CHAPTER - IV

MATERIALS AND METHODS

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ANIMAL CARE AND PREPARATION:

Wistar rats of either sex weighing 150 - 250 g. housed in separate cages were employed for the study. Rats had free access to water and food (Hindustan Lever - rat pellets) except 12 hrs. before and 2 hrs. after infliction of wound. Animals were weighed on wounding day as well as on the day they were sacrificed / study concluded.

Stringent aseptic measures were found not necessary. Therefore semi-aseptic care was undertaken throughout. No Local/systemic chemotherapeutics were used.

All surgical instruments and polypropylene tube meant for subcutaneous implantation were sterilized by dipping in 70% alcohol, rinsed and dried before use. Rats showing any sign of infection/severe hemorrhage on anticoagulants were excluded from study and treated.

 Animals were randomly selected for different groups for three wound models - incision, excision and dead space wound. Two to four hours before wounding dorsal skin hair was clipped off with clippers. No hair depilators were used.

ANAESTHESIA:

Wounds were inflicted under light anesthesia by pentobarbitone 30 mg/kg i.p. supplemented with ether if and when required.

Wounding day was taken as 'O' day.
Table 4-1: SHOWING ALLOCATION OF ANIMAL GROUPS TO VARIOUS WOUND MODELS AND TREATMENT:

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>INCISION WOUND</th>
<th>EXCISION WOUND</th>
<th>DEAD SPACE WOUND</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td>*</td>
<td></td>
<td>*</td>
</tr>
<tr>
<td>BOTROPASE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1 ml i.p.</td>
<td>*</td>
<td>*</td>
<td></td>
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<tr>
<td>BOTROPASE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.4 ml i.p.</td>
<td>*</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>HEPARIN s.i.</td>
<td>*</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>1000u/kg.</td>
<td>*</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>HEPARIN s.c.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2000u/kg.</td>
<td>*</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>WARFARIN p.o.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1mg/kg.</td>
<td>*</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>FIBRIN TOPICAL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 mg.</td>
<td>*</td>
<td>*</td>
<td>--</td>
</tr>
<tr>
<td>THROMBIN TOPICAL</td>
<td></td>
<td></td>
<td>--</td>
</tr>
<tr>
<td>25 u</td>
<td>*</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>MENADIONE i.p.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2mg/kg.</td>
<td>*</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>HEPARIN + FIBRIN</td>
<td></td>
<td></td>
<td>--</td>
</tr>
<tr>
<td>HEPARIN + THROMBIN</td>
<td></td>
<td></td>
<td>--</td>
</tr>
<tr>
<td>WARFARIN + FIBRIN</td>
<td></td>
<td></td>
<td>--</td>
</tr>
<tr>
<td>WARFARIN + THROMBIN</td>
<td></td>
<td></td>
<td>--</td>
</tr>
<tr>
<td>MENADIONE + WARFARIN</td>
<td></td>
<td></td>
<td>--</td>
</tr>
</tbody>
</table>

* Studied
— Not studied.
FIG. 4-1. **WOUND MODELS** - (a). INCISION, (b). EXCISION, (c). DEAD SPACE - POLYPROPYLENE TUBE IMPLANTATION.

PARAMETERS:
(a). WOUND BREAKING STRENGTH.
(b). 1. WOUND CONTRACTION.
2. EPITHELIZATION TIME.
(c). 1. GRANULOMA BREAKING STRENGTH.
2. TOTAL COLLAGEN.
3. HEXOSAMINE CONTENT.
4. HISTOLOGICAL EVALUATION.
GROUP AND ALLOCATION OF ANIMALS:

Rats were grouped into (Table 4-1) 35 groups n = 8-10, for physical, biochemical and histological evaluation of wound parameters.

WOUND MODELS: WOUNDING TECHNIQUES AND PARAMETERS STUDIED:

Three wound models were employed - (a) incision, (b) excision and (c) dead space wound to study the action of haemocagulants (Topical/systemic) and anticoagulants on the following parameters respectively: (a) wound breaking strength (b) wound contraction, epithelization period and (c) granuloma breaking strength, hexosamine and hydroxy proline content and histological features with haemotoxylin and eosin or special collagen staining Vangieson's or Masson's Trichrome.

INCISION WOUND:

On the depilated dorsal paravertebral region 6 cm. long incision wounds were made through full thickness of skin on either side of the mid-line (121) (Fig. 4-1-a). Wound edges were approximated by a cm. apart interrupted sutures with black silk thread no. 3. Sutures were removed on 7th post-wounding day. On 11th post-operative day rats were sacrificed and the breaking strength was measured by continuous water flow method (85).

METHOD OF DETERMINATION OF WOUND BREAKING STRENGTH:

(LEE - METHOD):

The principle of this method is to apply a tearing force at a constant rate and observing the force required to just gape the wound at the point of application of force (Fig. 4-2).
"W" - WOUND, DOTTED LINE.
"R" - WATER RESERVOIR (5 L. CAPACITY).
"T" - RUBBER TUBING.
"C" - POLYPROPYLENE GRADUATED CONTAINER.

"A\}' ALLIS FORCEPS APPLIED ON
"B}' PREDETERMINED LINES.
FORCEPS "A" IS FIXED TO STAND &
"B" FORCEPS IS CONNECTED TO
CONTAINER, "C" VIA PULLEY.

FIG. 4-2. BREAKING STRENGTH ASSEMBLY —
INSET — (1). HEALED WOUND WITH FIXED FORCEPS.
(2). GAPED WOUND — THE END POINT
FOR BREAKING STRENGTH MEASURE.
Two Alli's forceps were applied one on either side on a marked line, half-cm. away from the wound. One of the forceps was fixed to a stand and the other was connected over a pulley to a suspended light polypropylene graduated container. Water was allowed to go steadily from the reservoir into the container. The weight of the water collected just causing gaping of wound was noted. Three-four readings were taken for each incision wound and the average of such 6-8 readings for each animal was taken as a measure of individual wound breaking strength. From these average values the group mean and s.e. were calculated.

EXCISION WOUND:

This model was employed to study the progressive changes in wound area and time required for epithelization.

A 500 mm$^2$ circular piece of skin of interscapular region was excised, in its full-thickness after having marked it out with a rubber seal (Fig. 4-1-b) by the method of Morton and Malone (122). The progressive changes in the wound area were noted planimetrically. The wound size was measured regularly on alternate days from zero day. Wound tracings were taken on a transparent paper securing the animal gently in resting position, to avoid any distortion. Tracings were transferred to 1mm$^2$ graph sheet (Fig. 4-3). From the scaled paper the traced wound area was read out with provision that if the traced line cut 1 mm$^2$ box by 50% or more it was read as one and anything less than 50% was discarded. Wound area changes were expressed as percent change taking the inflicted wound size (500 mm$^2$) as 100%. The data were plotted on log-day-probit scale. Median wound closure time ($W_{c50}$) was calculated by Litchfield and Wilcoxon method (123) (appendix - e).
FIG. 4-3 SEQUENTIAL CHANGES IN WOUND AREA

DAY - 3

DAY - 5

DAY - 7

DAY - 9

DAY - 11

DAY 13

DAY 15

DAY - 17

DAY 19
The period of epithelization was measured in days required for falling of eschar leaving no raw wound behind.

**DEAD SPACE WOUND:**

This wound model consists of implanting of foreign body cotton pellet (68), viscose sponge (124), silicone implant (125) to harvest granulation tissue. In the present study 3 x 30 mm polypropylene tube was implanted beneath the paravertebral lumbar skin. A transverse 0.5 cm. incision was made in the upper part of lumbar region and through this slit the tube was implanted lengthwise about a cm. lateral to the midline (Fig. 4-1-c). On 11th post wounding day the harvested granulation tissue along with tube was carefully dissected out. Along the length, the tubular granuloma was cut vertically to obtain approximately two equal pieces of granulation tissue. Plate - 1 shows (a) harvested granuloma in situ, (b) enucleated granuloma ensheathing the tube (c) assembly to measure granuloma breaking strength. The breaking strength of each piece of granuloma was measured by the same method as in case of incision wound study. The average of two readings of each granuloma were taken to calculate mean and s.e. for all the groups.

The pieces of granulation tissue were dried for 24 hrs. at 60°C and their weight was noted. Hydroxyproline and hexosamine of this tissue were estimated spectro-photometrically by the method of Neuman and Logan (126) and Elson and Morgan (127) respectively. The values for these parameters are expressed as mg/g tissue (appendix - b).

A small piece of granulation tissue was fixed in a formalin and processed for histological evaluation. Paraffin sections were taken at 6 microns, stained with Haemotoxylin and Eosin, Vangieson's or Masson's trichome as special staining of collagen. Histological features were studied under light microscope (appendix - c & d).
DRUG ADMINISTRATION: (Table 4-2)

As alluded to in chapter-3, two topical procoagulants - thrombin and fibrin, a systemic haemocoagulant Botropase and two anticoagulants - heparin and warfarin were investigated for their effects on wound healing. In addition vitamin-K alone and with warfarin was also used to cover anticoagulant effects of warfarin so as to discern if any, direct effect of warfarin on healing process (appendix - f).

(i) Thrombin 25 units and fibrin - 100 mg were directly applied to incision, excision wounds only once after wounding - a common mode of their use. Since these haemogoagulants are not used systemically dead space wound model was not included for thrombin and fibrin.

(ii) Botropase a systemic haemogoagulant was administered once a day intraperitonially, at doses, 0.1 ml and 0.4 ml. The doses employed were based on pilot experiment results and by computing human doses for rats as recommended by Paget and Barnes (128). Since, Botropase was supplied in solution the vehicle used to dispense Botropase was also tested.

(iii) Clinically recommended (113) lower dose-range for subcutaneous heparin and oral warfarin were selected to administer these systemic anticoagulants. In pilot studies this dose (after computing for rats) its half and double quantities were tested to achieve adequate anticoagulation without any risk of hemorrhage. On this basis heparin was used in 1000 and 2000 u/kg. and warfarin 1 mg/kg.
### Table 4-2: Scheme of Drug Administration

<table>
<thead>
<tr>
<th>DRUG</th>
<th>DOSE</th>
<th>ROUTE</th>
<th>REMARKS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. BOTROPASE</td>
<td>0.1 ml</td>
<td>i.p.</td>
<td>one coagulant unit of Botropase clots standard fibringen solution in 30 seconds.</td>
</tr>
<tr>
<td></td>
<td>0.4 ml</td>
<td>i.p.</td>
<td></td>
</tr>
<tr>
<td>2. THROMBIN</td>
<td>25 u.</td>
<td>topical</td>
<td>once immediately after wounding.</td>
</tr>
<tr>
<td>3. FIBRIN</td>
<td>100 mg</td>
<td>topical</td>
<td>once immediately after infliction of wound.</td>
</tr>
<tr>
<td>4. HEPARIN</td>
<td>1000 u/kg.</td>
<td>s.c.</td>
<td>Clotting time monitored: as two parts, given at 10 hrs. interval, pretreatment 4 hrs. before wounding.</td>
</tr>
<tr>
<td></td>
<td>2000 u/kg.</td>
<td>s.c.</td>
<td>3 days pretreatment.</td>
</tr>
<tr>
<td>5. WARFARIN</td>
<td>1 mg/kg.</td>
<td>p.o.</td>
<td>prothrombin time monitored.</td>
</tr>
<tr>
<td>6. MENADIONE</td>
<td>2 mg/kg.</td>
<td>i.p.</td>
<td>(for excision wound alternative day-treatment)</td>
</tr>
</tbody>
</table>

(1) FOR INCISION & DEADSPACE WOUNDS: DRUG TREATMENT FOR - 10 DAYS.

(2) FOR EXCISION WOUND: TREATMENT TILL COMPLETE EPITHELIZATION.
To attain adequate anticoagulation prior to infliction of wound and to maintain this state throughout the study, heparin was started 4 hrs. before and warfarin 72 hrs. before surgery. Warfarin was given orally once a day thereafter. The dose of heparin was given as 2 parts at 10 hrs. interval. The dosage adequacy was monitored by frequent laboratory control—viz clotting time (Wright capillary tube method as described by Ranade (12), for heparin and one stage prothrombin time Quick's method (as described in 130) for warfarin.

The doses of heparin used increased the clotting time from basal range of 28-45 seconds to 96-115 seconds and warfarin dose increased the prothrombin time from 13 to 15 seconds to 33-52 seconds (appendix - a).

The dose of vitamin-K employed for the study totally masked the anticoagulant action of warfarin. On vitamin-K administration warfarin prothrombin time came back to basal level 11 to 13 seconds from 33-52 seconds.

For incision and dead space wound, systemically used drugs were administered for 10 consecutive days. The duration of drug treatment was variable for excision wound study and this depended on time taken for complete epithelization, where the treatment was stopped. Ordinarily, this duration was less than or upto 21 days.

Saline control was kept for all dose regimens.

**SATISTICAL ANALYSIS:**

Student's 't', test was employed for statistical analysis. In case of excision wound study for calculation of median wound closure time ($Wc_{50}$) Litchfield and Wilcoxon method (123) was used. Approximate s.e. for $Wc_{50}$ was calculated by using the formula (131):

$$S.E. = (\log \text{ of } Wc_{84} - \log \text{ of } Wc_{16})/\sqrt{2n}$$