CHAPTER III

THE PREAMBLE - REVIEW ON THE SCOPE OF WORK

1) PROCOAGULANTS:
   (a) Thrombin 46
   (b) Fibrin 48
   (c) Botropase 50

2) ANTICOAGULANTS:
   (a) Heparin 52
   (b) Warfarin 56

******
While it is true, that clotting factors and other procoagulants take part in the early events (eg: haemostasis) of injury. Perisurgical use of drugs that promote / inhibit blood coagulation is likely to affect fibrin deposition and / or fibrinolysis, which inturn disturb the orderly sequence of wound repair. Therapeutic object of such use of procoagulants and anticoagulants is focused somewhere else, than the surgical wound. Patients on longterm anticoagulant therapy may be exposed to tissue injury of different etiology. The implications of either enhanced or reduced blood coagulation and clotysis on tissue repair needs to be explored. Therefore, evaluation of commonly used procoagulants and anticoagulants on wound healing is undertaken. This study could be expected to throw light on: (i) link between blood coagulation and tissue repair. (ii) clinical implication of such drug therapy and (iii) possible ways to over-come adverse effects if any caused by these drugs on wound healing.

**THROMBIN:**

In general procoagulants circulate in plasma in a zymogen form and are activated in multistep amplifying system that ultimately results in thrombin generation. Thrombin is formed by proteolytic cleavages from the terminal half of its circulating zymogen - prothrombin - (factor - II).

Thrombin is a serine protease a natural coagulant of fibrinogen. There are at least three forms of thrombin - alpha, beta and gamma. Human gamma thrombin has essentially no clotting activity, found to activate factor - XIII (91). Thrombin participates in various physiological events which suggest that thrombin is not uniquely specific and has considerable range of enzymatic properties. Thus, actions of thrombin (89, 91) can be grouped into two, (1) actions on blood coagulation and (2) actions involved in tissue repair.
THROMBIN AND BLOOD COAGULATION:

Thrombin plays a multiple role in hemostasis; - Viz :-

(i) enzymatically activate other factors of the plasma clotting system: eg : Factor V, VII & VIII.

(ii) initiates platelet reaction and stimulates aggregation.

(iii) induce platelet - protein phosphorylation, complement dependent ultrastructural lesions on platelet surfaces.

(iv) take part in various endothelial cell functions - thrombin inactivates plasminogen activator inhibitor derived in endothelial cells in conditioned medium (80). Production of thrombin at the site of vascular injury stimulate prostaglandin - I₂ (PGL₂) synthesis by endothelial cell which limit the number of platelets involved in primary haemostatic response and help to localize clot/thrombus formation (92).

(v) Thrombin is the only physiologically relevant serine protease that convert protein - C into protein C₈ which is involved in the control of coagulation cascade (93).

THROMBIN AND TISSUE REPAIR:

Thrombin has diverse biological functions and a potent chemoattractant for human monocytes (89). Particularly, human L- thrombin is more potent as monocyte chemoattractant. Since, monocyte are ultimately transform into macrophages at the injured site, thrombin not only participate-inflammation but also wound repair. Many actions of thrombin are seemingly involved in tissue repair - (a) thrombin is a mitogen, stimulates the proliferation of cultured non-dividing normal human fibroblasts, mouse embryo fibroblast, line of chinese hamster lung cells and quiescent chick embryo fibroblast (94). Several studies have revealed
cell surface binding sites or receptors for thrombin on cells that are responsive
to its mitogenic action.

(b) Thrombin synergistically enhance the effects of epidermal growth factor (EGF)
on DNA synthesis (95).

(c) Platelet exposed to thrombin generate mitogenic source in the process of
tissue repair, Electronmicroscopy has shown that thrombin induced platelet
aggregation also result in disintegration of platelet granules and promotion of
platelet derived growth factor release.

(d) Thrombin activated platelets have the capacity to stimulate angiogenesis
and increased collagen synthesis as measured in the in-vivo rabbit corneal assay (8).

(e) Thrombin stimulates the production and release of a major surface associated
glycoprotein-fibronectin in culture of human fibroblast (96).

(f) Fibronectin is readily cleaved by thrombin (46).

Thrombin accelerates its own production. Appearance of fibrin follows
the generation of thrombin. Antithrombin - III neutralize thrombin : heparin
greatly enhances the reaction rate of the enzyme antithrombin - III and thrombin.

Thrombin clots blood and is the essential procoagulant. The cellular
response to injury is intimately associated with the interaction of thrombin, platelets
and collagen. Therefore, it may be referred that thrombin could be one of the
active agents that trigger wound repair. Hence, as a perspective pharmacological
study, the effects of topical thrombin on wound healing is undertaken in normal
as well as anticoagulated rats.

FIBRIN :

Local fibrin deposition is an early event common to both wound and
tumours (90). Extra vascular fibrin gel serves as a provisional 3-dimensional
matrix that support cell migration (89). Fibrin has long been recognised as a histologic feature of both acute and chronic inflammatory processes (6). Fibrin formation and turnover are intimately associated with inflammation and wound healing.

As said earlier, substances released during clot formation, platelet activation and clot resolution have the capacity to recruit inflammatory cells and other cells and also exerts mitogenic activity. The composition of clot is important in its recolonization of fibroblasts. Fibrin not only acts as glue but bioactive as well. Fibrin may interact with blood and tissue cells. Fibrin deposition appears to be a major factor in the healing of wound (10, 97). In-vivo the action of fibrin is probably supplemented by cell and plasma derived mitogens and chemoattractant - growth factors such as PDGF, EGF, TGF and complement (90). Macrophages are first activated by fibrin (97). The interaction of fibrin and fibronectin which are covalently linked by plasma transglutaminase-factor - XIII is critical for tissue repair (98).

In recent years a large number of clinical studies both in tissue repair and in transplant surgery have described fibrin as 'surgical glue' to bind the edges of wound. Fibrin as sealant has been used in such studies in various forms like fibrin-collagen-mesh, (99, 100) fibrin antibiotic combination (101) etc. It is now recognized, that fibrin application assure uncomplicated post-operative care, lower incidence of infection and grossly improved healing (102, 103). Yet, it appears that in all these studies the focus has been on sealant properties of fibrin and its probable and significant role in wound healing remains less emphasized.
Moreover, it is true that number of molecular and cellular events occur during wound repair. Fibrin, fibrinopeptides and fibrin degradation products (FDP$^5$) produce leucocyte chemotaxis (8, 17, 97) and induce angiogenesis (90). Degradation of fibrinogen and fibrin yield a core of fragments that take part in reparative changes and neovascularization. Fibrin degradation products are known to affect collagen synthesis and stimulate prolyl hydroxylase activity (86). Further, there are conflicting reports about the actions of generated FDP$^5$ on collagen synthesis. FDP$^5$ whose Mol.wt. $> 50,000$ at high concentration inhibit fibrogenesis and smaller FDP$^5$ fragments increase collagen synthesis (86). Clearly, fibrin deposited in-vivo and its degradation by plasmin presents a biological system of extraordinary complex. It appears that fibrin has both structural and regulating function during the process of healing.

**BOTROPASE (R) : (Reptilase : Haemocoagulase Batroxobin*)**

Snake venom has either one or many effects on blood coagulation. In the past, procoagulant, anticoagulant, fibrinolytic or other effects of snake venoms have been documented (104). Botropase is a non-toxic systemic haemocoagulant fraction of venom obtained from Brazilian snake - BOTHROPS JARARACA or ATROX - family CROTALINAE. Since 1954 Botropase is marketed by Pentapharma Ltd. Basel in Brazil, Europe & Asia under different trade names - viz: REPTILASE, Hemostase. The haemocoagulant action of Botropase is predominantly thrombinic in nature. Botropase is an enzyme preparation, its structure and chemistry are not yet well defined, but the preparation possesses thrombin like action which is sometimes referred to as "thromboserpentin" activity. Thrombin like enzyme fraction of snake venom have certain applications in clinical field.

* W.H.O. approved
Table 3-1: COMPARATIVE FEATURES OF BOTROPASE & THROMBIN.

[Compiled from data in Seegers and Ouyang (104)]

<table>
<thead>
<tr>
<th>Source</th>
<th>THROMBIN</th>
<th>BOTROPASE</th>
</tr>
</thead>
<tbody>
<tr>
<td>generated in vivo</td>
<td></td>
<td>fractionated venom of Bothrops Jararaca or Atrox of Brazil.</td>
</tr>
<tr>
<td>Factor IIa</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Structure &amp; Chemistry</th>
<th>serine protease</th>
<th>not known</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 forms</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cleaves both</td>
<td></td>
<td>only 'A' chain</td>
</tr>
<tr>
<td>A &amp; B chain</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Effects on Fibrinogen</th>
<th>thrombinogen protease</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>cleaves both</td>
<td></td>
<td>only 'A' chain</td>
<td></td>
</tr>
<tr>
<td>A &amp; B chain</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Structure of induced clot</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>$(\alpha \beta \gamma)_n$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>vonWillebrand Factor (VWF) Interaction</th>
<th>Yes</th>
<th>Yes</th>
</tr>
</thead>
<tbody>
<tr>
<td>interact with VWF</td>
<td></td>
<td>mainly end to end.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Nature of Fibrin</th>
<th>molecules are aligned end to end and side to side form visible gel</th>
<th>no visible gel.</th>
</tr>
</thead>
<tbody>
<tr>
<td>reaction induce aggregability</td>
<td></td>
<td>do not initiate platelet release reaction.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Effects on Platelet Aggregability</th>
<th>augment platelet release</th>
<th>do not initiate platelet release</th>
</tr>
</thead>
<tbody>
<tr>
<td>reaction induce aggregability</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Clot Retraction</th>
<th>active retraction</th>
<th>No.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Effects on Heparin-Nized Blood</th>
<th>no clot</th>
<th>coagulate heparinized blood.</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Factor XIII</th>
<th>Activation</th>
<th>Activation</th>
</tr>
</thead>
<tbody>
<tr>
<td>factor X</td>
<td>-</td>
<td>activation</td>
</tr>
</tbody>
</table>

| Fibrinolysis & nature of FDPS | - | more susceptible for fibrinolysis. |

| Bradykinin Release | - | Yes. |
Botropase directly acts on fibrinogen, converts it into fibrin and reduce bleeding time in various hemorrhagic conditions. It can be given intravenously or intramuscularly or applied locally*. Local application of botropase prevent oozing of blood. Thrombin like action of Botropase is characterized primarily by the fact, that blood coagulation takes place even in the absence of Ca²⁺ prothrombin and plasma platelet factors. There is no reported incompatibility of Botropase with antibiotics, electrolyte, hormones or immunogenic antigens.

Clot formed after Botropase is structurally not similar to thrombin induced clot. There are many differences between procoagulant role of thrombin and Botropase (Table 3-1). Heparinized blood clots with Botropase. Antithrombin does not interfere with Botropase action. Unlike thrombin Botropase is not absorbed by the fibrin clot. Thrombin-like fraction of Botropase is thought to be excreted in urine following systemic administration.

As blood coagulation has recognized functional link with tissue repair, it is interesting to study the effects of Botropase on wound repair. Since surgical use of Botropase is not uncommon, the study of action of Botropase on reparative process may throw new thoughts and emphasize additional advantage of Botropase therapy.

HEPARIN:

Heparin has been used universally as an essential medical aid from 1938. Commercial heparin is prepared from beef or pork, intestine. Heparin is present in tissues chiefly in mast cells, a biochemical representative of the distinctive class of compounds known as 'linear anionic polyelectrolytes'. Polyelectrolytes are polymers with ion containing groups attached to the polymer.

* Manufacturer's hand out.
Heparin is heterogenous, electronegative and hydrophilic strong acid. Structurally, it contains five different amine group. The longest component of heparin is 2, 6, disulphoglucosamine which interacts with uranic acid residue. Linear anionic heparin do not appear to be present in tissue as proteoglycan.

Natural heparin which has an average mol.wt. of 15,000 daltons, consists of structural moieties of different mol.wt. between 4,000 and about 25,000 daltons. It has long been known that heparin is not a homogenous substance but consists of glycosaminoglycans in a wide range of different molecular sizes and of different affinity to antithrombin - III (105).

Heparin is a directly acting anticoagulant both in vivo and in vitro and given parenterally. The best known property of heparin as an enzyme inhibitor is its anticoagulant activity. At pH 7 - 8 and 37°C heparin catalytically activates antithrombin-III to inhibit thrombin. Antithrombin-III (AT - III) antagonise serine protease procoagulants-thrombin and other factors XII, XI, X and IX. Heparin binds to lysyl residues on AT-III producing a conformational change that makes the active site on AT-III more accessible to the serine protease procoagulants. This action of heparin enhances thrombin inactivation by 750 folds and factor Xa inactivation to an greater degree (106). 'Low - dose heparin' inhibits the contact phase of the intrinsic coagulation system and reduce platelet adhesiveness (107).

**PHARMACODYNAMICS:**

Heparin produce wide range of biological effects which is due to its action on enzymes, cell surface (see below) and hormones at various tissue level.
PROTECTIVE ROLE:

Heparin protects the whole organism against,

(a) toxic conditions : peritonitis : burns
Russel Viper Venoms tissue thromboplastin.

(b) trauma : hypoxia, radiation and hypotension.

(c) stress : lymphopenia : spontaneous hemorrhage general adaptation syndrome. Enhance fibrinolysis.

(d) drugs : d-Tubocurarine : digitalis :
neomycin : paracetamol.

EFFECTS ON HORMONES:

Heparin releases thyroxine and insulin. It activates parathyroid hormone; suppresses somatotrophic and aldosterone production. It inhibit ACTH, cortisone, vasopressin and angiotension production.

ON ENZYMES:

ACTIVATE FORM ADDUCTS INHIBIT
blood arginine Factor IX hyaluronidases
esterase.
DNA polymerase collagenase ribonucleases
Factor XII to RNA polymerases produce kallikrein.

Liberates in vivo: diaminoxydase (histamine) from intestine, procollagenase - bone explants.
SOME KNOWN ACTIONS OF HEPARIN:

(a) on cells.
(b) inhibition of sensitivity reactions.
(c) protective role in whole animal.
(d) effects on hormones.
(e) on enzymes.

(a) ON CELLS:

1. markedly increases negative electrical potential of vascular wall.
2. inhibits proliferation of arterial endothelial & smooth muscle cells.
3. activates fibronectin opsonic for phagocytosis by hepatic reticuloendothelial cells.
4. induces thrombocytopenia.
5. mobilizes eosinphils.
6. displaces DNA from isolated nuclei.
7. activates macrophages.
8. produces lymphocytosis.
9. increase B Lymphocyte migration : inhibit T & B lymphocytes.
10. binds Red blood cell initiation factor.
11. antimitotic.

ON SENSITIVITY REACTION:

1. antiallergic : anti-inflammatory: Antihistaminic
2. inhibits generation and amplification of convertase of complement cascade (108).
3. inhibit binding of C₁ & C₂.
4. reverse anaphylactic shock.
5. reduce increased vascular permeability caused by PGF₂α, bradykinin and histamine.
Heparin through its action on cell membrane inhibits mitosis at the stage of transition from late telophase to interphase of the next cell cycle. Thus, heparin becomes rapidly associated with the vessel wall and cellular components of tissues to produce many pharmacological actions.

Fibrinolysis is enhanced by heparin administration (109). At therapeutic dose levels heparin increases the release of plasminogen activator from endothelial cells. It may promote fibrinolysis of non-cross-linked fibrin clots via a process that does not involve plasmin (110).

From the foregoing, it is clear that heparin has many actions - the biological significance of these, is yet to be elucidated. However, pertinent to the present study, its anticoagulant, profibrinolytic, anti-inflammatory and antiproliferative actions are of interest, since inhibition of cell proliferation could be expected to suppress healing.

It has been reported that heparin suppresses healing process (111) while Saliba et al. have found that heparin as promoter of granulation, epithelization in burn wounds (112). Further, as said earlier fibrin degradation products influence healing; heparin as anticoagulant and by its action on fibrinolysis may interfere with tissue repair. Since, heparin is used as anticoagulant perisurgically (113) in a number of operative therapies; it is of interest to investigate heparin effects on wound repair. This will enable to answer about the pros and cons of heparin use in relevant surgical conditions.

**WARFARIN:**

Warfarin was first synthesized by Ikawa et al. in 1944, the name of the drug is an acronym for the patent holder. (Wisconsin Alumni research foundation-
FIG. 3-1. REDUCTION OF K-EPOXIDE BY COUMARIN SENSITIVE EPOXIDE REDUCTASE

GLUTAMIC ACID

GLUTAMIC ACID

PROTHROMBIN

DESCARBOXY PROTHROMBIN

CHLORO-K

KH₂

REDUCED HYDROQUINONE FORM

WARFARIN

NAD⁺

NADH

KO
plus the coumarin derived suffix). In 1959 Link established its clinical efficacy and shown that warfarin can be safely used in human beings. Structurally warfarin is a bishydroxy coumarin compound with an asymmetrical carbon atom; official preparations of warfarin contain the mixture of the two optical isomers.

Warfarin is the most commonly used oral anticoagulant. Over a decade it is known that warfarin is vitamin-K antagonist. Like all bishydroxy-coumarins warfarin is an indirect anticoagulant acts only in-vivo. In presence of warfarin, liver cells are unable to carboxylate glutamic acid residues of factors II, VII, IX, X and to synthesize protein-\(C\)(Fig. 3-1, 114). Thus warfarin causes an acquired deficiency of prothrombin group of factors and of protein - C.

Administration of dicumarol derivatives is associated with an increase in antiplasmin levels (115). Oral anticoagulants even seem capable of counter-balancing the increase in fibrinolytic activity induced by bacterial pyrogens in man (116).

In addition, warfarin is known to produce many other actions:

Viz:

(a) Inhibits the production of procoagulants by macrophage (117).

(b) Salivary gland tissue factor (Thromboplastin) activity is inhibited by warfarin (118).

(c) Warfarin depresses cellular immune response, is a potent inhibitor of oxidative phosphorylation and increases lymphocyte and macrophage infiltration (119).

(d) In tissue culture, warfarin acts as DNA intercalant, block DNA synthesis and produce cytotoxicity (120).
(e) Warfarin can induce morphological differentiation in cancer cells.

Since, a decade interesting data have been generated about the predictable effects of anticoagulant therapy on tumour growth and metastasis (119, 120). Tumour has been described as non-healing wound (31). Warfarin has anticoagulant and alleged to have antifibrinolytic and antimetastatic actions in addition to its action on macrophage procoagulant activity. Warfarin is known to interfere with cancer cell proliferation (120). So, it is natural to suspect that drugs like warfarin by these effects modify healing sequence. Hence, it would be interesting to know if and in what direction warfarin affects wound healing. Findings in this direction would be of some clinical importance.