± 0.13 ng/ml in Brown Swiss, Holstein

and White Fulani, respectively. Plasma progesterone levels on 1 and 2 days before estrus ranged from 0.5 to 4.3 ng/ml with significantly higher levels in Brown Swiss and Holstein than in White Fulani cattle.

Vaca *et al.* (1983) reported the progesterone levels in Zebu cows were below 0.5 ng/ml from days 0-4, then increased reaching a maximum level of 3.1 ng/ml on days 9 and 10. Values declined sharply to less than 0.5 ng/ml on day 18.

Glencross and Abeywardene (1983) studied the plasma progesterone concentration and the pattern was similar showing a normal, gradual luteal phase rise and a sharp fall 3 to 4 days before estrus.

Byerley *et al.* (1987) studied the peripheral serum progesterone concentration in beef heifers following breeding at pubertal (E1, n=63) or 3rd estrus (E3, n=45). Results of this study indicated that luteal function differed between heifers that failed to conceive at this pubertal estrus and heifers that failed to conceive at third estrus. However, the concentration of progesterone did not differ between heifers that conceived at pubertal or third estrus. The relationship of change in luteal function for pubertal through the 3 estrous cycles and pregnancy is not clear.

Kamimura *et al.* (1990) suggested that post-partum recovery of reproductive function is confirmed by plasma progesterone in cows. The interval from calving to ovulation averaged 19.4 ± 4.6 days, ovulation was followed by a peak level of plasma progesterone of 7.1 ± 2.2 ng/ml.

Kamboj and Prakash (1993) reported that the mean ± SEM of plasma progesterone samples of Sahiwal cows were 0.86 ± 0.21 ng/ml on 0 to 4 days, 4.01 ± 0.41 ng/ml on 6th and 14th day and 2.13 ± 0.92 ng/ml on –6 to –2 days preceding estrus, respectively.

Buffalo

Kamonpatana *et al.* (1976) suggested that none progesterone values in buffaloes during estrous cycle was lower than 0.5 ng/ml and generally the same low values were found in case of non-pregnancy at days 24, 27 and 30 post insemination.

Batra *et al.* (1979) reported that plasma progesterone concentration in buffaloes at estrus was 0.1 ng/ml when rose to a peak of 3.6 ng/ml on day 13. It continued to increase in animals that conceived but dropped to 0.6 ng/ml on 3 days before next estrus in those that failed to conceive.

Naqvi (1982) suggested that mean progesterone concentration was lowest 0.4 ± 0.6 ng/ml on day of estrus and remained low up to day 3 post-estrus. The progesterone levels then increased gradually during luteal phase. The values were 1.28 ± 0.5, 2.13 ± 0.38 and 3.75 ± 0.70 ng/ml on 4^{th}, 8^{th} and 12^{th} day. The overall mean plasma progesterone was 3.9 ± 0.68 ng/ml four days prior to estrus which declined to a mean level of 0.75 ± 0.14 ng/ml one day prior to estrus. He also reported that average concentration of progesterone on day 7, 15, 22, 30, 37 and 415 days post-partum was 0.59 ± 0.10, 0.46 ± 0.17, 0.52 ± 0.18, 0.45 ± 0.13, 0.92 ± 0.23 and 1.29 ± 0.43 ng/ml, respectively. The mean post-partum progesterone was higher on day 7^{th} than on 15^{th} and remained low up to day 30^{th}. Beyond this day, post-partum progesterone showed significant increase in levels.

Jainudeen *et al.* (1983) studied the progesterone concentration in Swamp buffaloes for an estrous cycle in relation to *corpus luteum*. The mean concentration of progesterone with or without CL were 1.49 ± 0.78 ng/ml (n=31) and 0.24 ± 0.09 ng/ml (n=14), respectively. Plasma progesterone levels reflected age-dependent changes occurring in cyclic luteum. Ovarian contents and plasma progesterone levels during estrous cycle in Swamp buffalo are shown below:

Ovarian structure	Plasma progesterone	Rectal findings	Days of cycle
Graafian follicle	0.14 ± 0.09	Soft fluctuation	0
Ovulation point	0.44 ± 0.17	Depression	1-2
Corpus luteum early	0.64 ± 0.60	Not palpable	2-4
Developing	1.60 ± 0.85	Soft protrusion	4-8
Mature	1.74 ± 0.58	Firm well defined protrusion	8-16
Regressing	0.10 ± 0.03	Yellow to white protrusion shrunken, no vasculature	16

Avenell *et al.* (1985) measured plasma concentration of progesterone in 13 mature Swamp buffaloes at 4 h intervals from 36 to 40 h after onset of estrus. Progesterone concentration remained around 0.1 ng/ml throughout the sampling period.

Chua *et al.* (1985) estimated the progesterone levels in 4 carabao (buffalo, *Bos bubalis*) heifers. Plasma progesterone concentration was lowest at estrus (0.24 ± 0.187 ng/ml). Four anoestrous heifers had a pattern of progesterone concentration similar to that of cycling heifers or had a low level (0.164 ± 0.718 ng/ml) throughout 70 day period.

Dobson and Kamopatana (1986) estimated the concentration of progesterone in peripheral blood of Swamp and Murrah buffaloes and found it low at estrus (day 0: 0.13-0.27 ng/ml) which did not rise until after day 5. Values increased until day 14-16 with a lower mean value of 1.5 – 2.6 ng/ml in Swamp buffaloes compared to values of 4.0 to 4.26 ng/ml in Murrah buffaloes.

Kuruvita *et al.* (1987) measured the progesterone levels in buffalo plasma and reported the progesterone levels to be ranging from 0.04 to 0.35 ng/ml in post-partum cycle buffaloes.

Khattab *et al.* (1988) measured progesterone levels in 20 buffaloes that had not conceived for 4 months. Periods of ovarian activity were indicated by low plasma progesterone concentration (<0.2 ng/ml) and persistence of *Corpus luteum* was indicated by a concentration of 1.2 ng/ml.

El-Sobhy *et al.* (1988) measured concentration of progesterone in 19 pregnant and 21 non-pregnant buffaloes. Blood progesterone concentration on the day of ovulation was undetectable or ranged from 0.12 to 0.52 ng/ml, 1.50 ± 0.33 and 1.90 ± 0.50 ng/ml in short (14-17 days), normal (18-24 days) and long (25-28 days) cycles, respectively. In pregnant animals, the concentrations averaged 3.79 ± 0.13 ng/ml throughout pregnancy. The concentrations peaked at days 7-8, 13-14 and 19-20 in short, normal and long

cycles were –3.48 ± 0.41, 3.18 ± 0.40 and 3.86 ± 0.30 ng/ml, respectively, and during pregnancy between days 20 and 60 (3.6-4.7 ng/ml) with a secondary increase starting on day 200 of pregnancy (4.8-5.0 ng/ml).

Jain (1988) measured plasma progesterone concentrations in cycling and non-cycling buffaloes which ranged from 0.27 ± 0.07 to 0.61 ± 0.15 ng/ml and was significantly higher in non-cycling heifers.

Niansheng and Peichian (1988) estimated the progesterone levels in 9 buffaloes during estrous cycle. The plasma progesterone level during long and short estrous cycle was <1.0 ng/ml throughout the cycle and peaked at 2.14 to 3.92 ng/ml in normal cycles.

Kamboj and Prakash (1993) reported that mean ± SEM of plasma progesterone in buffaloes was 0.41 ± 0.2 ng/ml on 0 to 4 days, 2.58 ± 0.24 ng/ml on 6 to 14 days and 1.68 ± 0.34 ng/ml on –6 to –2 days preceding estrus. The mean progesterone levels declined from 1.28 ng/ml three days prior to estrus to <0.4 ng/ml on day of estrus. Progesterone levels in the early luteal, luteal, late luteal were 0.97, 2.01 and 1.01 ng/ml respectively (Kumud, 1999).

2.3.3.2 Progesterone during early pregnancy

Cow

The plasma P_4 concentration in early pregnancy is reported to be similar with the peak values found during the luteal phase of the estrus cycle in cows (Glencross *et al.*, 1973; Wettemann and Hafs, 1973; Sreenan and Gosling, 1975; Agarwal *et al.*, 1977; Haraszti *et al.*, 1977).

The plasma progesterone level above 0.3 µg/100 ml on day 19 after ovulation was indicative of the presence of a live conceptus (Shemesh *et al.*, 1968) and the concentration was found lower on day 9 after mating in heifers that returned to estrus 18-20 days after mating (Henricks *et al.*, 1971) increased from 1.2 ng/ml to 8.2 ng/ml on day 3 and 9.9 ng/ml on day 12, and increased thereafter to 13.9 ng/ml (Henricks *et al.*, 1972). Shemesh *et al.*

(1973) suggested that P_4 concentration exceeding 2.5 ng/ml in cows and 1.5 ng/ml in ewes during 19-22 days and 16-21 days, respectively, after insemination could be used as an index of pregnancy.

Aggarwal *et al.* (1977) reported that cows, which settled to service, had a similar trend to that of non-pregnant cows up to day 15 of the estrous cycle but thereafter, the concentration gradually increased till day 21. The overall mean concentration of P_4 for the pregnant and non-pregnant groups of cows was 1.40 ± 0.10 and 2.46 ± 0.24 ng/ml based on 99 and 49 observations, respectively. The statistical comparison of the two groups revealed highly significant difference; the pregnant group had significantly higher values than as non-pregnant group. Stage to stage comparison between these two groups showed that only two stages, viz., day 18 and day 21, were significantly higher in pregnant group than its counterpart in the non-pregnant group. Aggarwal *et al.* (1980) further reported the P_4 level of 0.578 ± 0.075, 2.207 ± 0.345 and 0.525 ± 0.075 and 0.528 ± 0.089, 3.45 ± 0.560 and 3.183 ± 0.591 ng/ml in cows of Hariana breed on day 0, 14 and 19 in non-pregnant and pregnant cows, respectively.

Buffalo

Agarwal and Agarwal (1979) measured blood plasma levels of P_4 in buffaloes during early pregnancy and reported that the levels ranged from 0.4 to 2.4 ng/ml. The values gradually increased from the lowest value on day 1 and 3 through day 7 and day 11, and attained a peak value on day 15, which was maintained till day 21. A slightly higher concentration of progesterone (approx. 4 ng/ml) at day 19-20 of pregnancy was reported by Arora *et al.* (1979) and Batra *et al.* (1979).

2.4 ORIGIN AND ROLE OF EMBRYONIC INTERFERONS IN CL MAINTENANCE

Pig

Pig conceptuses secrete a complex array of proteins, during the period of maternal recognition of pregnancy. Among these proteins, the trophoblastic interferons have been found and identified (La Bonnardiere, 1993). A

significant antiviral activity was found in uterine flushings and conceptus conditioned culture media on days 11 to 17 of pregnancy (Cross and Roberts, 1989) in pigs.

These pig conceptus secretory proteins (PCSP) from day 15 conceptuses include both type I, interferon-α (IFN-α) and type II, inteferon-γ (IFN-γ). And, it was revealed that the IFN-γ is the predominant species (Lefevre *et al.*, 1990; Mirando *et al.*, 1990). It was also revealed that the interferon secretion by the pig conceptus begins on days 12-13 of pregnancy and reached a maximum titre on days 15-16 (Mirando *et al.*, 1990). The coexpression of type I and type II interferon suggested a unique tissue specific regulation of interferon genes. A possible synergistic effect on target tissues since synergy between interferon-γ and type I interferon-α *in vivo* culture systems is well documented (DeMaeyer and DeMaeyer-Guignard, 1992). This argues for a strong requirement for presence of highly active interferons at the exact period of implantation (La Bonnardiere, 1993).

The uterine endometrium of pig secretes the luteolysin, $PGF_{2\alpha}$ (Prostaglandin F_2 alpha). The conceptuses secrete estrogens, which are antiluteolytic in nature. The prostaglandin alpha ($PGF_{2\alpha}$) is secreted in an endocrine direction, towards the uterine endometrium vasculature in cyclic gilts and transported to the *corpus luteum* to exert its luteolytic effect. However, in pregnant pigs, the secretion in $PGF_{2\alpha}$ is in an exocrine direction, into the uterine lumen, where it is sequestered to exert its biologic effect in utero or gets metabolized to prevent luteolysis (Bazer, 1989).

It is also reported that the pig conceptus secretory proteins (PCSPs) enhances the effectiveness of estrogen in redirecting the secretion of prostaglandins from the uterine vasculature (endocrine direction) towards the uterine lumen (exocrine direction). This is essential for the establishment of pregnancy in pigs (Dubois and Bazer, 1988).

Recently by using a novel microdialysis system (MDS) *in vivo* it has been established that, in pigs, γ-interferon is luteotrophic and antiluteolytic. Application of lower interferon concentrations of $1x10^{-7}$ M exhibited greater

luteotrophic and antiluteolytic effect on the *corpus luteum* in earlier days (day 10) of the cycle in comparison to higher interferon concentrations of $2x10^{-7}$ M (day 11) and $4x10^{-7}$ M (day 12) applied on subsequent days. The diminishing luteotrophic response in aging *Corpus luteum* even with higher dose levels suggests that its requirement for luteotrophic effects as well as arresting the luteolytic process increases with the age of the *corpus luteum* (Prakash *et al.*, 1997).

Sheep

In the ewe, the source of embryonic signal for maternal recognition of pregnancy is the conceptus trophoectoderm. This embryonic signal is better known as the ovine trophoblast protein (oTP-1), a type I interferon (Godkin *et al.*, 1984). In structural homology, it resembles the interferon alpha (IFN-α) group. An embryo secretes about 100-500 μg of this protein per day (Ashworth and Bazer, 1989). Maximal secretion occurs mid-way between days 13 to 21 of early pregnancy. Suboptimal secretion of this interferon and poor conceptus growth rate, result in an inability to regulate endometrial oxytocin receptor mediated $PGF_{2\alpha}$ secretion, which may be central to pregnancy failure in the early post-partum ewe (Wallace *et al.*, 1995). The antiluteolytic effects of ovine interferon are to repress endometrial oestradiol receptor gene expression, thereby inhibiting formation of oxytocin receptors which prevents the generation of $PGF_{2\alpha}$ pulses and maintains the CL (Spencer *et al.*, 1995). Systemic administration of rbIFNα in ewes enhanced antiluteolytic effects by activating 2′5′-A synthetase system, in the uterus (Mirando *et al.*, 1991). The supplementation of rbIFNα in ewes has shown to increase both pregnancy rates and early embryonic survival. Hence, rbIFNα is an effective tool in the fertility enhancement of ewes (Nephew *et al.*, 1990; Mirando *et al.*, 1991; Spencer *et al.*, 1995).

Goats

Goat conceptuses exert an antiluteolytic effect similar to that of sheep conceptuses. Goat conceptuses survive and extend CL lifespan when transferred to ewes (Warwick and Berry, 1989). The goat conceptuses also

secrete proteins with biochemical characteristics similar to those of oTP-1 (Gnatek *et al.*, 1989). The uterine luteolysin in goats is $PGF_{2\alpha}$ and the conceptus interferes with oxytocin induced pulsatile release of $PGF_{2\alpha}$ (Homeida, 1986). In goats, the luteolytic pulses of $PGF_{2\alpha}$ are associated with pulses of oxytocin and its neurophysin during luteolysis in cyclic goats (Homeida, 1986) and luteolysis can be delayed by intra-arterial administration of an oxytocin antagonist (Homeida and Khalafolla, 1987). Removal of goat conceptuses from the uterine lumen between days 13 and 15 does not affect the intra-oestrous interval, but their removal on day 17 does extend luteal lifespan by 7 to 10 days (Gnatek *et al.*, 1989). This suggests that the maternal recognition of pregnancy in goats occur around day 17. Between days 16 and 21, goat conceptuses secrete Conceptus Trphoblast Protein (CTP) that can be immuno-precipitated with antiserum to oTP-1, and may be the antiluteolytic protein (Gnatek *et al.*, 1989).

Intrauterine administration of rbIFN-τ (80, 160 or 320 μg/day) between days 12 and 18 of the oestrous cycle, or concomitantly (80 μg/day) with oxytocin between days 3 and day 7, delayed luteolysis. It was thus inferred that ovine interferon τ can act as an anti-luteolytic agent in goats (Homeida and Al-Afaleq, 1994). Recently, two proteins (17 and 22-24 kDa) were identified from the media in which day 17, goat conceptuses were cultured. Analysis of their amino acid sequences and antiviral activity confirmed that, the proteins belonged to the family of ruminant trophoblastic interferons. By day 18, as implantation proceeded, interferon was no longer detected. It was concluded that the goat conceptus secretes interferons during the period of maternal recognition of pregnancy and its supplementation during the critical period enhances fertility in this species (Homeida, 1986; Gnatek *et al.*, 1989; Homeida and Al-Afaleq, 1994; Guillomot *et al.*, 1998).

Cattle

Maternal recognition of pregnancy in the cow results from antiluteolytic signalling between the trophoectoderm of the conceptus and the uterus. This signalling molecule in cattle was identified as a glycoprotein (Mw

22,000-24,000) secreted between days 14 to 16, with maximal levels on day 15 post-conception. The protein was named as bovine trophoblast protein-1 (bTP-1) (Helmer, 1987). bTP-1 infusion in cyclic cows increased the intra-oestrous interval (Thatcher *et al.*, 1989). Later, it was found that bTP-1 has extensive sequence and functional homology with interferons of the α group. The recombinant bovine interferon alpha (rbIFN$_\alpha$) was shown to mimic the characteristic effects of bTP-1 on the reproductive system (Newton *et al.*, 1990).

Prostaglandin $F_{2\alpha}$ is the luteolytic signal in cattle. It is released from the surface epithelium of the uterus. Pregnancy recognition signals from the trophoblast of the cow are paracrine anti-luteolytic substances, which act on the uterine epithelium to inhibit release of luteolytic prostaglandin. The timing of the endocrine events during the maternal recognition is critical; one event of vital importance is the suppression of the expression of uterine oxytocin receptors, which in turn suppresses the release of prostaglandins (Thatcher *et al.*, 1997). Oxytocin secreted from the *Corpus luteum* (Wathes and Denning-Kendall, 1992) and probably also from the neurohypophysis, birds to endometrial oxytocin receptors, initiating $PGF_{2\alpha}$, pulsatile secretion and consequently luteal regression (Flint and Sheldrick, 1983). The expression of oxytocin receptors (OTR) in the uterine endometrium plays an important role in the initiation of luteolysis. During early pregnancy, the conceptus secretes interferons which inhibits OTR upregulation and luteolysis. It is well-established in cattle that the presence of embryonic signals on day 16 post-conception suppresses the expression of oxytocin receptors (Robinson *et al.*, 1999).

In this critical period (day 14-16), insufficient secretion of the embryonic interferon (bTP-1) may be attributed to high embryonic losses (Martal *et al.*, 1997). Therefore, due to the structural and functional homology of bTP-1 with rbIFN-α, it has been tried as an exogenous agent for improving reproductive efficiency in cattle. Both intrauterine and intramuscular administration of rbIFN-α have shown to enhance CL lifespan and intra-oestrous intervals in

cattle (Plante *et al.*, 1989). Intramuscular administration of rbIFN-α to cattle between days 14 to 17 or 14 to 19 extended the CL lifespan (Newton *et al.*, 1990). Interferon doses ranging from 2 to 10 mg per day significantly increased the estrous cycle length in cattle (Meyer *et al.*, 1995). While interferon administration has been shown to improve pregnancy rates in sheep (Mirando *et al.*, 1990; Nephew *et al.*, 1990) and goats (Homeida, 1986; Gnatek *et al.*, 1989; Homeida and Al-Afaleq, 1994; Guillomot *et al.*, 1998), the same could not be seen in a single study conducted in cattle (Barros *et al.*, 1992) suggesting species specific variation in response to treatment. Further, up to 10 mg per day intramuscular administration of interferon did not influence the body temperature of cattle although higher doses did transiently increase the body temperature (Barros *et al.*, 1992a; Meyer *et al.*, 1995). It was established that rbIFN-α can reduce the oxytocin induced PGFM release and may, therefore, extend the lifespan of the CL by interfering with events leading to luteolytic release of prostaglandins from the uterus (Plante *et al.*, 1991).

2.5 MATERNAL RECOGNITION OF PREGNANCY

Implantation allows the conceptus and uterine endometrium to achieve intimate contact for nutrient exchange and endocrine communication. At a specific time post conception, the conceptus must produce protein or hormonal signals to signal its presence in the maternal system. This signal is essential for *corpus luteum* (CL) maintenance, production of progesterone and continued endometrial development and secretory activity. Continuous secretion of progesterone from CL is essential for the establishment and maintenance of pregnancy. This is one of the earliest maternal responses that distinguish a normal ovarian cycle from pregnancy, and it is collectively called maternal recognition of pregnancy (Short, 1969). Two patterns of maternal recognition of pregnancy or the maintenance of the CL life span exist among species: luteotrophic and antiluteolytic. In primates and horses, trophoblasts secrete chorionic gonadotropins (CG), while in rodents, the act of mating

induces a prolactin surge from the posterior pituitary, both produce luteotrophic effects that stimulate CL and maintain production of progesterone (Hearney, 1986).

In ruminant animal species, an antiluteolytic rather than luteotrophic factor sustains the production of progesterone. During the critical period of maternal recognition of pregnancy, the ruminant conceptus must produce this antiluteolytic factor in sufficient quantities, for CL maintenance. This antiluteolytic factor affects the secretory pattern of $PGF_{2\alpha}$, which is known to be a physiological initiator of luteal regression in ruminants and porcine species. In ruminants and pigs, the pregnancy signals belong to the family of proteins collectively known as trophoectodermal interferons, are implicated in the maternal recognition of pregnancy (Martal *et al.*, 1979; Godkin *et al.*, 1982; Imakawa *et al.*, 1987; Bazer *et al.*, 1994).

2.5.1 Mechanism of Embryonic Signal Mediated CL Maintenance in Ruminants

Maintenance of the CL by the conceptus derived signal proteins or IFNαs during the early stages of pregnancy involves the non-establishment of the sequence of events during normal luteolysis. In the luteolytic process pulsatile release of $PGF_{2\alpha}$ from the endometrium induces CL release regression during late diestrus (Days 12-15). However, the mechanism which regulates initiation of $PGF_{2\alpha}$ pulses as well as pulse frequency and amplitude remain controversial (Flint *et al.*, 1990; Silvia *et al.*, 1991). Normal luteolysis involves obligatory formation of endometrial oxytocin receptors. A period of progesterone stimulation (approximately 10 days) is required for the synthesis of $PGF_{2\alpha}$ by the endometrium (McCracken *et al.*, 1984). During the period of progesterone dominance, the endometrium is non-responsive to estrogen. Following this period the inhibitory influence of progesterone is lost and the endometrium regains responsiveness to estrogen stimulation, which results in the up regulation of endometrial oxytocin receptors. Oxytocin released from either the CL or posterior pituitary can then interact with its receptor to stimulate the release of $PGF_{2\alpha}$.

Endometrial release of $PGF_{2\alpha}$ establishes a positive feedback loop with the CL, which reinforces oxytocin secretion for the continued support of the episodic release of $PGF_{2\alpha}$ (Flint *et al.*, 1990; Bazer *et al.*, 1991; Silvia *et al.*, 1991). The role of oxytocin in the release of $PGF_{2\alpha}$ remains central to the luteolytic process (McCracken *et al.*, 1984). Oxytocin receptor numbers are greatest at estrus (Flint *et al.*, 1990) suggesting that changes in oxytocin responsiveness result from declining serum progesterone levels (Lau *et al.*, 1992; Vallet *et al.*, 1990). But progesterone support does not appear to be essential for oxytocin receptor synthesis since endometrial oxytocin receptors increase late in the estrous cycle even when progesterone concentrations are maintained through prostaglandin synthesis inhibitor (Lau *et al.*, 1992). Estrogen is believed to sustain and enhance episodic release of $PGF_{2\alpha}$ necessary for complete luteolysis (Vallet *et al.*, 1990). Recently, it has been established that progesterone receptors are intense in the stroma and epithelium at estrus and are maintained until day 5. Thereafter, progesterone receptors decline in the epithelium with complete loss of progesterone receptors after day 11 of the estrous cycle. The oxytocin receptors develop in the luminal epithelium after day 12 of the estrous cycle, after the loss of progesterone dominance over the epithelium. So, it is the length of progesterone exposure that controls the timing for down regulation of progesterone receptors in the uterine epithelium which in turn permissive to synthesis of oxytocin receptors (Wallace *et al.*, 1991). The inhibition of CL regression following the administration of an oxytocin receptor antagonist during the late luteal phase of the ruminant estrous cycle (Jenkin, 1992).

Maternal recognition of pregnancy in domestic ruminants has evolved as a physiological mechanism by which the conceptus inhibits luteolysis through alteration in endometrial $PGF_{2\alpha}$ synthesis, thereby offering the normal luteolytic process.

CL maintenance in ruminants during early pregnancy is dependent upon conceptus polypeptide synthesis and secretion, and these proteins that embryonic interferons (IFN) (Bazer *et al.*, 1991). Ovine, bovine and caprine

trophoblast protein-1 are the conceptus signals for maternal recognition of pregnancy and CL maintenance in ruminants (Bazer *et al.*, 1991; Roberts *et al.*, 1992).

The ruminant trophoblast proteins are known to influence estrous cycle lengths, inhibit estrogen, and oxytocin mediated uterine $PGF_{2\alpha}$ release and suppress inositol phosphate / diacyl glycerol second messenger pathways for PGF2α synthesis (Bazer *et al.*, 1991). The ruminant trophoblast proteins involved in regulating luteolysis during early pregnancy are type-I trophoblast interferons like IFN_{α} and IFN_{β}, a subgroup of IFN-ω (α_{11}) family (Roberts *et al.*, 1991; Leaman and Roberts, 1992). The ruminant trophoblast IFNs exhibit potent antiviral antiproliferative and immunosuppressive activities typical of IFN (Roberts *et al.*, 1991). The embryonic trophoblast IFNs are believed to inhibit an endometrial inhibitor to prostaglandin synthesis, which is expressed early but greatly reduced late in the estrous cycle in the ewe and cow (Thatcher *et al.*, 1989). oTP-1 and bTP-1 inhibit basal and oxytocin induced secretion of $PGF_{2\alpha}$ in cultured uterine stromal and epithelial cells (Charpigny *et al.*, 1990; Danet-Desnoyers *et al.*, 1991). The ovine trophoblastic interferons have also antiprostaglandin synthatase activity that occurs through inhibition of phospholipase A_2 and cycloendoperoxidase activity (Tamby *et al.*, 1991).

This pattern of inhibition of endometrial prostaglandin synthesis interferes with luteolysis (Bazer *et al.*, 1991). Signal transduction of embryonic IFNs is proposed to act through stimulation of receptor coupled phospholipase A_2 and arachidonic acid metabolism (Hannigan and Williams, 1991).

The effects of ruminant type-1 IFNs may be compartmentalized to the luminal epithelium which may allow for stromal synthesis of prostaglandins, especially PGE_2 (Thatcher *et al.*, 1989). The embryonic IFNs are not known to enter the uterine vasculature. So compartmentalization of ruminant type-1, IFNs accounts for increased prostaglandin secretion by stromal cells, while cells involved in luteolysis, the epithelium, are suppressed (Bazer *et al.*, 1991).

Inhibition of luteolysis also involves regulation of endometrial oxytocin receptors, which are significantly lower on days 13 to 16 of pregnancy (Flint *et al.*, 1990). The embryonic IFNs are ineffective in inhibiting $PGF_{2\alpha}$ release once endometrial oxytocin receptors have formed (Charpigny *et al.*, 1990; Bazer *et al.*, 1991).

Therefore, the embryonic IFNs must be secreted synthesized and secreted well in advance of oxytocin receptor synthesis by the endometrium. Accordingly, the embryonic type-I IFN m-RNA is present within the trophoectoderm of the ovine conceptus between days 10 and 12 with greater intensity observed on day 16 (Roberts *et al.*, 1991).

Therefore, the embryonic IFNs must be secreted synthesized and secreted well in advance of oxytocin receptor synthesis by the endometrium. Accordingly, the embryonic type-I IFN m-RNA is present is present within the trophoectoderm of the ovine conceptus between days 10 and 12 with greater intensity observed on day 16 (Roberts *et al.*, 1991). These ruminant type-I trophoblast IFN may interfere with luteolysis by maintaining the inhibitory effects of progesterone on oxytocin receptor synthesis. Down regulation of progesterone receptors permits oxytocin receptor synthesis after day 11 or 12 of the cycle, embryonic type-I IFNs may enhance or stabilize progesterone receptors to present oxytocin receptor synthesis. The estrogen release is inhibited by inhibition of increase in estrogen receptor protein and estrogen receptor mRNA that occurs during days 13 to 16 of estrous cycle, is inhibited (Cherny *et al.*, 1991) by the embryonic IFNs. Thus, the enhanced release of $PGF_{2\alpha}$ stimulated by estrogen, possibly acting through its stimulation of oxytocin receptor, is blocked by the embryonic IFNs.

The type-I embryonic IFNs regulates transcription factors that affect the prostaglandin synthetase inhibitor and formation of oxytocin receptors (Thatcher *et al.*, 1989; Bazer *et al.*, 1991). The secretion of these IFNs allows the conceptus to control the uterine environment and the time of secretion corresponds to the time at which luteolysis occurs during the estrous cycle

(Bazer *et al.*, 1991). In cattle, secretion of bTP-1 and in ewe the secretion of oTP-1 continues from day 14 onwards of early pregnancy (Thatcher *et al.*, 1989).

These trophoblastic type-I IFNs in this period are essential for production of endometrial $PGF_{2\alpha}$, prevention of $PGF_{2\alpha}$ release from the endometrium resulting in prevention of luteolysis and maintenance of CL function (Thatcher *et al.*, 1989; Geisert *et al.*, 1990; Bazer *et al.*, 1991; Danet-Desnoyers *et al.*, 1991). But if these embryonic type-I IFNs are not produced in adequate amounts, the CL undergoes luteolysis resulting in luteal insufficiency.

2.5.2 Failure of Maternal Recognition of Pregnancy : Luteal Insufficiency

It is evident now in most ruminant species and bovines the ultimate objective of maternal recognition of pregnancy is to provide for uninterrupted synthesis and secretion of the most vital hormone for CL maintenance, progesterone. This process involves adequate secretion of embryonic IFN's at the critical time of maternal recognition of pregnancy and biochemical communication between the conceptus and its maternal system. Success of their communication or process of maternal recognition of pregnancy results in the maternal system recognizing the presence of the conceptus (O'Neill, 1985; Hearney, 1986; Cavanagh and Morton, 1994; Meyer *et al.*, 1995). But whenever the adequate amounts of embryonic IFNs are not secreted, the CL is not rescued and the process of luteolysis or structural and functional demise of CL ensues. It involves the dual process of loss of the capacity to synthesize and secrete progesterone with loss of cells that comprise the CL (Knickerbocker *et al.*, 1988; McGuire *et al.*, 1994; Pate, 1994). As progesterone, secreted by the CL is essential for embryo survival, the functional demise of the CL, leads to pregnancy failure. This luteal insufficiency has been believed to cause infertility like embryonic mortality and repeat breeding (Arthur *et al.*, 1989).

2.5.3 Consequences of Luteal Insufficiency : Repeat Breeding

Repeat breeding bovines have apparently normal genitalia without any abnormal discharge from genital tract with normal estrous cycle and estrus period but fails to conceive after 3 consecutive inseminations or services with fertile semen / bull and the animals should not be more than 10 years of age (to exclude those with senile changes).

Repeat breeding in cattle or buffalo is one of the most frustrating reproductive problems at field condition. The incidence of repeat breeding in cattle and buffaloes ranges from 14 to 27 percent and 7 to 10 percent, respectively (Rao, 1997). Causes of repeat breeding is mainly divided into : (1) Failure of fertilization, which may be due to defective gamete transport, abnormalities in ovulation and egg pick up or defective gametes, (2) Embryonic mortality due to chromosomal abnormalities, dietary deficiency, high environmental temperature, adverse uterine environment like infection or inflammation and above all the most important factor is hormonal imbalances resulting from luteal insufficiency. Twenty to thirty percent of repeat breeding causes are due to embryonic mortality in bovines and approximately 25 percent of bovine embryos are lost during the first 3 weeks of life (Sane *et al.*, 1994). The repeat breeding incidences in buffaloes are frequently treated assuming that it is due to luteal insufficiency resulting in failure of CL maintenance and repeat breeding due to early embryonic mortality (Arthur *et al.*, 1989).

The economic and biological significance of embryo mortality in cattle and other bovine species is well recognized. In the absence of infectious disease of the reproductive system, it is the most important factor limiting reproductive efficiency in bovines. The cost due to losses to farmers has been estimated as high as £ 250 million per year in the United Kingdom alone (Peters and Ball, 1995) and $ 1.4 billion in the USA (Gerritts *et al.*, 1976). Though no extensive studies in this regard have been carried out in India, the global cost of embryo mortality in the world cattle population (*Bos taurus* and *Bos indicus*) is estimated to be approximately 1.28×10^{12} (FAO, 1994) million

dollars annually. In the Indian context, it is perceived that the buffaloes are having a much higher incidence of embryonic mortality than cattle, caused by luteal-insufficiency in the early embryonic life (Singh and Gangwar, 1976; Chatterjee *et al.*, 1985; Tomar and Verma, 1987; Kaul and Prakash, 1994; Sane *et al.*, 1994).

2.5.4 Prospects for Prevention of Embryo Mortality

Recent research, both in terms of physiological mechanisms and pharmacological treatments has mostly focused on the period of maternal recognition of pregnancy or the anti-luteolytic effect. It has been estimated that 13 to 15 percent of pregnancies are lost around day 14 to 19 probably related to a failure of anti-luteolytic embryonic IFN secretory mechanism. Therefore, the application of embryonic IFN or related molecules provide the most logical antiluteolytic agents during the critical period of maternal recognition of pregnancy (Thatcher *et al.*, 1995).

2.5.5 Rationale for Application of Recombinant Bovine Interferon Alpha rbIFN$_\alpha$

Maternal recognition of pregnancy in ruminants involves secretion of embryonic trophoblast IFNs, which inhibit the luteolytic mechanisms and maintain CL function in early pregnancy around day 15 (Short, 1969; Roberts *et al.*, 1991). oTP-1 and bTP-1 belong to the family of IFN-α having similar antiviral, immunomodulatory and anti-proliferative properties. There is 70 percent amino acid sequence homology between embryonic trophoblastic IFNs and IFN-α group of interferons (Imakawa *et al.*, 1987; Stewart *et al.*, 1987; Roberts *et al.*, 1992). Both embryonic trophoblastic IFNs and IFN-α are known to bind to the same receptor, type-I IFN receptor, which consists of at least two subunits at the uterine endometrium. Therefore, exogenous administration of these IFNs at the critical period of maternal recognition of pregnancy, are expected to influence the CL life span bovines.

2.5.6 Application of Recombinant Cytokines for CL Maintenance in Ruminants

Summary of IFN Supplementation experiments (*in vitro*)	
Authors	**Experimental findings**
Godkin *et al.*, 1988	Characterization of bovine conceptus proteins during peri and post implantation periods of early pregnancy , demonstrating for the first time the continued production of these proteins beyond day 24 post breeding
Newton *et al.*, 1988	Characterisation of high molecular weight glycoproteins related to IFN family during peri-implantation period of bovine conceptus determination of the similar embryonic protein signals in the bovine andddd ovine species
Gross *et al.*, 1988	Bovine conceptus secretory protein related to IFN-α regulates endometrial protein and prostaglandin biosynthesis to prevent luteolysis and promote embryonic devlopment
Roberts, 1989	Bovine trophoblastic protein (bTP-1) and ovine trophoblastic proteins (oTP-1) are structurally related to IFN- α family play important role in early pregnancy recognition due to antiluteolytic nature
Short *et al.*, 1991	Bovine trophoblast protein (bTP-1) has IFN-α like properties
Hernandez-Ledezma *et al.*, 1992	Bovine trophoblast protein (bTP-1) having IFN-alpha characteristics successfully expressed in conceptuses produced IVM-IVF techniques
Davidson *et al.*, 1994	Effect of IFN-tau and IFN-on endometrial cells may promte its growth during period of pregnancy
Stojkovic *et al.*, 1995	Invitro derived bovine tropoblastic tissue secretes biologically active IFN-tau
Spencer *et al.*, 1995	oIFN-tau regulates estrogen gene expression in endometrial cells thereby inhibiting luteolytic process.
Naivar *et al.*, 1995	Bovine endometrial cells can distinguish between closely related type-1 IFN's and secrete uterine proteins in response.
Kerbler *et al.*, 1997	Maternal progesterone concentration in bovines are positively correlated with IFN-tau synthesis by the conceptus.
Prakash *et al.*, 1997	Early conceptus in pigs signal there presence by means of IFN-γ for CL maintenance
Kubisch *et al.*, 1998	Secretion of IFN-tau by bovine blastocyst invitro is an useful indicator of developmental competence.

Emond *et al.*, 2000	IFN-tau stimulates granulocyte-macrophage colony-stimulating factor gene expression in bovine lymphocytes and endometrial stromal cells, thereby mediating uterine accomodation mechanism during early pregnancy
Binelli *et al.*, 2000	IFN-tau inhibit intracellular mechanisms responsible for luteolysis in endometrial cells
Binelli *et al.*, 2001	IFN-tau stimulates JAK-STAT pathway involved in antiluteolytic effects on bovine endometrial cells.
Johnson *et al.*, 2001	IFN-tau regulates ovine uterine function in presence of progesterone
Pru *et al.*, 2001	IFN-tau induced proteins prepare the bovine endometrium for implantation
Kubisch *et al.*, 2001	Bovine blastocysts secrete IFN-tau which do not vary significantly over a variety of culture conditions and blastocysts invitro produce similar amounts of IFN-tau as in vivo conditions

Summary of IFN Supplementation experiments in ruminants (*invivo*)

Author	Species	IFN used	Route	Effects
Lau *et al* 1996	Sheep	Ovine conceptus secretpry protein	i.u	Reduces oxytocin induced PGF2α
Parkinson *et al* 1992	Sheep	rbIFNα	i.m	Extension of CL life span
Parkinson *et al* 1992	Sheep	rbIFNα	i.u	Extension of CL life span Decrease of PGFM release
Schalue-Francis *et al* 1991	Sheep	rbIFNα	i.u	Extension of CL life span
Schalue-Francis *et al* 1991	Sheep	rbIFNα	i.m	Improved pregnancy rate
Stewart *et al* 1989	Sheep	rbIFNα	i.u	Extension of CL life span
Knickerbocker *et al* 1986	Cattle	bTP-1	i.u	Extension of estrous cycle length Decrease in uterine PGFM release
Plante *et al* 1988	Cattle	rbIFNα	i.u	Extension of CL life span

Helmer *et al* 1989	Cattle	bTP-1	i.m	decreased PGFM release
Plante *et al* 1989	Cattle	rbIFNα	i.u	Extension of estrous cycle length
Plante *et al* 1989	Cattle	rbIFNα	i.m	Extension of estrous cycle length
Thatcher *et al* 1989	Cattle	rbIFNα	i.u	Extensio of CL life span
Nephew *et al* 1990	Sheep	rbIFNα	i.m	Improved pregnancy rate
Newton *et al* 1990	Cattle	rbIFNα	i.m	Increase in rectal temperature associated temporally with decrease in lpasma progesterone
Newton *et al* 1990	Goats	rbIFNtau	i.u	Different effects of different routes of administration Extension of life span i.u route of administration decreased expression of estradiol receptors and oxytocin receptors involved in luteolysis
Martinod *et al* 1991	Sheep	rbIFNα	i.m	Improved pregnancy rate
Plante *et al* 1991	Cattle	rbIFNα	i.u	Extension of CL life span Reduced oxytocin mediated PGFM release
Plante *et al* 1991	Cattle	rbIFNα	i.m	Extensio of CL life span Reduced oxytocin mediated PGFM release Acute changes in body temperature
Siegenthaler & Martinod 1991	Cattle	rbIFNα	i.m	Extension of CL lifespan in cyclic heifers
Barros *et al* 1992	Cattle	rbIFNα	i.m	Reduction of pregnancy rate
Barros *et al* 1992	Cattle	rbIFNα	i.m	Decreased LH
Garverick *et al* 1992	Cattle	rbIFNα	i.u	Delayed luteolysis of CL anticipated to have short life span
Meyer *et al* 1995	Cattle	rbIFNα	i.u	Decreased PGFM Extended luteal phase
Salfen *et al* 1995	Cattle	rbIFNα	i.u	Decreased PGFM Extended luteal phase

High rates of embryonic loss are a major cause of reproductive failure during peri-implantation period when signaling for pregnancy recognition is critical for pregnancy recognition in ruminants (Bazer *et al.*, 1994). As the embryonic trophoblastic proteins secreted as signalling molecules are type-I IFNs, many related compounds have been used in sheep and cattle for CL maintenance *in vivo*. The substances include embryonic homogenates, conceptus secretory proteins (oCSP, oTP, bTP-1) are recombinantly derived IFNs (rbIFNα and IFN-τ).

Studies utilizing embryo removal and transfer established that ovine embryos affect luteal life span by the 13th day after mating (Moor and Rowson, 1966). Furthermore, homogenates of 13 and 14 day old ovine embryos extended CL life span in non-pregnant ewes (Rowson and Moor, 1967). Application of ovine blastocyst and progesterone production during early pregnancy in sheep (Rowson and Moor, 1967; Moor, 1968; Martal *et al.*, 1979; Ellinwood *et al.*, 1979; Godkin *et al.*, 1982; Godkin *et al.*, 1984). Conceptus proteins like oTP-1 when infused into the uterine lumen of cyclic ewes have known to prolong luteal maintenance (Godkin *et al.*, 1984). This effect was believed to involve the effect of oTP-1 on uterine endometrium (Moor, 1968; Ellinwood *et al.*, 1979; Findlay, 1981).

The intrauterine infusions of homogenized 17 and 18 days old embryos delayed CL regression and extended intraestrus intervals in non-pregnant Holstein-Friesian heifers. The results of this experiment were first of all strongly suggestive of the fact that bovine embryo derived substances were effective in prolonging CL life span during early pregnancy (Northey and French, 1980).

Intrauterine injections of day 16 to day 18 bovine conceptus proteins on days 15.5 through 21 post-estrus was found to significantly extended CL life span and intraestrus intervals through attenuation of endometrial $PGF_{2\alpha}$ production in Holstein cows (Knickerbocker *et al.*, 1986). A 20 kd protein called trophoblastin was found to appear during the estrous cycle of ewes and had endometrial receptors. Injected into the uterine lumen in the purified

trophoblastin inhibited cyclic luteolysis. Daily intrauterine injections of 14-16 day old ruminant embryo homogenates into ewes and heifers from day 12 of the estrous cycle maintained progesterone secretion (Martal *et al.*, 1987).

Intrauterine infusion of rbIFNα, type-1 IFN into cyclic cows from day 15. to 21 post-estrus delayed luteolysis. It suggested the use of rbIFNα to supplement endogenous bTP production in cows to decrease embryonic mortality (Plante *et al.*, 1988). Later intrauterine and intramuscular administration of interferon was tested for effectiveness in extending luteal life span in cattle. Intrauterine infusion of 1 mg of rbIFNα twice daily, to lactating dairy cows from day 14 to 21 post-estrus extended intraoestrous interval (30.4 ± 1.91 d versus 24.8 ± 0.58 d) and functional life span of the CL (28.4 ± 2.01 d versus 23.6 ± 0.75 d). In another experiment, twice daily intramuscular injection of 20 mg rbIFNα to heifers from day 15 to 19 extended intraestrus intervals (24.6 ± 1.36 d versus 20.6 ± 0.49 d) and functional life span of the CL (23.2 ± 0.37 d versus 20.2 ± 0.73 d). In a similar experiment, pubertal dairy heifers received twice daily intramuscular injections of 0, 2.5, 5 or 10 mg rbIFNα injection from day 14 through 21 post-estrus. The three rbIFNα treated groups had longer intraestrus intervals and functional CL life spans than the control group of animals. Intraestrus intervals were 22 ± 0.68, 24 ± 1.14, 24.6 ± 1.17 and 25.4 ± 0.97 d, respectively. These experiments strengthened the theory that rbIFNα can regulate CL maintenance in cattle (Plante *et al.*, 1989). Consequent to these findings, experiments were conducted to evaluate effect of rbIFNα on neutral temperature and progesterone levels in heifers. In four experiments intramuscular administration of rbIFNα (range 1.25 to 20 mg) caused hyperthermia. Average peak body temperatures of 40 to 40.4°C occurred 2.5 to 6 hours after injection. Temperatures returned to baseline 12 to 16 hours later. This rise in rectal temperature could be reduced but not totally alleviated, with concomitant administration of an inhibitor of prostaglandin synthesis. However, the hyperthermic response decreased with repeated daily exposure to rbIFNα. This showed gradual adaptation of the animals to rbIFNα treatment. The increase in rectal temperatures was

associated temporarily with a decrease in serum progesterone (Newton *et al.*, 1990). rbIFNα infused through intrauterine route in ewes was also effective in extending CL life span (Stewart *et al.*, 1989). The time of rbIFNα treatment also had influence on its activity. In ewes, 2,000 µg of rbIFNα infused into the uterus daily from days 12 to 15 significantly extends cycle length with a significant suppression of PGFM concentrations on days 14 through 15, and that intramuscular administration of 2.5 mg rbIFNα twice daily from days 9 through 15 had similar effect. However, a similar intramuscular regimen from days 12 through 15 was not effective, the degree of estrous cycle length extension being intermediate between that of control ewes and the day 9 through 15 rbIFNα intramuscular treatment. In case of ewes, both intramuscular and intrauterine treatments both caused an increase in rectal temperature. In both cases, this increase occurred only after the initial rbIFNα treatment; subsequent treatments had no effect on temperature. Intramuscular injection of rbIFNα caused a 1.25°C to 1.5°C increase with the peak occurring 3 hours after injection, whereas intrauterine infusion of rbIFNα resulted in an increase of 0.75°C with the peak occurring at 6 hours post-treatment (Flint *et al.*, 1991).

Systemic administration of rbIFNα has been shown to prolong CL life span and estrous cycle length in cattle. Dosage and timing of treatment are critical factors for its use as a fertility enhancing agent. This fact was evident in the experiments conducted on Simmental heifers. Daily intramuscular injections of 20 mg rbIFNα/heifer from day 14 through day 20 post-estrus extended the estrous cycle lengths by 24.08 ± 3.38 days versus 20.74 ± 3.22 for controls. In the subsequent experiment, 3 different doses of rbIFNα of 5 mg/heifer, 10 mg/heifer and 20 mg/heifer on days 13 to 20, 14 to 19 and 14 to 17 post-estrus was administered through the intramuscular route. These treatments extended CL life span (22.62 ± 1.65, 22.89 ± 1.00 and 21.87 ± 1.05 days, respectively versus 19.87 ± 1.36 days). In a related experiment, dairy heifers received intramuscular rbIFNα as a dose of 40 mg on day 13 post-estrus, 40 mg on day 13, followed by 20 mg on days 14-16, 40 mg on

day 13, followed by 20 mg on days 14-19 or a placebo solution. These respective inter-estrus intervals were 21.6 ± 1.69, 22.5 ± 1.75, 23.1 ± 1.83 and 20.22 ± 1.75 days. Likewise the intervals from estrus until progesterone concentrations in the plasma declined to undetectable levels were 20.58 ± 1.32, 21.1 ± 1.22, 22 ± 2 days and 19.33 ± 1.76 days for the control group (Siegenthaler and Martinod, 1991).

In sheep, supplementation of rbIFNα through the intramuscular route on days 12 through 16 increased intraestrus intervals in non-pregnant sheep and increased both pregnancy rate and embryonic rate significantly (Nephew *et al.*, 1990). A similar beneficial effect of exogenously supplemented rbIFNα was found to increase oestrous cycle lengths and pregnancy success in sheep (Schalue-Francis *et al.*, 1991). Significant enhancement of fertility and intraestrus intervals were evident in another experiment using twice rbIFNα injections on days 12 through 14 of the cycle (Davis *et al.*, 1992). rbIFNα administration either through intramuscular or intrauterine routes conclusively proved its role as an antiluteolysin apparently by inducing changes analogous to those reported for ovine trophoblast protein-1 (Parkinson *et al.*, 1992). The influence of IFN on luteolytic mechanism was further confirmed in sheep, as the local action on endometrial oxytocin and estrogen receptors. It was suggestive of the reduced oestrogen receptor function mediation of the inhibitory effect of IFNs on oxytocin receptors involved in the luteolytic process (Lamming *et al.*, 1995). Intrauterine infusion of ovine conceptus secretory proteins reduced oxytocin induced PGFM release in ewes, without affecting endometrial oxytocin receptor concentrations. Thus, the rbIFNα and related molecule supplementation in sheep conclusively proved its antiluteolytic and luteolytic role in this species.

In cattle, rbIFNα administration in intrauterine and intramuscular routes was seen to reduce oxytocin induced PGFM release and may, therefore, extend the life span of the *Corpus luteum* by interfering with the events leading to luteolytic release of $PGF_{2\alpha}$ from the uterus. Administration of rbIFNα can cause acute changes of body temperature and circulating

concentrations of progesterone that becomes less severe after repeated exposure to rbIFNα (Plante *et al.*, 1991). In post-partum beef cows expected to have short or normal luteal phases, intrauterine infusion of rbIFNα delayed luteolysis of *Corpora lutea* anticipated to have a short life span (Garverick *et al.*, 1992). However, in cattle, it was found that rbIFNα systemic ad ministration may act to reduce progesterone secretion by interfering with the pituitary support for luteal progesterone synthesis (Barros *et al.*, 1992a). It was also found that heifers receiving 20 mg of rbIFNα on days 14-17 or a single injection of 40 mg of rbIFNα on day 13 post-estrus after being inseminated had reduced pregnancy rates as when compared the control group (Barros *et al.*, 1992b). At the same time, it was also reported that intrauterine infusion of rbIFNα in post-partum beef cows, increased luteal phase lengths and reduced oxytocin induced PGFM release, anticipated to have short luteal phases (Salfen *et al.*, 1995).

The efficacy of recombinant bovine and interferon tau were compared for their effect on CL life span, intraestrus interval and oxytocin induced uterine secretion of $PGF_{2\alpha}$. Cows received intrauterine injections of 100 µg of recombinant ovine interferon tau (treatment group) plus 1.4 mg of BSA or 1.5 mg BSA alone (Control group) and 200 µg of recombinant bovine interferon plus 1.3 mg of BSA (treatment group) or 1.5 mg BSA alone (control group), through the intrauterine route in twice daily injections from day 14 to 21 via experimental estrous cycle. On day 17, cows were injected with 100 IU of oxytocin, to investigate the oxytocin mediated PGFM release. In this experiment, recombinant ovine interferon extended the life span of *Corpus luteum* (27.5 versus 19.2 days) and intraestrus interval (30.5 versus 20.6 day) and abolished oxytocin induced PGFM release. Recombinant bovine interferon tau also extended the life span of the *Corpus luteum* (29.0 versus 21.4 days) and intraestrus interval (31.5 versus 22.6 days) while abolishing oxytocin mediated PGFM release suggesting both recombinant ovine and bovine interferon tau can be effective antiluteolytic agents in cattle (Meyer *et al.*, 1995a). The recombinant bovine interferon tau in utero was also found to attenuate secretion of PGFM from cultured endometrial epithelial cell *in vitro* (Meyer *et al.*, 1995b).

It was evident from the intramuscular administration of recombinant bovine interferon tau on days 9, 12 or 15 of the estrous cycle in doses of 0, 1 or 5 mg that the 1 mg dose did not cause any elevation in body temperature, or decrease in plasma progesterone concentrations (Meyer *et al.*, 1995a).In summary, the supplementation of recombinant IFNs in sheep have shown to improve pregnancy and conception rates and significantly reduce embryonic mortality in this species. However in cattle, though recombinant IFNs have increased intraestrus intervals and CL lifespan no significant improvement in pregnancy rates were observed in spite of their antiluteolytic role. The administration of IFNs caused a temporary rise in rectal temperatures associated with depression of circulatory progesterone profiles. In none of the Indian livestock species however, the prospects of application of IFNs for augmenting reproduction have been evaluated.

2.6 GAPS IN KNOWLEDGE

In light of the available literature, the following gaps in knowledge are conspicuous

1. No studies have evaluated recombinant IFNs potential in the Indian context for CL maintenance.
2. No studies have been carried out in this regard in buffaloes, addressing the problem of premature luteolysis leading to embryonic mortality and repeat breeding.
3. No studies have been done to evaluate the influence of recombinant IFNs on estrous cycle lengths, on circulatory levels of major hormones (LH, PGFM and Progesterone) related to CL maintenance.

2.7 STATEMENT OF THE PROBLEM

A large portion of the pregnancies are lost in the pre implantation stage only after breeding in bovines, due to the detrimental effects of uterine $PGF_{2\alpha}$, resulting in embryonic mortality and repeat breeding in bovines (Sreenan and Diskin, 1983). In the Indian context, buffaloes, the premiere dairy animals of

the country, suffer from the reproductive anomalies of embryonic mortality and repeat breeding leading to huge economic burden on the livestock farmer (Singh and Gangwar, 1976; Chatterjee *et al.*, 1985; Tomar and Verma, 1987; Kaul and Prakash, 1994; Sane *et al.*, 1994). The present investigation was, therefore, planned to assess the potential of rbIFN$_\alpha$ on CL lifespan in buffaloes and investigate the endocrine and physiological changes brought about by its administration and provide first hand information on its potential use in livestock production. As a prerequisite for carrying out the study, a simple, sensitive and specific assay for estimation of 13,14-dihydro-15-keto PGF$_{2\alpha}$ (PGFM) is required. So the present investigation was planned with the objectives (a) standardization and validation of a simple, sensitive and specific enzyme immunoassay for 13,14-dihydro-15-keto PGF$_{2\alpha}$ (PGFM) estimation in buffalo plasma, and (b) study the influence of rbIFNα administration on CL life span and physiological and endocrine and changes brought about by its application.

CHAPTER - 3

Materials and Methods

3. MATERIALS AND METHODS

3.1 LOCATION OF THE STUDY

The experiment was conducted at the National Dairy Research Institute farm. The farm is located 249 meters above the mean sea level in the Indo-Gangetic Plain on 29°42′N latitude and 72°02′E longitude. The farm is situated in the dry tropical northern belt in Haryana State of India. All experiments were conducted from November 1999 to October 2000. During the experimental period, the environmental temperature ranged from 24.8°C to 43.4°C in summer months (April to September) and 12.0°C to 34.4°C during winter months (October to March). The vapour pressure ranged from 6.4 to 23.9 mm Hg. The annual rainfall ranged from 760 to 960 mm. The relative humidity ranged from 40 to 85 percent with an average of 69 percent. The data regarding environmental parameters were obtained from the observatory of Central Soil Salinity Research Institute, Karnal, which is situated in North-West direction about 1 km away from the NDRI farm.

3.2 EXPERIMENTAL ANIMALS

For the present study, 15 non-lactating, cyclic Murrah buffaloes, which were free of any anatomical, physiological or infectious disorders, were selected from the NDRI herd. In addition, more buffaloes were taken for short term experiments required to fulfill the objective of PGFM enzyme immunoassay validation, details of which are furnished in the specific section. The animals selected were between two to four lactations. At the beginning of the experiment, the animals were shifted to a separate paddock and maintained throughout the experimental period.

3.2.1 Housing

The buffaloes were kept in a loose housing system. The experimental paddock had brick flooring and asbestos roofing. The shady trees in the

paddock provided additional loafing area for the animals. A water enclosure was constructed for providing for wallowing in summer months of the year. In the summer months, the animals were also provided with water sprinklers to prevent heat stress.

3.2.2 Feed

The buffaloes were fed on a ration consisting of concentrate mixture and roughage. The calculated amount of concentrate mixture as per the practice followed in the farm was fed at 10.00 a.m. in the morning. The roughage was provided *ad libitum* at 9.30 a.m. and 3.30 p.m. during the day. Mineral mixture was provided on alternate days. The animals had access to fresh drinking water throughout the day.

EXPERIMENT – 1

3.3 DEVELOPMENT AND VALIDATION OF A SENSITIVE ENZYMEIMMUNOASSAY (EIA) FOR 13,14-dihydro-15-keto $PGF_{2\alpha}$ (PGFM) DETERMINATION IN BUFFALO PLASMA

3.3.1 PGFM Antibody

The bovine PGFM antiserum used in the present investigation was specific for estimation of PGFM. It was generously gifted by Dr. H.H.D. Meyer, Institut fuer Physiologie, Freising-Weihenstephan, Germany.

3.3.2 Preparation of PGFM-Horse Radish Peroxidase (HRP) Conjugate

The PGFM-HRP conjugate was prepared as following. Solution A was prepared by dissolving 500µg of PGFM in 0.5 ml N,N – dimethylformamide (Sigma,USA) and the resulting solution was cooled to 0°C.To this solution 6.25µl 4-methylmorpholine(Sigma,USA) was added and the solution cooled to -15°C.

Solution B was prepared by slowly adding (dropwise) 375 µl dimethylformamide to 500µl of HRP (Boehringer, West Germany; 10 mg/ml water) and the solution cooled to 0°C.

The coupling reaction was performed by first adding 6.25µl isobutylchloroformate (Sigma, U.S.A.) to solution A and the solution stirred continuously for 3 minutes at -15°C. This was followed by the addition of solution B to A under continuous stirring at -15°C and the pH of the resulting mixture was immediately corrected to pH 8.0 with 1 N NaOH . The mixture was initially incubated for 1 h at -15°C followed by 2 h at 0°C. After addition of 10 mg of $NaHCO_3$ the product was dialysed overnight and further purified by gel chromatography (Sephadex G25fine; column : 1.6×50 cm). The HRP-PGFM conjugate obtained in this manner was immediately tested for its titre and stored at - 60°C.

3.3.3 Preparation of Affinity Purified Goat IgG Antirabbit IgG

About 40 ml plasma from a goat immunized against rabbit IgG containing 20 IU heparin/ml of blood was vortexed with rabbit IgG agarose and was loaded onto a small column. First non-specific proteins were eluted with PBS (0.15 M NaCl, pH 7.2) buffer. Proteins bound specifically were eluted with 15 ml of 0.1 M glycine-HCl (pH 2.0). All steps were performed at room temperature. The eluted fractions (3 ml each) were collected in vials containing 0.2 ml of 1 M Tris-HCl (pH 8.0). The eluted IgG was dialysed overnight against PBS and the protein content determined by measuring the absorbance spectrophotometrically at 260 nm and 280 nm, and extrapolated from a normograph.

3.3.4 EIA Procedure

3.3.4.1 First coating

The first coating was performed by adding 1 µg of goat IgG dissolved in 100 µl of coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) per well of the microtiter plate (Nunc, Roskilde, Denmark). The plates were subsequently incubated overnight at 0°C.

3.3.4.2 Second coating

For saturation of the remaining binding sites, 300 µl of PBS containing one percent BSA was added to all the wells and incubated for 40 minutes at room temperature under continuous shaking on a plate shaker.

3.3.4.3 Washing

The coated plates were washed twice with 350 µl of washing solution (0.05% Tween 20, 10% PBS) per well using an automated microtiter plate washer (Model ELX 50 8MS, USA).

3.3.4.4 Assay protocol

Duplicates of 20 µl of unknown plasma or PGFM standards prepared in hormone stripped charcoal-dextran treated plasma ranging from 10 to 1250 pg/ml were simultaneously pipetted into respective wells along with 100 µl of PGFM antibody diluted 1:5000 in assay buffer (50 mM Na_2HPO_4 0.15 M NaCl, 0.1% BSA, 0.02% Thiomersal, pH 7.4) with the aid of a dilutor dispenser. Plates were incubated at 4°C overnight under constant agitation.

3.3.4.5 Substrate reaction

The plates were washed four times with washing solution and incubated further in the dark for 40 min after addition of 150 µl of substrate solution / well (substrate buffer: 0.05 M citric acid, 0.11 M Na_2HPO_4, 0.05% ureum peroxide pH 4.0 with 5 N HCl; substrate solution: 17 ml of substrate buffer plus 340 µl 3,3', 5,5'-tetramethyl benzidene; 12.5 mg/ml dimethylsulfoxide (Sigma, Germany). The reaction was stopped by addition of 50 µl 4 N H_2SO_4 and the intensity of colour measured at 450 nm with a 12-channel microtiter plate photometer (Model Microscan, India).

3.3.5 Biological Validation of PGFM EIA

For conducting various experiments for the biological validation of PGFM, animals of different physiological states were taken.

Group I : Three multiparous, non-lactating, cyclic Murrah buffaloes; Blood samples were collected at 4 hourly intervals by means of jugular venipuncture, 120 hours prior to the expected estrus till 80 hours post-estrus. In addition to these 4 hourly samples jugular blood samples were drawn at 1 hour interval 42 hours before and 10 hours after observed estrus.

Group II : Fifteen multiparous, non-lactating, cyclic Murrah buffaloes. Blood samples were collected every 12 hours from these animals for 60-80 days. Estrus was recorded by visual observations as well as by bull parading at 6 hourly intervals every day. The day of estrus was designated as day 0. In all, blood samples were collected during 47 estrous cycles from these buffaloes.

Group III : Seven multiparous Murrah buffaloes were taken post insemination in this group. Blood samples were collected from the day of insemination at 12 hours interval for 23 days.Of these seven animals 5 were later declared pregnant, the blood samples of which were analysed .

Group IV : Five Murrah buffaloes having chronic reproductive tract infections were taken in this group. Blood sampling was carried out at 24 hours interval for 42 days.

Group V : Three Murrah buffaloes diagnosed as having ovarian cysts. Blood sampling was carried out at 12 hours interval for 24 days.

Blood samples were collected in heparinized test tubes and immediately cooled on ice and centrifuged at 4°C at 1500xg as soon as possible for 30 minutes. The plasma was frozen at –20°C till analysis of PGFM and progesterone by sensitive enzymeimmunoassay (EIA) and radioimmunoassay (RIA) respectively.

3.3.6 Assay Sensitivity

The lowest PGFM detection limit significantly different from 0 concentration (OD_{450} range 1.1-1.2) was 20 pg/ml of plasma, which corresponded to 0.4 pg/20µl plasma/well (Figures 1 and 2).

3.3.7 Intra and Inter Assay Variation

Intra and inter assay coefficients of variation were determined using pooled plasma containing 68 ± 2.54 and 215 ± 3.45 pg/ml PGFM in 76 assays were found to be 6.3 and 11.6 and 7.5, and 12.7 percent, respectively.

3.3.8 Specificity

The antiserum used in these assays was specific for PGFM (Gueven and Ozsar, 1993) and is stated to have minimal cross reaction with any of the related prostaglandins, PGE_2, PGEM, PGA_2, PGAM and $PGF_{2\alpha}$ (<0.01%).

EXPERIMENT – 2

3.4 INFLUENCE OF RECOMBINANT BOVINE INTERFERON ALPHA (rbIFN$_\alpha$) DOSE RESPONSE ON ESTROUS CYCLE LENGTH AND ENDOCRINE CHANGES IN CYCLING BUFFALOES

This investigation was carried out to determine the effects of different doses of recombinant bovine interferon alpha (rbIFN$_\alpha$, Novartis, Switzerland) on estrous cycle length in 15 cycling, non-lactating buffaloes, which were free of any anatomical, physiological or infectious disorders. The buffaloes were divided into three groups of 5 each. The treatments given to the groups are detailed below:

Group I : Consisted of five buffaloes, which received (i.m. route) 4 mg of rbIFN$_\alpha$ in divided morning and evening doses (twelve hours apart, i.e., 6.00 a.m. and 6.00 p.m.) on days 14 through 16 of

Fig.1. Standard curve of PGFM in buffalo plasma

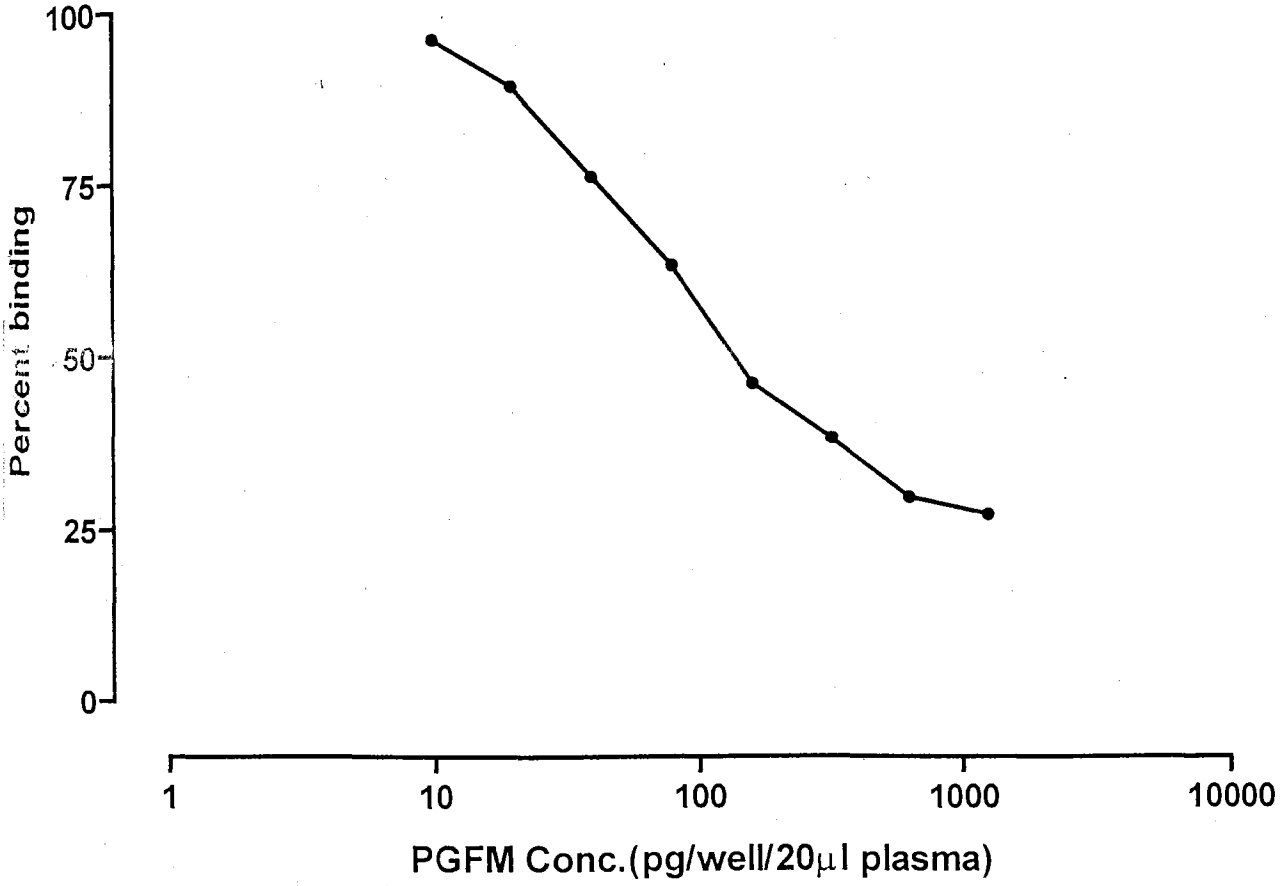


Fig.2a. Effect of different volumes of plasma on standard curve of PGFM

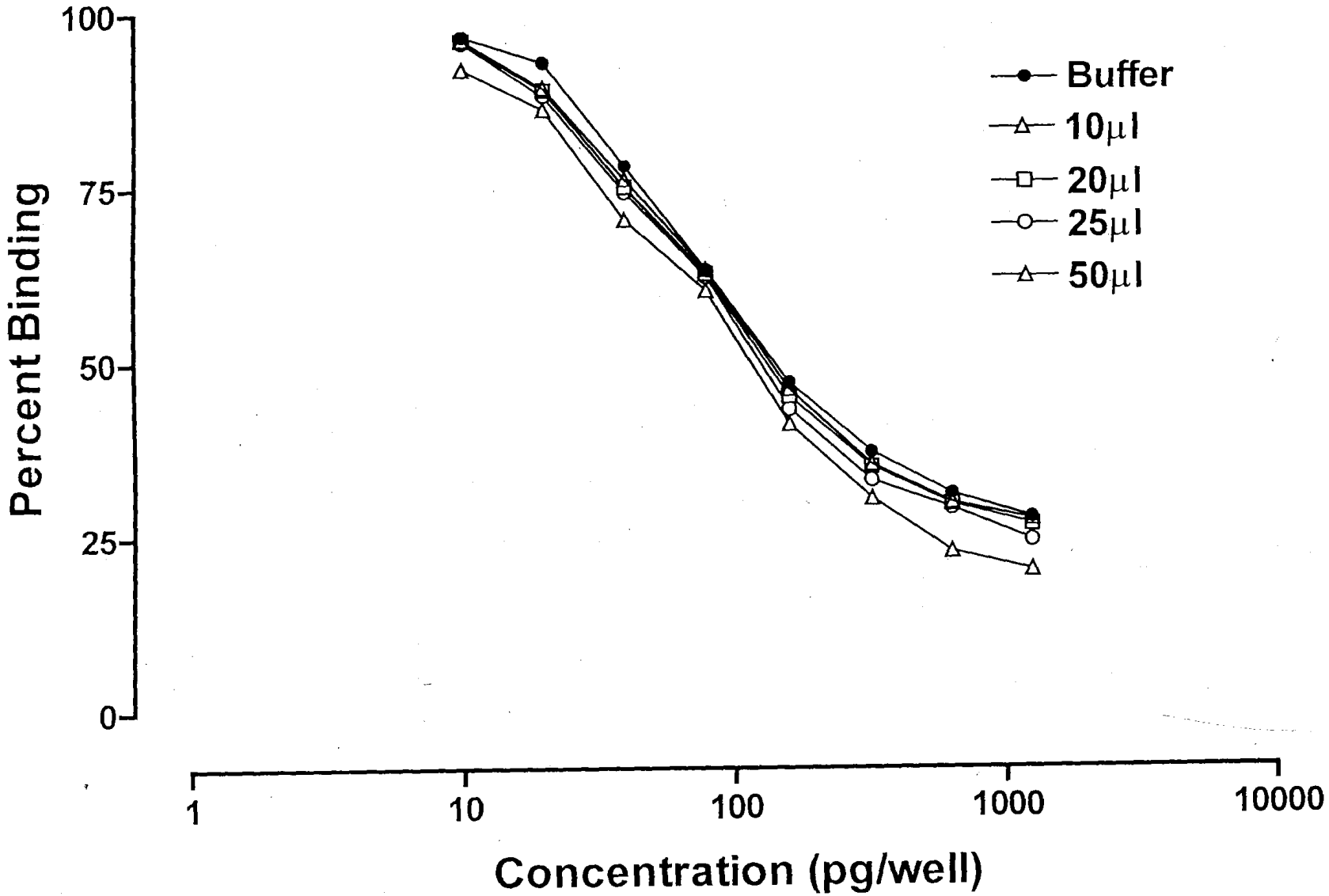
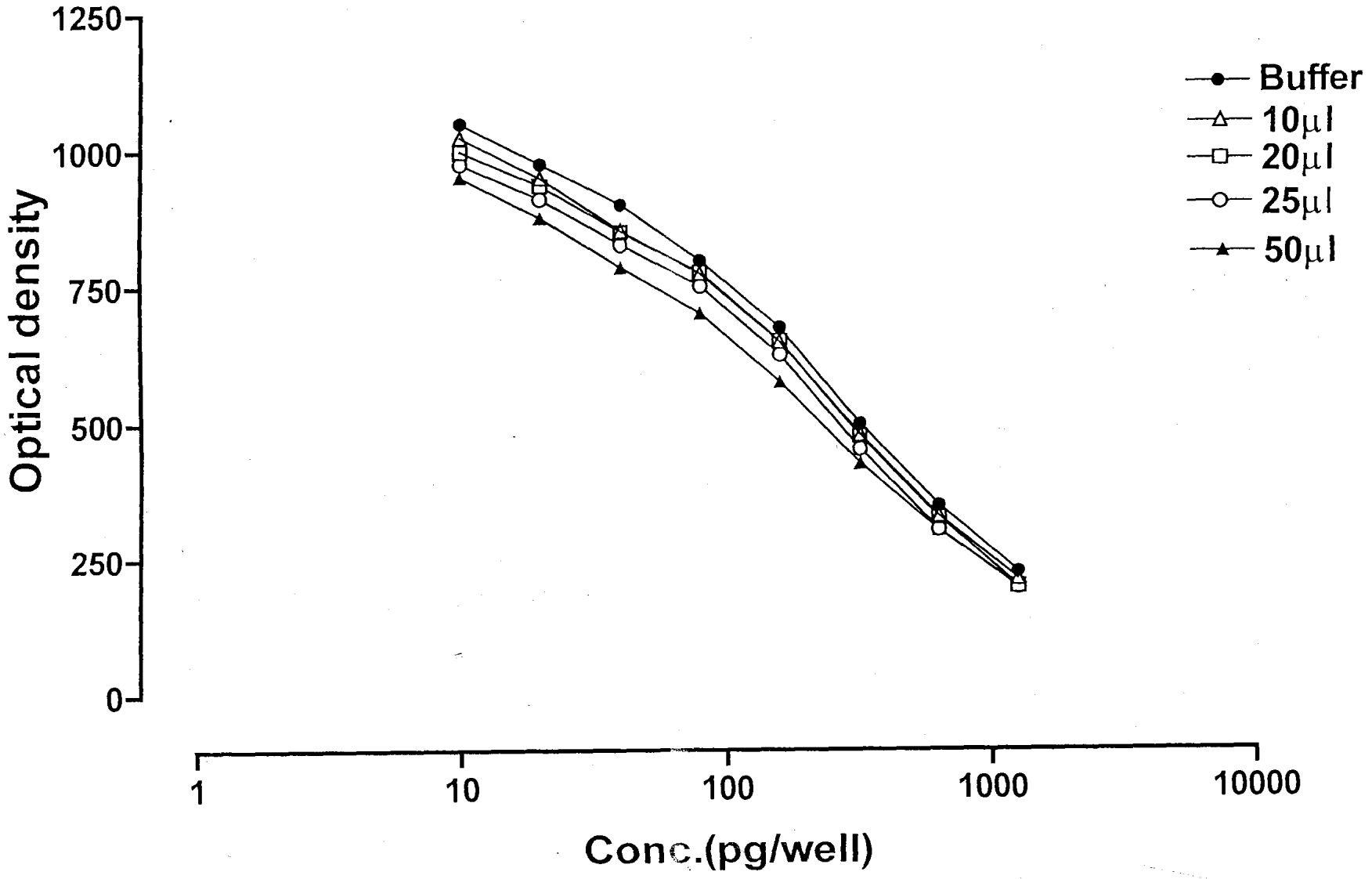


Fig.2b. Effect of different volume of plasma on standard curve of PGFM



the estrous cycle. The treatment cycle lengths were compared with lengths of two cycles prior to and two cycles post-treatment that served as the control cycles.

Group II : The 5 buffaloes of this group received (i.m. route) 8 mg of rbIFN$_\alpha$ in divided morning and evening doses (twelve hours apart, i.e., 6.00 a.m. and 6.00 p.m.) on days 14 through 16 of the estrous cycle. The treatment cycle lengths were compared with cycle lengths of two cycles prior to and two cycles post-treatment that served as the control cycles.

Group III : The highest dose of rbIFN$_\alpha$ of 16 mg in divided morning and evening doses (twelve hours apart, i.e., 6.00 a.m. and 6.00 p.m.) was administered (i.m. route) to the 5 buffaloes of this group. As for previous groups, the treatment cycle lengths were compared with cycle lengths of two cycles prior to and two cycles post-treatment that served as the control cycles.

3.4.1 Estrous Cycle Monitoring

Estrous cycle monitoring was carried out as follows; Blood samples were collected on alternate days prior to rbIFN$_\alpha$ administration and daily post rbIFNα administration up to the next observed estrus. All the blood samples were centrifuged within 30 to 60 minutes post collection to obtain plasma. Plasma obtained was stored at -20°C till further analysis for progesterone by sensitive RIA procedures. Estrus was detected by bull, visual observations as well as plasma progesterone analysis.

3.4.2 Preparation and Administration of Interferon

Recombinant bovine interferon alpha (rbIFN$_\alpha$) was supplied as a formulated lyophilized substance, prepared by Novartis, Switzerland (Formerly CIBA-GEIGY, Basel, Switzerland). A single vial contained 125 mg of lyophilized interferon. The stock preparation of interferon had a specific

antiviral activity of 1.4 x 10^7 units/mg protein determined by viral plaque inhibition of vesicular stomatitis virus grown on Madin-Darby bovine kidney (MDBK) cells (Horisberger and Stavitzky, 1985) (Table 1). For intramuscular administration, sterile pyrogen free water was added to a vial containing 125 mg rbIFN$_\alpha$ to produce final concentrations of 2, 4 and 8 mg/ml. The diluted rbIFN$_\alpha$ of three concentrations were dispensed into three sterile vials and sealed before being taken to the experimental paddock for administration to animals. All dilutions were done under sterile conditions in a laminar flow hood.

3.4.3 rbIFN$_\alpha$ Schedule

The 3 different doses of rbIFN$_\alpha$ (i.e., 2, 4 and 8 mg/ml) were administered through the intra-muscular route by means of sterilized disposable syringes. During the interval from the preparation of rbIFN$_\alpha$ dilutions till administration, the rbIFN$_\alpha$ solutions were kept at 4°C. The doses were administered in equal morning and evening doses of 1 ml of each were administered on days 14 through 16 of the estrous cycle.

3.4.4 Rectal Temperature Measurements

The rectal temperature measurements were made from 12 hours prior to 80 hours post the first injection of rbIFN$_\alpha$ in all groups at hourly intervals encompassing days 14 through 16 of the treatment cycle. Similar measurements were done on identical days in 5 untreated buffaloes (control cycles) as well. All recordings were done by means of a digital thermometer (Microidea, Leesburg, FL; Model : MT3001-BF, China) in degree Fahrenheit. The objective of the measurements was to investigate the influence of rbIFN$_\alpha$ on body temperature of the animals.

3.4.5 Blood Sampling

Blood samples were collected from animals of all groups by means of jugular catheter at 2 hours before and 8 hours after the first injection of rbIFN$_\alpha$ at 15 minutes interval on day 14 of the estrous cycle (as per catheterization

Table 1. Material specifications of Recombinant bovine interferon alpha ($rbIFN_\alpha$)

Type	Recombinant bovine interferon alpha	
Code	CGA 206 091	
Origin	Novartis, Switzerland	
Lot No.	13 / 872 / 1	
Date of Manufacture	March 2, 1989	
Sample Size(s)	Vial @ 125 mg protein (nominal) *	
Formulation Particulars	Formulated lyophilizate	
Protein Content	119.2 mg per vial	(Lowry)
Purity	98.6 % IFN	(SDS – PAGE)
Endotoxin Concentration	< 156 EU per vial	(LAL Assay)
(Specific) Activity **	4.6×10^7 IRU / mg protein	(MDBK / VSV)
Storage Recommendation	2 – 8 °C	
Precautions	Keep reconstituted material and dilutions always sterile (sterile filtration)	
Reference / Date	E 89009305, 24 Apr 89	

protocol, Table 2). Further daily blood samples were collected throughout the cycle. The blood samples collected on alternate days were analysed for progesterone up to day 14 of the cycle and daily samples taken thereafter were analysed for progesterone till the next estrus. The blood samples were collected in an identical manner in five control cycles as well. The blood was collected in heparinized tubes (20 IU/ml of blood) put in the ice bucket and carried back to the laboratory immediately. Once in the laboratory, all the blood samples were centrifuged at 1500xg for 30 minutes, plasma was separated and stored in properly labeled vials at –20°C till analysis for LH and progesterone.

3.4.6 Heat Detection

Heat detection was carried out by visual observations, vasectomized bull parading four times a day (6.00 a.m., 12.00 noon, 6.00 p.m. and 12.00 a.m.) and plasma progesterone analysis by Radio Immuno Assay Procedure (Kamboj and Prakash, 1993).

3.4.7 Hormone Analysis

The plasma samples collected were analyzed for progesterone (Kamboj and Prakash, 1993) and LH (Prakash *et al.*, 2002) to study the influence of rbIFN$_\alpha$ administration on peripheral LH mediated progesterone levels.

EXPERIMENT – 3

3.5 INFLUENCE OF rbIFN$_\alpha$ ON OXYTOCIN MEDIATED PGFM RELEASE

Six cyclic, non-lactating buffaloes, which were free of any anatomical, physiological and infectious disorders, were used for this experiment. Estrous cycle was monitored by plasma progesterone analysis, and estrus was

Table 2. Catheterization Protocol

Animal No:
Date of the experiment:
General observation:
Details of the catheterization protocol: Time of catheterization: Sedation: Medication: Length of the catheter: Heparin concentration per ml of plasma: Amount of blood sample collected: Time of the first sample collection: Frequency of the sample collection: Post catheterization medication:

identified as described in the previous sections. The animals were implanted with a jugular catheter on day 17 of the cycle. Each animal was given an exogenous oxytocin challenge at the rate of 50 IU per animal on day 17 of the cycle. Blood samples were collected at 15 minutes interval from 2 hours before the administration of oxytocin till 6 hours after administration.

In the subsequent cycle, the same animals were administered rbIFN$_\alpha$ (i.m. route) at the rate of 16 mg/day in divided morning and evening doses of 8 mg on days 14 through 16. The oxytocin challenge was given on day 17 at the rate of 50 IU per animal. Blood samples were collected in an identical manner as described above.

3.5.1 Catheterization and Blood Sampling

The blood samples were collected by means of a jugular catheter. The catheterization was done under the supervision of the veterinarian under hygienic conditions. The animals were administered local anesthesia prior to catheterization and antibiotics after removal of catheter. The blood samples (5 ml each) were collected in heparinized tubes (20 IU heparin/ml of blood). The tubes were put in ice bucket and carried back to the laboratory, immediately after collection. All the samples were centrifuged within 30 minutes of collection at 1500xg for 30 minutes and plasma was separated. Then, the plasma samples were stored at –20°C till hormone analysis.

3.5.2 Heat Detection

Heat detection was carried out by visual observations, bull parading and plasma progesterone analysis from blood samples collected on alternate days before and daily after rbIFN$_\alpha$ administration throughout the experimental period. The estrous cycle length in the oxytocin administered cycle and rbIFN$_\alpha$ plus oxytocin administered cycle, were compared with one cycle prior to and one cycle post-treatment.

3.6 BLOOD PLASMA ANALYSIS FOR PROGESTERONE QUANTIFICATION

3.6.1 Materials for RIA

3.6.1.1 Radiochemicals

The radiochemical in the assay namely (1, 2, 6, 7-^{3}H) progesterone (100 μ Ci/mmol) was purchased from M/s Radio Chemical Centre, Amersham, England.

3.6.1.2 Chemicals (non-radioactive)

Non-radioactive chemicals used in this investigation were either imported from M/s Sigma Chemical Company, St. Louis, USA or purchased locally from reputed firms. The chemicals obtained from abroad included the chemicals like 2,5-diphenyloxazole (PPO), 1,4-bis-2-(5-phenyloxazolyl) benzene (POPOP), dextran (approx. molecular weight 70,000) and thiomersal. The chemicals obtained locally were sodium chloride, sodium dihydrogen phosphate [NaH_2PO_4, $2H_2O$], sodium phosphate dibasic [Na_2HPO_4], BSA (all used for preparation of phosphate buffer saline) and toluene (for use in scintillation cocktail).

3.6.1.3 Antisera

The progesterone antibody used in the present study was BSP NR #2 (20-10-93) raised at National Dairy Research Institute (Prakash and Madan, 2001) (Table 3).

3.6.2 Radioimmunoassay of Progesterone in Plasma

Progesterone was estimated by an RIA procedure developed for our laboratory conditions as detailed by Kamboj and Prakash (1993). The assay procedure developed excludes the extraction step. The assay method followed in present investigation is as follows:

1) Duplicate 20 μl plasma samples were taken in 12 x 100 mm test tubes along with the addition of PBS buffer to make up the volume up to 320 μl.

Table 3. Specificity of the progesterone antiserum

Steroids	Percent cross reaction
4 – pregnane – 3, 20 dione	100
11 α – hydroxyl progesterone	90
Corticosterone	<0.2
Cortisone	< 0.01
Hydrocortisone	< 0.01
β - estradiol	< 0.001
Estriol	< 0.001
Testosterone propionate	< 0.001

2) 0.1 ml of progesterone specific antiserum was added (1:10,000) to each tube followed by addition of 0.1 ml of tracer (8,500 CPM).

3) All tubes were incubated at room temperature for 2 h for the immune reaction.

4) Free and antisera bound hormone were separated by addition of a freshly prepared cold charcoal-dextran suspension (0.625% activated charcoal plus 0.1% dextran in buffer).

5) The tubes were then stirred, incubated at 0°C in ice water bath for 10 min and then centrifuged at 1500xg at 4°C for 15 min.

6) The supernatant containing the bound progesterone was decanted into scintillation vials and counted in 5 ml of scintillation fluid.

7) Along with the assay, standard progesterone samples in hormone free plasma were run ranging from 8 pg to 250 pg/20 µl.

3.6.2.1 Assessment of radioimmunoassay

In the process of estimation of hormones and evaluation of the assay system, quality control in terms of sensitivity, specificity and precision was carried out for each of the hormone.

3.6.2.1.1 Sensitivity

Sensitivity is generally regarded as hallmark of good assay. It is defined as the least amount of hormone distinguishable from zero concentration. The sensitivity of the assay was calculated on the basis of different standard curves. The sensitivity of the assay for progesterone in plasma at minimum detection limit was 8 pg/tube and the 50 percent binding limit being 56.5 pg/tube (Fig. 3).

3.6.2.1.2 Precision

It is often referred to "reproducibility", a measure of variation observed between repeated determinations of the same sample. In other words, it can be defined as a degree to which a number of measurements of an antigen in the same sample agrees with the mean concentration. It was assessed by

calculating intra- and inter- assay coefficient of variation from measurements of pooled plasma in the same as well as in different assays. The intra-assay and inter-assay coefficients of variation of plasma progesterone were 8.7 and 11.2 percent, respectively.

3.6.2.1.3 Specificity

Specificity of an assay is defined as "the degree of which an assay responds to substances other than that for which the assay was designed". The specificity of the progesterone antiserum raised against different compounds related to progesterone are given in Table 3.

3.7 BLOOD PLASMA ANALYSIS FOR LH QUANTIFICATION

Quantification of LH was carried out by EIA (Prakash and Anandlaxmi, 2000).

3.7.1 Preparation of Biotinyl-LH Conjugate

To 10 µg bovine LH dissolved in 50 µl of phosphate buffered saline (PBS; 50 mM $NaPO_4$; 0.15 M NaCl, pH 7.2), 12 µl biotinamidocaproate-N-hydroxysuccinimide ester (biotin, Sigma, Germany) dissolved in dimethyl sulfoxide (1 ng/ml, Sigma, Germany) was added and the mixture was immediately vortexed and incubated further for 3 hours at room temperature under constant agitation. The coupling reaction was stopped by the addition of 20 µl NH_4Cl (IM) and the reaction mixture incubated for a further 30 minutes before addition of 2 ml of a solution of 1 percent bovine serum albumin (BSA) in PBS. For the isolation of the biotin-LH conjugate, the mixture was dialysed, overnight at 0°C with three changes in PBS. After dialysis, the conjugate was mixed with an equal volume of glycerol to prevent freezing and preserved at -20°C in 1 ml aliquots.

3.7.2 bLH Antibody

The bovine LH antisera used in the present investigation (USDA-309-684P) was very specific for LH and is stated to have minimal cross reactivity with TSH and FSH (Schams *et al.*, 1972) (Table 4).

Fig.3. Standard curve of progesterone in buffalo plasma

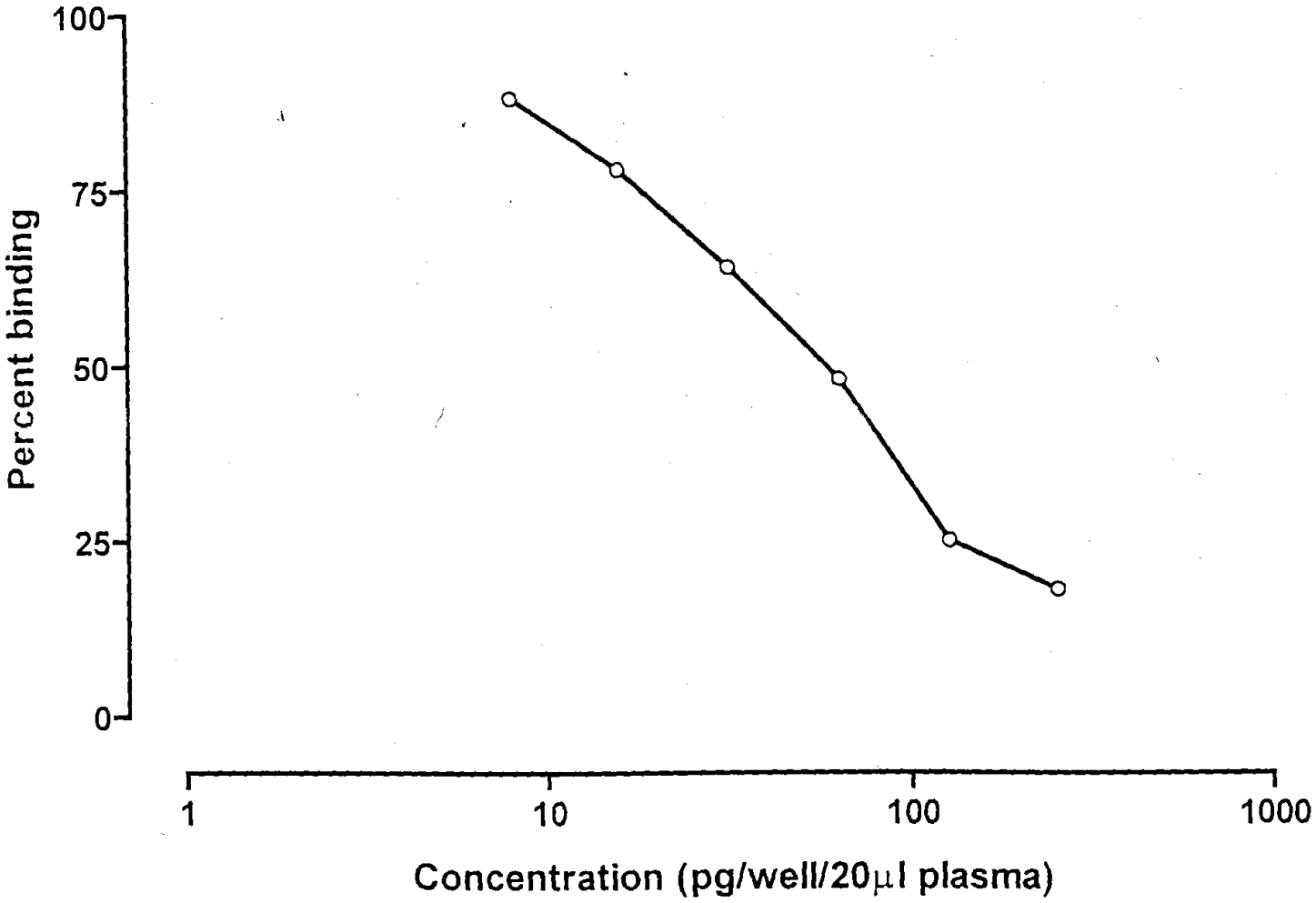


Table 4. Specificity of LH antiserum

Related compounds	Percent cross reaction
bLH	100
bFSH	0.7
bGH	0.7
bPRL	0.01
bTSH	0.2

3.7.3 Preparation of Affinity Purified Goat IgG Antirabbit IgG

The IgG antirabbit IgG was purified in an identical manner as described earlier for PGFM EIA.

3.7.4 EIA Procedure

3.7.4.1 First coating

The first coating was performed by adding 1 µg of goat IgG dissolved in 100 µl of coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) per well of the microtitre plate (Nunc, Roskilde Denmark, No.439454). These plates were subsequently incubated overnight at 0°C.

3.7.4.2 Second coating

For saturating the remaining binding sites, 300 µl of PBS containing 1 percent BSA was added to all the wells and incubated for 1-2 hours at room temperature.

3.7.4.3 Washing

The coated plates were washed twice with 350 µl of washing solution (0.05% Tween 20, 10% PBS) per well using an automated microtitre plate washer (ELX50 8MS,USA)

3.7.4.4 Assay protocol

Duplicates of 20 µl of unknown plasma or bovine LH standards (prepared in hormone stripped charcoal dextran treated plasma) ranging from 0.31 to 10 ng/ml were simultaneously pipetted into respective wells along with 100 µl of LH antibody diluted 1:1,60,000 in assay buffer (50 mM $NaPO_4$ 0.15 M NaCl, 0.1% gelatin, 0.02% thiomersal) with the aid of a dilutor-dispenser. Plates were incubated overnight under constant agitation. They were then decanted and washed two times with washing solution before addition of 100 µl of biotinyl-LH conjugate diluted 1: 800 in assay buffer. The plates were further incubated for 30 minutes, decanted and washed twice. Then, 20 ng streptovidin-peroxidase (Sigma, Germany) in 100 µl of assay buffer was

added to all the wells and plates were wrapped in aluminium foils and incubated for a further 30 minutes under constant agitation. All steps were performed at room temperature.

3.7.4.5 Substrate reaction

The plates were washed four times with washing solution and incubated further in the dark for 40 min after addition of 150 µl of substrate solution / well (substrate buffer : 0.05 M citric acid, 0.11 M Na_2HPO_4, 0.05% ureum peroxide pH 4.0 with HCl; substrate solution : 17 ml of substrate buffer plus 340 µl 3,3',5,5'-tetramethyl benzidene; 12.5 mg/ml dimethylsulfoxide (Sigma, Germany). The reaction was stopped by addition of 50 µl 4 N H_2SO_4 and the colour intensity was measured at 450 nm with a 12-channel microtiter plate photometer (Model Microscan, India).

3.7.4.6 Assessment of enzymeimmunoassay

In the process of estimation of hormones and evaluation of the assay system, quality control in terms of sensitivity, specificity and precision was carried out for each of the hormone.

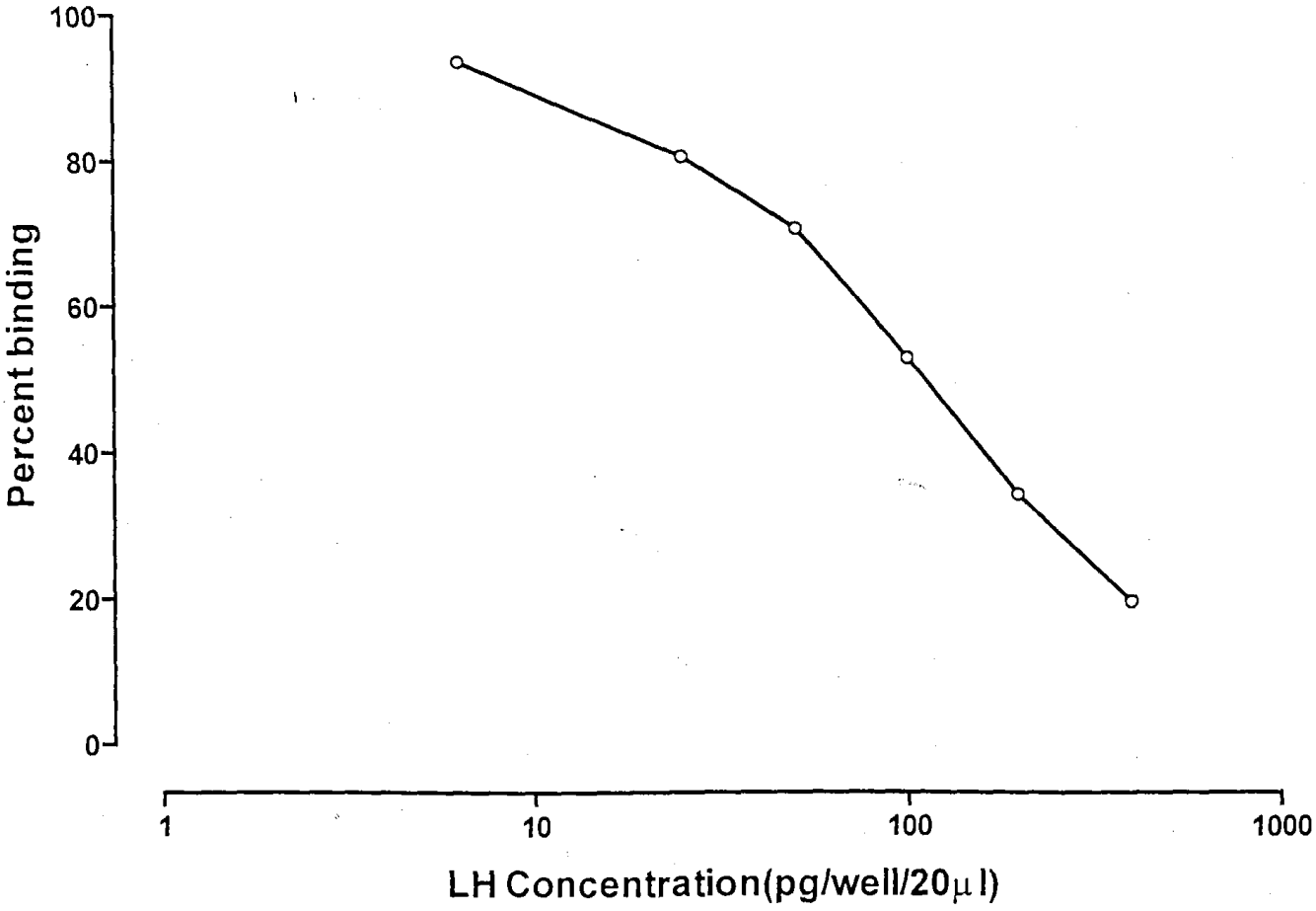
3.7.4.6.1 Sensitivity

High sensitivity is generally regarded as hallmark of good assay. It is defined as the least amount of hormone distinguishable from zero concentration. The sensitivity of the assay was calculated on the basis of different standard curves. The sensitivity of the assay for LH in plasma at minimum detection limit was 6.25 pg/tube or 0.31ng/ml (Fig. 4).

3.7.4.6.2 Precision

It is often referred to "reproducibility", a measure of variation observed between repeated determinations of the same sample. In other words, it can be defined as a degree to which a number of measurements of an antigen in the same sample agree with the mean concentration. It was assessed by calculating intra- and inter- assay coefficients of variation from measurements of pooled plasma in the same as well as in different assays. The intra-assay

Fig.4. Standard curve of LH in buffalo plasma



and inter-assay coefficients of variation were determined using pooled plasma containing 0.61 ± 0.25 and 1.7 ± 0.75 ng/ml of bovine LH in 39 assays were 6.9 and 12.5, and 8.7 and 13.2 percent, respectively.

3.7.4.6.3 Specificity

Specificity of an assay is defined as "the degree to which an assay responds to substances other than that for which the assay was designed". The specificity of the LH antiserum raised against different compounds related to LH are given in Table 4.

3.8 STATISTICAL ANALYSIS

The data obtained was analyzed by Graphpad Prism software package, 1995. Paired t test was employed to test the difference between oestrous cycle lengths as influenced by 3 doses of rbIFN$_\alpha$ treatments. Paired t-test was also employed to test the difference between rectal temperatures in 3 different groups treated with rbIFNα and effect of rbIFN$_\alpha$ doses on oxytocin mediated PGFM release.

To test the effect of the three treatments in different hormonal levels, analysis of variance technique was used in the following statistical model:

$$X_{ijk} = \mu + T_i + T_j + e_{ijk}$$

Where,

μ = Overall mean,

X_{ijk} = Dependent variables,

T_i = Effects of treatments,

T_j = Interval effect, and

e_{ijk} = Error.

Results

4. RESULTS

4.1 BIOLOGICAL VALIDATION OF PLASMA PGFM ENZYME IMMUNOASSAY (EIA)

During the physiological stages of periestrus, cyclicity, early pregnancy, and reproductive disorders of reproductive tract infection and ovarian cystic conditions, the peripheral PGFM levels were measured. The results obtained with respect to the different physiological stages are presented as follows:

4.1.1 Plasma PGFM Levels During Periestrus

Changes in the mean plasma PGFM levels during the periestrus period is presented in Tables 5 and 6 along with the corresponding progesterone values. The temporal changes in the mean levels of both these hormones are depicted in Figures 5 and 6, which present the hormone profiles at 4 and 1 hour intervals, respectively.

The plasma PGFM levels (Mean ± SEM) 120 h prior to estrus were 139.5 ± 49.5 pg/ml, which rose to 591.6 ± 23.3 at 36 hours prior to estrus ($P<0.01$; Fig. 5). When one considers the PGFM profile obtained from blood samples collected 6 times (4 hourly intervals) a day. However, the hourly samples collected from 42 hours prior to estrus clearly depict a pulsatile pattern of PGFM secretion (Fig. 6). About 9 pulses of PGFM were observed during the period of sampling. The peak PGFM levels varied between 286.60 ± 24.18 to 591.60 ± 23.30 pg/ml (Fig. 6; Table 6). The lowest PGFM levels of 69.00 ± 60.56 pg/ml PGFM were obtained during estrus.

There was no significant difference ($P>0.01$) observed between PGFM levels post-estrus up to 80 hours, which fluctuated around basal levels ranging from 95.63 ± 17.33 to 113.57 ± 31.35 pg/ml (Table 5; Fig. 5). Also no

Table 5. PGFM and Progesterone (Mean ± S.E.) profiles in cyclic buffaloes during periestrus period (at 4 hour intervals)

Hours	PGFM	Progesterone
-120	139.5± 49.5	2.96± 0.123
-116	161.5± 64.5	2.10± 0.145
-112	145.5± 63.5	2.56± 0.145
-108	186.5± 70.5	2.29± 0.235
-104	201.0± 89.0	2.10± 0.245
-100	212.5± 55.5	2.31± 0.231
-96	202.0± 38.0	2.10± 0.124
-92	204.0± 65.0	1.95± 0.116
-88	213.0± 37.0	1.97± 0.115
-84	230.0± 46.0	1.82± 0.125
-80	222.0± 58.0	1.98± 0.127
-76	283.3± 64.8	1.87± 0.129
-72	280.3± 78.0	1.56± 0.119
-68	284.3± 41.5	1.85± 0.119
-64	318.3± 52.0	1.45± 0.126
-60	376.6± 40.2	1.39± 0.128
-56	368.3± 39.7	1.30± 0.129
-52	378.6± 83.3	1.25± 0.131
-48	480.3± 57.8	1.19± 0.134
-44	498.0± 78.0	1.10± 0.126
-40	564.6± 53.5	1.26± 0.127
-36	591.6± 23.3	1.18± 0.154
-32	484.0± 58.0	1.13± 0.164
-28	443.6± 63.6	1.10± 0.129
-24	401.6± 58.8	1.09± 0.159

Contd.....

Contd.....(Table 5)

Hours	PGFM	Progesterone
-20	386.6± 85.0	1.25± 0.125
-16	334.3± 36.6	1.16± 0.127
-12	286.6± 24.1	0.98± 0.124
-8	210.0± 31.4	0.78± 0.129
-4	175.6± 60.1	0.56± 0.128
0	69.0± 60.5	0.44± 0.127
4	78.0± 32.7	0.44± 0.145
8	95.0± 17.3	0.51± 0.168
12	115.0± 20.4	0.45± 0.167
16	124.0± 31.6	0.56± 0.357
20	134.0± 32.0	0.47± 0.359
24	139.0± 14.0	0.49± 0.214
28	146.0± 48.9	0.57± 0.125
32	152.0± 50.8	0.57± 0.124
36	162.0± 38.1	0.68± 0.129
40	196.0± 53.6	0.79± 0.128
44	204.0± 48.1	0.62± 0.127
48	226.0± 30.7	0.87± 0.129
52	158.0± 25.8	0.98± 0.127
56	194.3± 36.7	0.96± 0.125
60	200.6± 35.9	0.78± 0.128
64	187.6± 29.2	0.86± 0.137
68	204.3± 31.3	0.82± 0.138
72	211.6± 39.2	0.89± 0.139
76	197.6± 56.0	0.65± 0.145
80	165.0± 35.0	0.67± 0.153

Fig.5. Peri - estrus plasma PGFM and progesterone profiles (Mean±S.E.) in buffaloes (n=3) at 4 h intervals

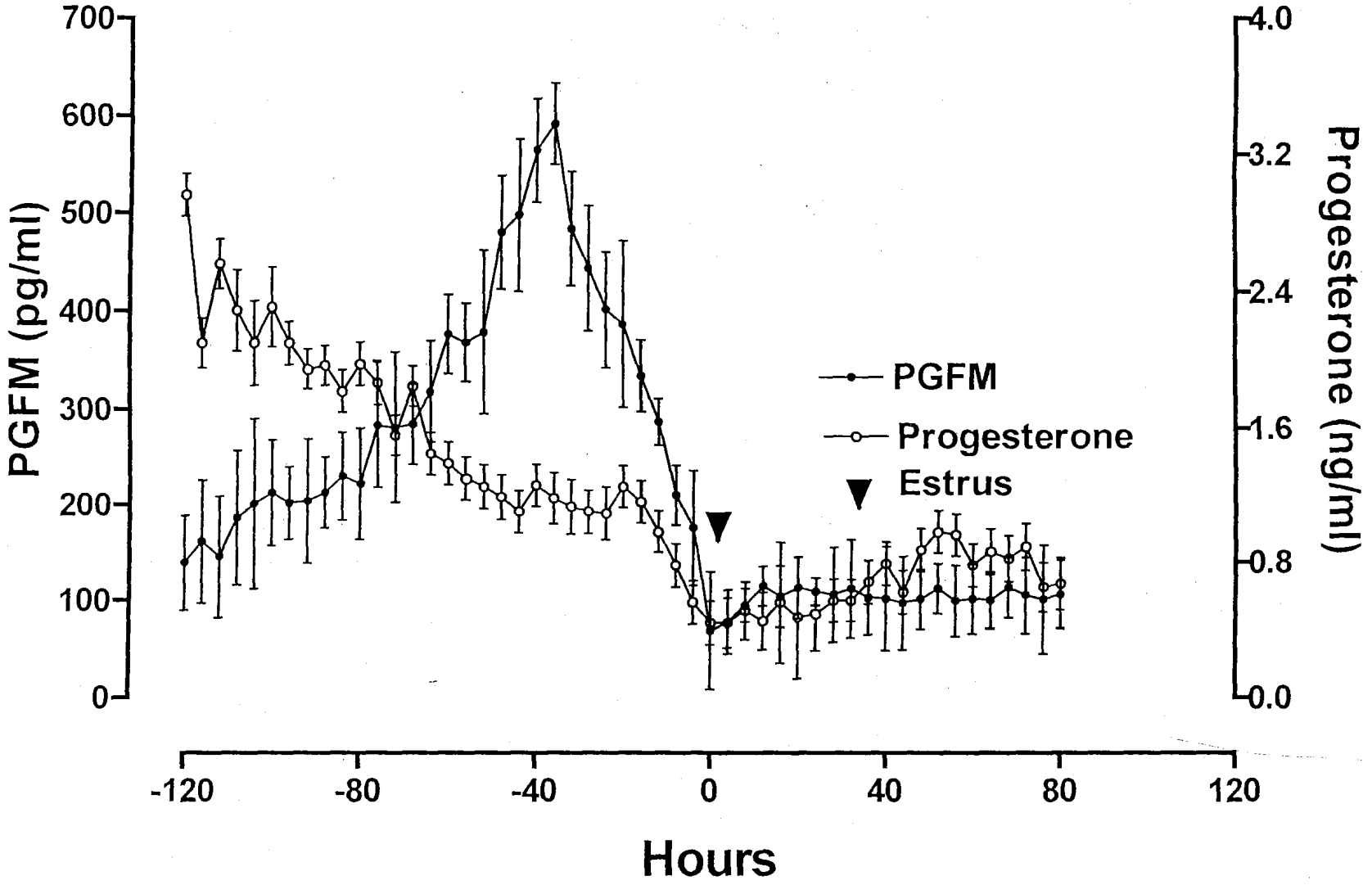


Table 6. PGFM and Progesterone profiles (Mean ± SE) in buffaloes during periestrus period (at 1 hour intervals)

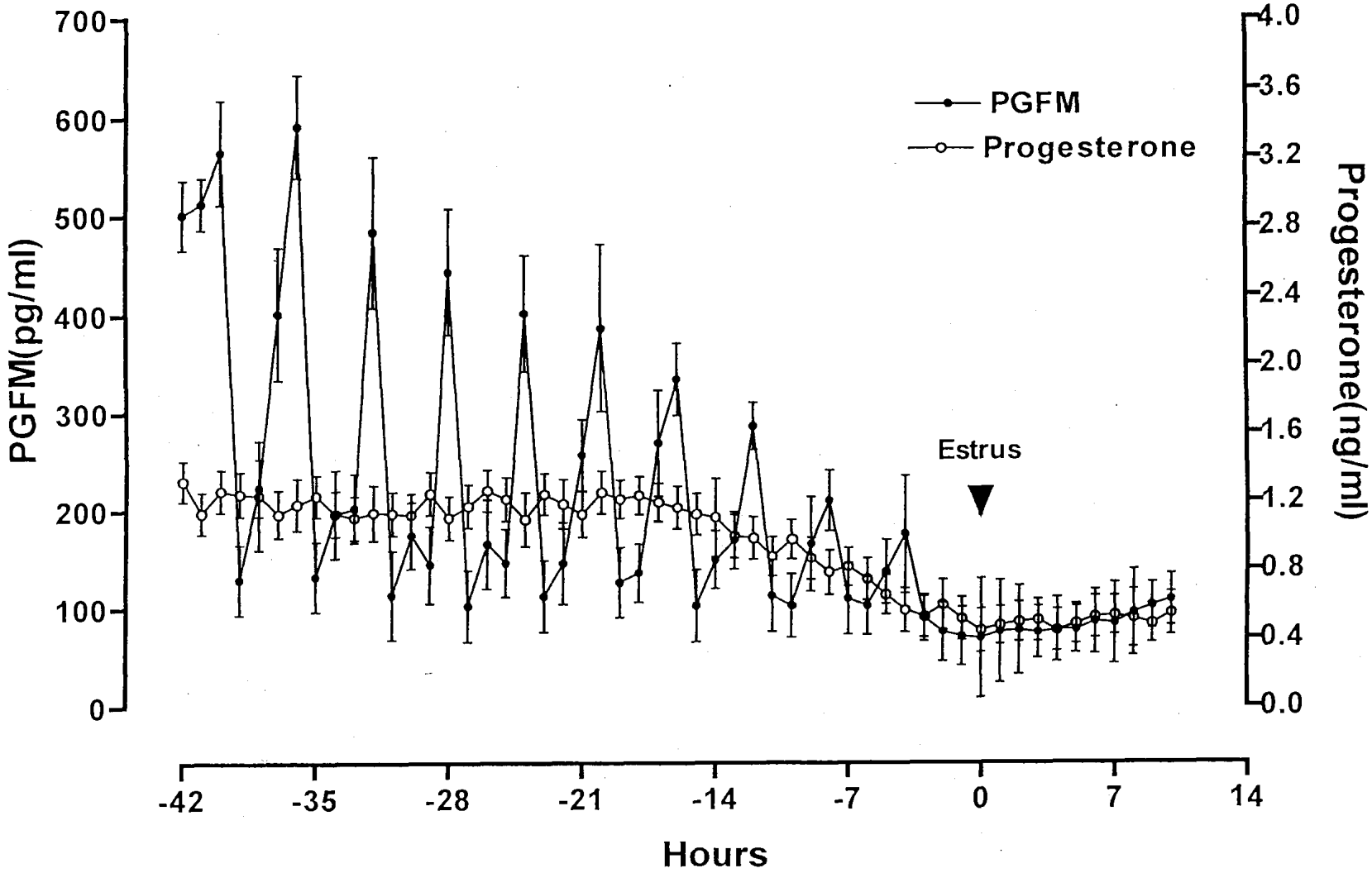
Hours	PGFM (pg/ml)	Progesterone (ng/ml)
-42	501.23±35.26	1.32±0.11
-41	512.25±26.31	1.29±0.12
-40	564.60±53.51	1.26±0.12
-39	129.35±35.64	1.24±0.13
-38	224.12±29.63	1.23±0.32
-37	401.26±67.23	1.12±0.13
-36	591.60±52.63	1.18±0.15
-35	132.54±35.64	1.23±0.12
-34	196.56±45.36	1.12±0.13
-33	202.35±35.63	1.10±0.12
-32	484.00±76.39	1.13±0.16
-31	113.56±45.32	1.12±0.12
-30	174.56±34.26	1.11±0.12
-29	144.25±39.65	1.24±0.12
-28	443.60±63.66	1.10±0.12
-27	102.36±36.45	1.17±0.12
-26	165.39±45.63	1.26±0.12
-25	146.21±34.78	1.21±0.12
-24	401.60±58.89	1.09±0.15
-23	112.36±36.59	1.24±0.12
-22	145.63±41.56	1.18±0.14
-21	256.98±36.54	1.12±0.13
-20	386.60±85.03	1.25±0.12
-19	126.31±35.68	1.21±0.01
-18	135.69±29.63	1.23±0.11
-17	268.96±54.32	1.19±0.11

Contd....

Contd.... (Table 6)

Hours	PGFM (pg/ml)	Progesterone (ng/ml)
-16	334.30±36.67	1.16±0.12
-15	102.36±36.56	1.12±0.12
-14	149.23±29.36	1.10±0.23
-13	168.96±29.36	0.99±0.12
-12	286.60±24.18	0.98±0.12
-11	112.31±36.54	0.87±0.11
-10	102.36±32.16	0.97±0.11
-9	165.49±48.36	0.86±0.12
-8	210.00±31.43	0.78±0.12
-7	109.36±36.24	0.81±0.11
-6	102.35±29.36	0.74±0.12
-5	136.98±32.56	0.65±0.11
-4	175.60±60.16	0.56±0.12
-3	89.65±23.65	0.52±0.11
-2	75.69±29.86	0.59±0.14
-1	71.26±29.86	0.51±0.12
0	69.00±26.86	0.44±0.12
1	75.63±52.36	0.47±0.11
2	77.23±45.36	0.49±0.11
3	75.29±26.35	0.50±0.12
4	78.01±32.71	0.44±0.12
5	77.89±23.65	0.48±0.11
6	86.23±32.68	0.52±0.12
7	84.56±41.26	0.53±0.11
8	95.01±43.65	0.51±0.16
9	102.36±23.56	0.48±1.11
10	108.39±26.35	0.54±0.12

Fig.6. Peri-estrus plasma PGFM and progesterone profiles(Mean±S.E.) in buffaloes(n=3) at 1 h interval



pulsatility was observed in PGFM profiles immediately post-estrus expression (Table 5; Fig. 5).

4.1.2 PGFM Levels During Cyclicity

The changes in mean levels of plasma PGFM (two samples collected daily) with the corresponding levels of progesterone 7 days prior to estrus up to 14 days post-estrus have been depicted in Table 7 and Figure 7. The plasma PGFM levels registered an increasing trend beginning day 4 prior to estrus (140.9 ± 19.29 pg/ml) reaching peak levels of 399.00 ± 43.42 pg/ml on day 2 prior to estrus. Subsequently, the PGFM concentration declined ($P<0.01$) to lowest levels of 74.30 ± 11.97 pg/ml at estrus, which stayed low thereafter during the luteal phase. The PGFM levels showed an increasing trend along with declining levels of progesterone during late luteal phase as was evident in case of periestrus period described earlier.

4.1.3 Plasma PGFM and Progesterone Levels During Early Pregnancy

Mean PGFM and progesterone levels in twice daily samples collected from pregnant buffaloes post-insemination up to 23 days is depicted in Table 8 and Figure 8. The progesterone levels during days 14 to 23 post-insemination were maintained at significantly ($P<0.01$) higher levels than the luteal phase during cyclicity. In contrast to cyclic buffaloes in the pregnant buffaloes, no increase of PGFM was observed during days 15-18 or the expected days of luteolysis (Table 8; Fig. 8). There was a gradual rise in plasma PGFM levels from around 70 pg/ml at estrus to a mean concentration of 120.00 ± 14.56 pg/ml on day 6 post-insemination. Thereafter, there was an increase in PGFM concentrations to 233.65 ± 12.86 pg/ml by day 8 post-insemination after which the hormone fluctuated within narrow limits up to the end of sampling period (day 23). The basal plasma PGFM levels ranged between 224.51 ± 14.72 to 278.56 ± 15.50 pg/ml during this period (day 8 to day 21) post-insemination.

Table 7. PGFM and Progesterone profiles (Mean ± SE) in cyclic buffaloes

Days	PGFM Mean (pg/ ml)	Progesterone (ng/ ml)
-7.0	108.0±15.63	1.29± 0.16
-6.5	115.0±12.69	
-6.0	117.0±19.67	1.48± 0.17
-5.5	110.0±16.61	
-5.0	121.4±19.54	1.56± 0.12
-4.5	131.7±28.31	
-4.0	140.9±19.29	1.51± 0.10
-3.5	173.9±20.79	
-3.0	231.4±30.86	1.34± 0.06
-2.5	280.1±22.01	
-2.0	399.0±43.42	1.05± 0.04
-1.5	209.7±28.61	
-1.0	134.9±31.8	0.76± 0.02
-0.5	106.3±23.12	
0.0	74.30±11.97	0.45± 0.08
0.5	96.10±12.04	
1.0	106.9±21.03	0.53± 0.01
1.5	103.0±12.24	
2.0	105.6±12.83	0.67± 0.02
2.5	106.4±12.92	
3.0	107.0±13.35	0.81± 0.03
3.5	101.2±12.99	
4.0	101.2±13.22	0.91± 0.03
4.5	104.4±13.38	
5.0	107.2±13.25	1.13± 0.04
5.5	107.3±13.94	
6.0	101.2±14.44	1.27± 0.06
6.5	100.3±14.68	
7.0	112.3±14.20	1.45± 0.07
7.5	101.3±24.39	
8.0	100.2±19.19	1.63± 0.08
8.5	104.2±18.87	
9.0	107.2±15.0	1.86± 0.10
9.5	106.0±14.42	
10.0	100.2±15.08	2.07± 0.13
10.5	103.2±14.90	
11.0	101.3±17.10	2.31± 0.10
11.5	104.3±15.03	
12.0	106.3±18.23	2.21± 0.12
12.5	109.6±15.10	
13.0	101.3±17.53	2.19± 0.13
13.5	100.3±19.21	
14.0	108.4±17.21	1.82± 0.09

Fig.7. Peripheral PGFM and progesterone profiles (Mean±S.E.) in Murrah buffaloes (n=47) during cyclicity

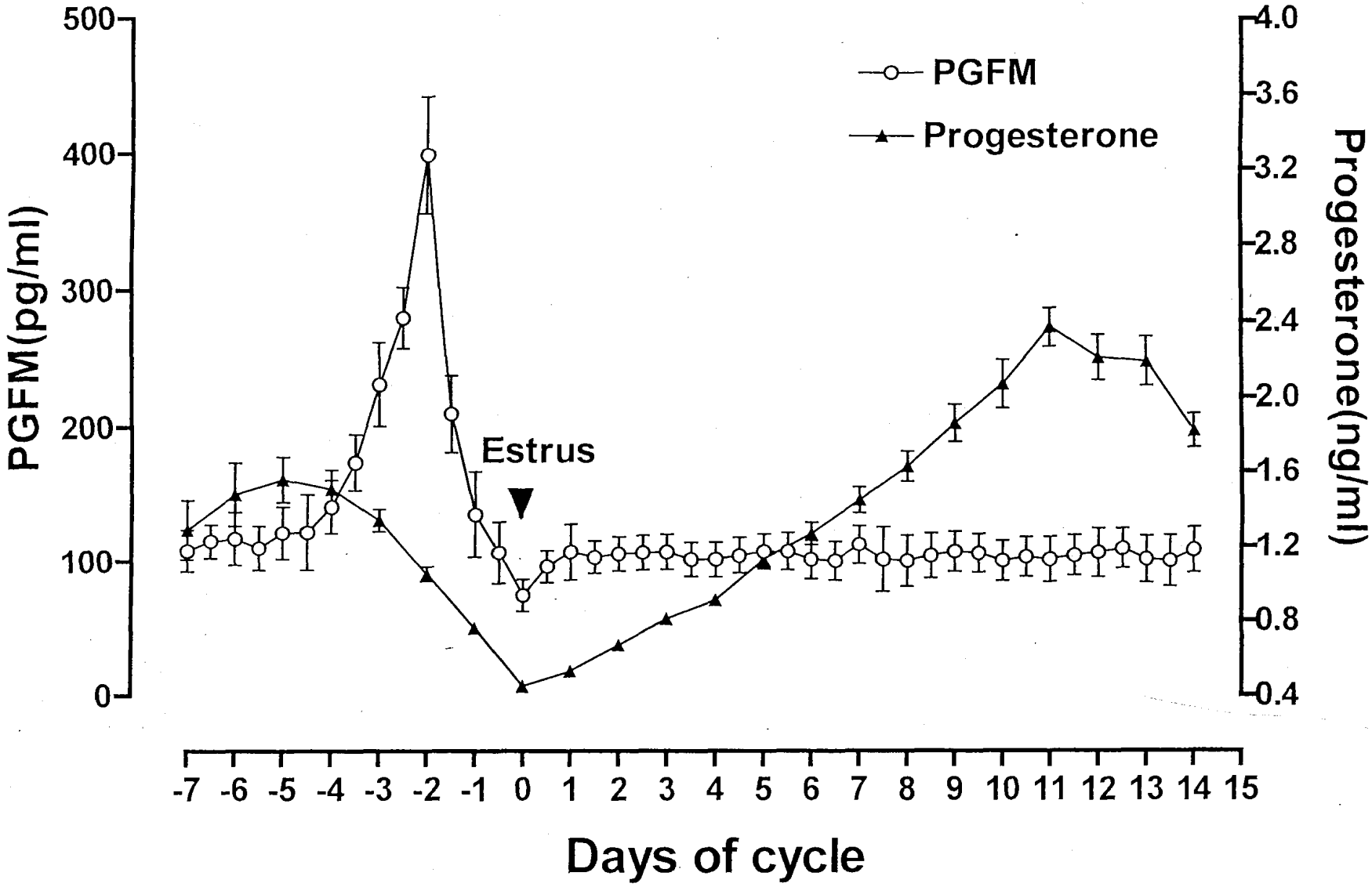
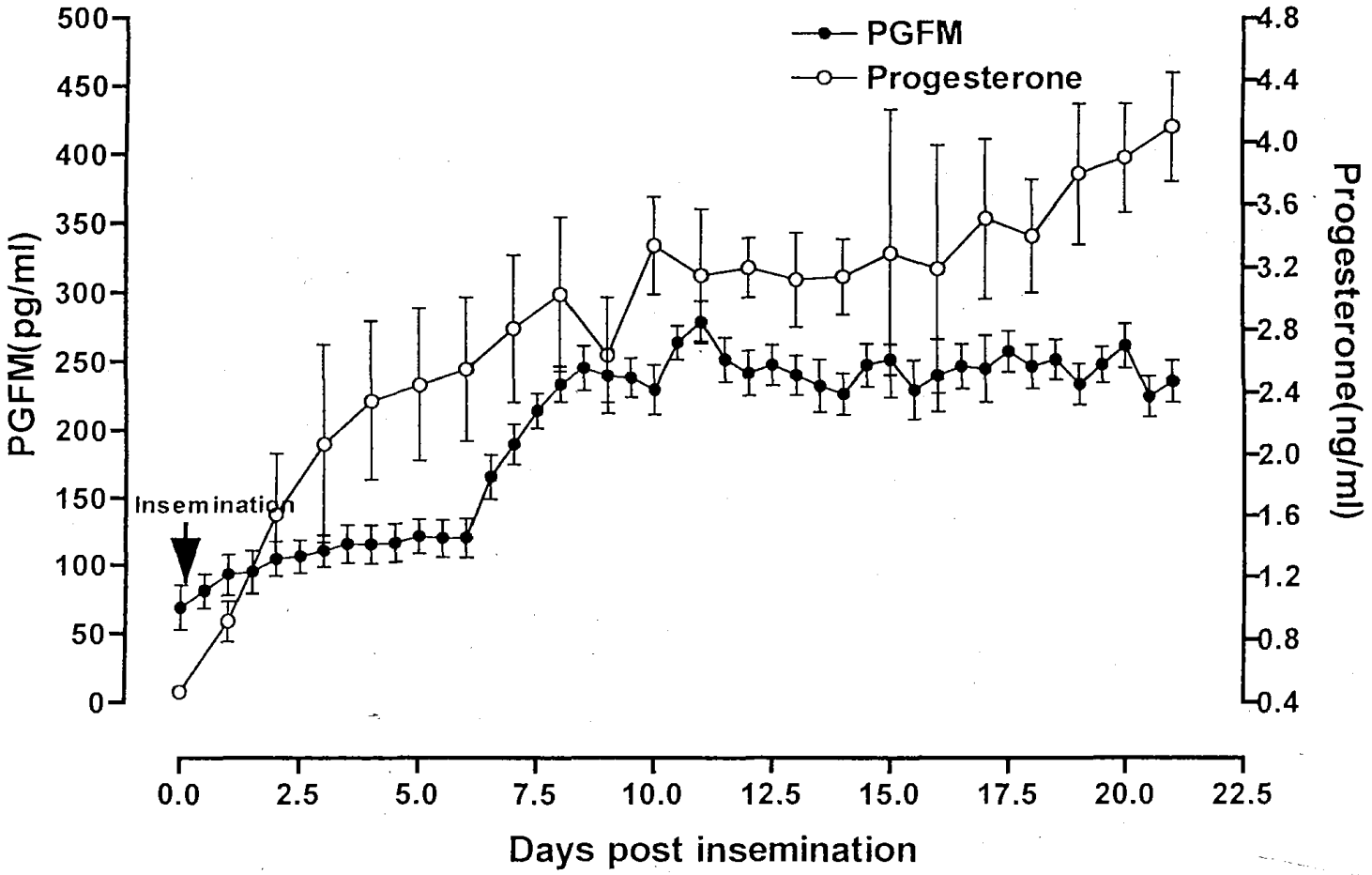


Table 8. PGFM and Progesterone profiles (Mean ± SE) during early pregnancy in buffaloes

Days	PGFM (pg/ml)	Progesterone (ng/ml)
0	69.20±16.20	0.47±0.01
0.5	81.20±12.35	
1.0	93.00±14.69	0.92±0.13
1.5	95.00±15.81	
2.0	104.60±12.38	1.61±0.40
2.5	106.40±12.37	
3.0	110.40±11.72	2.07±0.64
3.5	115.80±14.16	
4.0	115.40±14.33	2.35±0.51
4.5	116.60±14.20	
5.0	121.00±12.70	2.45±0.49
5.5	119.60±13.81	
6.0	120.00±14.56	2.55±0.46
6.5	165.32±16.34	
7.0	189.63±15.04	2.81±0.47
7.5	214.35±12.83	
8.0	233.65±12.86	3.03±0.49
8.5	245.32±16.02	
9.0	239.65±19.27	2.64±0.37
9.5	238.51±14.21	
10.0	229.63±18.08	3.34±0.31
10.5	263.54±12.26	
11.0	278.56±15.50	3.15±0.42
11.5	256.56±15.50	
12.0	241.57±16.06	3.20±0.19
12.5	252.01±16.28	
13.0	247.00±14.69	3.12±0.30
13.5	232.12±19.09	
14.0	226.32±15.09	3.14±0.24
14.5	247.31±15.51	
15.0	251.34±11.24	3.29±0.92
15.5	229.38±21.47	
16.0	239.62±26.31	3.19±0.79
16.5	246.32±16.20	
17.0	244.53±24.21	3.51±0.51
17.5	257.16±14.91	
18.0	246.32±15.92	3.40±0.36
18.5	251.36±14.43	
19.0	233.65±14.80	3.80±0.45
19.5	247.52±13.04	
20.0	261.32±16.11	3.90±0.35
20.5	224.51±14.72	
21.0	235.61±15.34	4.10±0.35

Fig.8. PGFM and progesterone profile (Mean±S.E.) in buffaloes (n=5) during early pregnancy



4.1.4 Plasma PGFM Levels During Reproductive Disorders

4.1.4.1 Plasma PGFM levels in buffaloes with reproductive tract infections

The mean plasma PGFM profiles along with corresponding progesterone profiles in buffaloes suffering from reproductive tract infections are presented in Table 9 and Figure 9. The plasma PGFM levels ranged from 447.20 ± 18.10 to 633.40 ± 85.75 pg/ml, while the progesterone profiles ranged from 0.40 ± 0.08 to 0.82 ± 0.02 ng/ml. It is apparent from the data that animals suffering from reproductive tract infections had consistently high plasma PGFM levels that suppressed luteal function as indicated by the low progesterone levels leading to acyclicity in these animals.

4.1.4.2 PGFM levels in buffaloes with ovarian cystic conditions (luteal cysts)

The mean values of plasma PGFM concentrations along with corresponding progesterone levels in buffalo with luteal cysts are presented in Table 10 and Figure 10. PGFM levels on different days of sampling stayed low ranging from 56.67 ± 13.72 to 76.33 ± 12.90 pg/ml ($P>0.01$). The progesterone levels were, however, maintained at high levels, which was sustained throughout the sampling period.

4.2 INFLUENCE OF RECOMBINANT BOVINE INTERFERON ALPHA (rbIFN$_\alpha$) ON ESTROUS CYCLE LENGTH

The influence of rbIFN$_\alpha$ on estrous cycle length in buffaloes subjected to each of the doses of 4 (group 1), 8 (group 2) and 16 (group 3) mg/day on days 14 through 16 of the estrous cycle are depicted in Table 11 and Figures 11 to 14. For each treatment group, 10 control cycles (2 per buffalo) prior to treatment cycle and 10 control cycles (2 per buffalo) after the treatment cycles were observed and compared with the 5 treatment cycles. The pre-treatment, treatment and post-treatment estrous cycle lengths (in days) were 21.8 ± 2.59, 21.4 ± 1.95, 21.7 ± 2.17 (4 mg/day), 22 ± 2.89, 23.0 ± 1.87, 21.9 ± 1.95 (8 mg/day), and 21.1 ± 2.16, 27.6 ± 2.07, 21.8 ± 2.19 days for groups I, II and

Table 9. PGFM and Progesterone profiles (Mean ± S.E.) in buffaloes with reproductive infections

Days	PGFM (pg/ml)	Progesterone (ng/ml)
1	633.4±85.75	0.68±0.05
2	605.2±89.38	0.61±0.05
3	632.6±96.64	0.61±0.03
4	609.4±87.91	0.63±0.06
5	592.8±87.68	0.72±0.06
6	571.4±83.64	0.62±0.05
7	573.8±82.43	0.66±0.06
8	581.6±67.76	0.65±0.09
9	579.4±66.79	0.64±0.04
10	536.2±65.12	0.70±0.04
11	527.8±59.97	0.61±0.02
12	538.8±59.29	0.63±0.08
13	549.0±49.32	0.72±0.09
14	554.6±70.40	0.66±0.07
15	564.2±56.63	0.73±0.10
16	520.4±64.02	0.40±0.08
17	530.8±62.45	0.54±0.06
18	542.4±35.03	0.58±0.07
19	554.6±39.06	0.62±0.02
20	546.0±37.26	0.66±0.07
21	540.6±27.45	0.65±0.05
22	524.6±13.83	0.66±0.04
23	521.4±53.65	0.61±0.02
24	504.0±30.26	0.64±0.02
25	535.2±51.02	0.73±0.06
26	527.0±32.88	0.52±0.11
27	504.6±37.83	0.67±0.10
28	476.2±31.87	0.68±0.19
29	449.4±36.46	0.71±0.06
30	487.4±22.08	0.84±0.11
31	447.2±18.10	0.74±0.02
32	498.2±32.89	0.75±0.07
33	401.0±15.91	0.60±0.14
34	469.2±17.73	0.82±0.02
35	461.2±24.39	0.75±0.10
36	465.2±27.72	0.66±0.07
37	483.4±28.05	0.68±0.08
38	496.8±23.19	0.69±0.08
39	472.2±20.71	0.72±0.06
40	458.6±39.08	0.73±0.02
41	451.4±37.64	0.63±0.06
42	447.4±31.98	0.62±0.03

Fig.9. PGFM and progesterone profiles (Mean±S.E.) in acyclic buffaloes (n=5) with reproductive tract infections

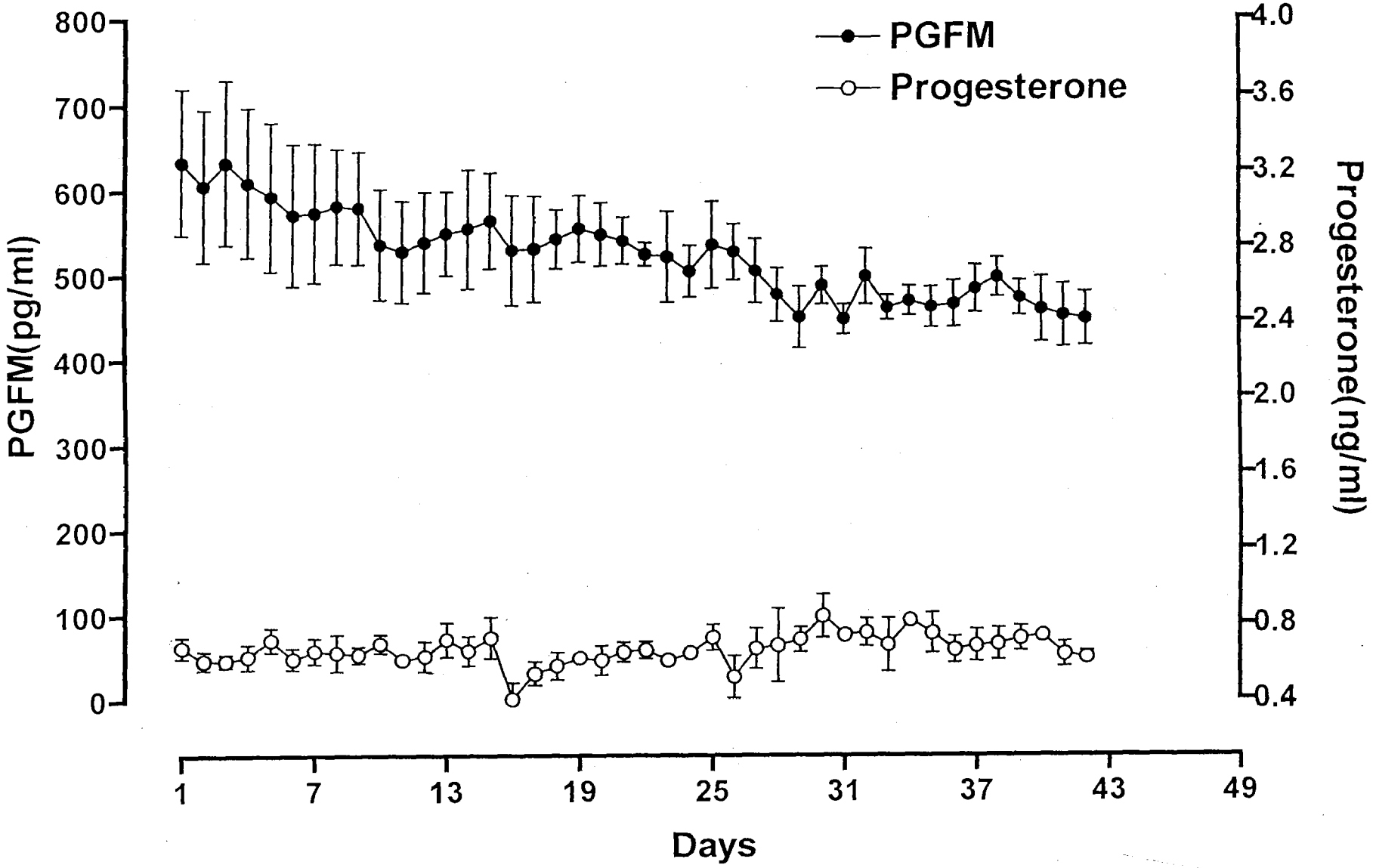


Table 10. PGFM and Progesterone profile (Mean±S.E.) in buffaloes with Ovarian Cystic Conditions

Days	PGFM (pg/ml)	Progesterone (ng/ml)
0.5	60.67±12.38	3.12±0.12
1.0	59.01±14.29	2.98±0.12
1.5	56.67±13.71	3.10±0.13
2.0	60.67±1636	3.00±0.13
2.5	57.67±16.69	3.15±0.12
3.0	60.02±13.51	2.98±0.12
3.5	64.01±19.64	3.15±0.12
4.0	59.33±12.60	2.99±0.12
4.5	64.67±14.25	2.97±0.24
5.0	64.33±12.84	3.19±0.12
5.5	65.01±12.02	3.40±0.15
6.0	66.01±13.05	2.69±0.12
6.5	57.67±15.02	2.98±0.12
7.0	62.33±17.75	2.94±0.12
7.5	60.33±17.68	2.93±0.14
8.0	72.0±16.65	2.36±0.12
8.5	66.67±17.83	2.87±0.11
9.0	69.67±12.84	2.94±0.11
9.5	76.33±12.90	2.67±0.11
10.0	75.01±19.81	2.59±0.11
10.5	71.33±10.41	2.75±0.11
11.0	70.33±19.56	2.67±0.11
11.5	59.33±17.33	2.59±0.12
12.0	62.67±17.26	2.78±0.12
12.5	71.67±12.72	2.90±0.12
13.0	66.03±14.72	3.10±0.11
13.5	63.87±14.41	3.40±0.12
14.0	68.02±17.81	2.60±0.11
14.5	69.01±17.21	2.40±0.12
15.0	64.48±16.22	2.10±0.23
15.5	68.67±16.17	2.70±0.32
16.0	72.67±17.66	2.60±0.13
16.5	72.60±15.20	2.50±0.14
17.0	71.33±15.50	2.10±0.12
17.5	74.67±17.33	2.30±0.21
18.0	70.67±17.12	2.70±0.31
18.5	69.01±15.03	2.80±0.36
19.0	66.67±12.66	2.60±0.35
19.5	65.38±13.18	2.50±0.32
20.0	63.67±12.33	3.13±0.32
20.5	62.67±13.93	3.40±0.35
21.0	62.67±13.48	3.50±0.12
21.5	66.33±14.66	2.99±0.12
22.0	64.01±11.73	2.79±0.26
22.5	59.33±12.66	2.85±0.25
23.0	62.67±12.31	2.67±0.23
23.5	64.67±12.84	2.74±0.12

Fig.10. PGFM and progesterone profile (Mean±S.E.) in buffaloes (n=3) having luteal cysts

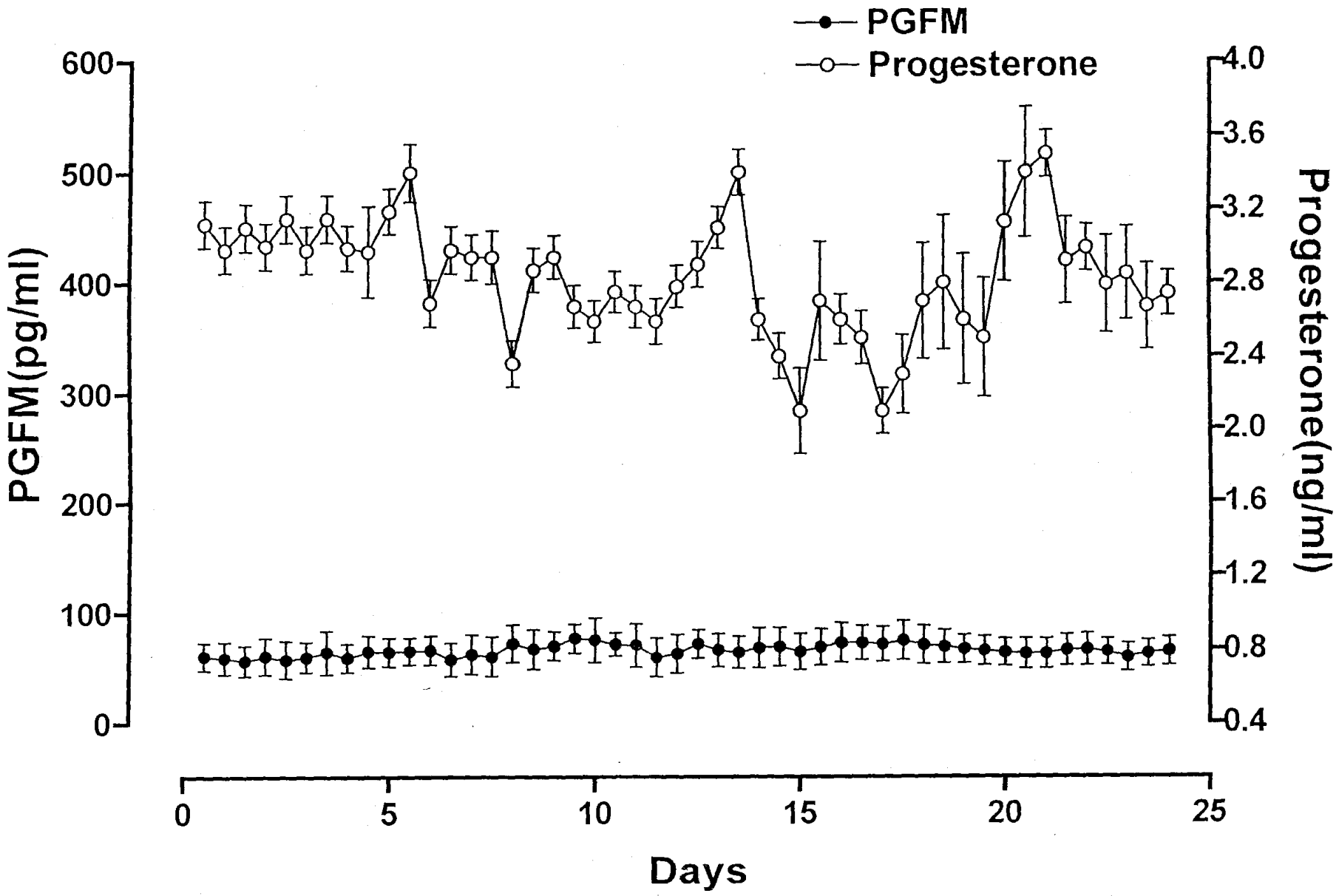


Table 11. Influence of rbIFN$_\alpha$ on Estrous cycle lengths (Mean±S.D.) in buffaloes

Group	Estrous cycle length					
	Pre treatment cycle*		Treatment cycle		Post treatment cycle*	
	n	Mean ± S.D.	n	Mean ± S.D.	n	Mean ± S.D.
I (4 mg / day)	10	21.8 ± 2.59	5	21.4 ± 1.95	10	21.7 ± 2.17
II (8 mg / day)	10	22.0 ± 2.89	5	23.0 ± 1.87	10	21.9 ± 1.95
III (16 mg / day)	10	21.1 ± 2.16[a]	5	27.6 ± 2.07[b]	10	21.8 ± 2.19[a]

* Each animal was observed for two estrous cycles

a & b superscripts showing significant difference between treatments

n denotes the number of oestrous cycles

Fig.11. Estrous cycle lengths before, during and after rbIFNα treatment (@4mg/day on days 14-16 of cycle)

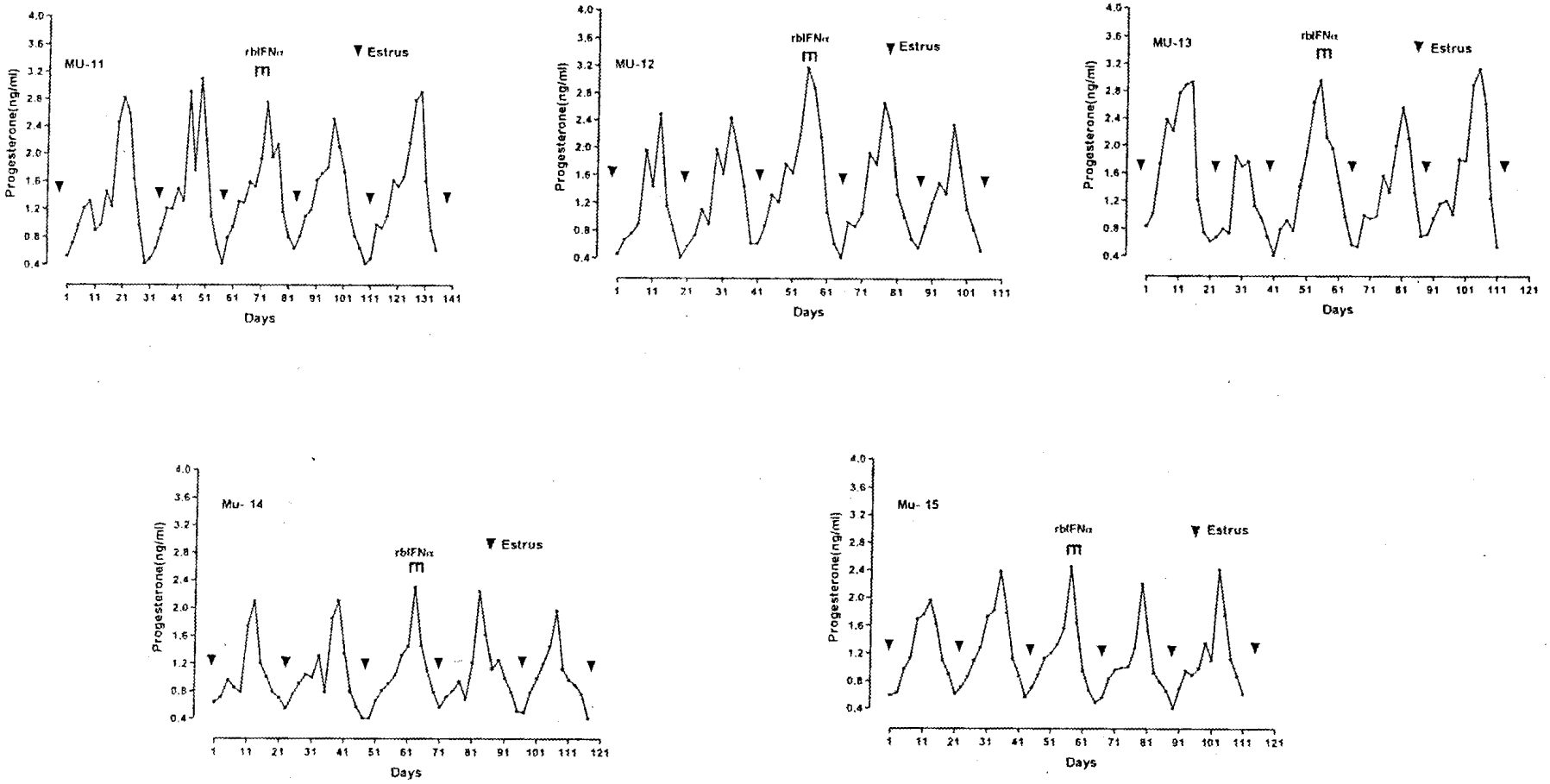
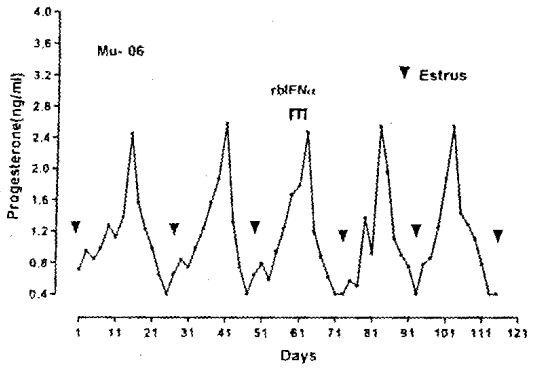
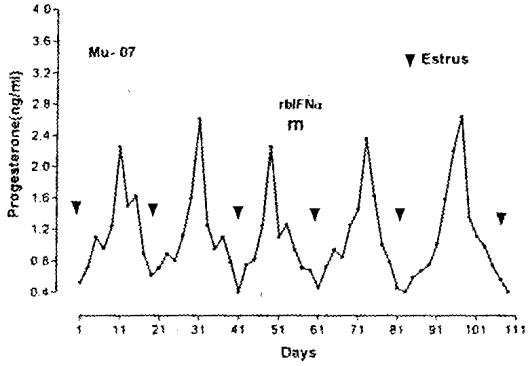
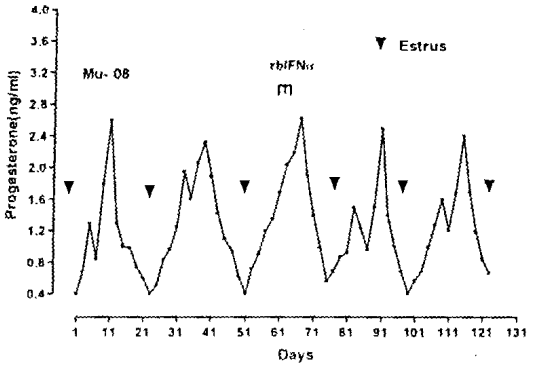
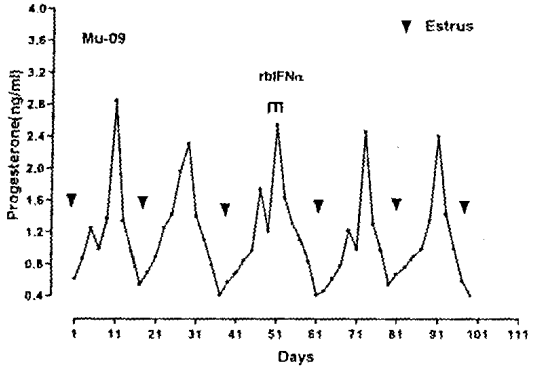


Fig.12. Estrous cycle lengths before, during and after rbIFNαtreatment (@8mg/day on days 14-16 of cycle)









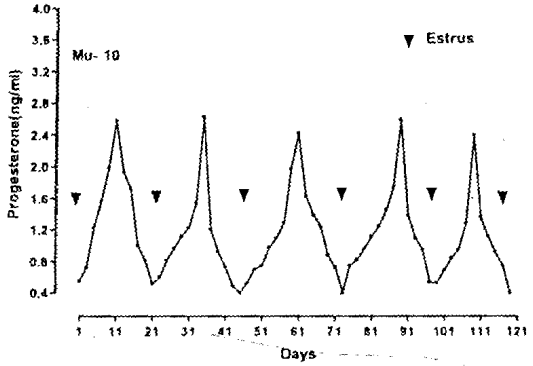


Fig.13. Estrous cycle lengths before, during and after rbIFNα treatment (@16mg/day on days 14-16 of cycle)

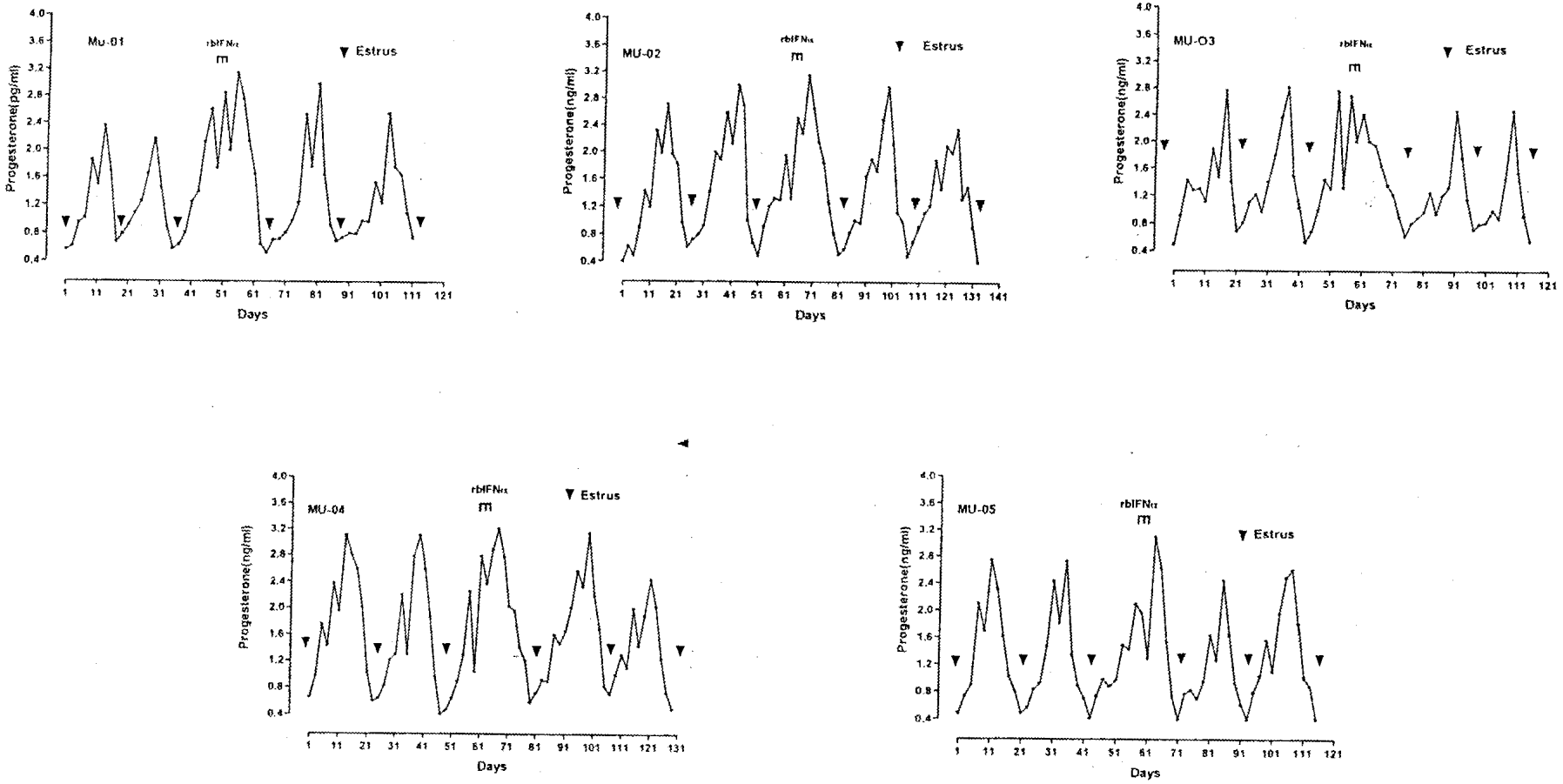
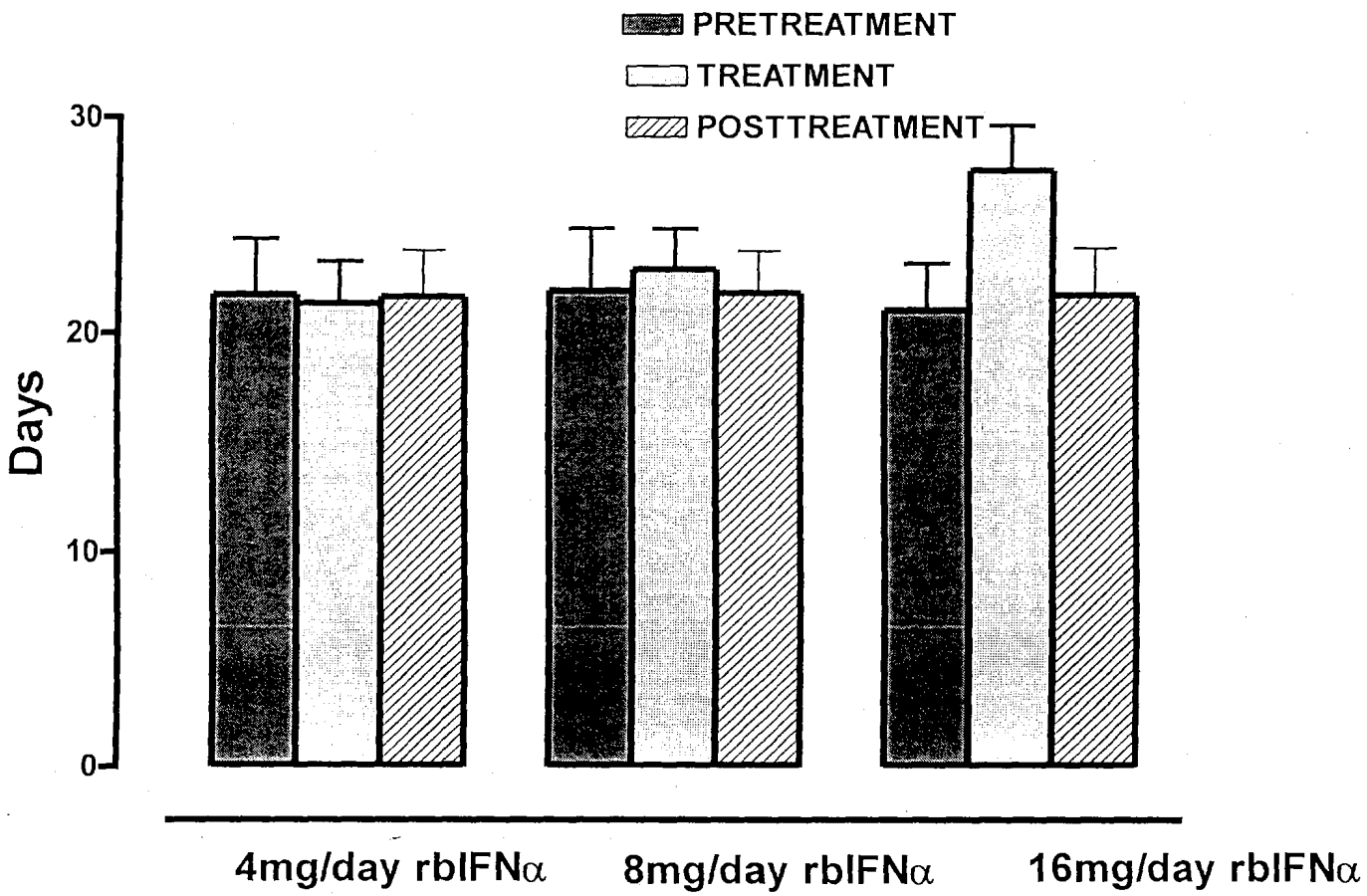


Fig.14. Dose response effect of rbIFNα on estrous cycle lengths (Mean±S.D.) in Murrah buffaloes (n=5 in each group)



III, respectively. The rbIFN$_\alpha$ dose of 16 mg/day (Group III) was found to significantly ($P<0.01$) extend the luteal life span, which was also reflected by the progesterone profiles (Figs. 11-13). The lower doses of rbIFN$_\alpha$ administration (4 and 8 mg/day) did not influence the estrous cycle length ($P>0.01$). Hence, for the subsequent experiments to study the oxytocin mediated PGFM release, the animals were administered at a dose of 16 mg/day on days 14-16 of estrous cycle.

4.3 INFLUENCE OF rbIFN$_\alpha$ ON RECTAL TEMPERATURES

The changes in rectal temperature with (treatment groups I, II and III) and without (control group) administration of rbIFN$_\alpha$ doses are presented in Table 12. The mean rectal temperature profiles are depicted graphically in Figures 15 to 18 for control and groups I, II and III, respectively. The results indicate a transient rise in the rectal temperatures in all the 3 treatment groups post rbIFN$_\alpha$ administration which lasted for 7, 6 and 6 hours, respectively, for doses of 4, 8 and 16 mg/day, respectively, before returning back to the pre-administration values. The mean rectal temperature increased ($P<0.01$) from 98.52 ± 0.05 to 98.98 ± 0.07, 98.42 ± 0.09 to 98.80 ± 0.08 and 98.32 ± 0.05 to 98.58 ± 0.03°F in buffaloes belonging to groups I, II and III, respectively, after the initial interferon injection. However, the increase in rectal temperature was seen only for a brief period and subsequent interferon administration did not have any effect on rectal temperature (Figs. 15-18). The mean rectal temperature profile in untreated control buffaloes did not display any specific trend, and fluctuated narrowly during the course of sampling.

4.4 INFLUENCE OF rbIFN$_\alpha$ ON RECTAL TEMPERATURE AND PLASMA LH LEVELS

The mean rectal temperature recorded at hourly intervals and the changes in the LH levels at 15 minutes intervals beginning two hours before and up to 8 hours after rbIFN$_\alpha$ treatment (first injection) on day 14 of the cycle for 3 doses (4, 8 and 16 mg/day) are depicted in Figures 20 to 22. Both these parameters for control animals is depicted in Figure 19. No significant

Table 12. Temporal changes in rectal temperatures (Mean ± S.E. ^{o} F) in buffaloes treated with different doses of rbIFN$_{\alpha}$

Hours	Control Group n = 5	Group I (4 mg rbIFN$_{\alpha}$ / day) n = 5	Group I (8 mg rbIFN$_{\alpha}$ / day) n= 5	Group I (16 mg rbIFN$_{\alpha}$ / day) n = 5
-12	98.40± 0.07	98.36± 0.08	98. 62± 0.05	98. 40± 0.07
-11	98.42± 0.05	98.66± 0.05	98. 35± 0.34	98. 36± 0.05
-10	98.51± 0.04	98.58± 0.08	98. 54± 0.10	98. 34± 0.04
-9	98.56± 0.05	98.36± 0.02	98. 56± 0.15	98. 56± 0.05
-8	98.46± 0.05	98.58± 0.10	98. 40± 0.11	98. 46± 0.05
-7	98.51± 0.07	98.58± 0.03	98. 30± 0.16	98. 31± 0.07
-6	98.40± 0.03	98.60± 0.04	98. 20± 0.17	98. 40± 0.03
-5	98.56± 0.06	98.59± 0.08	98.44± 0.07	98. 38± 0.06
-4	98.42± 0.02	98.46± 0.09	98. 40± 0.11	98. 42± 0.02
-3	98.51± 0.05	98.61± 0.03	98. 31± 0.10	98. 31± 0.05
-2	98.45± 0.04	98.52± 0.03	98. 21± 0.06	98. 34± 0.04
-1	98.51± 0.05	98.52± 0.05	98. 42± 0.09	98. 32± 0.05
0	98.49± 0.07	98.62± 0.03	98. 21± 0.12	98. 49± 0.07
1	98.54± 0.05	98.98± 0.07	98. 70± 0.05	98. 54± 0.05
2	98.44± 0.05	98.96± 0.08	98. 80± 0.08	98. 44± 0.05
3	98.46± 0.05	98.14± 0.03	98. 40± 0.07	98. 46± 0.05
4	98.58± 0.03	98.40± 0.03	98. 50± 0.06	98. 58± 0.03
5	98.50± 0.03	98.67± 0.05	98. 40± 0.05	98. 50± 0.03
6	98.62± 0.05	98.74± 0.08	98. 31± 0.05	98. 46± 0.05
7	98.46± 0.06	98.58± 0.03	98. 32± 0.05	98. 46± 0.06
8	98.54± 0.05	98.60± 0.08	98. 32± 0.05	98. 54± 0.05
9	98.40± 0.03	98.54± 0.05	98. 31± 0.07	98. 40± 0.03
10	98.48± 0.04	98.50± 0.04	98. 31± 0.05	98. 48± 0.04
11	98.50± 0.04	98.62± 0.05	98. 31± 0.04	98. 50± 0.04
12	98.50± 0.03	98.54± 0.03	98. 32± 0.05	98. 50± 0.03
13	98.56± 0.05	98.41± 0.03	98. 39± 0.05	98. 56± 0.05
14	98.48± 0.03	98.48± 0.03	98. 38± 0.08	98. 48± 0.03
15	98.54± 0.05	98.58± 0.03	98. 40± 0.05	98. 54± 0.05
16	98.56± 0.05	98.48± 0.03	98. 29± 0.09	98. 56± 0.05
17	98.50± 0.03	98.58± 0.03	98. 31± 0.07	98. 50± 0.03
18	98.71± 0.04	98.60± 0.04	98. 40± 0.05	98. 34± 0.04
19	98.52± 0.03	98.52± 0.03	98. 42± 0.15	98. 52± 0.03
20	98.42± 0.03	98.56± 0.05	98. 41± 0.09	98. 42± 0.03
21	98.52± 0.02	98.55± 0.05	98. 54± 0.12	98. 52± 0.02
22	98.42± 0.03	98.52± 0.05	98. 41± 0.15	98. 48± 0.03
23	98.56± 0.05	98.56± 0.05	98. 30± 0.20	98. 56± 0.05
24	98.30± 0.07	98.54± 0.05	98. 45± 0.05	98. 30± 0.07
25	98.61± 0.06	98.48± 0.08	98. 43± 0.12	98. 36± 0.06
26	98.48± 0.03	98.54± 0.06	98. 43± 0.12	98. 48± 0.03
27	98.50± 0.03	98.54± 0.03	98. 41± 0.07	98. 50± 0.03
28	98.52± 0.05	98.52± 0.03	98. 32± 0.05	98. 52± 0.05
29	98.56± 0.03	98.58± 0.08	98. 35± 0.10	98. 56± 0.03
30	98.54± 0.03	98.42± 0.11	98. 40± 0.08	98. 46± 0.03
31	98.44± 0.05	98.54± 0.02	98. 32± 0.04	98. 44± 0.05
32	98.34± 0.04	98.53± 0.05	98. 18± 0.22	98. 34± 0.04

Contd.....

Contd.....(Table 12)

Hours	Control Group n = 5	Group I (4 mg rbIFN$_\alpha$ / day) n = 5	Group I (4 mg rbIFN$_\alpha$ / day) n = 5	Group I (4 mg rbIFN$_\alpha$ / day) n = 5
33	98.41± 0.05	98.46± 0.02	98. 50± 0.04	98. 28± 0.05
34	98.41± 0.07	98.50± 0.03	98. 36± 0.08	98. 41± 0.07
35	98.48± 0.03	98.54± 0.03	98. 32± 0.12	98. 48± 0.03
36	98.61± 0.05	98.56± 0.03	98. 23± 0.16	98. 36± 0.05
37	98.42± 0.03	98.66± 0.06	98. 32± 0.03	98. 42± 0.03
38	98.53± 0.05	98.58± 0.05	98. 36± 0.16	98. 53± 0.05
39	98.42± 0.03	98.50± 0.04	98. 41± 0.07	98. 42± 0.03
40	98.50± 0.04	98.47± 0.04	98. 31± 0.14	98. 50± 0.04
41	98.58± 0.03	98.56± 0.03	98. 46± 0.14	98. 58± 0.03
42	98.29± 0.08	98.50± 0.03	98. 29± 0.08	98. 44± 0.06
43	98.48± 0.03	98.52± 0.03	98. 48± 0.03	98. 50± 0.07
44	98.36± 0.08	98.54± 0.05	98. 36± 0.08	98. 40± 0.03
45	98.35± 0.03	98.54± 0.05	98. 35± 0.03	98. 38± 0.08
46	98.32± 0.10	98.46± 0.03	98. 32± 0.10	98. 44± 0.08
47	98.31± 0.21	98.60± 0.04	98. 31± 0.21	98. 50± 0.05
48	98.46± 0.22	98.52± 0.05	98. 46± 0.22	98. 65± 0.07
49	98.51± 0.05	98.52± 0.03	98. 51± 0.05	98. 40± 0.07
50	98.32± 0.06	98.50± 0.04	98. 32± 0.06	98. 52± 0.05
51	98.21± 0.12	98.58± 0.03	98. 21± 0.12	98. 46± 0.05
52	98.31± 0.14	98.52± 0.03	98. 31± 0.14	98. 42± 0.07
53	98.56± 0.10	98.52± 0.06	98. 56± 0.10	98. 43± 0.02
54	98.32± 0.22	98.50± 0.04	98. 32± 0.22	98. 41± 0.07
55	98.41± 0.15	98.56± 0.05	98. 41± 0.15	98. 40± 0.03
56	98.32± 0.03	98.53± 0.05	98. 32± 0.03	98. 42± 0.07
57	98.30± 0.14	98.54± 0.02	98. 30± 0.14	98. 58± 0.03
58	98.54± 0.05	98.53± 0.05	98. 54± 0.05	98. 45± 0.05
59	98.31± 0.09	98.51± 0.03	98. 31± 0.09	98. 34± 0.05
60	98.44± 0.14	98.58± 0.03	98. 44± 0.14	98. 34± 0.05
61	98.30± 0.05	98.60± 0.04	98. 30± 0.05	98. 42± 0.03
62	98.41± 0.13	98.44± 0.05	98. 41± 0.13	98. 38± 0.03
63	98.50± 0.08	98.51± 0.04	98. 50± 0.08	98. 36± 0.05
64	98.41± 0.08	98.58± 0.03	98. 41± 0.08	98. 50± 0.04
65	98.52± 0.10	98.52± 0.03	98. 52± 0.10	98. 58± 0.03
66	98.32± 0.10	98.56± 0.05	98. 32± 0.10	98. 48± 0.03
67	98.40± 0.19	98.62± 0.03	98. 40± 0.19	98. 43± 0.02
68	98.26± 0.05	98.50± 0.03	98. 26± 0.05	98. 44± 0.05
69	98.32± 0.05	98.45± 0.04	98. 32± 0.05	98. 46± 0.03
70	98.21± 0.16	98.70± 0.05	98. 21± 0.16	98. 48± 0.03
71	98.40± 0.03	98.58± 0.03	98. 42± 0.03	98. 61± 0.09
72	98.32± 0.07	98.53± 0.05	98. 32± 0.07	98. 43± 0.05
73	98.41± 0.08	98.50± 0.05	98. 41± 0.08	98. 58± 0.08
74	98.41± 0.03	98.48± 0.03	98. 41± 0.03	98. 62± 0.03
75	98.44± 0.05	98.54± 0.03	98. 44± 0.05	98. 52± 0.05
76	98.42± 0.12	98.43± 0.06	98. 42± 0.12	98. 52± 0.03
77	98.31± 0.11	98.50± 0.04	98. 31± 0.11	98. 50± 0.07
78	98.13± 0.03	98.52± 0.04	98. 13± 0.3	98. 53± 0.05
79	98.31± 0.10	98.56± 0.02	98. 31± 0.10	98. 56± 0.05
80	98.21± 0.07	98.56± 0.05	98. 21± 0.07	98. 68± 0.06

(*Mean*±S.E.) *in* untreated control buffaloes on day 14-16 of the cycle

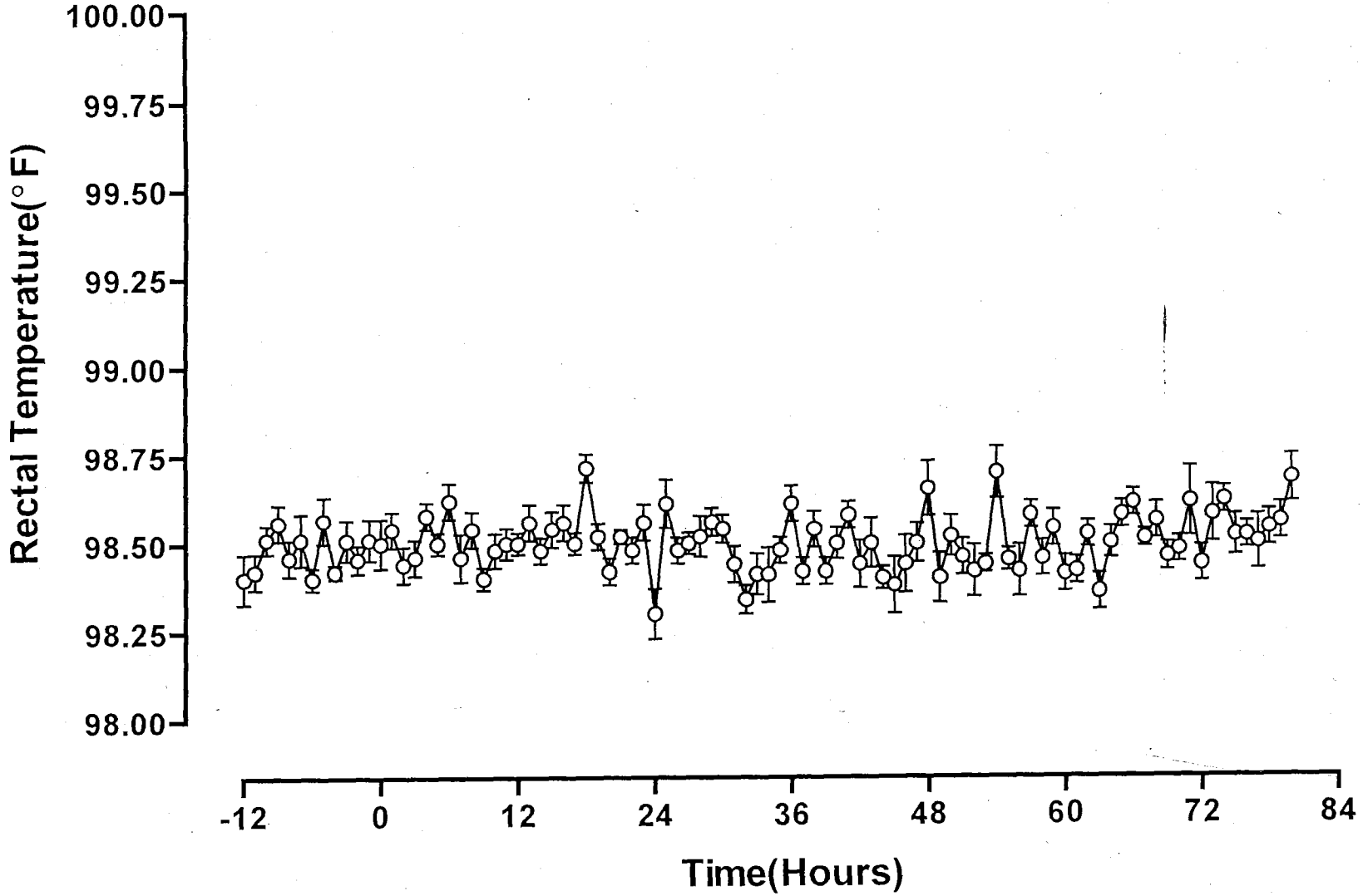


Fig.16. Temporal changes in rectal temperatures (Mean±S.E.) post-rblFNα (4mg/day) administration on days 14-16 of cycle

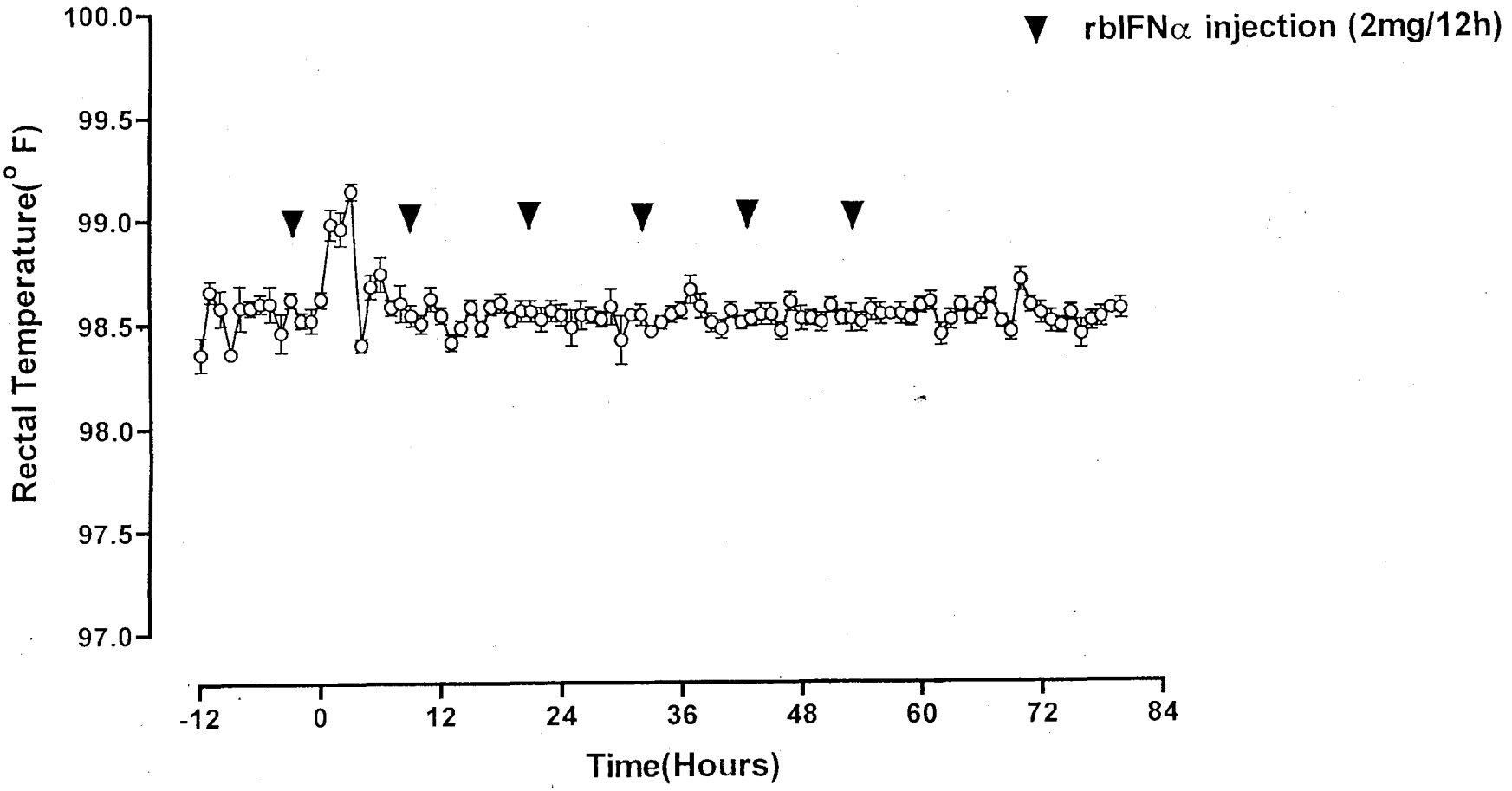


Fig.17. Temporal changes in rectal temperature (Mean±S.E) post- rbIFNα (8mg/day) administration on days 14-16 of cycle

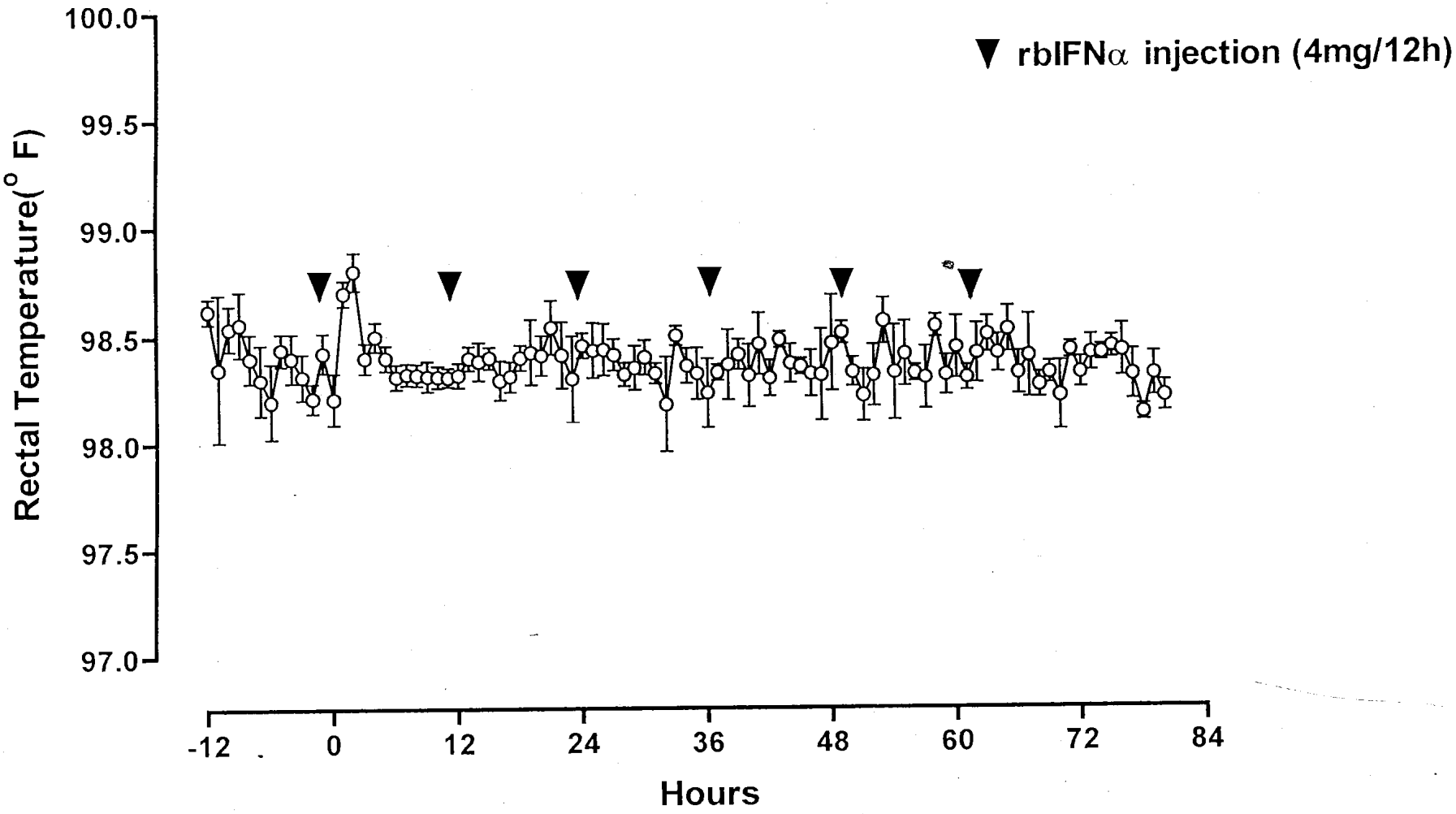
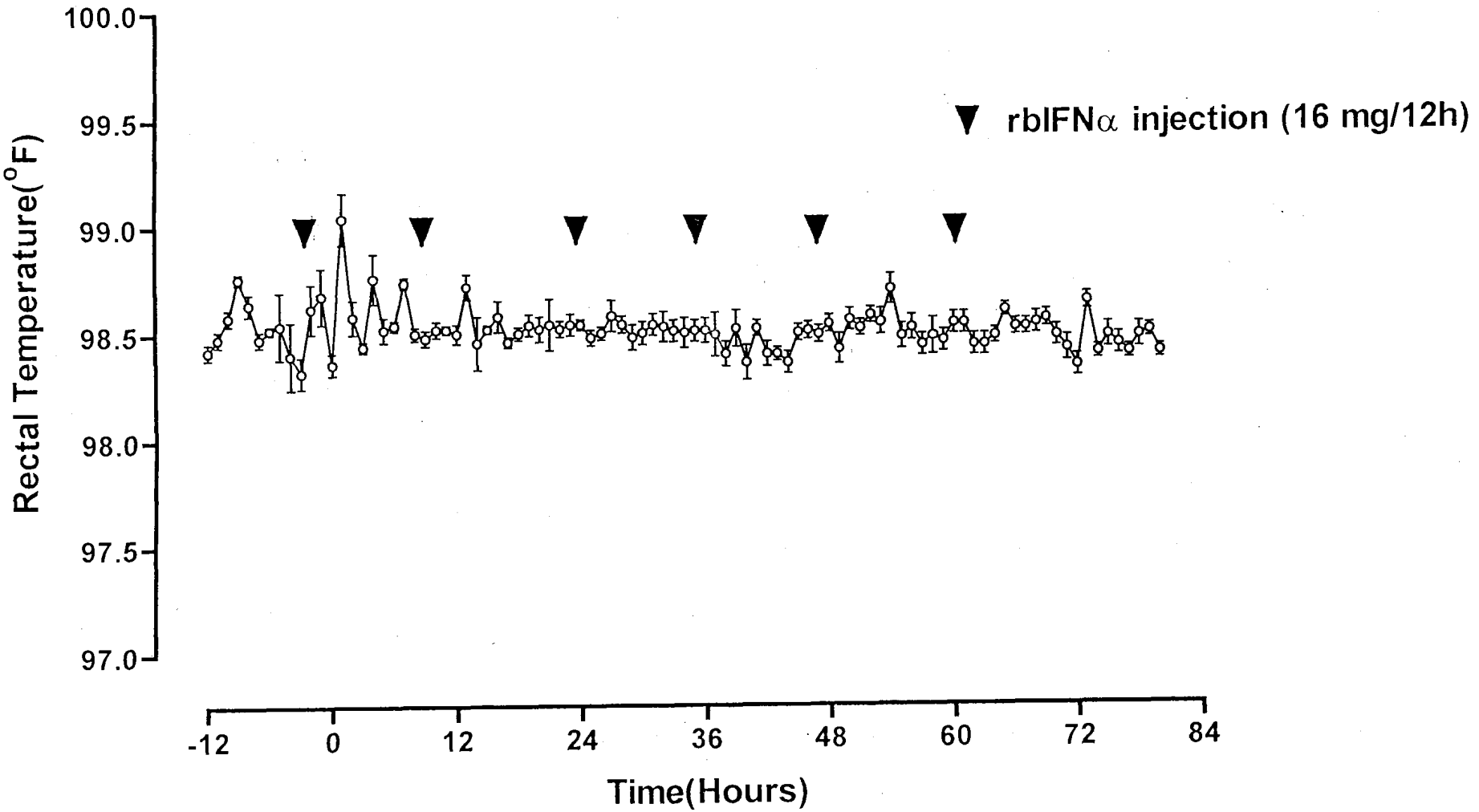


Fig.18. Temporal changes in rectal temperature (Mean±S.E.) post rbIFNα (16mg/day) administration on days 14-16 of cycle



(P>0.01) change was observed in the LH levels post- rbIFN$_\alpha$ treatment in any of the 3 groups of buffaloes. Also, there was no significant (P>0.01) difference in the LH levels between the control and the rbIFN$_\alpha$ treated animals. As depicted previously, rectal temperatures show a significant (P<0.05) increase after the first injection in case of each dose of rbIFN$_\alpha$ while no such effect was evident in untreated control animals. From the results, it is apparent that both these effects are probably disassociated.

4.5 INFLUENCE OF rbIFN$_\alpha$ ON TEMPORAL CHANGES IN PLASMA PROGESTERONE AND LH LEVELS

The influence of the 3 doses of rbIFN$_\alpha$ on transient changes of mean progesterone and LH levels are presented in Tables 13 and 14 and Figures 23 to 26 along with the changes in the untreated control animals. It is evident from the results that the rbIFN$_\alpha$ doses had no significant effect (P>0.01) on plasma LH profiles (range 1.0 to 2.4 ng/ml). However, the progesterone profiles subsequent to rbIFN$_\alpha$ administration registered a decrease (P<0.05) that lasted for 240 minutes in case of 4 mg/day of rbIFN$_\alpha$ dose, 270 minutes in case of 8 mg/day of rbIFN$_\alpha$ dose, and 210 minutes in case of 16 mg/day of rbIFN$_\alpha$ dose before returning back to pre-administration levels. The progesterone levels declined from 2.11 ± 0.03 to 0.09 ± 0.04, 2.23 ± 0.14 to 1.01 ± 0.03 and 1.63 ± 0.04 to 1.12 ± 0.08 ng/ml in the animals of the 3 treatment groups, respectively.

4.6 INFLUENCE OF rbIFN$_\alpha$ ON OXYTOCIN MEDIATED PLASMA PGFM RELEASE

The individual plasma PGFM levels as induced by oxytocin challenge on day 17 of the estrous cycle, without and with prior rbIFN$_\alpha$ treatment on days 14 through 16 of the estrous cycle are depicted in Figure 27.

The mean plasma PGFM levels as induced by oxytocin challenge (50 IU) on day 17 of the estrous cycle subsequent to rbIFN$_\alpha$ (16 mg/day dose) treatment on days 14 through 16 of the estrous cycle (treatment cycle) as well

Fig.19. Temporal Changes in LH and rectal temperature (Mean±S.E.) in untreated control buffaloes on day 14 of cycle

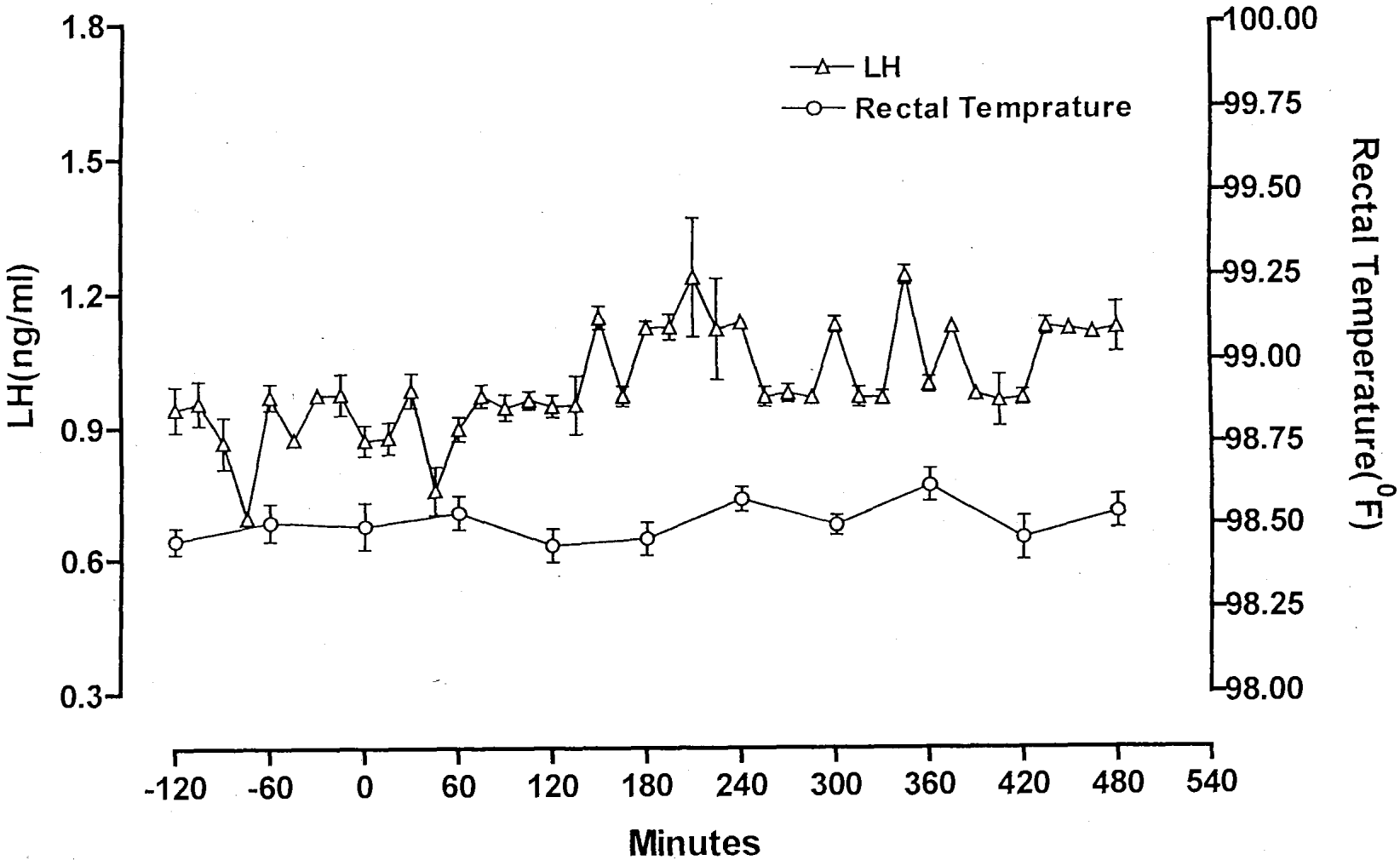


Fig.20. Temporal Changes in LH and Rectal Temperature (Mean±S.E.) in buffaloes administered rbIFNα@4mg/day on day14 (first injection)

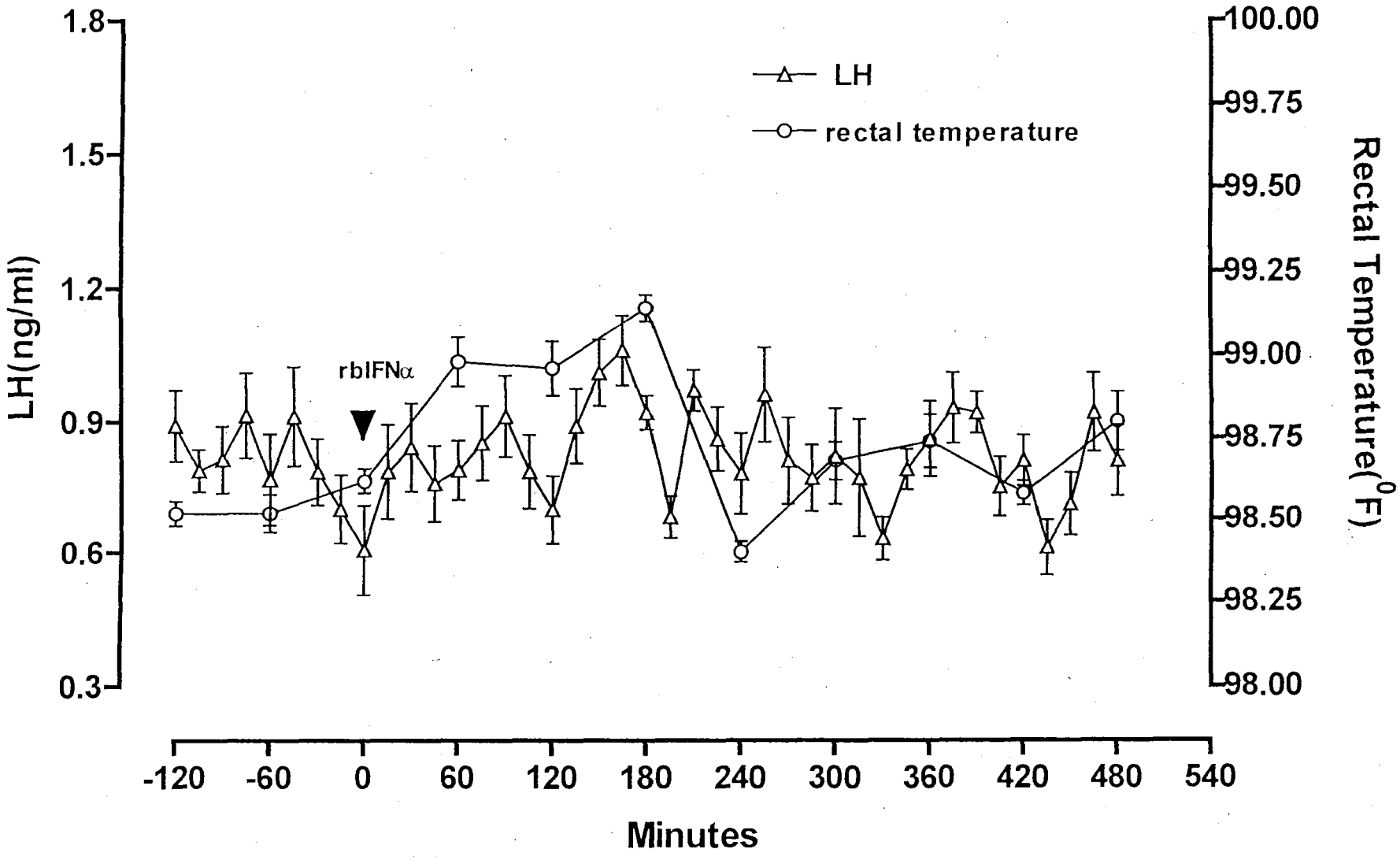


Fig.21. Temporal changes in LH and rectal temperature (Mean±S.E.) in buffaloes administered rbIFNα @ 8mg /day on day 14(first injection)

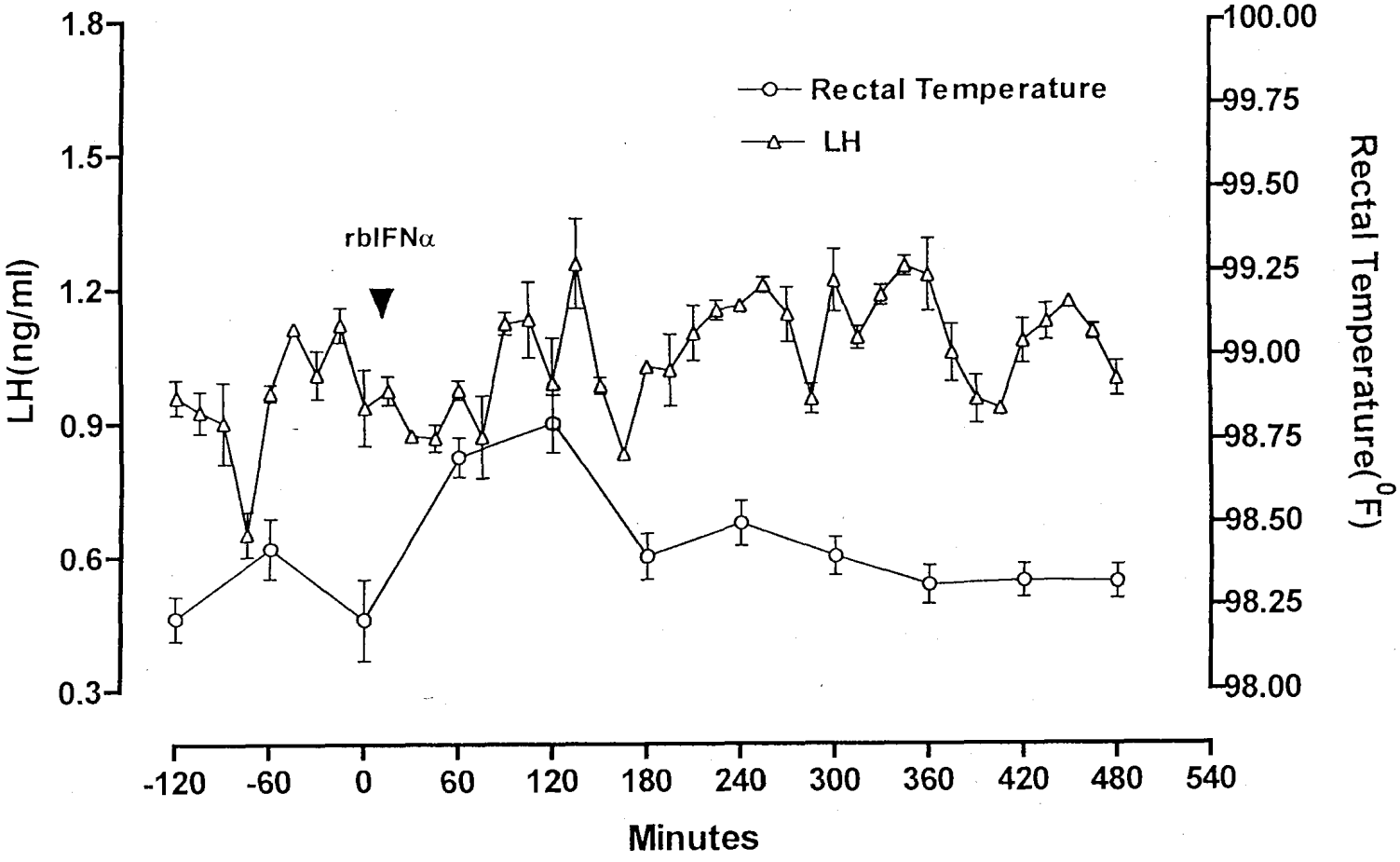


Fig.22. Temporal Changes in LH and rectal Temperatures(Mean±S.E.) in buffaloes administered rbIFNα @16mg/day on day 14(first injection)

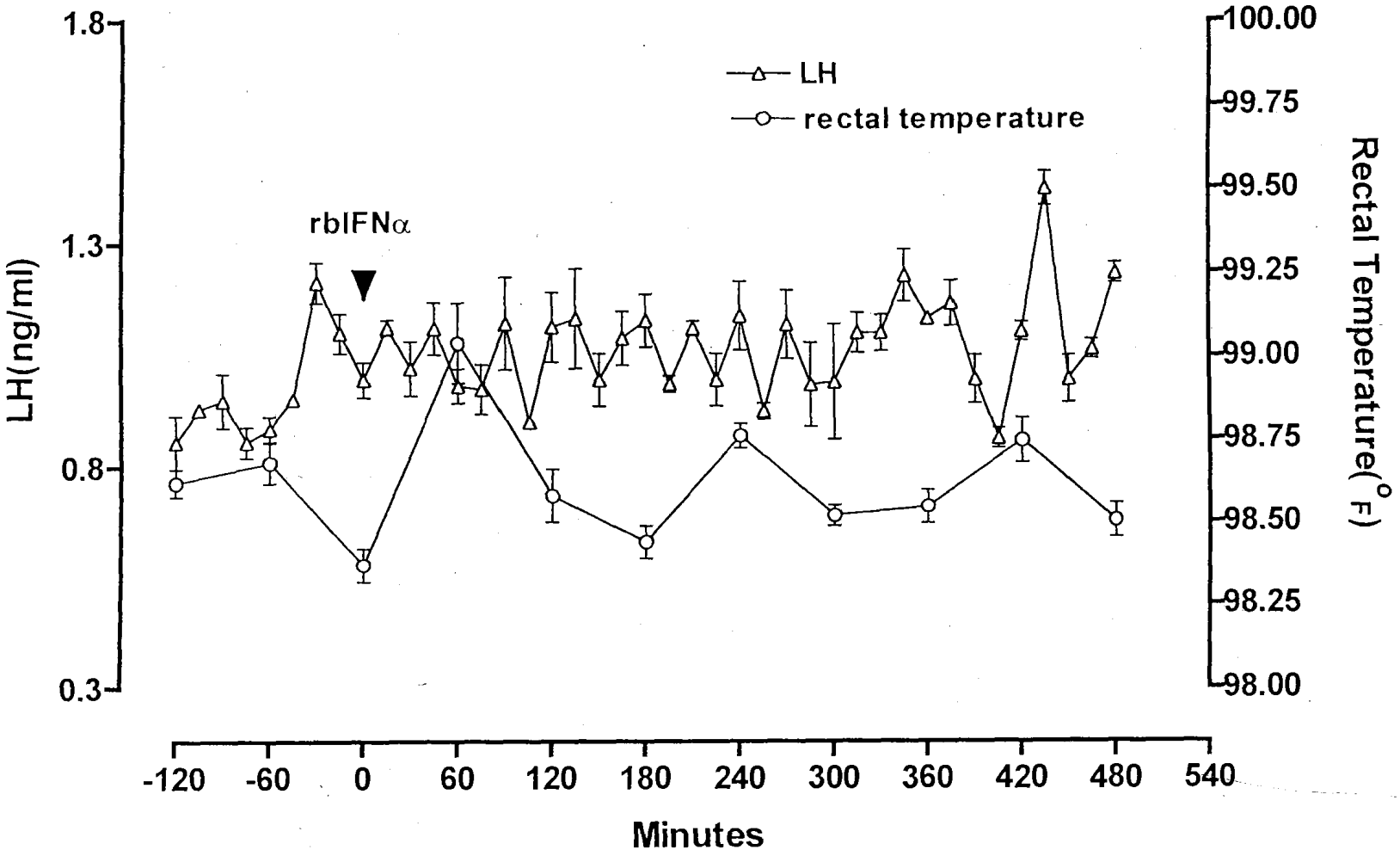


Table 13. Temporal changes in Progesterone (Mean ± S.E.) in buffaloes treated with different doses of rbIFN$_\alpha$

Minutes	Control Group (ng/ml) n = 5	Group I (ng/ml) (4 mg rbIFN$_\alpha$ / day) n = 5	Group II (ng/ml) (8 mg rbIFN$_\alpha$ / day) n = 5	Group III (ng/ml) (16 mg rbIFN$_\alpha$ / day) n = 5
-120	1.87±0.07	2.12± 0.02	2.23± 0.04	1.87± 0.05
-105	1.92±0.08	2.42± 0.03	2.11± 0.08	1.92± 0.07
-90	1.91±0.02	2.10± 0.03	2.13± 0.12	1.90± 0.03
-75	1.90±0.06	2.21± 0.03	2.11± 0.16	1.77± 0.05
-60	1.77±0.09	1.99± 0.06	2.11± 0.03	1.85± 0.03
-45	1.85±0.06	2.23± 0.04	2.12± 0.16	1.83± 0.05
-30	1.83±0.01	2.34± 0.04	2.11± 0.07	1.72± 0.03
-15	1.72±0.02	2.11± 0.03	2.23± 0.14	1.63± 0.04
0	1.63±0.04	2.21± 0.03	2.11± 0.14	1.71± 0.03
15	1.71±0.09	1.86± 0.03	1.98± 0.08	1.80± 0.06
30	1.80±0.03	1.69± 0.05	1.80± 0.03	1.74 ± 0.07
45	1.74±0.02	1.84± 0.05	1.35± 0.08	1.83 ± 0.03
60	1.83±0.01	1.56± 0.03	1.01± 0.03	1.12 ± 0.08
75	1.12±0.05	1.23± 0.04	1.13± 0.10	1.92 ± 0.08
90	1.92± 0.06	1.12± 0.05	1.40± 0.21	1.78 ± 0.05
105	1.78± 0.03	1.45± 0.03	1.30± 0.22	1.87 ± 0.07
120	1.87± 0.08	0.99± 0.04	1..05± 0.05	2.12 ± 0.07
135	2.12± 0.09	1.34± 0.03	1.60± 0.06	1.99± 0.05
150	1.99± 0.07	1.51± 0.03	1.10± 0.12	2.14± 0.05
165	2.14± 0.06	1.61± 0.06	1.45± 0.14	2.14± 0.07
180	2.14± 0.05	1.71± 0.04	1.23± 0.10	1.81± 0.02
195	1.81± 0.04	1.98± 0.05	1.60± 0.22	1.82± 0.07
210	1.82± 0.05	1.12± 0.05	1.02± 0.15	1.77± 0.03
225	1.77± 0.04	1.23± 0.02	1.45± 0.03	1.97± 0.07
240	1.97± 0.06	1.14± 0.05	1.24± 0.14	1.87± 0.03
255	1.87± 0.07	1.93± 0.03	1.22± 0.05	1.80± 0.05
270	1.80± 0.08	2.29± 0.03	1.42± 0.09	1.79± 0.05
285	1.79± 0.09	2.28± 0.04	1.72± 0.14	2.14± 0.05
300	2.14± 0.01	2.12± 0.05	1.94± 0.05	1.87± 0.03
315	1.87± 0.03	2.24± 0.04	2.29± 0.13	1.65± 0.03
330	1.65± 0.02	1.99± 0.03	2.11± 0.08	1.71± 0.05
345	1.71± 0.03	2.12± 0.03	2.35± 0.08	1.98± 0.04
360	1.98± 0.07	2.02± 0.05	2.35± 0.10	2.12± 0.03
375	2.12± 0.04	2.11± 0.03	2.43± 0.10	1.99± 0.03
390	1.99± 0.08	2.23± 0.03	2.21± 0.19	1.97± 0.02
405	1.97± 0.02	2.11± 0.04	2.11± 0.05	1.97± 0.05
420	1.97± 0.03	1.98± 0.05	2.23± 0.05	1.97± 0.03
435	1.97± 0.05	2.46 ± 0.03	2.11± 0.16	2.31± 0.03
450	2.31± 0.06	2.52± 0.05	2.02± 0.03	2.11± 0.09
465	2.11± 0.07	2.35 ± 0.05	2.35± 0.07	2.22± 0.05
480	2.24± 0.09	1.92± 0.05	2.11 ± 0.08	2.14± 0.08

Table 14. Temporal changes in LH (Mean ± S.E.) in buffaloes treated with different doses of rbIFN$_\alpha$

Minutes	Control Group (ng/ml) n = 5	Group I (ng/ml) (4 mg rbIFN$_\alpha$ / day) n = 5	Group II (ng/ml) (8 mg rbIFN$_\alpha$ / day) n = 5	Group III (ng/ml) (16 mg rbIFN$_\alpha$ / day) n = 5
-120	0.94± 0.05	0.89± 0.08	0.95± 0.03	0.85± 0.0.6
-105	0.95± 0.05	0.77± 0.05	0.92± 0.40	0.93± 0.01
-90	0.86± 0.05	0.81± 0.08	0.90± 0.09	0.95± 0.06
-75	0.69± 0.01	0.91± 0.04	0.65± 0.05	0.85± 0.03
-60	0.97± 0.02	0.76± 0.07	0.96± 0.01	0.88± 0.02
-45	0.87± 0.01	0.91± 0.09	1.11± 0.01	0.95± 0.07
-30	0.97± 0.01	0.78± 0.10	1.01± 0.05	1.21± 0.03
-15	0.97± 0.04	0.69± 0.11	1.12± 0.06	1.10± 0.06
0	0.87± 0.03	0.60± 0.07	0.93± 0.08	0.99± 0.02
15	0.87± 0.03	0.70± 0.07	0.97± 0.03	1.11± 0.05
30	0.98± 0.03	0.78± 0.10	1.27± 0.02	1.02± 0.04
45	0.75± 0.05	0.84± 0.10	0.86± 0.03	1.11± 0.05
60	0.89± 0.02	0.75± 0.08	0.97± 0.02	0.97± 0.07
75	0.97± 0.02	0.78± 0.06	0.87± 0.09	1.23± 0.05
90	0.94± 0.02	0.85± 0.08	1.12± 0.02	0.90± 0.05
105	0.96± 0.04	0.91± 0.09	1.13± 0.08	1.11± 0.05
120	0.94± 0.06	0.78± 0.08	0.99± 0.10	1.13± 0.03
135	0.95± 0.02	0.69± 0.07	1.25± 0.01	0.99± 0.03
150	1.14± 0.02	0.89± 0.08	0.98± 0.07	1.09± 0.05
165	0.97± 0.01	1.01± 0.07	0.83± 0.06	1.12± 0.06
180	1.12± 0.02	1.06± 0.03	1.02± 0.01	0.98± 0.05
195	1.12± 0.13	0.92± 0.04	1.01± 0.07	1.11± 0.03
210	1.23± 0.11	0.68± 0.04	1.10± 0.06	0.99± 0.04
225	1.12± 0.01	0.97± 0.07	1.15± 0.02	1.14± 0.04
240	1.13± 0.02	0.86± 0.09	1.16± 0.01	0.92± 0.03
255	0.96± 0.02	0.78± 0.10	1.20± 0.01	1.12± 0.05
270	0.97± 0.01	0.96± 0.09	1.14± 0.06	0.98± 0.03
285	0.96± 0.01	0.81± 0.07	0.95± 0.03	0.99± 0.05
300	1.12± 0.02	0.77± 0.10	1.21± 0.06	1.10± 0.12
315	0.96± 0.01	0.82± 0.13	1.09± 0.03	1.10± 0.03
330	0.96± 0.02	0.77± 0.04	1.18± 0.05	1.23± 0.04
345	1.23± 0.01	0.63± 0.04	1.25± 0.02	1.13± 0.03
360	0.99± 0.01	0.79± 0.08	1.23± 0.08	1.16± 0.03
375	1.12± 0.02	0.86± 0.07	1.05± 0.06	0.99± 0.02
390	0.97± 0.05	0.93± 0.04	0.95± 0.05	0.86± 0.03
405	0.95± 0.01	0.92± 0.06	0.93± 0.01	1.10± 0.05
420	0.96± 0.01	0.75± 0.05	1.08± 0.04	1.42± 0.07
435	1.12± 0.01	0.84± 0.06	1.12± 0..01	0.99± 0.06
450	1.11± 0.05	0.61± 0.07	1.17± 0.12	1.06± 0.03
465	1.11± 0.02	0.71± 0.03	1.10± 0.07	1.23± 0.03
480	1.12± 0.07	0.92± 0.08	0.99± 0.05	1.11± 0.05

Fig.23. LH and Progesterone profiles (Mean±S.E.) in untreated control buffaloes on day 14 of cycle

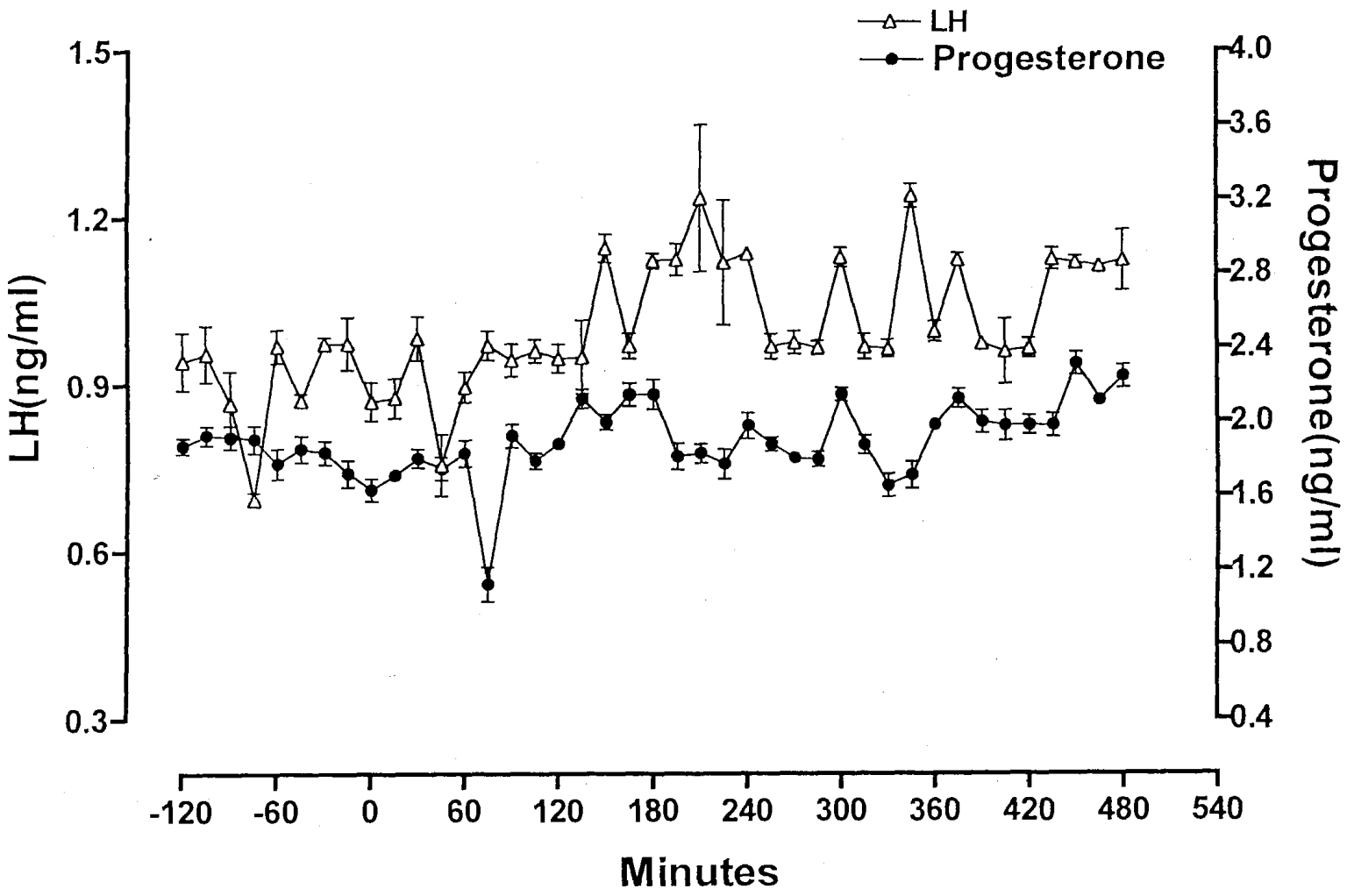


Fig.24. LH and progesterone profiles (Mean±S.E.) in buffaloes administered rbIFNα @4mg/day on day 14 (first injection)

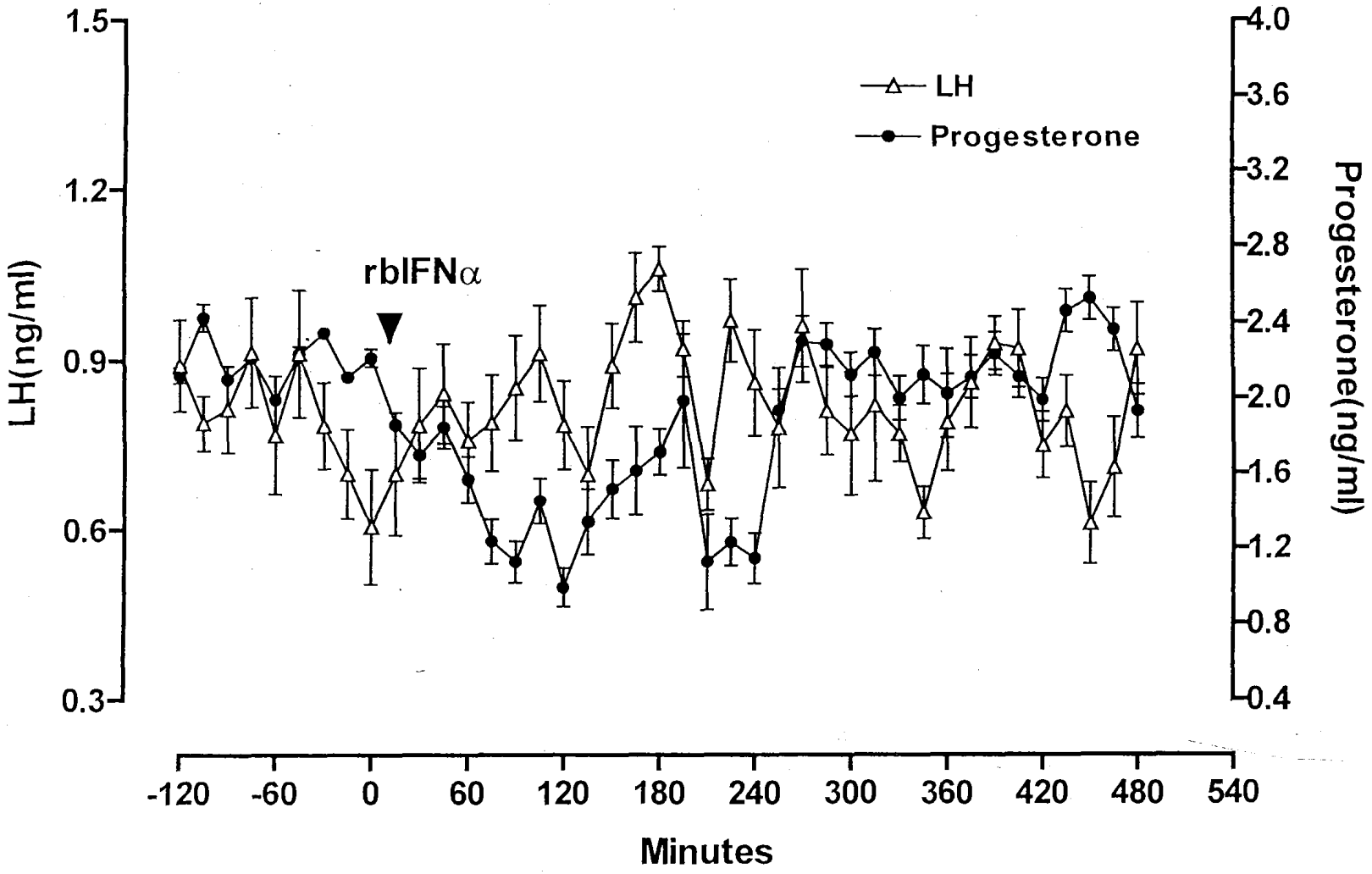


Fig.25. LH and Progesterone profiles(Mean±S.E.)in 5 buffaloes administered rbIFNα@8mg/day on day14 (first injection)

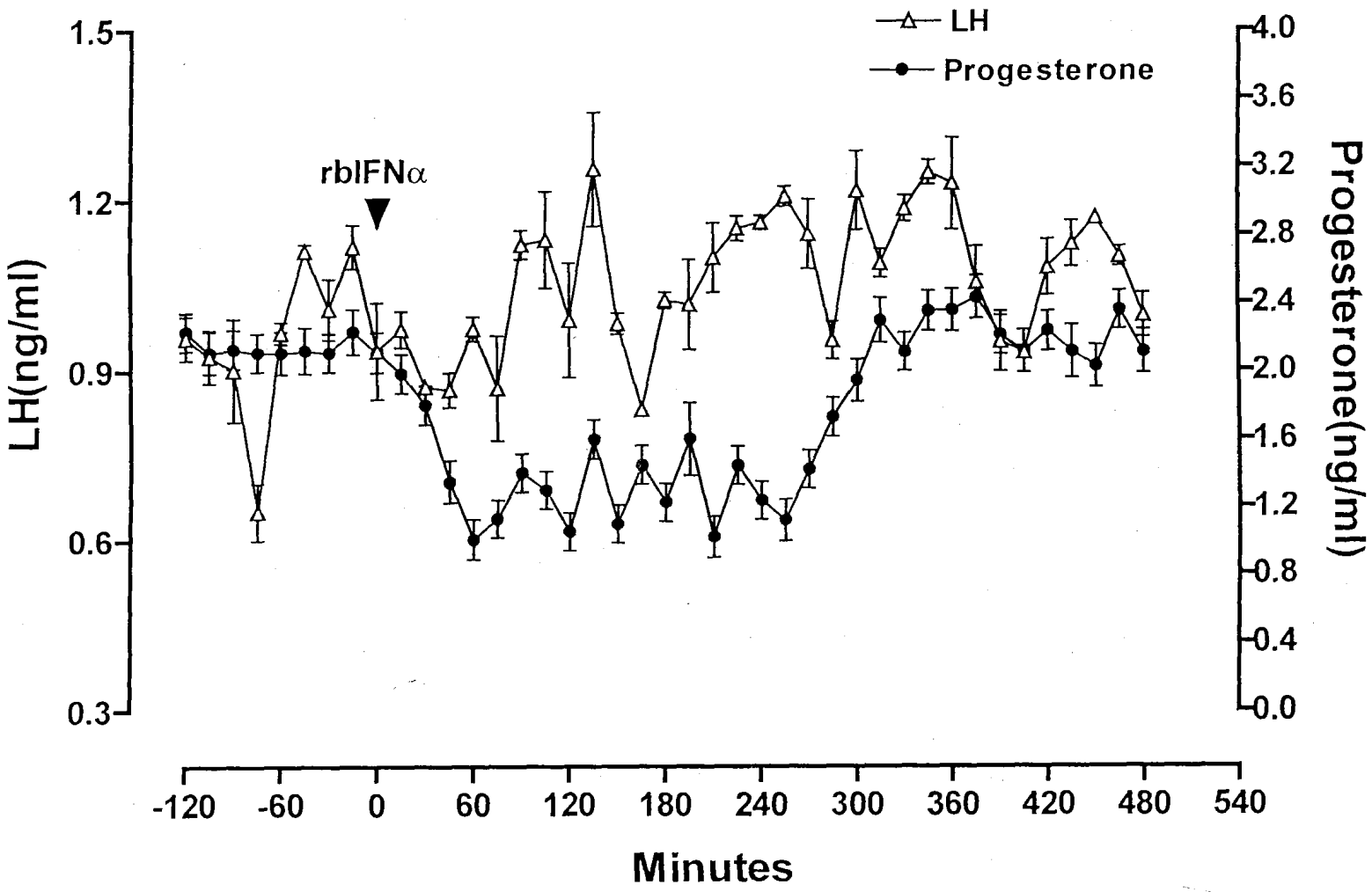


Fig.26. LH and progesterone profiles (Mean±S.E.) in buffaloes administered @16mg rbIFNα on day 14 (first injection)

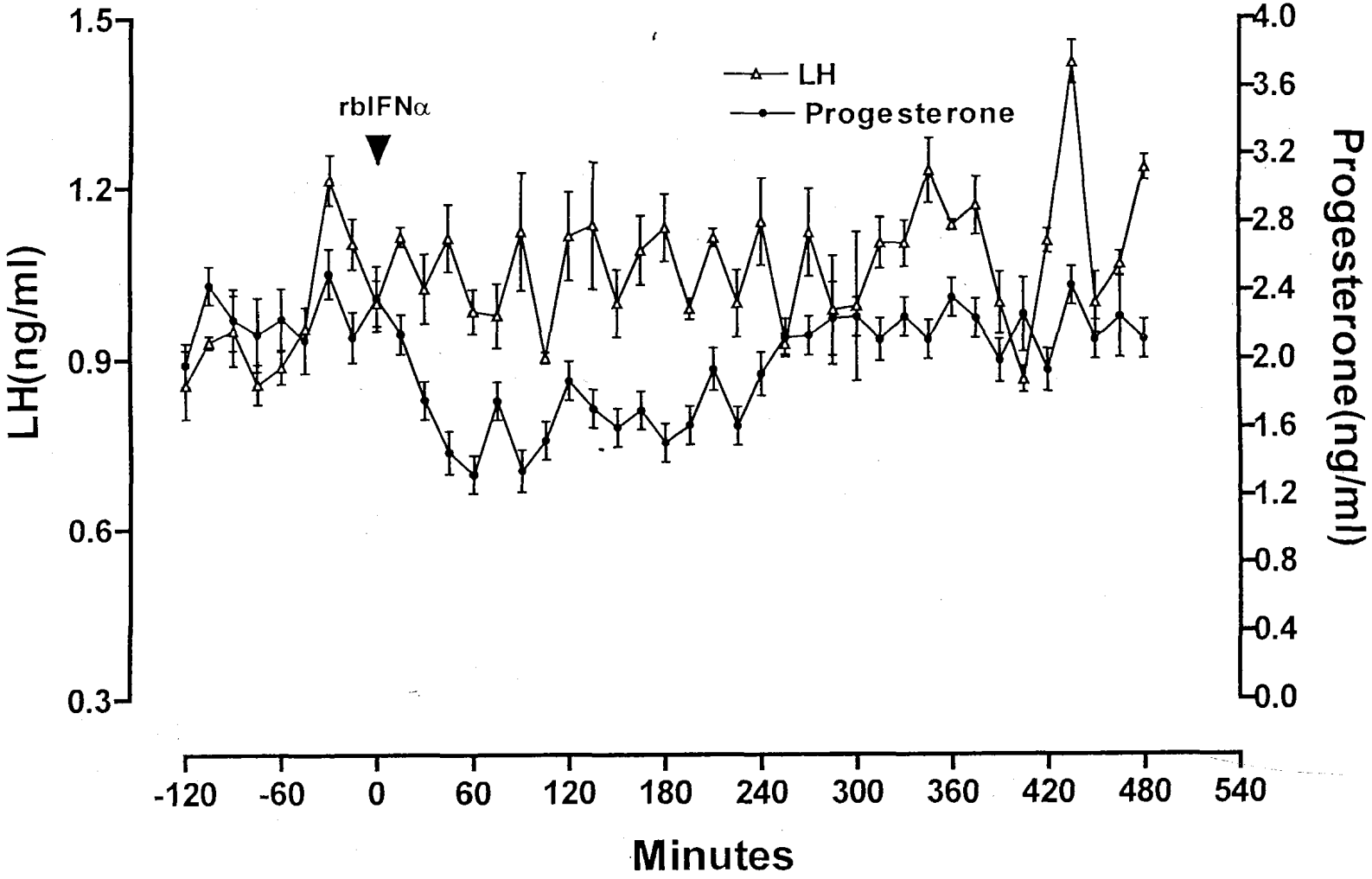


Table 15. PGFM (pg/ml) profiles (Mean±S.E.) in Oxytocin and Oxytocin plus rbIFNα treated buffaloes

Minutes	Oxytocin	rbIFNα+ Oxytocin
-120	88.66±4.01	86.83±3.11
-105	93.66±1.78	90.16±2.57
-90	89.16±3.35	87.00±2.64
-75	94.33±3.58	91.50±3.67
-60	98.33±4.49	98.83±2.66
-45	95.66±3.77	93.16±4.09
-30	97.33±7.03	93.00±6.49
-15	92.16±5.23	92.50±2.66
0	90.50±4.08	96.50±2.29
15	130.50±8.66	120.33±4.45
30	153.00±11.47	127.83±4.11
45	183.16±14.68	138.00±3.17
60	213.66±11.30	143.66±3.94
75	214.00±24.63	140.00±10.23
90	149.33±17.76	118.33±9.07
105	118.50±10.31	101.83±3.02
120	105.83±3.82	101.00±4.29
135	103.83±4.40	101.83±2.52
150	102.16±6.64	99.66±1.56
165	95.83±1.77	95.16±2.54
180	98.50±1.64	93.83±2.57
195	97.33±1.81	94.16±2.41
210	97.83±1.60	93.00±2.38
225	96.50±2.40	90.50±3.39
240	98.83±2.53	90.50±3.06
255	98.33±2.04	93.66±1.33
270	99.50±2.24	95.66±1.33
285	98.50±1.38	95.16±1.68
300	97.50±1.76	93.66±2.70
315	96.16±1.13	92.83±2.49
330	96.16±2.63	93.33±1.60
345	94.20±2.24	96.00±1.70
360	93.60±2.61	94.01±1.81

Fig.27. Changes in peripheral PGFM concentrations in buffaloes treated with oxytocin and oxytocin plus rbIFNα

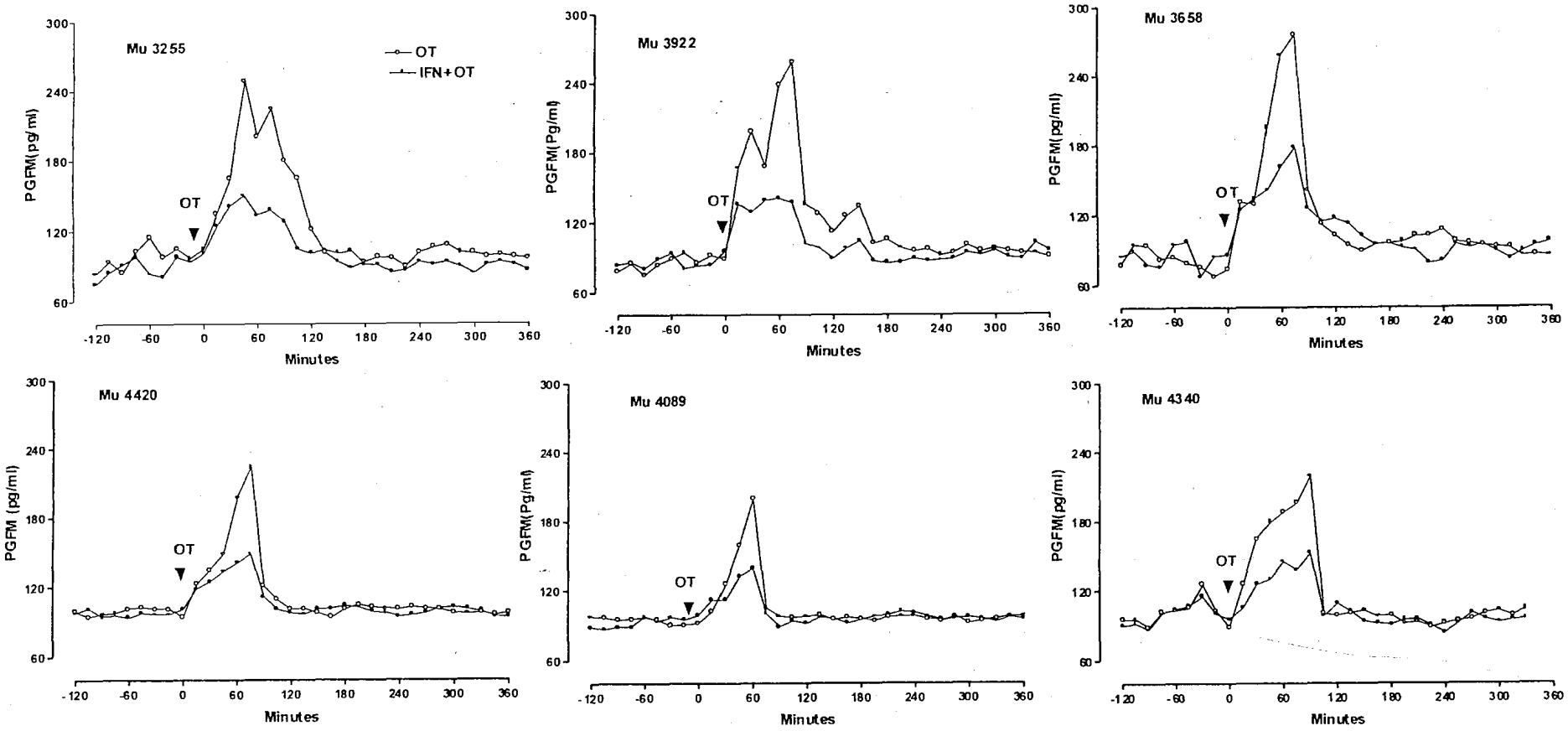


Fig.28. Peripheral PGFM profiles (Mean±S.E.) in buffaloes treated with oxytocin and rbIFNα plus oxytocin

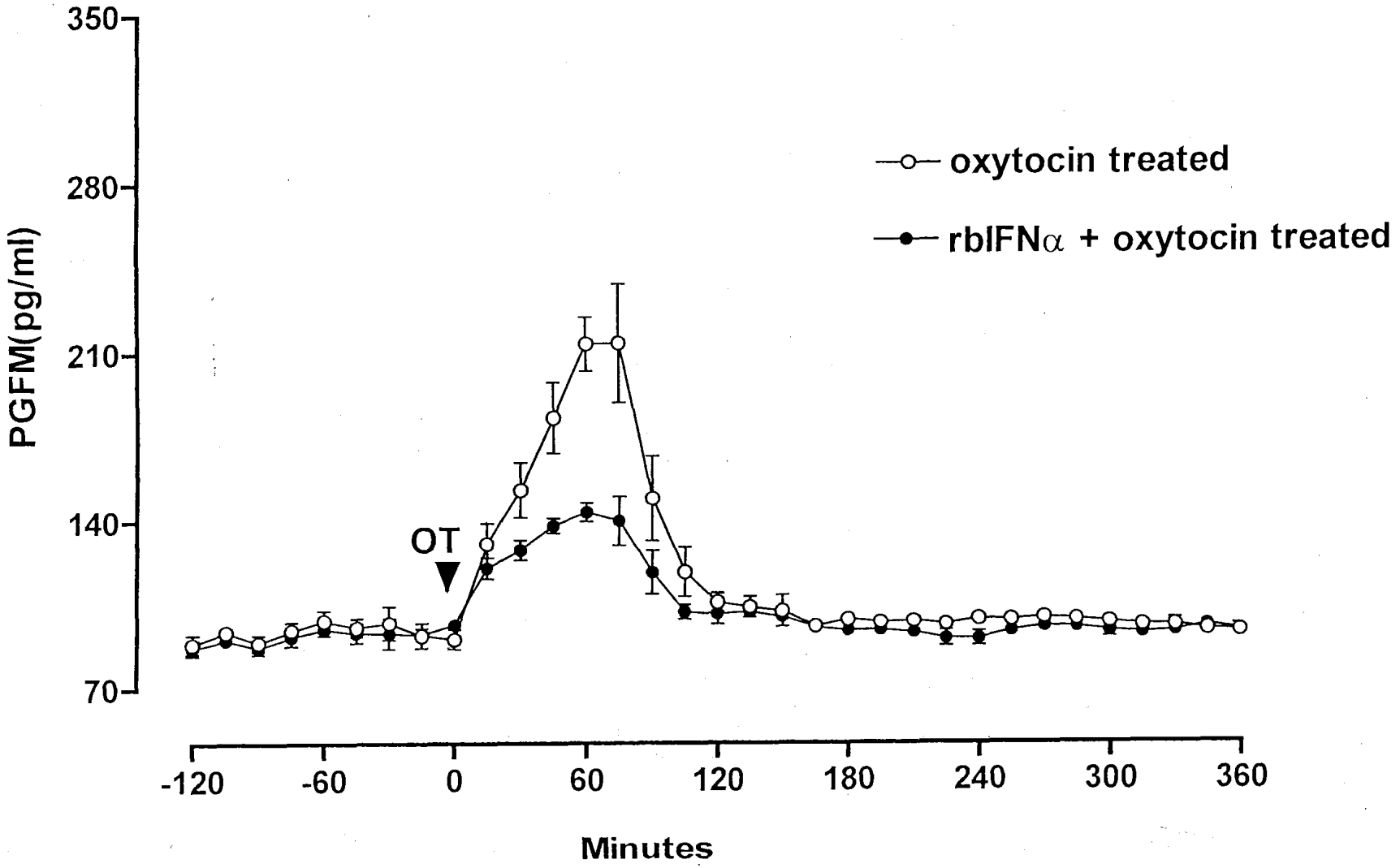
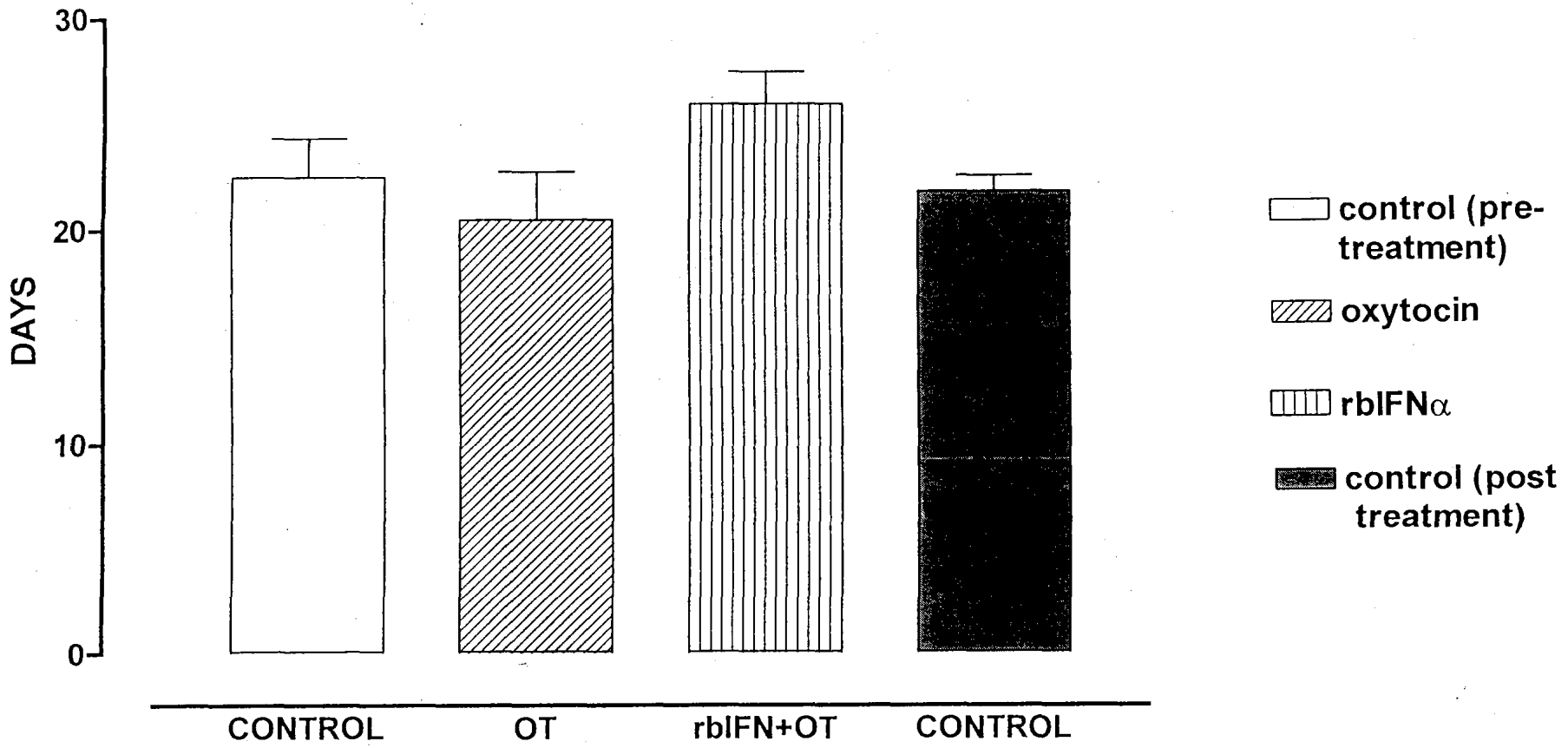


Fig.29. Estrous cycle lengths(Mean±S.D.) in buffaloes treated with oxytocin and rbIFNα plus oxytocin (n=6)



as in untreated control animals administered oxytocin alone on day 17 of the cycle are depicted in Table 15 and Figure 28.

Oxytocin administration to buffaloes on day 17 of the untreated control cycle brought about a sharp increase ($P<0.05$) in plasma PGFM concentration from 90.50 ± 4.08 pg/ml (range 89 to 105 pg/ml) prior to oxytocin administration to 214.00 ± 24.63 pg/ml (range 105 to 276 pg/ml) within 75 minutes after administration. When the animals were subjected to rbIFN$_\alpha$ treatment (16 mg/day) during days 14 to 16, oxytocin administration on day 17 produced an increase of plasma PGFM from 96.50 ± 2.29 pg/ml (range 79 to 112 pg/ml) prior to oxytocin administration to 143.60 ± 3.94 pg/ml (range 86 to 101 pg/ml) within 60 minutes (range 134 to 162 pg/ml) after oxytocin administration. Comparison of the oxytocin mediated plasma PGFM profiles (Fig. 27) in the animals of control and interferon treatment clearly indicated a decrease in the amplitude of plasma PGFM release ($P<0.05$) in the animals belonging to interferon treated group. Results, therefore, suggest that interferon treatment suppressed oxytocin mediated PGFM release in buffaloes.

The influence of rbIFN$_\alpha$ on the oxytocin treated and untreated as compared to the untreated pre- and post- treatment cycle lengths (Mean ± S.D.) is depicted in Figure 29. The estrous cycle lengths for pre-treatment, oxytocin treated, rbIFN$_\alpha$ plus oxytocin treated and post treatment cycles were 22.50 ± 1.87 (range 20 to 25 days), 20.50 ± 2.25 (range 18 to 24 days), 26.00 ± 1.84 (range 25 to 29 days) and 21.83 ± 0.75 (range 21 to 23 days), respectively. The rbIFN$_\alpha$ treated estrous cycle lengths were significantly ($P<0.01$) longer in comparison to the pre-, post- and oxytocin treated estrous cycle lengths.

CHAPTER - 5

Discussion

5. DISCUSSION

5.1 STANDARDIZATION AND VALIDATION OF PGFM ASSAY

5.1.1 Assay Standardization

Based on the studies on the biological roles of $PGF_{2\alpha}$ *in vivo*, its initial metabolite, 13,14-dihydro-15-ketoprostaglandin $F_{2\alpha}$ (PGFM) is considered as the most useful marker of $PGF_{2\alpha}$ in the peripheral circulation (Piper *et al.*, 1970; Granstrom and Samuelsson, 1978). PGFM has a longer half-life in the peripheral circulation than its parent compound; it occurs in higher concentrations and no arti-factual formation of the compound takes place during collection and handling of the sample. Many assay procedures have been developed for estimation of this metabolite. However, to the best of our knowledge, the PGFM-EIA standardized and described in the Materials and Methods section is the first report of direct EIA in unextracted buffalo plasma. The use of the second antibody for coating the microtitre plate wells instead of hormone-specific antibody is preferred as it reduces assay variabilities associated with uneven binding of the latter antibody to the wells (Meyer, 1986).

There was a slight drop in the optical densities with increasing plasma volumes although the sensitivities and the relative binding percentages did not change (Figs. 2a,b). This effect of plasma volumes on optical densities can be compensated for by using the same plasma volumes for standards and unknown samples. A high assay sensitivity of 0.4 pg/well PGFM was obtained from 76 assays, while taking 20 µl plasma for estimation. This was sufficient for determining low physiological levels of PGFM during cystic conditions (Fig.10), pulsatile release of PGFM during periestrus period (Figs. 5 and 6) as well as high PGFM levels in Murrah buffaloes suffering from reproductive tract infections (Fig. 9).

5.1.2 Assay Validation

5.1.2.1 PGFM Levels in the periestrus period

In non-pregnant cyclic ruminants, luteal regression is caused by $PGF_{2\alpha}$ secreted from the uterus (McCracken *et al.*, 1970, 1972; Inskeep and Murdoch, 1980). Even though there is species specific variation of $PGF_{2\alpha}$ release, the release of $PGF_{2\alpha}$ occurs in a pulsatile manner. Onset of pulsatile pattern of $PGF_{2\alpha}$ secretion probably initiates luteal regression (Silvia *et al.*, 1991). Peripheral measurements of the major metabolite of $PGF_{2\alpha}$, viz., 13,14-dihydro-15-keto- $PGF_{2\alpha}$ (PGFM) are assumed to be an accurate reflection of the uterine $PGF_{2\alpha}$ release (Granstrom and Kindahl, 1982). In the present study, PGFM concentration in blood plasma of the 3 buffaloes in the peri-estrus period were sampled at 4 hourly intervals starting 120 hours prior to estrus up to 80 hours post-estrus, revealed a rise in PGFM levels coincident with falling progesterone levels. Though the 4 hourly sampling pattern (Fig. 5) did not reveal any conspicuous pulsatile release of PGFM, the sampling at short time intervals (Fig. 6) clearly showed a pulsatile release of PGFM with as many as 9 pulses of PGFM being exhibited in the sampling period of 30 hours prior to observed estrus. The levels were basal at estrus and did not change significantly ($P<0.01$) till 80 hours post-estrus. The peak of PGFM levels reached around 36 to 40 hours prior to detected estrus (Fig. 5). This is similar to the timing of reported peak levels in buffaloes of 48.83 ± 1.30 h prior to estrus (Batra and Pandey, 1983). The sudden increase in the peripheral PGFM levels prior to estrus with declining levels of progesterone is consistent with earlier investigations in buffaloes and cattle (Batra *et al.*, 1979; Pahwa and Pandey, 1983; Batra and Pandey, 1983; Kindahl *et al.*, 1984). The pulsatile release of PGFM observed in the present investigation was more pronounced than those reported by Batra and Pandey (1983). This can be attributed to the sampling interval being shorter (1 hour in the present investigation as compared to 6 hours) in their study. In this regard, these results showed a similar trend to that observed in cows in earlier investigations (Kindahl *et al.*, 1976a,b). The peak levels prior to estrus ranged

from 268.96 ± 54.32 to 591.6 ± 52.63 pg/ml as compared to 150 to 570 pg/ml reported in cattle (Kindahl *et al.*, 1976c). These observations clearly demonstrate that the assay could measure the physiological variations in plasma PGFM levels during peri-estrus periods.

5.1.2.2 Plasma PGFM levels in cyclic buffaloes

In the present investigation, plasma PGFM levels were maintained at basal levels and registered a rise beginning 4 days prior to the observed estrus. It maintained an increasing trend till it reached peak levels 2 days prior to estrus, followed by a gradual declining trend till lowest levels were observed at estrus (Figs. 5 and 6). The progesterone profiles in the present investigation showed a declining trend concomitant with rise of PGFM levels. In the present investigation, PGFM levels varied widely depending upon the stage of estrous cycle from lowest levels (74.3 ± 11.97 pg/ml) at estrus and highest levels (399.00 ± 43.42 pg/ml) two days prior to estrus. These results are comparable with the levels reported in cattle by Kindahl *et al.* (1976c). Although the PGFM profiles were similar to those recorded by Batra and Pandey (1983) in buffaloes, the levels in the present study were lower. This can be attributed to differences in the assay system and specificity of the antisera used in the assays.

5.1.2.3 Plasma PGFM levels in early pregnant buffaloes

The present investigation revealed a higher basal level of PGFM in early pregnant buffaloes after the initial rise as compared to those seen during the mid luteal phase. This result is in agreement with earlier reports (Kindahl *et al.*, 1976b) in cattle.

However, PGFM levels did not show any significant variation ($P>0.01$) between days of the sampling period. This agreed with the reports of Kindahl *et al.* (1976b) for cows, Batra and Pandey (1983) for buffaloes and Peterson *et al.* (1976) for ewes.

This may be attributed to the luteotrophic effect of early embryo on alteration of uterine prostaglandin release (Hansen *et al.*, 1985). During the

period of study, the progesterone levels were maintained at luteal phase levels suggesting maintenance of *corpus luteum*. Thereby showing a similar trend to that of cattle (Bulman and Lamming, 1978; Shelton and Sumners, 1983) and buffaloes (El-Sheikh *et al.*, 1969; Batra *et al.*, 1979; Kamonpatana *et al.*, 1979).

5.1.2.4 Plasma PGFM Levels in Buffaloes with Reproductive Tract Infections

The PGFM levels in the buffaloes having reproductive tract infections had significantly ($P<0.01$) higher levels throughout the period of sampling and did not show any specific trend as seen in cycling buffaloes. The consistently low levels of progesterone during the course of experimentation indicated that *corpus luteum* was not formed in these buffaloes. The results are consistent with that observed by Kindahl *et al.* (1986) in cows. It is known that in animals with uterine infection, the presence of bacterial endotoxins act as a powerful stimulus for release of $PGF_{2\alpha}$ release (Roberts, 1975; Edqvist *et al.*, 1978). The sustenance of high levels of PGFM in the peripheral plasma may be attributed to stimulation of inflammatory process in the endometrium and release of endotoxin (Peter and Bosu, 1987). It is also known that Gram-negative bacterial endotoxins are known to be potent stimulators of prostaglandin synthesis and prolonged increased levels of PGFM have been associated with endometritis (Watson *et al.*, 1984). In the present investigation, a similar trend was revealed resulting in high levels of PGFM in animals suffering from reproductive tract infections which results in basal levels of progesterone.

In conclusion, the biological validation of PGFM-EIA is confirmed through the present findings since PGFM changes during 1) peri-estrus, 2) cyclicity, 3) early pregnancy, 4) reproductive tract infections and 5) cystic conditions occur along expected lines as seen in bovines. The validated PGFM-EIA offers a reliable alternative to RIA. Apart from being non-radioactive in nature, the EIA procedure has several other advantages over RIA namely, (a) It is many times more sensitive than the RIA procedures

described in buffalo plasma, (b) It requires less plasma volumes as compared with the conventional RIA methods, (c) It does not involve hazardous extraction solvents required for extraction procedures, (d) It is less labour intensive and relatively simple to perform. Further, since the procedure requires less capital investment in terms of instruments, the PGFM-EIA provides a safe, cost-effective and less time taking alternative to the conventional RIA procedures and can, therefore, be adopted in laboratories, where financial constraints limit the adoption of RIA.

5.2 INFLUENCE OF DIFFERENT DOSES OF rbIFNα ON ESTROUS CYCLE LENGTHS IN BUFFALOES

Maternal recognition of pregnancy in ruminants followed by CL maintenance involves biochemical communication between the conceptus and the maternal system to provide an uninterrupted synthesis and release of progesterone (Bazer *et al.*, 1991; Niswender *et al.*, 2000). There exists a high degree of sequence homology between the ruminant trophoblastic proteins (bTP-1 in cattle and oTP-1 in sheep) and interferon of the α class (Imakawa *et al.*, 1987; Charpigny *et al.*, 1988; Imakawa *et al.*, 1989). Successful maternal recognition of pregnancy in ruminants involve the embryonic trophoblastic protein mediated CL maintenance, through alteration of normal luteolytic mechanisms (Plante *et al.*, 1989). The extension of luteal function beyond the length of the normal estrous cycle is the index of successful maternal recognition of pregnancy and CL maintenance by blockade of luteolysis (Hodgen and Istkovitz, 1988; Martal *et al.*, 1997). Administration of rbIFN$_\alpha$ has been used to extend CL function in cattle and sheep (Plante *et al.*, 1988, 1989; Nephew *et al.*, 1990; Newton *et al.*, 1990; Mirando *et al.*, 1991; Siegenthaler and Martinod, 1991; Davis *et al.*, 1992; Garverick *et al.*, 1992; Parkinson *et al.*, 1992; Spencer *et al.*, 1995). No studies had been carried out in buffaloes so far. Hence, for dose standardization, three different doses of 4, 8 and 16 mg/day were administered (i.m.) to the buffaloes on day 14 through 16 of the estrous cycle. This period is critical to the processes that initiate the luteolytic mechanism leading to the regression of CL, in bovines (Bazer *et al.*,

1994; McCracken *et al.*, 1999). The results of the present investigation suggest a dose dependent response of rbIFN$_\alpha$ on CL maintenance and estrous cycle lengths. The dose of 16 mg/day of rbIFN$_\alpha$ significantly prolonged the estrous cycle lengths ($P<0.01$) in all the buffaloes of this group (Fig. 14).

The present study showed that rbIFN$_\alpha$ administered through systemic route which is easily accessible and practical can extend CL life span in buffaloes, which agrees with similar studies in cattle (Plante *et al.*, 1989) and sheep (Nephew *et al.*, 1990; Parkinson *et al.*, 1992). The study further reaffirms the efficacy of rbIFN$_\alpha$ in extending the functional life span of the CL as detected by the peripheral progesterone concentrations in buffaloes in a dose-dependent manner (Figs. 11 to 13). This is in agreement with the established role of rbIFN$_\alpha$ in CL life span regulation in sheep and cattle (Plante *et al.*, 1988, 1989; Stewart *et al.*, 1989; Thatcher *et al.*, 1989; Schalue-Francis *et al.*, 1991). Among the three groups of buffaloes administered rbIFN$_\alpha$ the buffaloes receiving 4 and 8 mg/day had estrous cycle lengths which were similar to the pre-treatment and post-treatment control cycle lengths ($P>0.01$). The only plausible explanation can be that the dosage of rbIFN$_\alpha$ was inadequate for extending the life of CL in buffaloes subjected to these treatments. Administration of rbIFN$_\alpha$ or similar molecules like it (bTP-1) are known to inhibit PGF$_{2\alpha}$ secretion by bovine endometrium under *in vitro* conditions (Barros *et al.*, 1990), by increasing the activity of an endometrial prostaglandin synthetase inhibitor, thereby reducing endometrial PGF$_{2\alpha}$ synthesis (Gross *et al.*, 1988a,b). The rbIFN$_\alpha$ administration has also been known to inhibit the upregulation of the uterine oxytocin receptors, which normally occurs during the late luteal phase when luteal oxytocin secretion is high. The reduction in the number of oxytocin receptors caused by interferon-α has been found to inhibit the pulsatile secretion of PGF$_{2\alpha}$ thereby preventing the occurrence of luteolytic mechanisms (Flint *et al.*, 1991), and resulting in CL maintenance. Though the exact mechanism involved in CL maintenance in buffaloes is unclear, the results suggest that 16 mg/day of rbIFN$_\alpha$ dosage in buffaloes was sufficient to influence the anti-luteolytic mechanisms.

The results of this experiment are in accordance with the concept of alteration of luteolytic mechanisms *in vivo* by rbIFN$_\alpha$ resulting in CL maintenance in buffaloes as reported by several investigators in cattle (Plante *et al.*, 1988, 1989). It is also apparent from the results that the dosage and time of administration are critical to the antiluteolytic effect of rbIFN$_\alpha$. In buffaloes, luteal regression followed by commencement of estrous cycle post-breeding results in a major decrement of fertility in this species (Singh and Gangwar, 1976; Chatterjee *et al.*, 1985; Tomar and Verma, 1987; Kaul and Prakash, 1994). The present investigation is indicative of a potential for practical application of exogenous rbIFN$_\alpha$ supplementation in inseminated buffaloes for CL maintenance and hence augmenting fertility as has been suggested also for cattle (Plante *et al.*, 1989; Thatcher *et al.*, 1997).

5.3 INFLUENCE OF rbIFN$_\alpha$ DOSES ON PROGESTERONE AND LH PROFILES IN BUFFALOES

5.3.1 Plasma Progesterone Changes as Influenced by rbIFN$_\alpha$ Administration

The primary function of the CL is secretion of progesterone and the peripheral levels of this hormone is indicative of the functional status of the CL as well as serves as an index of CL maintenance (Dobson and Kamonapatana, 1986; Niswender *et al.*, 2000) in ruminants. In all the 3 doses of rbIFN$_\alpha$ application, the progesterone profiles did not differ significantly ($P>0.01$) from the pre-treatment and post-treatment cycles on days preceding luteolysis (days 14-18). However, the rate of decline of progesterone on days following day 19 of the cycle when functional luteolysis normally occurs was much slower in buffaloes receiving 16 mg/day of rbIFN$_\alpha$ (on days 14 through 16). The maintenance of progesterone levels beyond the expected days of luteolysis in this group of buffaloes clearly brings out the antiluteolytic role of rbIFN$_\alpha$ in buffaloes. This is the first report of interferon application in buffaloes, and the results are consistent with the reported antiluteolytic role of rbIFN$_\alpha$ or related molecules in sheep, cattle and pigs (Godkin *et al.*, 1984; Plante *et al.*,

1988, 1989; Stewart *et al.*, 1989; Nephew *et al.*, 1990; Siegenthaler and Martinod, 1991; Garverick *et al.*, 1992; Prakash *et al.*, 1997). It is established that the time of administration and dosage of rbIFN$_\alpha$ are critical factors for its antiluteolytic effects (Siegenthaler and Martinod, 1991). This study further strengthens the hypothesis that the luteal phase in cyclic buffaloes can be extended by administration of rbIFN$_\alpha$ at a time equivalent or prior to the maternal recognition of pregnancy as reported by other investigations in cattle (Betteridge *et al.*, 1980; Northey and French, 1980; Humbolt and Dalla-Porta, 1984; Plante *et al.*, 1988, 1989). In the present investigation, the sustained levels of progesterone in buffaloes receiving 16 mg/day of rbIFN$_\alpha$ was absent in-groups receiving 4 mg/day and 8 mg/day of rbIFN$_\alpha$ doses. The only plausible explanation for this effect can be that the rbIFN$_\alpha$ dosage was not sufficient to elicit a response. It is suggested from the results that the endometrial luteolytic mechanism in buffaloes are somehow inhibited by 16 mg/day dose of rbIFN$_\alpha$ treatment, which accounts for maintenance of high progesterone levels and extension of luteal life span as reported in cattle (Plante *et al.*, 1988, 1989; Garverick *et al.*, 1992) and sheep (Stewart *et al.*, 1989).

The antiluteolytic effects of rbIFN$_\alpha$ apparent in the present experiment can be attributed to the high degree of sequence homology between IFN$_\alpha$ and bovine trophoblastic proteins (bTP-1) (Godkin *et al.*, 1988; Pontzer *et al.*, 1988; Newton *et al.*, 1989) and similarly in their biologic actions characteristic of interferons. The biologic actions of rbIFN$_\alpha$ and related molecules are known to influence PGF$_{2\alpha}$ release by the uterine endometrium by inhibiting endometrial synthesis (Gross *et al.*, 1988a,b), secretion (Barros *et al.*, 1992) as well as down regulation of oxytocin receptors in the uterus which are normally involved in the pulsatile secretion of PGF$_{2\alpha}$ (Flint *et al.*, 1991). Another possible mechanism of rbIFN$_\alpha$ in blocking luteolysis may be through the decrease in ovarian follicular steroidogenesis, mediated luteolysis (Thatcher *et al.*, 1988). Employing microdialysis technique, Prakash *et al.* (1997) have also demonstrated the direct antiluteolytic and luteotrophic

effects of gamma interferon (the principal interferon secreted by the pig blastocyst) in *corpus luteum* maintenance in pigs. The results, therefore, point to the possibility of rbIFN$_\alpha$ use in buffaloes for preventing lysis of CL post-insemination, thereby augmenting fertility in this species by mimicking the endogenous signal for pregnancy recognition as reported in cattle (Garverick *et al.*, 1992).

5.3.2 Temporal Changes in LH and Progesterone Associated with rbIFN$_\alpha$ Administration

Interferons are known to have variety of effects on hormonal secretions by endocrine cells (Kaupilla *et al.*, 1982; Orava, 1986; Branca *et al.*, 1987; Goldstein *et al.*, 1987; Orava, 1989). Therefore, it was important to assess the effect of rbIFN$_\alpha$ administration on progesterone and LH levels, two most important hormones for luteal maintenance. In the present investigation, all the doses of rbIFN$_\alpha$ caused a transient decrease in the progesterone levels (Figs. 24 to 26), which reached pre-administration levels at 4, 4.5 and 3.5 hours for 4, 8 and 16 mg/day doses, respectively, after which the progesterone levels were similar to the pre-injection levels. Similar results in progesterone levels subsequent to rbIFN$_\alpha$ administration have been reported in cattle (Newton *et al.*, 1990; Plante *et al.*, 1991; Barros *et al.*, 1992a). However, the durations of decline in progesterone levels for these 3 doses were comparatively lower (range 3.5-4.5 hours) than the duration reported (range 4-8 hours) in cattle (Newton *et al.*, 1990; Plante *et al.*, 1991).

For all the doses of interferon administration, no significant change ($P<0.01$) in LH levels were observed. The results are in contrast to Barros *et al.* (1992a) who reported a transient decrease in LH levels post-rbIFN$_\alpha$ administration in cattle following interferon administration. The reason for this is unclear. Perhaps, it could be a species-specific effect. However, this rules out the possibility of rbIFN$_\alpha$ action as a locally synthesized regulator of hypothalamic-hypophyseal function in buffaloes, as suggested by some earlier reports in other species (Ho-Yen and Carrington, 1987; Khan *et al.*,

1989). However, the results are consistent with an earlier report that like trophoblastic proteins, rbIFN$_\alpha$ is unlikely to influence hypothalamic-hypophyseal function (Godkin *et al.*, 1984).

The decrease in progesterone levels in all 3 doses of rbIFN$_\alpha$ was temporarily associated with an elevation of rectal temperature in treated buffaloes, similar to earlier reports in cattle (Newton *et al.*, 1990; Plante *et al.*, 1991; Barros *et al.*, 1992a). Both these effects become dissipated after repeated administration of rbIFN$_\alpha$ as seen in cattle (Newton *et al.*, 1990; Plante *et al.*, 1991). A similar effect of decreasing progesterone after rbIFN$_\alpha$ administration was observed by Newton *et al.* (1990). From their observations, the workers had suggested that this was probably due to a) redistribution of blood flow away from reproductive tract and b) catabolism of progesterone due to transient increase in the rectal temperatures. This postulation may also be applicable in buffaloes treated with rbIFN$_\alpha$ since similar observations were recorded in the present investigation. The progesterone decreasing effect of rbIFN$_\alpha$ was not observed in the studies of Plante *et al.* (1989) possibly due to infrequent blood sampling. Therefore, rbIFN$_\alpha$ like other luteal function extension agents (McMillan *et al.*, 1986; Thatcher *et al.*, 1986; Heyman *et al.*, 1987; Thatcher *et al.*, 1987) could be useful in augmenting fertility in buffaloes post-insemination.

5.3.3 Influence of rbIFN$_\alpha$ on Rectal Temperatures

Reproductive processes in bovines are sensitive to environment induced hyperthermia (Biggers *et al.*, 1987; Putney *et al.*, 1988). Administration of rbIFN$_\alpha$ is known to cause a hyperthermic response in cattle (Newton *et al.*, 1990; Plante *et al.*, 1991; Barros *et al.*, 1992) and sheep (Parkinson *et al.*, 1992). Therefore, rectal temperature recordings were carried out for longer duration and at shorter time intervals (1 hour), in the 3 groups of rbIFN$_\alpha$ treated buffaloes as well as control buffaloes. A sharp rise in the rectal temperatures was observed in all the 3 groups of rbIFN$_\alpha$ treated buffaloes (Figs. 16 to 18), after the first injection of rbIFN$_\alpha$. There was no

significant difference (P>0.01) between duration for which the rectal temperatures remained elevated for different doses of rbIFN$_\alpha$ administered. From the present results it is also evident that the rectal temperatures treatments of rbIFN$_\alpha$ differed significantly from the control group of buffaloes (P<0.01). However, there was no significant difference (P>0.01) between different rectal temperatures in the four groups of buffaloes with respect to time. It has also been demonstrated that the rectal temperatures subsequent to the first injection of rbIFN$_\alpha$ were not influenced by subsequent doses of rbIFN$_\alpha$. This effect may be attributed to the process of adaptation and development of refractoriness to the effects of rbIFN$_\alpha$ by the buffaloes. In the present investigation, the temporal rectal temperature measurements at hourly intervals indicate to the fact that the treatment of rbIFN$_\alpha$ caused an acute rise in rectal temperatures that subsided within 7 hours of treatment, irrespective of dosage, which is in contrast to the studies in cattle (Plante *et al.*, 1988, 1989; Newton *et al.*, 1990; Plante *et al.*, 1991) and sheep (Parkinson *et al.*, 1992), where the rectal temperatures returned to the basal levels after a longer duration of time. Further, no difference between doses was evident as 4 mg/day and 16 mg/day dose of rbIFN$_\alpha$ caused similar rise of rectal temperature subsequent to the first injection (Figs. 16 to 18). Elevated rectal temperatures in cattle are known to have a negative influence on embryonic development, secretion of bTP-1 from the embryos, and alteration of uterine proteins steroid secretion (Biggers *et al.*, 1987; Putney *et al.*, 1988). However, in the present study the rbIFN$_\alpha$ administration do not seem to cause elevation of rectal temperatures to levels that are beyond the normal physiological range in this species. Further, the effect is rapidly dissipated and there is no recurrence after subsequent rbIFN$_\alpha$ injections in all dose groups indicating similarity in the actions of rbIFN$_\alpha$ in buffaloes as found in cattle (Newton *et al.*, 1990; Plante *et al.*, 1991).

In summary, it can be inferred from the present investigation that the reported hyperthermic effect of rbIFN$_\alpha$ in cattle (Plante *et al.*, 1988, 1989; Newton *et al.*, 1990; Plante *et al.*, 1991; Barros *et al.*, 1992) and sheep

(Garverick *et al.*, 1992) is less pronounced in buffalo. The transient increases in rectal temperature subsequent to the first injection of rbIFN$_\alpha$ with dissipation of the effect within a shorter duration of time (4 to 6 hours) suggests that this effect of rbIFN$_\alpha$ may not be sufficient to override the luteotrophic effect of rbIFN$_\alpha$ in this species as also been seen in cattle (Plante *et al.*, 1991).

5.4 INFLUENCE OF rbIFN$_\alpha$ ON OXYTOCIN MEDIATED PGFM RELEASE

PGF$_{2\alpha}$ of uterine origin is the primary luteolytic agent in domestic farm animals and most ruminants (Knickerbocker *et al.*, 1988). In ruminant species, an obvious potential strategy for blocking luteolysis is the alteration of PGF$_{2\alpha}$ secretion, through secretion of embryonic interferons or ovine or bovine trophoblastic protein-1 (Flint *et al.*, 1991; Salamonsen *et al.*, 1991; Bazer *et al.*, 1991; Roberts *et al.*, 1992). On account of its high degree of sequence homology with embryonic trophoblastic proteins (Imakawa *et al.*, 1989) rbIFN$_\alpha$ is known to inhibit PGF$_{2\alpha}$ secretion thereby maintaining CL function (Bazer *et al.*, 1991; Roberts *et al.*, 1992). The release of PGF$_{2\alpha}$ can be assessed by measuring concentrations of prostaglandin following injection of oxytocin (NewComb *et al.*, 1977). It is well established that a reflection of PGF$_{2\alpha}$ secretion can also be assessed by measuring the stable metabolite of PGF2$_\alpha$ namely PGFM (Kindahl *et al.*, 1976; Guilbault *et al.*, 1984).

It is known that cattle injected with oxytocin in the late luteal phase of normal length estrous cycles had increased levels of PGFM levels (Zollers *et al.*, 1989). This model, therefore, is widely accepted and used to indicate and determine the influence of rbIFN$_\alpha$ on oxytocin mediated PGFM release (Plante *et al.*, 1991; Garverick, 1992) in bovines. In the current study, it is evident that rbIFN$_\alpha$ (16 mg/day) administration on days 14 through 16 suppressed oxytocin mediated PGFM release (on day 17) significantly ($P<0.05$; Fig. 28). The results are in agreement with reports in cattle (Bazer *et al.*, 1986; Knickerbocker *et al.*, 1988; Plante *et al.*, 1990, 1991; Garverick *et al.*, 1992). These results further complement CL life span prolonging effect of rbIFN$_\alpha$ as

evident in the earlier experiments. The dose of interferon found optimal in prolonging the estrous cycle lengths was used in this experiment. The time of rbIFN$_\alpha$ administration is apparently critical in evoking this antiluteolytic effect as in bovines. The secretion of bTP-1 is maximum around day 16-19 of early pregnancy (Bartol *et al.*, 1985). These results further confirm that the antiluteolytic effect of embryonic interferons is mediated through a suppression of PGFM release during early pregnancy as seen in cattle (Robinson *et al.*, 1999; Mann and Lamming, 2001) and sheep (Fincher *et al.*, 1984; Vallet *et al.*, 1988). It is probable that rbIFN$_\alpha$ may decrease PGF$_{2\alpha}$ secretion by either interfering with upregulation of oxytocin receptors (Flint *et al.*, 1991), inhibition of prostaglandin synthesis (Gross *et al.*, 1988a) or through synthesis of an endogenous prostaglandin synthetase inhibitor (Gross *et al.*, 1988b). The conclusive evidence of rbIFN$_\alpha$ overriding the oxytocin mediated PGFM release in the current study is the extension of CL life span in all the 6 buffaloes administered rbIFN$_\alpha$ (Figs. 27 to 29). No such effect was found in cycles with only oxytocin treatment that resulted in CL life span comparable to that of the pre- and post- control cycles in these animals (Fig.29). Though no comparable studies are found in buffaloes, the results presented a situation similar to that of early pregnancy which is associated with suppression of endometrial release of PGF$_{2\alpha}$ induced by oestradiol (Thatcher *et al.*, 1984) and oxytocin (Lafrance and Goff, 1985; Putney *et al.*, 1989). Exogenous oxytocin is also known to induce a rise in PGF$_{2\alpha}$ secretion in *in vitro* bovine endometrial culture systems (Tysseling *et al.*, 1998), while interferons related to rbIFN$_\alpha$ suppressed PGF$_{2\alpha}$ secretion (Salamonsen *et al.*, 1988; Helmer *et al.*, 1989; Salamonsen *et al.*, 1989). However, the difference in mean PGFM levels post oxytocin administration between rbIFN$_\alpha$ treated and untreated groups demonstrated inhibitory action on PGF$_{2\alpha}$ release by rbIFN$_\alpha$ in this species. In conclusion, it can be inferred from the present investigation that rbIFN$_\alpha$ extends luteal life span in buffaloes by inhibiting secretion of PGF$_{2\alpha}$ as has also been seen in earlier investigations carried out in cattle (Plante *et al.*, 1990, 1991; Garverick *et al.*, 1992; Salfen *et al.*, 1995) and sheep (Parkinson *et al.*, 1992).

5.5 GENERAL DISCUSSION

The standardization and validation of PGFM enzyme immunoassay for the first time in buffaloes has been achieved for the first time in buffalo plasma. The development of a sensitive enzyme immunoassay for PGFM was an essential pre-requisite for conducting the objectives of the study. The high sensitivity of the assay ensured that a very small quantity of blood plasma (20 µl) was required for estimation of PGFM. The standard curve of PGFM assay encompassed the complete range of physiological variations of PGFM levels in buffalo. The development of an EIA for PGFM also is advantageous in several ways. Apart from being non-radioactive in nature, the assay does not require any prior extraction of the plasma sample. The assay is relatively quicker and the results are available in a day's time.

The validation of the enzyme immunoassay was performed biologically in 5 different ways. Secretion of PGFM during (a) cyclicity, (b) peri-estrus period, (c) early pregnancy, (d) in buffaloes suffering from reproductive tract infections and (e) in buffaloes having cystic *corpora lutea*, indicated clearly that the profiles of the hormone in all cases, followed the patterns along expected lines as indicated in literature. Some more additional information was generated for the first time in buffaloes, viz., the occurrence of the pulsatile release of PGFM during luteolysis when the hormone was estimated in blood samples collected at short intervals of time. This finding is significant in establishing pulsatile secretion of PGFM in buffaloes, which was not observed in a study in this species (Batra and Pandey, 1983) due to a longer sampling intervals of 6 hours as compared to 1 hour in the present study. The pulsatility was absent when the hormone was estimated in blood samples collected at 4 hours or 12 hours. The difference in PGFM levels in physiologically different states suggested the possible use of PGFM levels as a marker for monitoring reproductive health in this species. Further, the conformation of the continued secretion of high PGFM levels in buffaloes suffering from reproductive tract infections as well as cystic ovarian conditions also suggested the possibility of using this hormone assay procedure from the practical view point of identifying cases with reproductive disorders.

Further, the confirmation of the continued secretion of high PGFM levels in buffaloes suffering from reproductive tract infections as well as cystic *corpora lutea* also suggests the possibility of using this hormone assay procedure from the practical view point of identifying cases with reproductive disorders.

The present study clearly brings out for the first time the significant role played by interferon-α (rbIFN$_\alpha$) in *corpus luteum* maintenance in Murrah buffaloes. This effect of rbIFN$_\alpha$ was evident in the extension of estrous cycle lengths and progesterone profiles, in the present investigation. The *corpus luteum* secretes progesterone which is an important regulator of estrous cycle length and is essential for maintenance of pregnancy (Niswender and Nett, 1988). The synthesis and secretion of progesterone is coordinated by luteotrophic and luteolytic mechanisms. As both of these mechanisms are present concurrently during the estrous cycle and early pregnancy, the regulation of progesterone synthesis and secretion may be dependent upon a balance of these stimuli (Garverick *et al.*, 1992). In the present investigation, out of the three doses of rbIFN$_\alpha$ (4, 8 and 16 mg/day on days 14 to 16 of cycle) the dose of 16 mg/day significantly ($P<0.01$) extended the estrous cycle lengths, favouring a slower decline of progesterone in the treated animals, thereby resulting in *corpus luteum* maintenance. These results are suggestive of the fact that due to the similarity in sequence homology and receptor affinity between rbIFN$_\alpha$ and ruminant trophoblastic proteins (oTP-1 and bTP-1), this effect may have resulted. The administration of rbIFN$_\alpha$ around the time of maternal recognition of pregnancy (day 14 to 16 of cycle) can maintain *corpus luteum* function in buffaloes. This view is supported by earlier investigations in sheep (Stewart *et al.*, 1989) and cattle (Plante *et al.*, 1989; Garverick *et al.*, 1992; Geshi *et al.*, 2001).

The present investigation also for the first time provides information on the temporal changes in rectal temperatures in buffaloes incident to rbIFN$_\alpha$ administration at short intervals of time of 1 hour, for all the three doses of rbIFN$_\alpha$ administered (on days 14 through 16 of cycle), beginning 12 hours

before up to 80 hours after the first injection of rbIFN$_\alpha$. The earlier studies in cattle (Newton *et al.*, 1990; Barros *et al.*, 1992; Meyer *et al.*, 1995) and sheep (Parkinson *et al.*, 1992) differed from the present investigation in rectal temperature measurements for shorter duration of time. All the three doses of rbIFN$_\alpha$ elicited an acute response subsequent to the first injection of rbIFN$_\alpha$.This transient rise in rectal temperature could possibly be due to the antiviral property of interferons (Pontzer *et al.*, 1988, Roberts *et al.*, 1989; Pontzer *et al.*, 1991). However, the present investigation clearly demonstrated that this transient and acute response was limited only to the initial injection of rbIFN$_\alpha$ and did not appear in subsequent injections as also reported by investigations in cattle (Newton *et al.*, 1990). This refractoriness may be due to changes in prostaglandin synthesis as suggested by this study.

The results of the present investigation suggest that rbIFN$_\alpha$ administration in buffaloes does not elevate rectal temperatures to the levels comparable with the reports in cattle (Newton *et al.*, 1990; Barros *et al.*, 1992; Meyer *et al.*, 1995) or sheep (Parkinson *et al.*, 1992) which may not limit its *corpus luteum* regulating function in this species, or influence early embryonic development and secretion as reported for cattle (Biggers *et al.*, 1987; Geisert *et al.*, 1988).

The present study also brings out the influence of rbIFN$_\alpha$ treatment on temporal changes in the peripheral levels of progesterone and LH, which are indicative of luteal function. All the three doses of rbIFN$_\alpha$ caused a transient depression in the plasma progesterone profiles similar to the reports in cattle (Newton *et al.*, 1990; Plante *et al.*, 1991; Barros *et al.*, 1992), however, for a shorter duration. But surprisingly, no changes in the peripheral levels of LH was noticed. These results suggest that rbIFN$_\alpha$ in buffaloes may not influence progesterone secretion by interfering with the LH support for luteal synthesis of progesterone as reported in cattle (Barros *et al.*, 1992a). However, the transient progesterone suppressing effect of rbIFN$_\alpha$ may be attributed to the changes in vascular dynamics and progesterone catabolism, caused by associated increase in rectal temperatures as also suggested in studies on

cattle (Newton *et al.*, 1990). It has been also evident in the present investigation that the transient changes elicited by the first injection of rbIFN$_\alpha$, is dissipated with the subsequent injections. This response can be attributed to the gradual adaptation resulting in refractoriness to the antiviral property of rbIFN$_\alpha$. The hypothesis that the progesterone suppressing effect is a subsidiary effect of rbIFN$_\alpha$ administration is supported by the studies in ovariectomized cattle with progesterone releasing implants, where rbIFN$_\alpha$ failed to decrease plasma progesterone levels (Barros *et al.*, 1992). No change in the levels of LH subsequent to the rbIFN$_\alpha$ administration rules out the possibility of interferon alpha indirectly influencing CNS functions through opiate system or directly influencing function of pituitary gonadotrophs in this species as reported by investigations on biological effects of interferon-α (Nakashima *et al.*, 1987; Kuriyama *et al.*, 1990; Vankelecom *et al.*, 1990; Blatteis *et al.*, 1991). This view is also supported by the investigations in sheep (Godkin *et al.*, 1984). The present findings suggest that rbIFN$_\alpha$ administration in buffaloes may not limit its effectiveness as a fertility enhancing molecule as suggested by studies carried out in cattle (Barros *et al.*, 1992a,b).

Finally in the current study, the oxytocin-induced release of PGFM in buffaloes administered rbIFN$_\alpha$ through the intramuscular route (on days 14 through 16 of the cycle) was significantly reduced ($P<0.05$) as compared to the untreated control cycles. Though the precise mechanism of suppression of PGFM release is unknown, this effect may be attributed to a) A reduction in oxytocin receptor numbers as the increase in oxytocin receptor expression is known to be associated with an increased responsiveness of the non-pregnant cyclic cows to an oxytocin challenge in terms of PGFM release. Both PGFM release and oxytocin m-RNA expression are known to be suppressed by the presence of the embryo (Robinson *et al.*, 1999). Due to the high sequence homology between ruminant trophoblastic proteins and rbIFN$_\alpha$ this effect may have been elicited; b) A direct inhibition of prostaglandin synthesis (Gross *et al.*, 1988a) or a production of prostaglandin synthetase inhibitor

(Gross *et al.*, 1988b). As there is a species specific difference in the regulation of $PGF_{2\alpha}$ reflected by PGFM release any one or both of the above mechanisms may be effective in reduction of PGFM release in this species. However, it seems that the PGFM suppressing effect of $rbIFN_{\alpha}$ may be due to the disruption of release mechanisms rather than biosynthetic mechanisms as reported by other investigators (Roberts *et al.*, 1992). In the present investigation, $rbIFN_{\alpha}$ treated cycles were significantly ($P<0.01$) extended as compared to the oxytocin or treated or pre- and post- treatment control cycles.

These results raise several intriguing questions regarding the $rbIFN_{\alpha}$ mode of action in buffaloes. It is known that the critical time for presence of a conceptus to exert its antiluteolytic effect is day 16 of cycle in bovines (Northey and French, 1980). Therefore administration of $rbIFN_{\alpha}$ initiated on day 14 was possibly effective in attenuating the PGFM release, thereby maintaining the *corpus luteum* and extending intraoestrus intervals, through antiluteolytic mechanisms. This view is supported by investigations in sheep (Parkinson *et al.*, 1992) and cattle (Meyer *et al.*, 1995; Salfen *et al.*, 1995).

In conclusion, the earlier reports of interferon alpha administration in bovines prolonging the life span of *corpus luteum* in bovines (Plante *et al.*, 1989) has also been corroborated in the present studies in buffaloes. The evidence that this extension of life span of *corpus luteum* is brought about by some mechanisms probably similar to that seen in cattle, which reduced the secretion of PGFM in circulation has been amply provided by the reduction of oxytocin stimulated PGFM release in buffaloes administered 16 mg/day dose of interferon-α. These results provide an opening for practical application of using interferon alpha administration of 14-16 days post-insemination for improving fertility in this species.

CHAPTER - 6

Summary and Conclusions

6. SUMMARY AND CONCLUSIONS

A study to investigate the influence of recombinant bovine interferon alpha (rbIFN$_\alpha$) on maintenance of *corpus luteum* (CL) life span and function was undertaken on riverine Murrah buffaloes.

In order to meet the objectives of the study, a simple, sensitive and direct enzyme immunoassay was standardized and validated in buffalo plasma. The PGFM-EIA was further applied to quantitate peripheral PGFM levels, during various reproductive states in buffaloes, viz., a) peri-estrus period, b) cyclicity, c) early pregnancy, d) reproductive tract infections, and e) ovarian cystic conditions.

Influence of intramuscular administration of 3 doses (4, 8 and 16 mg/day) of rbIFN$_\alpha$ on days 14-16 post-estrus on CL life span was studied.

Changes in rectal temperatures associated with the rbIFN$_\alpha$ administration was studied comprehensively by recording rectal temperatures at hourly intervals beginning 12 hours before up to 80 hours after the first injection of rbIFN$_\alpha$.

The endocrine profile associated with administration of 3 doses of rbIFN$_\alpha$ was studied with respect to peripheral progesterone and LH levels using sensitive radio immunoassay and enzyme immunoassays standardized for our laboratory conditions.

Jugular blood samples were drawn daily for LH quantitation. Blood samples were also collected on every alternate day before rbIFN$_\alpha$ administration and daily after rbIFN$_\alpha$ administration during the estrous cycle. Quantitation of LH and progesterone was also carried out in frequent blood samples collected at 15 minutes interval beginning 2 hours before until 8 hours after the first injection of rbIFN$_\alpha$ for all 3 doses.

Influence of rbIFN$_\alpha$ on oxytocin mediated PGFM release was studied by exogenous administration of 50 IU of oxytocin on day 17 of the estrous cycle without prior rbIFN$_\alpha$ treatment as well as groups of experimental animals after rbIFN$_\alpha$ treatment on days 14 through 16 of the estrous cycle. Blood samples were collected at 15 minutes interval 2 hours before and 6 hours after oxytocin administration from buffaloes belonging to both the experimental groups.

Standardization and Biological Validation of PGFM

A direct enzyme immunoassay for PGFM was standardized in unextracted buffalo plasma using 20 µl of plasma sample, with high sensitivity (20 pg/ml) and reproducibility. The intra- and inter- assay variations of the assay were 6.3 and 11.6 percent, respectively.

The PGFM EIA standardized in the buffalo plasma was further used to quantitate physiological levels of PGFM during various physiological states of peri-estrus, cyclicity, early pregnancy, reproductive infections and ovarian cystic conditions for the purpose of biologically validating the PGFM assay.

For the first time in buffaloes, a pulsatile pattern of PGFM was observed in buffaloes in frequent blood samples collected during the peri-estrus period. The basal levels of PGFM ranged from 95.63 ± 17.33 to 113.57 ± 31.35 pg/ml, while the peak levels ranged from 286.60 ± 24.18 to 591.60 ± 23.30 pg/ml. These results suggest that PGFM release is pulsatile prior to luteolysis in buffaloes. The pulses of PGFM coincide with decreasing progesterone levels during the peri-estrus period.

In cyclic animals, the PGFM levels remained basal during early and mid luteal phase increasing gradually from day 4 prior to estrus to reach peak levels 2 days prior to estrus, declining thereafter to lowest levels during estrus. The PGFM levels increased simultaneously with the decreasing progesterone levels, similar to the trend observed during peri-estrus period.

In early pregnant buffaloes, the PGFM levels did not show any significant ($P>0.01$) increase during days 15-18 of cycle or the expected days

of luteolysis. Simultaneously, the progesterone levels were sustained at significantly ($P<0.01$) higher levels during days 14 to 23 as compared to the luteal phase levels of estrous cycle. The results clearly depicted the influence of early embryo on the pattern of progesterone and PGFM release.

Buffaloes with chronic reproductive tract infections had significantly ($P<0.01$) higher plasma PGFM levels throughout the sampling period showing no decline as seen in cycling buffaloes during the luteolysis. The corresponding progesterone levels stayed very low throughout the sampling period. The levels of progesterone remained basal throughout the period of sampling indicating the suppression of luteal functions due to the influence of high levels of PGFM.

Buffaloes with cystic conditions had significantly elevated progesterone levels along with basal levels of PGFM. It was evident that low levels of PGFM could not cause luteal regression, thereby resulting in significantly ($P<0.01$) higher levels of progesterone as compared to the luteal phase levels of cyclic buffaloes.

Influence of rbIFN$_\alpha$ on estrous cycle length

Among the three doses of rbIFN$_\alpha$ administered exogenously to the buffaloes on days 14 through 16 of the estrous cycle, the dose of 16 mg/day of rbIFN$_\alpha$ was found to significantly ($P<0.01$) extend the estrous cycle length of buffaloes, as compared with the pre- and post- treatment control cycles. The results suggest that rbIFN$_\alpha$ administration extended the life of *corpus luteum* which is also reflected by the plasma progesterone profiles. The possible mechanism for the extension of luteal function could be through activation of anti-luteolytic processes similar to those recorded during early pregnancy in cattle by earlier investigators.

Influence of rbIFN$_\alpha$ on rectal temperature

The three different doses of rbIFN$_\alpha$ increased the rectal temperature after the first injection of rbIFN$_\alpha$. No significant difference ($P>0.01$) was

observed between the duration for which the rectal temperatures remained elevated. The rectal temperature after registering a small rise returned back to the pre-administration levels in 6 to 7 hours. However, no increase in rectal temperature was seen after subsequent injections of rbIFN$_\alpha$ in animals irrespective of rbIFN$_\alpha$ the dose used. The transient increase in rectal temperature was within the physiological range. So, the brief duration for which the rectal temperature registered an increase may be due to the antiviral and anti-inflammatory properties of rbIFN$_\alpha$.

Influence of rbIFN$_\alpha$ on temporal changes in plasma progesterone and LH levels

The very first injection of rbIFN$_\alpha$ in each of the three experimental groups transiently decreased plasma progesterone levels for a brief period of 3.5 to 4.5 hours. No significant ($P>0.01$) difference was observed between the pre-administration and post-administration levels of progesterone during subsequent injections of rbIFN$_\alpha$. Administration of rbIFN$_\alpha$ did not influence peripheral plasma LH levels irrespective of the dose applied. It is evident from the study that rbIFN$_\alpha$ does not interfere with the LH support for CL function, while the progesterone suppression for a brief period after the first injection of rbIFN$_\alpha$ may be due to the rearrangement of vascular dynamics or catabolism of progesterone on account of associated transient elevation of the rectal temperature as suggested by earlier reports on similar observations in cattle.

Influence of rbIFN$_\alpha$ on Oxytocin Mediate PGFM-Release

The mean plasma PGFM release as induced by exogenous oxytocin challenge, exhibited a significant ($P<0.05$) elevation post-oxytocin administration. The dose of 16 mg/day of rbIFN$_\alpha$ on day 14 through 16 significantly suppressed the oxytocin mediated PGFM release when compared with the PGFM increase following oxytocin administration (143.66 ± 3.94 vs. 214.00 ± 24.63 pg/ml). Further, the estrous cycle lengths of rbIFN$_\alpha$ plus oxytocin treated buffaloes were significantly higher ($P<0.01$) than those recorded for oxytocin treatment alone. This clearly suggested the inhibitory

influence of systemic administration of rbIFN$_\alpha$ on the amplitude of PGFM release. It is apparent from this investigations that in buffaloes the CL maintenance and resulting extension of estrous cycle lengths is mediated through the anti-luteolytic effect of rbIFN$_\alpha$.

In conclusion, from the present study, the following salient findings emerge :

- The PGFM EIA standardized and validated in the study, is sufficiently reliable and specific for estimation of PGFM levels in buffaloes that can serve as an useful indicator of reproductive health.
- Exogenous administration of recombinant cytokines like rbIFN$_\alpha$, through the systemic route can extend *corpus luteum* life span in buffaloes.
- Only the first injection of rbIFN$_\alpha$ transiently decreased plasma progesterone levels associated with rise in rectal temperatures for a brief period. This effect on either progesterone decline or rectal temperature rise was not seen in buffaloes during subsequent injections irrespective of interferon dose administered.
- Administration of rbIFN$_\alpha$ did not suppress the peripheral LH levels, implying therefore, that rbIFN$_\alpha$ administration did not interfere with luteotrophic support for CL function.
- rbIFN$_\alpha$ significantly ($P<0.01$) reduced oxytocin mediated PGFM release, thereby maintaining CL functions through an antiluteolytic mechanism.

The above findings suggest that the mechanism governing early recognition of pregnancy through release of proteins similar to interferon alpha as seen in cattle, sheep and goats by earlier investigations may also apply in case of buffaloes.

The present investigation opens a distinct possibility that administration in buffaloes on days 14 to 16 post-insemination could help improve fertility in this species.

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