CHAPTER 3
MATERIALS AND METHODS

Apparently healthy female dogs of different breeds, presented for elective neutering, a part of Animal Birth Control\(^1\), at the veterinary hospital, at Thiruvananthapuram, Kerala, formed the base of this study. The period of study was from April 2011 and April 2015, 49 months.

3.1 Selection of Animals

All the female dogs of different breeds and age groups presented for sterilisation procedures were screened for general health status and suitability for neutering procedures. Out of the 46 dogs screened, 24 dogs were selected finally for the study. Their health status was assessed by physical examination and history taken prior to subjecting them to the sterilisation procedure, ovaro-hysterectomy.

3.2 Design of Study

The dogs were categorised based on the age group and laparotomy site. The twenty four female dogs were broadly divided into 2 groups each consisting of 12 animals based on attainment of puberty viz. pre-pubertal and pubertal. The pre-pubertal group included dogs that were 7 months of age or less, which had not yet experienced their first oestrum. Six of these dogs were subjected to bilateral ovaro-hysterectomy through the right flank incision (Group I). They were identified as 1.1(1) to 1.1(6). The other six animals also underwent ovaro-hysterectomy but through the ventral mid line incision (Group II). The second group dogs were identified as 1.2(1) to 1.2(6). The post pubertal group included dogs that were above 7 months of age and who had experienced one or more oestrum. They were also

\(^1\) Part of responsible ownership
subjected to the same surgical procedure, bilateral ovaro-hysterectomy, six dogs through the flank incision (Group III) and remaining six through the ventral mid line incision (Group IV). Group III dogs were identified as 2.1(1) to 2.1(6) and Group IV dogs were identified as 2.2(1) to 2.2(6). The team of surgeon and assistants and the theatre remained the same throughout the study in order to facilitate uniformity.

Table 1: Design of the study

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of Dogs</th>
<th>Age</th>
<th>Stage</th>
<th>Site of celiotomy</th>
<th>Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>6</td>
<td>≤7 months</td>
<td>Pre-pubertal(1)</td>
<td>Right flank(1)</td>
<td>1.1(1) to 1.1(6)</td>
</tr>
<tr>
<td>II</td>
<td>6</td>
<td>≤7 months</td>
<td>Pre-Pubertal(1)</td>
<td>Ventral mid line(2)</td>
<td>1.2(1) to 1.2(6)</td>
</tr>
<tr>
<td>III</td>
<td>6</td>
<td>&gt;7 months</td>
<td>Post-pubertal(2)</td>
<td>Right flank(1)</td>
<td>2.1(1) to 2.2(6)</td>
</tr>
<tr>
<td>IV</td>
<td>6</td>
<td>&gt;7 months</td>
<td>Post-Pubertal(2)</td>
<td>Ventral mid line(2)</td>
<td>2.2(1) to 2.2(6)</td>
</tr>
</tbody>
</table>

3.3 Clinical Study

All the dogs were operated upon to remove both the ovaries and uterus, aseptically through either a flank incision (Group I & III) or ventral mid line incision (Group II & IV). The signalment and blood values were taken prior to surgery on the same day. They were monitored during the surgery as routine. All treatment and follow up procedures were the same in all the four groups. Following surgery, all the dogs were evaluated physically and clinically with complete blood counts, serum biochemistry study, hormonal evaluation and ultrasonographs 6 months after surgery. The animal behaviour was assessed 6 months after the surgery.
3.3.1 Signalment and anamnesis

All details pertaining to age, breed, history of previous illness, concurrent disease and treatment of each animal were recorded. It was ensured that at the time of surgery the animal was in proper health and free from any sickness.

3.3.2 General clinical examination

Physiological parameters like rectal temperature, pulse rate, rate of respiration, and colour of visible mucous membranes were checked and ensured to be normal. Body condition was assessed as good, fair, poor, emaciated and only those dogs whose condition were good were included in the study.

3.3.3 Preparatory procedures

The dogs were fasted for 8 hours prior to the procedure. They were given a shampoo bath about 4 - 5 hours before presenting for surgery. Their weight was taken on presentation for the surgical procedure and owner's informed consent received.

3.3.4 Anaesthetic regime

The dogs were premedicated with Atropine sulphate\(^2\) (Plate 1) at the dose rate of 0.04mg/kg body weight and with Xylazine\(^3\) (Plate 2) at the dose rate of 1mg / kg body weight intramuscularly (Plate 3).

General anaesthesia was induced with Ketamine\(^4\) (Plate 4) at the rate of 5 - 10mg/kg body weight and Midazolam\(^5\) (Plate 5) at the dose rate of 0.01 mg/kg.

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\(^2\) Atropine, Neon Laboratories, Andheri E, Mumbai

\(^3\) Xylaxin, Indian Immunologicals, Hyderabad

\(^4\) Zokent, Aesmira, Andheri E, Mumbai

\(^5\) Medzol, Themis medicare Ldt, Uttarakand
The animal was observed and a combination of Ketamine and Midazolam was administered in a 1:1 ratio intravenously as per requirement to maintain anaesthesia. The dogs were intubated to maintain a patent airway.

An IV fluid line was connected for administration of necessary IV fluids, maintenance dose of Ketamine and Midazolam, Ceftraixone sulbactam\(^6\)(Plate 6), Meloxicam\(^7\) (Plate 7) and Metaclopromide hydrochloride\(^8\)(Plate 8) on the operating table by fixing a scalp vein set in the cephalic vein and applying a micro pore tape to ensure stable positioning during the surgical procedure. The sterile surgical pack was opened and laid out (Plate 9).

**3.3.5 Preparation of site**

The site of surgery was prepared for aseptic surgery.

**Group I & III: Flank approach:** The right flank region, from costal arch to the anterior one third of the lateral aspect of the thigh, was shaved (Plate 10), scrubbed with Chlorhexidine Gluconate 1.5%w/v and cetrimide IP 3%\(^9\) solution (Plate 11), and painted with Povidone Iodine 5% w/v\(^{10}\) solution (Plate 12 & 13).

**Group II & IV: Ventral mid line approach:** The entire ventral abdominal region, from sternal region to the caudal border between the hind limbs, was shaved (Plate 14), scrubbed with Chlorhexidine Gluconate 1.5%w/v and cetrimide IP 3% solution, and painted with Povidone Iodine 5% w/v solution (Plate 15).

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\(^6\) Sulbavet C, Provet Animal Health Pvt Ltd, TNRWA 50, Tagore Nagar, Kadavanthara, Cochin, Kerala

\(^7\) Melonex, Intas Pharmaceutical Ltd, Matoda- 382 210, Ahmedabad

\(^8\) Emenorm, Intas Pharmaceutical Ltd, Matoda- 382 210, Ahmedabad

\(^9\) Savlon, Johnson & Johnson, Bangalore Rural

\(^{10}\) Drez Solution, Stedman Pharmaceuticals, Tamil Nadu
3.3.6 Surgical procedure

3.3.6.1: Incision

Group I & III: Flank approach: The animal was placed on left lateral recumbency. An incision was placed on the right flank, at 45 degree angle backward, starting 2 fingers away from the last floating rib (Plate 16). This was extended for about 2 cm to 2.5cm backward (Plate 17&18). The muscle layers (Obliquus Externus Abdominis, Obliquus Internus Abdominis and Transverse Abdominis) were separated by blunt dissection, in the line of the muscle fibres of each layer until the abdominal cavity was reached.

Group II & IV: Ventral mid line approach: An incision was placed on the ventral mid line caudal to the umbilicus (Plate 19) extending backward for 2.5cm (Plate 20 & 21). The incision was deepened into the deeper layer of Rectus Abdominis, Linea Alba, till the abdominal cavity was reached. In some cases the incision was extended backward or forward for proper placement of ligatures on the uterus or ovaries.

3.3.6.2 Removal of ovaries and uterus

Flank Approach: On entry into the abdominal cavity the right ovary was located at 45 degree angle between the plane of the vertebral column and floating rib at the caudal border of the kidney. It was exteriorized (Plate 22 & 23) and confirmed by locating the body of uterus and left ovary. The suspensory ligament of the ovaries was released. The ovarian vessels were first located and ligated anterior to the ovary with anchorage using the two clamp method (Plate 24 - 30). The ovarian pedicle was checked for bleeding point (Plate 31). The left ovary was removed similarly (Plate 32). The uterine vessels and base of uterus were then ligated at the junction of
the body of uterus and cervix and transfixed (Plate 33, 34). Both the ovaries, cornua and body of uterus were removed.

**Ventral mid line approach:** On entry into the abdominal cavity, the body of uterus is located below (dorsal to) the bladder. It was exteriorized using a spay hook (Plate 35). Both ovaries and the uterus were located and their presence confirmed. The procedure of ligation and removal of ovary and uterus was the same as above (Plate 36 - 45).

### 3.3.6.3 Closure of laparotomy incision

**Abdominal muscles**

The abdominal cavity was checked and observed to rule out any haemorrhaging prior to closure of the site (Plate 46).

**Flank approach:** The abdominal muscle opening was closed using Braided Coated Polygalactin 910\(^\text{11}\). The peritoneum and Transverse Abdominis together, Obliquus Internus Abdominis and Obliquus Externus Abdominis were sutured separately using continuous sutures (Plate 47).

**Ventral mid line approach:** The incision was closed using Braided Coated Polygalactin 910. A layer of continuous suture pattern (Plate 48 & 49) was applied at level of linea alba and surrounding tissue. A second layer of Connel’s suture (Plate 50) was applied on rectus abdominis as a reinforcement using the same suture material.

**Skin and subcutis**

**Flank approach:** Subcutaneous continuous sutures of the cross mattress pattern (Plate 51) followed by intradermal sutures (Plate 52) on the skin with Braided Coated Polygalactin 910 were applied to close the incision (Plate 53 & 54).

\(^\text{11}\) Vicryl NW 2346, Johnson and Johnson, Aurangabad
Ventral mid line approach: Subcutaneous continuous sutures of the cross mattress pattern (Plate 55) followed by vertical mattress sutures at tension points (Plate 56) and horizontal mattress sutures in between on the skin, using Braided Coated Polygalactin 910 were used to close the incision (Plate 57).

3.3.7 Post-operative care

The suture line was then cleaned with Chlorhexidine Gluconate 1.5% w/v and cetrimide IP 3% solution (Plate 58), Povidone Iodine spray\textsuperscript{12} was applied and a sterile paraffin dressing\textsuperscript{13} (Plate 59) was applied using micropore adhesive tape. A cotton protective coat was then prepared and fitted on to cover the body and surgical site (Plate 60). The procedure was the same for both approaches (Plate 61 - 63). This dressing was repeated every alternate day if the coat remained unsoiled and fresh clean coats were put on. Ceftraixone sulbactam\textsuperscript{14} was given once daily at a dose rate of 25 mg/kg body weight, along with Serratiopeptidase\textsuperscript{15} tablets twice daily. The dressing is continued for one week or till complete healing was observed. All owners were given specific instructions to add fibre and control the carbohydrate and fat in the diet. Exercise was added as a compulsory activity either by playing with the pet or taking for walks or by letting loose for at least 8 to 10 hours if the household had more than one animal.

\textsuperscript{12} Drez Spray, Stedman Pharmaceuticals Ltd, Tamil Nadu

\textsuperscript{13} Jelonet, sminth & nephew healthcare Pvt Ltd, Andheri East, Mumbai

\textsuperscript{14} Sulbavet C, Provet Animal Health Pvt Ltd, TNRWA 50, Tagore Nagar, Kadavanthara, Cochin, Kerala

\textsuperscript{15} Lyser Forte, Comad Chemicals, Unit 3 village, Dassomajra, Baddi, Himachal Pradesh
3.4 Evaluation of Experiment

3.4.1 Clinical evaluation

After the surgery, all animals were examined and assessed clinically during the post operative period which was divided into two phases, (i) Phase 1: immediate post operative phase from day 0 up to day 7 and (ii) Phase 2: post operative phase from the 8th day up to 3 months. The first phase was assessed under the supervision of the veterinarian and recorded by way of a score card - Immediate Post Operative Clinical Assessment (Annexure 1) (Firth and Haldane, 1999; Duncan and Lascelles, 2004) which was answered by the owner, with reference to the following points: ease of movement (day 1, 7 and by 3 months), visibility and ease of dressing the site, cooperation of the pet in dressing, suture breakage, wound dehiscence, oozing from the site, ease to squat for urination (day 1, 7 and by 3 months), bleeding/pus from the vagina, occurrence of proestrual bleeding. The owner assigned scores from 0 to 4, signifying the lowest intensity to the highest intensity.

Similarly, beyond 3 months, the animals were assessed for pain, scar formation at the site, ease of squatting for urination, body score to assess weight gain, occurrence of any complications like involuntary urination, pus discharge from vagina etc. Incidence of long term complications like fractures, sicknesses or other concurrent diseases, attraction to male dogs especially during the breeding season, occurrence of proestrual bleeding or pseudo pregnancy were also noted. The assessment was recorded with the help of a score card - Long Term Clinical Assessment (Annexure 2) (Holton et al., 2001; Morton et al., 2005) which was scored by the owner. The answers were assigned scores from 0 to 4 (lowest intensity to highest intensity).
The scores of both score cards were then statistically analysed. These scores were in the Likart Scale and did not follow normality, hence non-parametric tests were employed. In order to compare the scores, between the groups (age and site of approach separately), the Wilcoxon – signed rank sum test was used.

3.4.2 Haematological evaluation

Blood samples were collected in sterile K3 EDTA blood collection tubes and Sodium Citrate 3.8% ESR vials and the values were assessed on the day of and prior to surgery and 6 months after surgery. The parameters that were assessed include Haemoglobin, Total leucocyte count, Differential count, Erythrocyte Sedimentary Rate, Platelet Count and Packed Cell Volume. The methods used for assessment were – SLS method for Heamoglobin (Oshiro et al., 1982), Flow Cytometry method for Total Leucocyte Count and Differential Count (MacKenzie and Pinder, 1987; Liu et al., 1993), Westergren method for Erythrocyte Sedimentary Rate (Westergren, 1957), Impedance technique using Coulter Principle for Platelet Count (Sandhaus et al., 2002) and Cumulative High detection method for Packed Cell Volume (Klee et al., 2000). In order to compare the values obtained, statistically before and after surgery with reference to different age groups and surgical approaches, the paired t – test was employed. Both the age groups and methods along with their interaction, was compared using the two way analysis of variance (Two Way ANOVA).

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16 CML Biotech (P) Ltd, Angamaly Kerala
17 CML Biotech (P) Ltd, Angamaly Kerala
3.4.3 Biochemical evaluation

Blood samples were collected in sterile clot activator vials on day of and prior to surgery and 6 months after surgery to assess the Serum Calcium and Serum phosphorus using the following methods: BAPTA technique for Serum Calcium assessment (Bourguignon et al., 2014) and Enzymatic method for Serum Phosphorus assessment (Berti et al., 1988). In order to compare the values obtained, statistically, before and after surgery with reference to different age groups and surgical approaches, the paired t–test was employed. Both the age groups and methods along with their interaction, was compared using the two way analysis of variance (Two Way ANOVA).

3.4.4 Hormonal evaluation

Blood samples were collected in sterile clot activator vials on day of and prior to surgery and 6 months after surgery to assess the Serum estrogen (estradiol) and Serum progesterone level. Electrochemiluminescence immune assay (ECLIA) technique (Prieto et al., 2010) was used to assess the both hormones mentioned. The values obtained, were compared statistically employing the paired t- test to assess the significant difference before and after surgery with reference to different age groups and surgical approaches. Both the age groups and methods along with their interaction, was compared using the Two way analysis of variance (Two Way ANOVA) was administered to compare the age groups and methods along with their interaction.

18 AcCuvet Plus, Labtech
3.4.5 Ultrasonographic evaluation

All animals were subjected to ultrasonographic scan at 6 months and beyond after surgery, to assess the condition of the ovarian pedicle, uterine stump and to record any other ultrasonographically visible changes within the abdominal cavity if present. The machine used was DP-2200 Vet\textsuperscript{19} digital Ultrasonic Diagnostic Imaging System using an electronic micro-convex array transducer with frequency from 3.5 MHz to 6 MHz.

The animals were kept on dorsal recumbency after shaving the hair coat. The ovarian position was imaged using 6MHz transducer to rule out functional ovarian remnant at the ovarian pedicle. The ovarian pedicle was normally not visible unless there were functional corpora lutea. The site of the ovarian pedicle was at the caudal border of the kidney near the 5\textsuperscript{th} lumbar vertebrae. At times the animal had to be tilted slightly to get the picture properly.

The uterine stump was normally viewed below the urinary bladder, sometimes indenting the bladder wall inwards. At times the stump could lie to the side or obliquely over the bladder. 3.5MHz transducer was also used to view these areas.

The abdominal organs were viewed with the 3.5MHz transducer to locate any changes which could have arisen consequent to the surgery like the possibility of strangulation of the bowel, fistula formation or changes in the kidney, liver or other organs which may have resulted directly from and after effect of the surgery.

The evaluation was directed to locate any hyperechoic, hypoechoic or mixed echogenicty changes at the above mentioned sites and to correlate such changes with the clinical, haematological, biochemical, hormonal and behavioural changes to aid in

\textsuperscript{19} Mindray, Shenzen, China
arriving at a conclusion that would achieve the aims of the study. The absence of ovarian and uterine tissue alone could not confirm the absence of the tissue.

3.4.6 Behaviour evaluation

The behaviour of all the animals was assessed beyond 6 months of surgery, in detail using a questionnaire similar to C Barq assessment – Behavioural Assessment (Annexure 3) (Kim et al., 2006 & Van den berg et al., 2006). The questionnaire was given at the last ultrasonography evaluation session. The behavioural aspects analysed were obedience, trainability, basic aggression, fear anxiety, separation anxiety, excitability, attachment level, acceptance of other species, exploring nature, attention seeking (destructive and non destructive), nervousness, activity level, boredom level, excessive barking and strange repetitive behaviour. Each behaviour was assessed using one or more situations which are commonly faced during the lifetime of the animal and recording its response using the scores mentioned above. The owners were required to assign scores, ranging from 0 to 4 (lowest to highest), for the different behavioural responses normally elicited by the situations. A zero indicated the least reaction and 4 indicated maximum reaction.

These scores were then statistically analysed using the Wilcoxon – signed rank sum test. Since the data was in the Likart scale, thus not following normality, this non-parametric test had to be employed. The values of the all ovaro-hysterectomised dogs of the study were considered to assess the basic behaviour of ovaro-hysterectomised dogs. Separate relation to age and method of approach was analysed to find out whether age and approach of ovaro-hysterectomy had an effect on the behaviour of the ovaro-hysterectomised dogs.