CHAPTER - III

Materials and Methods

The present investigation “Gynaeco-clinical ultrasonographic studies with reference to nutritional management of transition period on uterine involution and postpartum fertility in Jaffarabadi buffaloes” was carried out at the Department of Veterinary Gynaecology and Obstetrics in collaboration with Cattle Breeding Farm, Junagadh Agricultural University, Junagadh, with the support of “AICRP on Nutritional and Physiological Interventions for Enhancement of Reproductive Performance of Animals” of AAU, Anand during the period from June 2015 to December 2017.

3.1 Location and Climate of the Region

The geographical location of Cattle Breeding Farm, Junagadh is on 21.29°N latitude and 70.27°E longitudes. It has an average elevation of 107 meters (351 ft.) from mean sea level. It has a tropical climate, with three distinct seasons observed, a mild winter from November to February, a hot summer from March to June and a monsoon from July to October. The Arabian Sea and the Gulf of Cambay are also influential factors affecting the climate and weather of Cattle Breeding Farm. Junagadh faces adverse climatic condition in the summer months with the temperature ranging from 38°Celsius to 45°Celsius. In the winter months, the temperature ranges from 10° Celsius to 25° Celsius with rainfall of 1000 to 1200 mm annually during months of June to September.

3.2 Selection of Animals

For the present study, 40 advanced pregnant (~8.5 months) pluriparous buffaloes were selected from Cattle Breeding Farm. Pregnancy status of all the animals selected was confirmed through breeding records (AI dates) and gynaeco-clinical examinations. The experiment was initiated at about 1.5 month prepartum by dividing the selected animals randomly into control and supplementation (treatment) groups. All the 40 animals had calved normally without any complication.

3.3 Management of Animals on the Farm

All buffaloes were maintained in well ventilated hygienic sheds and were fed green fodder, hay and compounded concentrate, as per standard feeding schedule.
followed on the farm. The pregnant buffaloes were isolated 45 days before calving to facilitate proper observations. The buffaloes approaching the parturition were segregated and kept in calving pen. Immediately after calving the weight and sex of calves were recorded. These buffaloes were transferred from calving pen to the milking group of buffaloes two days after calving and were kept under stall feeding system. The buffaloes were fed with the jaggery 1 kg/day on the day of parturition till 4 to 5 days postpartum. They were hand milked twice daily in milking byre and suckling of the calves was the routine practice of the farm. The buffaloes were cleaned and sprinkled with water twice daily, particularly during summer, and they had free access to drinking water.

3.4 Health Care of Animals

All buffaloes were appropriately vaccinated against Foot and Mouth Disease, Haemorrhagic Septicaemia and Black Quarter. They were also tested annually for possibilities of Brucellosis, Johne’s disease and Tuberculosis. The animals were checked regularly for presence of ecto-parasites, if any. The faecal samples and blood smears were also screened periodically for detection of parasitic infestations and protozoan diseases, respectively. As a routine, all buffaloes were dewormed before and after monsoon.

3.5 Experimental Groups

A total of 40 advanced pregnant (~8.5 months) Jaffarabadi buffaloes of 2nd to 4th parity were selected at random from the animals maintained at Cattle Breeding Farm, Junagadh. These animals were maintained under optimum nutritional (20 kg seasonal green + ad lib dry fodder + concentrate mixture to meet the DCP & TDN requirement of animals), hygienic and managemental conditions. The animals were subdivided into two equal groups, each of 20, as control and treatment groups at random to evaluate the effect of area-specific multi-minerals and by-pass fat supplementation on the uterine involution, postpartum fertility and blood biochemical and hormonal profile and associated peridata.

Group - I: Control Group

A total of 20 advanced pregnant Jaffarabadi buffaloes were included in this group and were managed as per routine farm feeding schedule (concentrate, min mix, green fodder, dry fodder) till calving and then up to 90 days postpartum. These 20 animals were equally divided into 2 subgroups (C1 & C2). In C1 subgroup (n=10), Inj. Stimvet 5 ml (containing Se, Zn, Cu, Mn; 25, 200, 75 and 50 mg, respectively;
Wellcon Animal Health Pvt. Ltd., Mumbai) was given twice, first around 45 days before parturition and second on the day of parturition. Moreover, herbal ecbolic bolus (Exapar bolus, Ayurvet Ltd., Delhi) was given orally 2 boli bid for first 4 consecutive days postpartum in five buffaloes and the rest five were kept as herbal control. While buffaloes of C2 subgroup (Stimvet control, n=10) were kept as control without Inj. Stimvet 5 ml, but five buffaloes received herbal ecbolic bolus (Exapar bolus, Ayurvet) orally 2 boli bid for first 4 consecutive days postpartum and the rest five served as herbal control.

**Group - II: Treatment Group**

A total of 20 advanced pregnant Jaffarabadi buffaloes in addition to routine farm feeding schedule were additionally supplemented orally daily with 50 g of chelated area specific mineral mixture (developed by ANRS, AAU, Anand) and 150 g of by-pass fat (Sunergy, Polchem, Maleshiya) with concentrate for 6 weeks prepartum and 2 weeks postpartum, then the level of by-pass fat was increased as per the milk production @ 15 g/liter of milk produced maximum up to 200 g/head/day till 60 days postpartum. These 20 animals were also equally divided into 2 subgroups (T1 & T2), and were treated with either Stimvet injection or herbal ecbolic Exapar bolus as described above for subgroups C1 and C2, keeping respective controls.

The important puerperal events, viz., calf birth weight & sex, placental weight, placental expulsion time, prolapsed, retained fetal membranes, if any, etc. were recorded. Per-rectal (precalibrated operators’ hand) as well as ultrasonographic (5-7 MHz transrectal transducer) evaluation of uterine and cervical involution as well as ovarian follicular activities were carried out at day 7, 15, 30, 45 and 60 postpartum for randomly selected few animals in both the control and treatment groups. The animals were then followed till 120 days postpartum for postpartum first oestrus, breeding and service period/conception rate.

3.6 **Per-Rectal Observations**

The examination of the genitalia of selected animals was carried out by per-rectal palpation (hand-calibration method of the operator as per Dobson-Hill, 2009), wherein the diameter and width of both the cervix and the uterus were estimated by hand previously calibrated and correlated to a ruler. For uterine involution following criteria were considered by giving the non-parametric scores to the parameters.
1. **Position of**
   1. Cervix
   2. Gravid uterine horn
   3. Non-gravid uterine horn

   Uterine position was scored as per Scully *et al.* (2013) on a 0-3 scale [0= uterus and uterine horn returned to the previously non-gravid state (within the pelvic canal) and 1-3= uterine body and horns falling further over the pelvic brim i.e., between the pelvic brim and abdominal cavity].

2. **Size of uterine horns** (Comparative size; > cervix 1; = cervix 2; < cervix 3)

3. **Tone and consistency of uterine horns** (low tonicity 1; moderate tonicity 2; good tonicity 3)

### 3.7 Ultrasonographic Examination

The transrectal ultrasonography was performed using a real-time B-mode ultrasound scanner (DB355M, IMAGO.S, ECM, France) equipped with a 5.0-7.5 MHz rectal probe designed for intra-rectal placement on 7th, 15th, 30th, 45th and 60th day postpartum. During the scanning, the relevant images were frozen on the screen and the measurements were taken using an inbuilt calliper system.

#### 3.7.1 Preparation of Animals for Ultrasonography

Buffaloes were restrained in standing position for scanning. The rectum was evacuated by back racking prior to scanning. During the examination, safety precautions were taken with regards to the safety of the operator, the patient and the equipment to avoid injury or damage. For a clear visualization of the images, care was taken to avoid direct light on the monitor.

#### 3.7.2 Interpretation of Sonograms

The ultrasonic image is formed of various shades of gray, white and black dots depending upon the penetration and reflection of the sound waves. The penetration and reflection in turn depends upon the consistency of the tissue, and accordingly it was monitored as:

1) **Hyperechoic**: Brighter/white coloured image with more reflection of sound waves indicating the solid medium/tissue.

2) **Hypoechoic**: Greyish white image with moderate to less reflection of sound waves indicating the thick liquid or loose tissue.
3) Anechoic: Black coloured image indicating no reflection of sound waves suggesting watery or thin medium.

### 3.7.3 Technique of Scanning

The experimental animals were scanned as per the steps given here under.

1) Buffaloes were scanned trans-rectally using a rectal probe.

2) A protective transparent plastic sheath was used to cover the transducer and contact jelly was applied over the transducer beneath the sheath, before insertion.

3) After back racking, the transducer was inserted per-rectally and was placed on the postpartum cervix.

4) The transducer was positioned dorsally and perpendicular above the cervix and relevant measurements of cervical diameter and thickness were recorded.

5) The transducer was positioned dorsally and perpendicular over the uterus and sound beams were directed dorso-ventrally. These beams passed through the tissue beneath in a sagittal plane to get a relevant view of organ.

6) Approximately 10 cm ahead from the gross bifurcation, the uterine diameter and thickness of uterine wall were measured as per the guidelines of Melendez et al. (2004) for both the involuting gravid and non-gravid horns. Two diameters of the previously gravid and non-gravid uterine horns were evaluated. The first measurement was taken from serosa to serosa (A to B) to obtain the outer diameter of the uterine horn (Sheldon and Dobson, 2000). The second measurement was from mucosa to mucosa (C to D) to obtain the lumen diameter of the uterine horn. The difference between the first and the second measurements was divided by 2 to arrive at the estimate of the thickness of the uterine wall (Parikh et al., 2017).

7) Caruncles located approximately at the same position of scanning for uterus were measured for its length and width.

### 3.8 Factors Affecting Uterine Involution

Among 40 buffaloes under study, the following factors were studied during the experiment to evaluate its effect on involution of uterus.

a) Sex of calf (Male/Female)

b) Birth weight of calf (< and > 32 kg)
c) Placental expulsion time (< and > 5 hours)
d) Weight of placenta (< and > 6 kg)
e) Side of pregnancy (Right and left horn)
f) Gestational Length (< and > 309 days)

3.9 Blood Collection and Assay Procedure

All the Jaffarabadi buffaloes included in the study were subjected to blood collection (7 ml) from jugular vein in heparinized vacutainers at days -45, -30, -7, 0, 7, 15, 30, 45 and 60 (day 0 is day of parturition) in both the groups. The plasma was separated immediately after the collection of blood by centrifugation for 10 minutes at 3000 rpm. The plasma was stored in sterilized plastic storage vials of 5 ml capacity at -80°C with a drop of merthiolate as preservative until analyzed for various hormonal, metabolic and mineral attributes.

3.9.1 Estimation of Metabolic Parameters

The parameters like blood glucose and plasma total protein, total cholesterol, non-esterified fatty acids and beta-hydroxybutyrate were estimated using standard procedures and diagnostic assay kits.

Blood Glucose

The fresh blood samples were analysed for blood glucose by direct strip method using Accu-Chec Integra Kit (NIPRO Diagnostics, Mumbai). A drop of freshly collected blood was touched against the front edge of the strip near the black notch to draw up blood. As soon as a sufficient amount of blood was drawn up, the meter beeped and the test result displayed automatically.

Total Protein

Estimation of total protein was done by Biuret method (Doumas, 1975) using standard procedure and assay kits of Crest Biosystems, a Division of Coral Clinical Systems, Goa with the help of Chemistry Analyzer (Nova 2021, Analytical Technologies Pvt. Ltd., Vododara).

Plasma Total Cholesterol

Total plasma cholesterol was estimated by CHOD/PAP method (Allain et al., 1974) using standard procedure and assay kits procured from Crest Biosystems, a Division of Coral Clinical Systems, Goa, with the help of Chemistry Analyzer (Nova 2021, Analytical Technologies Pvt. Ltd., Vododara).
Non-Esterified Fatty Acids (NEFA):

Its estimation was carried out by colorimetric method devised by DeVries et al. (1976), using standard procedure and diagnostic kits (Randox Lab Ltd., Crumlin, UK) as under.

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<tr>
<th></th>
<th>Reagent Blank</th>
<th>Standard</th>
<th>Sample</th>
<th>Sample Blank</th>
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<tbody>
<tr>
<td>Distilled water</td>
<td>10 µl</td>
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<tr>
<td>Standard</td>
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<td>Sample</td>
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<tr>
<td>Reagent 1</td>
<td>200 µl</td>
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It was mixed and incubated for 5 minutes at 37°C

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<thead>
<tr>
<th></th>
<th>Reagent 2</th>
<th>Sample</th>
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<tbody>
<tr>
<td>Standard</td>
<td>400 µl</td>
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</tr>
<tr>
<td>Sample</td>
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<td>400 µl</td>
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The content was mixed again and incubated for 5 minutes at 37°C. The cuvettes were then inserted into the standard RX MONZA flowcell holder and the readings were taken against 550 nm wavelength spectrum on Eppendorf BioSpectrometer®, Eppendorf, India limited.

Beta-Hydroxybutyrate (BHBA)

The estimation of BHBA was carried out using standard procedure and ELISA assay kits (Cayman Chemicals, USA) as per the method of Galan et al. (2001). In standard BHBA wells, 50 µl of standard solution was added in two wells and in sample wells 50 µl of sample was added. The reaction was initiated by adding 50 µl of the developer solution to all wells being used and later incubated at 25°C in the dark for 30 minutes. The absorbance was then read at wavelengths of 445-455 nm using a plate reader. The average absorbance of each standard and sample was calculated. The absorbance value of the standard was subtracted from itself and all other values (both standards and samples). This was taken as the corrected absorbance. The corrected absorbance values of each standard were plotted as a function of the final BHBA concentration (mM). The values of the BHBA samples were calculated using the equation obtained from the linear regression of the standard curve by substituting the corrected absorbance values for each sample into the equation (using an online application, http://www.elisaanalysis.com/app).

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\text{BHBA (mM)} = \left[ \frac{\text{corrected absorbance}-(y \ \text{intercept})}{\text{slope}} \right] \times \text{dilution}
\]
### 3.9.2 Estimation of Macro-Minerals Profile

**Plasma Calcium**

Plasma calcium concentration was estimated by OCPC method (Bagainski, 1973) using standard procedure and assay kits procured from Crest Bio-systems, a Division of Coral Clinical Systems, Goa, with the help of Chemistry Analyzer (Nova 2021, Analytical Technologies Pvt. Ltd., Vadodara).

**Plasma Inorganic Phosphorus**

Inorganic phosphorus was estimated by Molybdate UV method (Fiske and Subbarow, 1925) using standard procedure and assay kits procured from Crest Biosystems, a Division of Coral Clinical Systems, Goa, with the help of Chemistry Analyzer (Nova 2021, Analytical Technologies Pvt. Ltd., Vadodara).

**Plasma Magnesium**

Magnesium was estimated by Calmagite method (Gindler et al., 1971) using standard procedure and assay kits procured from Crest Bio-systems, a division of Coral Clinical Systems, Goa, with the help of Chemistry Analyzer (Nova 2021, Analytical Technologies Pvt. Ltd., Vadodara).

### 3.9.3 Estimation of Micro-Minerals Profile

The blood plasma samples (0.5 ml each) were wet digested using mixture (4.5 ml) of perchloric-nitric and sulphuric acid in the ratio of 1:4:1 on hot plate in volumetric flasks till white transparent residues. The residues were then diluted with triple glass distilled water and volume was made up to 25 ml. These aliquots were then transferred to a 50 ml storage vials. The aliquots of all digested samples were stored at room temperature until analyzed. Plasma zinc, iron, copper, cobalt and manganese were determined on ICP-OES (Optical Emission Spectrometer; Model Optima 7000 DV; Perkin-Elmer, USA) machine against standard curves at the Micro-Nutrient Research Project (ICAR), BA College of Agriculture, AAU, Anand.

### 3.9.4 Estimation of Hormonal Parameters

The blood plasma samples were subjected to estimation of hormones, viz., Progesterone, Estradiol and Cortisol as well as PGF\(_2\alpha\) metabolites as follows.

**Plasma Progesterone Assay**

Plasma progesterone was estimated by employing standard RIA technique of Kubasic et al. (1984) at Radio-Immuno-Assay (RIA) Laboratory of the Veterinary College, AAU, Anand. Labelled antigen (with I\(^{125}\)), antibody coated tubes and
standards were procured from Immunotech-SA, Marsielle Cedex, France. Coated tubes after addition of samples and tracer were incubated for one hour at room temperature while continuously shaking (300-350 rpm). After incubation the material was decanted carefully on absorbing rack. These tubes were read in gamma counter (Multi well Gamma counter, PC-RIA MAS Stratec Biomedical AG Germany). The values were calculated against a standard curve of 0.1 to 60 ng on the logit log paper and represented as ng/ml. The sensitivity of the assay was 0.1 ng/ml. The intra- and inter-assay coefficients of variation were 5.4 and 9.1 per cent. The cross-reactivity of the antibody with progesterone, 20α-dihydro-progesterone and 17α-hydroxyprogesterone was 100, 0.96 and 0.13 per cent, respectively.

**Plasma Estradiol-17β Assay**

Plasma profile of estradiol-17β was estimated by employing standard RIA technique of Robertson (1979) at RIA Laboratory in Anand. Labelled antigen (with I\(^{125}\)), antibody coated tubes and standards were procured from Immunotech-SA, Marsielle Cedex, France. Coated tubes after addition of samples and tracer were incubated for three hours at room temperature while continuously shaking (300-350 rpm). After incubation, the material was decanted carefully on absorbing rack. These tubes were read in gamma counter (Multi well Gamma counter, PC-RIA MAS Stratec Biomedical AG Germany). The values were calculated against a standard curve of 10 to 5348 pg on the logit log paper and represented as pg/ml. The sensitivity of the assay was 9.58 pg/ml. The intra- and inter-assay coefficients of variation were 14.4 and 14.5 per cent. The cross-reactivity of the antibody with 17 α-estradiol, estradiol-3-glucuronide, estriol, estrone and testosterone was 0.002, 0.033, 0.26, 2.31 and <0.0038 per cent, respectively.

**Plasma Cortisol Assay**

Plasma cortisol concentration was estimated by employing standard RIA technique of Brock et al. (1978). Labelled antigen (with I\(^{125}\)), antibody coated tubes and standards were procured from Immunotech-SA, Marsielle Cedex, France. The assay procedure followed was same as used for plasma progesterone. Here the values were calculated against a standard curve of 10 to 2000 ng on the logit log paper and represented as ng/ml. The sensitivity of the assay was 0.1 ng/ml. The intra- and inter-assay coefficients of variation were 5.8 and 9.2 per cent. The cross-reactivity of the antibody with corticosterone, 17 α-hydroxy-progesterone and cortisone was 8.4, 3.5 and 1.5 per cent, respectively.
Plasma PGF$_2$$\alpha$ Metabolites (PGFM)

The levels of 13, 14-dihydro-15-keto PGF$_2$$\alpha$ metabolites (PGFM) were estimated from blood plasma samples using standard procedure and EIA diagnostic kits (Cayman Chemical Co., Ann Arbor, USA) as described by Mishra et al. (2003). To non-specific binding (NSB) wells 100 µl EIA buffer was added and 50 µl EIA buffer was added to B$_0$ wells. Fifty (50) µl of the aliquot was added from the already prepared #8 tube to both the lowest of the standard wells. Then 50 µl from tube #7 was added to each of the next two standard wells. This procedure was continued until all the standards were aliquot. Then 50 µl of the sample was added per well. All the samples were assayed at a minimum of two dilutions. Fifty (50) µl of 13, 14-dihydro-15-keto PGF$_2$$\alpha$ AChE tracer was added to each well, except the total activity well (TA) and blank (Blk). Fifty (50) µl of the 13, 14-dihydro-15-keto PGF$_2$$\alpha$ EIA antiserum was added to each well, except the TA and the Blk wells. The plate was covered with a plastic film and incubated for 18 hours at 4ºC. The plate was then developed using Ellman’s Reagent by reconstituting 20 ml of the reagent to develop 100 wells.

The plate readings were evaluated at wavelengths between 405 and 420 nm. The average readings from the plate NSB were subtracted from B$_0$ average which gave the corrected B$_0$ or corrected maximum binding. The B/B$_0$ was calculated for the remaining wells. The per cent B/B$_0$ was plotted for standards versus 13, 14-dihydro-15-keto PGF$_2$$\alpha$ concentration using a linear (Y) and log (X) axes and performed a 4 parameter logistic fit.

3.10 Statistical Analyses

The uterine and cervical involution in terms of diameters on different days and total time in animals of control and treatment groups were compared using ANOVA and ‘t’ test (Snedecor and Cochran, 1994). The data generated on plasma progesterone, estradiol-17β, cortisol and PGFM; and other biochemical and mineral constituents were analyzed using standard statistical procedures (ANOVA, DMRT and ‘t’ test) on SPSS software version 20.00 to know the variations within and between the groups for each trait and the results were interpreted.