THE EFFECT OF CHITOSAN DEXTRAN GEL AS A HAEMOSTATIC AND ANTI ADHESION AGENT IN THE CENTRAL NERVOUS SYSTEM AND EVALUATION OF HAEMOSTATIC MECHANISM OF SKELETAL MUSCLE TISSUE

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By

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ABSTRACT

INTRODUCTION

Haemostasis and adhesion prevention in surgery is of paramount importance to prevent complications. It is even more important in neurosurgical procedures where even minor complications can lead to devastating consequences. There is constant work in the direction of development of both haemostatic and anti-adhesion agents with recent research with the use of chitosan dextran gel and autologous muscle tissue showing promise.

Normal wound healing following surgery may lead to adhesion formation with the development of adhesions correlating to the presence and amount of blood clot. This linking of bleeding and adhesion formation is key to adhesion prevention. The amount and site of adhesion formation will often influence the postoperative course of the patient. While there have been many substances developed in an attempt to prevent adhesions, this thesis will examine Chitosan dextran (CD) gel and muscle for their potential role in neurological surgery. CD gel has previously been shown to be effective as a haemostat and as an anti- adhesion agent in endoscopic sinus surgery while autologous muscle has been shown to be effective with major vascular injury.

This thesis will examine Chitosan Dextran gel in central nervous system and also try to explore the potential mechanisms of action of muscle tissue in bleeding control.

METHODS

The haemostatic and anti adhesion potential of Chitosan Dextran gel was studied with the help of sheep models.

A neurosurgical burr hole model was used to assess the safety and efficacy of Chitosan Dextran gel on the dura and brain tissue. Bleeding control was tested at the level of bone, dura and brain separately with both Chitosan Dextran gel and Gelfoam paste on separate burr holes. Baseline bleeding was measured at the time of injury using the Boezaart scale, and then every two minutes after the application of each agent until complete haemostasis or 10 minutes, whichever was earlier. Safety was assessed through MRI scans and histopathological analysis.

To further assess the antiadhesion potential of Chitosan Dextran gel, a sheep model of spinal laminectomy was used. Gelfoam paste was again used as the control agent. Following the laminectomy procedure and exposure of dura, the test agent, i.e, Chitosan Dextran gel or Gelfoam or normal saline wash was applied on the dura and the wound was closed. Healing was allowed for three months. The efficacy of adhesion prevention was assessed by Peel test and MRI scans. Histopathology was performed to assess safety of the agent.

In vitro studies were performed to evaluate the haemostatic action of muscle tissue. Muscle extracts were prepared by dissolving crushed snap-frozen muscle tissue in saline. Plain saline was used as control. Prothrombin time, activated partial thromboplastin time (APTT), thrombin time, and platelet aggregation studies were performed on both muscle extract and saline. Prothrombin time and APTT were repeated using factor VII–deficient plasma, factor X–deficient plasma, lupus plasma, and contact pathway inhibited plasma.

RESULTS

1. The efficacy and safety profiles of Chitosan Dextran gel were comparable to those of Gelfoam in the neurosurgical burr hole study. The logistic regression model suggested that Chitosan Dextran gel was more effective at stopping bleeding after two minutes, the clinical significance may be small and this should be tested in a model with greater volume of bleeding with more intervention numbers.

2. With regards to antiadhesion efficacy of Chitosan Dextran gel in the sheep model of laminectomy there was a significant reduction in adhesions when compared to the untreated (normal saline) group. However when compared to the Gelfoam treated group there was no significant difference. MRI did not show any difference in the overall epidural fibrosis among the three groups.

3. In vitro muscle coagulation studies did not show any significant difference between muscle and saline except in the APTT using factor X-deficient plasma. Higher concentrations of muscle extract showed an increase in platelet aggregation.

CONCLUSION

Chitosan Dextran gel is an effective safe haemostatic and anti-adhesive agent in the central nervous system. Further work is needed to extend its use in neurosurgical procedures in humans.

Platelet aggregation appears to play an important role in the haemostatic action of muscle tissue and further study of this mechanism may improve the development of new topical haemostatic agents.

DECLARATION

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Sukanya Rajiv

PREFACE

A portion of the work described within this thesis has been submitted for publication, as listed below:

1. Rajiv, Sukanya, Marguerite Harding, Ahmed Bassiouni, Camille Jardeleza, Amanda Drilling, Craig James, Thanh Ha, Steve Moratti, Simon Robinson, and Peter-John Wormald. "The efficacy and safety of chitosan dextran gel in a burr hole neurosurgical sheep model." *Acta neurochirurgica* 155, no. 7 (2013): 1361-1366.
2. Rajiv S, Rodgers S, Bassiouni A, Vreugde S, Wormald PJ. "Role of crushed skeletal muscle extract in haemostasis." *Int Forum Allergy Rhinol*, 2015; In Press

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CHAPTER 1 AIMS

Studies to be performed

The following studies were performed:

1. To assess the safety and efficacy of Chitosan Dextran gel as a haemostatic agent on the dura and brain tissue in the sheep animal model
2. To assess the anti adhesion potential of Chitosan Dextran gel in a post laminectomy sheep animal model
3. To study the possible haemostatic mechanisms of muscle tissue when used as a haemostatic agent

CHAPTER 2 INTRODUCTION

Effective haemostasis is required to give a good surgical field for the surgeon to work safely and this in turn should result in a better outcome for the patient[1]. Surgical haemostasis affects different aspects of the health system from patient morbidity and mortality to overall cost reduction[2]. Significant intra-operative blood loss leads to blood transfusions that can have its own complications[3,4]. Greater blood loss can also lead to derangement in the coagulation mechanisms leading to Disseminated intravascular coagulation (DIC)[5] with morbidity and even mortality.

Haemostasis begins with preoperative assessment of the patient prior to the elective procedure. Medical conditions such as anemia and various bleeding disorders can increase the likelihood of intraoperative bleeding leading to blood transfusions in the perioperative period[6-9]. Medications like aspirin, antiplatelet drugs and warfarin also increase bleeding risk during surgery[6,10]. With the development of newer agents like direct thrombin inhibitors and factor Xa inhibitors, it has become more difficult to reverse the anticoagulant state[11].

The other important factor in intra-operative haemostasis is good anesthetic technique providing an optimal surgical field for the procedure. There are a variety of anesthetic agents available and their use depends on the type of surgery. With regards to neurosurgery, the effect of a drug on cerebral blood flow (CBF), cerebral metabolic rate (CMR), cerebral blood volume (CBV) and intracranial pressure (ICP) determine its use[12,13]. Volatile agents like Sevofluorane are more commonly used since their effect on the neural homeostasis is minimal[12,13].

Meticulous surgical technique is the single most important step to reduce overall blood loss[14,15]. Various methods like electro cautery, pressure application, suture ligation and tourniquet use, are available to arrest bleeding[16]. However these techniques are not without complications. Topical haemostatic agents can be used to control various types of bleeding including diffuse and individual bleeders and their ease of use has made these agents very popular[17]. However it is essential to understand the normal haemostatic mechanisms, the mechanism of action of the topical agent and directions to use them, to help in deciding the appropriate agent for a particular situation. Care should also be taken about the side effects of the various topical agents.

Gelatin based, cellulose based and collagen based agents are very widely used, but these products can swell up and can cause pressure damage to nearby structures and hence should be used with caution in closed cavities[17]. Fibrin and thrombin based agents though extremely effective to control bleeding in a variety of surgeries but run the risk of allergic reactions and antibody formation[15,18]. Albumin derived haemostats, polysaccharide based haemostats and inorganic agents work independent of the coagulation mechanism[17]. Vasoconstrictors like adrenalin are used intraoperative to transiently control bleeding[19] and anti-fibrinolytics are used as clot stabilizing agents[20]. Hence there are many different types of agents available commercially and their use is dictated by the surgical situation and surgeons understanding of the product.

The central nervous system in human beings is very complex. It comprises both the brain and spinal cord which are continuous with each other. These neuroanatomical organs control the very essence of the human body and mind. The adult human brain is a bilaterally symmetrical semi-solid tissue inside the bony cranium of the head. It is

mainly divided into three parts: Cerebrum, Cerebellum and Brain stem[21,22]. The cerebrum is the largest part of the brain and consists of two symmetrical halves –the right and left cerebral hemispheres[21,22]. Each half of the cerebral hemisphere is further divided into 5 further lobes: frontal, parietal, temporal, occipital and insula. The cerebellum lies beneath the cerebral hemispheres and is connected below with the brainstem[21,22]. The brain is also covered by the protective meninges, dura, arachnoid and pia mater[21,22]. These different parts of the brain control all functions of the body and along with spinal cord help perform all motor activities and perceive various sensations. This ranges from speech, vision, hearing, autonomic functions, respiration, movement, emotional control, bowel and bladder functions, etc[21,22]. Hence damage to any part of the central nervous system can cause devastating consequences. Thus bleeding control during a surgical procedure in these organs is of utmost priority.

Increased blood loss/ bleeding in any surgical procedure makes the operation more risky since difficult visualization of the structures can cause inadvertent damage to the surrounding tissue. Increased blood loss can also cause shock[23], increases blood transfusion requirements, which have their own complications like TRALI (transfusion related acute lung injury)[24], increases inpatient length of stay and thus contributes to increased costs to the healthcare system[25].

With any surgical procedure, there is associated scar tissue formation, which is a normal wound healing process. However, excessive healing is a complication seen following spinal and back surgeries, leading to peridural and epidural fibrosis[26]. The extent of this fibrosis depends on the amount of intra-operative bleeding or poor haemostasis[27]. The theory of postoperative laminectomy membrane suggests, that increased bleeding leads

to a larger hematoma formation in the laminectomy site. This is subsequently replaced by fibrous tissue in the remodeling phase leading to peridural adhesions[28].

Postoperative epidural and peridural adhesions are a common cause for persistent pain and may lead to failed back surgery syndrome[29]. These adhesions also increase the risk for a repeat procedure at the same location[30]. One of the methods used to decrease peridural adhesions is by use of barrier agents, which prevent contact between the dura and surrounding muscles[14]. There are many agents available, and their use should be guided by the nature of surgery and knowledge of the product.

The subject of this thesis is a newly developed chitosan-dextran gel which has shown in the nose and sinuses to be both haemostatic and decrease adhesion formation. Chitosan is a polysaccharide compound obtained from crustaceans[31]. Different forms of chitosan have been shown to be effective as a haemostatic agent[32-34]. It has been shown that chitosan achieves bleeding control independent of the haemostatic mechanisms of the body[35]. It decreases adhesions and also improves wound healing[36,37].

In our department we have also developed a significant interest in the use of autologous muscle patch as a haemostatic agent. There are reports dating back to the early 1900s where it was used as a haemostatic agent in published case reports. It is now re-emerging, with recent studies showing effective bleeding control in high -pressure carotid artery bleeding models.[38] With a greater understanding of the mechanism behind this highly effective haemostatic agent, we may be able to further improve current haemostatic agents either by incorporating this mechanism into the CD gel or by utilizing the muscle patch action in a stand alone topical haemostat.

In our studies we have used Chitosan Dextran gel to assess bleeding control in brain and also to study its anti-adhesion properties in spinal surgery. We have also sought to further understand the role of skeletal muscle tissue as a haemostatic agent.

Thus, in the next few chapters we discuss normal haemostatic mechanisms in the body, different types of topical haemostatic agents available for bleeding control, normal wound healing, pathophysiology of epidural scarring and agents available to prevent scarring. We then present our studies to assess the haemostatic and anti-adhesion properties of Chitosan Dextran gel in an animal model and in-vitro studies performed to assess the haemostatic property of muscle tissue.

CHAPTER 3 NORMAL HAEMOSTASIS

It is of paramount importance that blood maintains its fluid state and a clot (haemostatic plug) is formed only when required. There are several haemostatic mechanisms that are in operation in vivo to help this happen and they play an essential role in stopping bleeding when there is a loss of integrity of the vessel wall. These mechanisms also ensure that the haemostatic plug remains confined to the area of vascular injury and when the injured site is healed, the plug is removed from the site. There is a constant balance that is maintained between the procoagulant and anticoagulant factors, as an imbalance will lead to either unwanted thrombosis or haemorrhage[39].

Important structures/ factors that play a role in maintaining the normal haemostatic mechanism includes blood vessel, platelets[40-46], coagulation factors[47], inhibitors of coagulation[39,48-55], and the fibrinolytic system[56-61].

BLOOD VESSEL/ ENDOTHELIAL CELL

The blood vessel wall is made up of the intima, media and adventitia. The intima is lined by a layer of endothelial cells that covers the sub endothelial connective tissue [62].

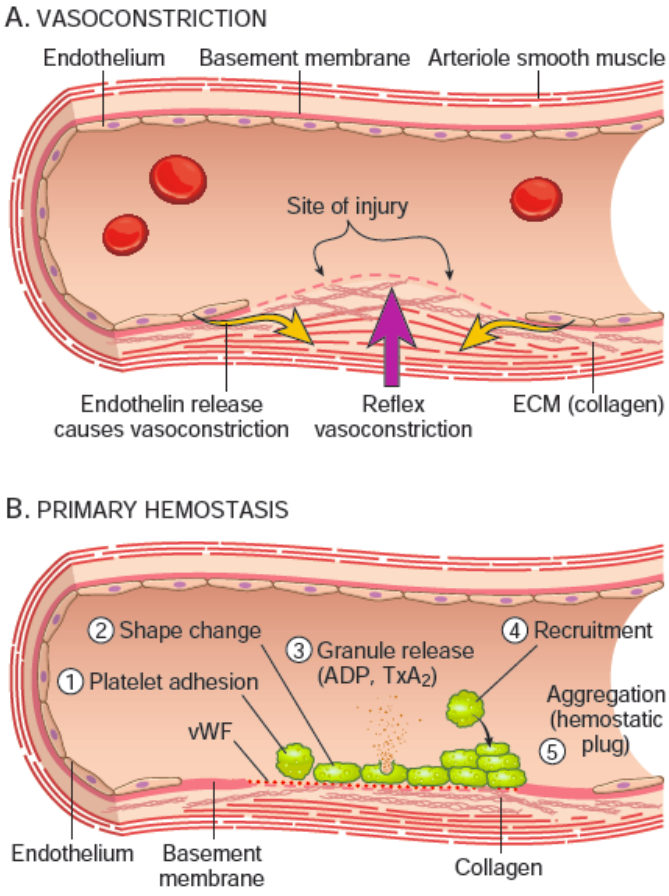
Endothelial cells: Endothelial cells form a continuous internal lining along the blood vessel. They are generally non thrombogenic when the vessel integrity is intact[63]. These cells contain both pro-thrombotic and antithrombotic properties. The relative balance between the pro and the anti-thrombotic factors expressed by the endothelial cells at any point in time depends on the vascular integrity and the extent of local inflammation. This determines whether there will be active clot formation, or clot propagation or clot dissolution[64].

Important pro-thrombotic factors/ substances within the endothelial cells include von Willebrand factor (vWF), tissue factor (TF) and Plasminogen activator inhibitors (PAI)[63,64]. A damage to the vessel wall leads to the exposure of the sub endothelial connective tissue. The platelets come in direct contact with the sub endothelial connective tissue, activating vWF and leading to active localised clot formation[65]. Tissue factor exposure to the blood stream is the major activator of the extrinsic pathway (discussed in detail below)[66]. PAIs inhibit the activation of plasminogen, thus inhibiting fibrinolysis (discussed in further detail below)[63].

On the other hand, the endothelial cells also express a number of factors that inhibit clot formation under normal circumstances. The intact endothelium prevents any interaction between the coagulation factors in the blood and the thrombogenic sub endothelial connective tissue[63]. Anti-thrombotic substances in the endothelial cell include prostacyclin, ADPase, Tissue Factor Pathway Inhibitor (TFPI), Tissue Plasmniogen Activator (t-PA)[39,67]. Prostacyclin (PGI2) and Nitric oxide (NO) and act by vasodilating the blood vessel and inhibiting platelet binding to the endothelial surface[53-55]. ADPase degrades ADP which is a potent activator of platelet aggregation[63]. TFPI inhibits various factors in the coagulation cascade and t-PA degrades plasminogen to plasmin. Plasmin degrades fibrin and thus dissolves a clot[63].

Vasoconstriction is a phenomenon that happens locally at any site where there is a loss of vessel integrity due to an interplay between the various humoral and inflammatory factors[63,67] (Figure 1)[63,67]. Vasoconstriction plays an important role in reducing the blood flow in the affected region which helps in the formation of a stable haemostatic plug and allows the coagulation factors to accumulate to reach a critical point to aid in the

process of coagulation. Vasoconstriction is seen in both, vessels that have a muscular coat and in the microcirculation, in vessels without a muscular wall[67]. Further, the endothelial cells by themselves produce substances that cause vasoconstriction such as angiotensin II (AT II) and thromboxane A2 (Tx A2)[63]. Figure 1 depicts the various steps in clot formation, namely, vasoconstriction, primary haemostatic plug, secondary haemostasis and finally regulation of extent of thrombosis.



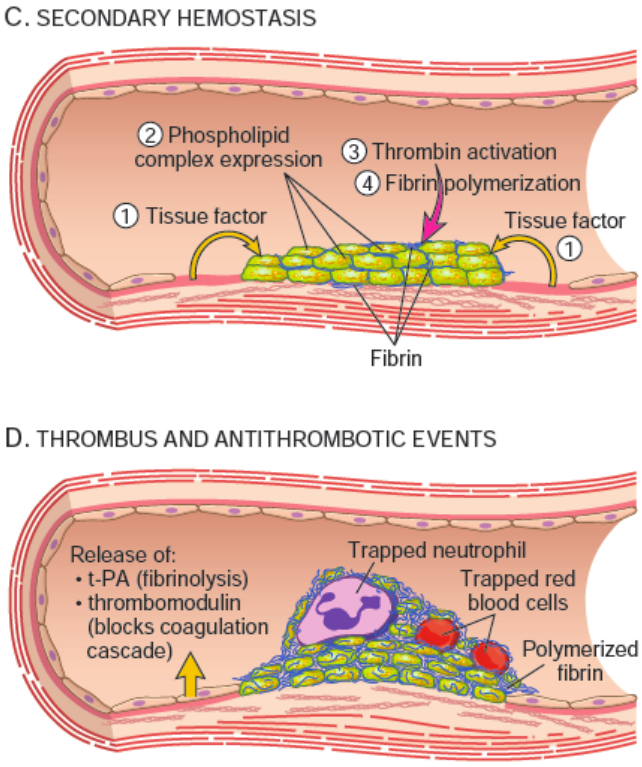


Figure 1: Normal haemostasis: A: There is immediate local vasoconstriction at the site of vascular injury due to release of neuro-humoral factors. B: Platelets bind to the von Willebrand Factor on exposed extracellular matrix via the glycoprotein 1b receptor. This causes platelet activation and conformational changes that leads to platelet granule release (such as Thromboxane A2 and leading to additional platelet recruitment and aggregation (via gp IIb-IIIa receptor), thus forming the primary haemostatic plug. C: activation of the coagulation cascade results in the formation of fibrin and thus the secondary haemostatic plug. D: Counter-regulatory mechanisms act via tissue plasminogen activator (t-PA) and thrombomodulin ensuring that the haemostatic plug remains confined to the area of injury.
(Taken from: Kumar, V., Abbas, A. & Aster, J. *Robbins & Cotran Pathologic Basis of Disease*. (Elsevier Health Sciences, 2014).)

PLATELETS

Platelets are an integral part of the haemostatic plug. Platelet granules and platelet membrane are the chief components of a platelet that play an important role in the formation of a haemostatic plug. Platelets first adhere, then activate by changing their shape before aggregating to form a clot[68]. As shown in Figure 2, platelets adhere to vWF via the surface receptor Glycoprtein 1b (Gp1b)[63]. This leads to a conformational

change and platelet activation (associated with an increase in the surface glycoprotein IIb/IIIa receptors and release of platelet granules). There is a simultaneous release of a number of pro-thrombotic agents from the platelets and endothelial cells which leads to recruitment of more platelets to the area. Finally there is active platelet aggregation due to interaction between the GpIIb/IIIa receptors, leading to the formation of an active haemostatic plug[63,68]. Deficiency of various factors in the above process is responsible for different disorders such as Bernard-Soulier syndrome (deficiency of Gp1b receptor), Glanzmann thrombasthenia (deficiency of GpIIb-IIIa receptor) and Von Wiilebrand disease (deficiency of vWFactor) (as illustrated below in Figure 2)[63,67].

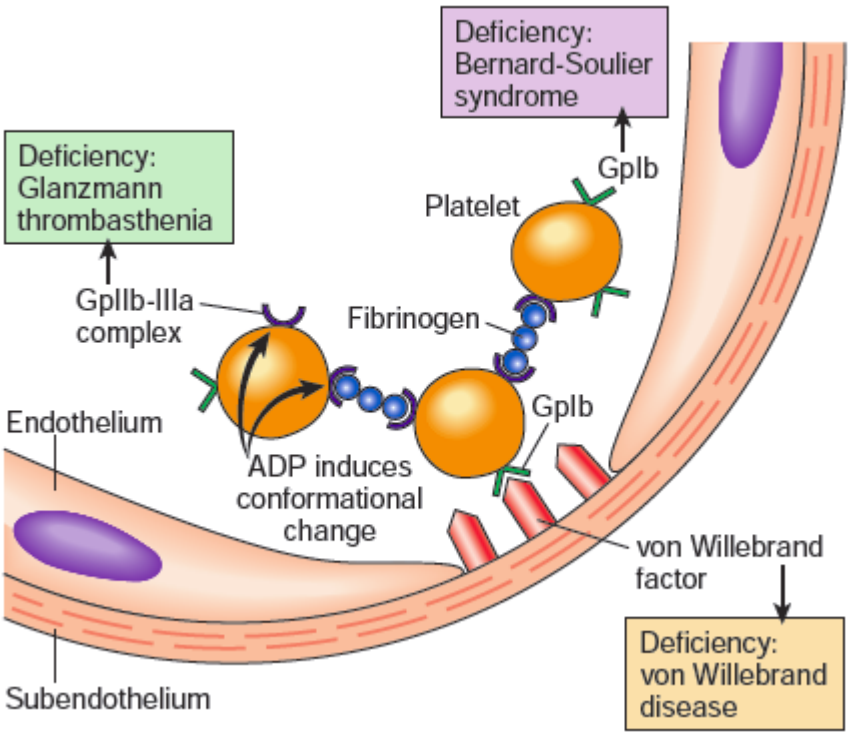


Figure 2: Platelet adhesion and aggregation. Platelet adhesion to the subendothelial connective tissue is via the interaction between the GpIb receptor on the platelet surface and the vWF on the sub endothelial surface. Platelet aggregation is via the fibrinogen that acts as a bridge between the GpIIb-IIIa receptors on the platelet surface. The figure also illustrates the different diseases that can be caused due to the deficiency of different factors in the above process.
Taken from: Kumar, V., Abbas, A. & Aster, J. *Robbins & Cotran Pathologic Basis of Disease*. (Elsevier Health Sciences, 2014).)

COAGULATION CASCADE

The coagulation cascade essentially refers to a series of enzymatic reactions that culminates with the formation of a fibrin clot. It has traditionally been divided into the extrinsic and intrinsic pathways, although there is increasing evidence that in vivo there is evidence of an interaction between the two pathways (Figure 3)[67]. Deficiency of certain clotting factors (such as Factor VIII, V, IX, VII) leads to several bleeding disorders, while other such as Factor II deficiency may be incompatible with life. Certain factor deficiency only leads to mild bleeding (such as factor XI deficiency) and Factor XII deficiency does not lead to any bleeding manifestation at all (and may actually cause increased thrombotic tendency)[69].

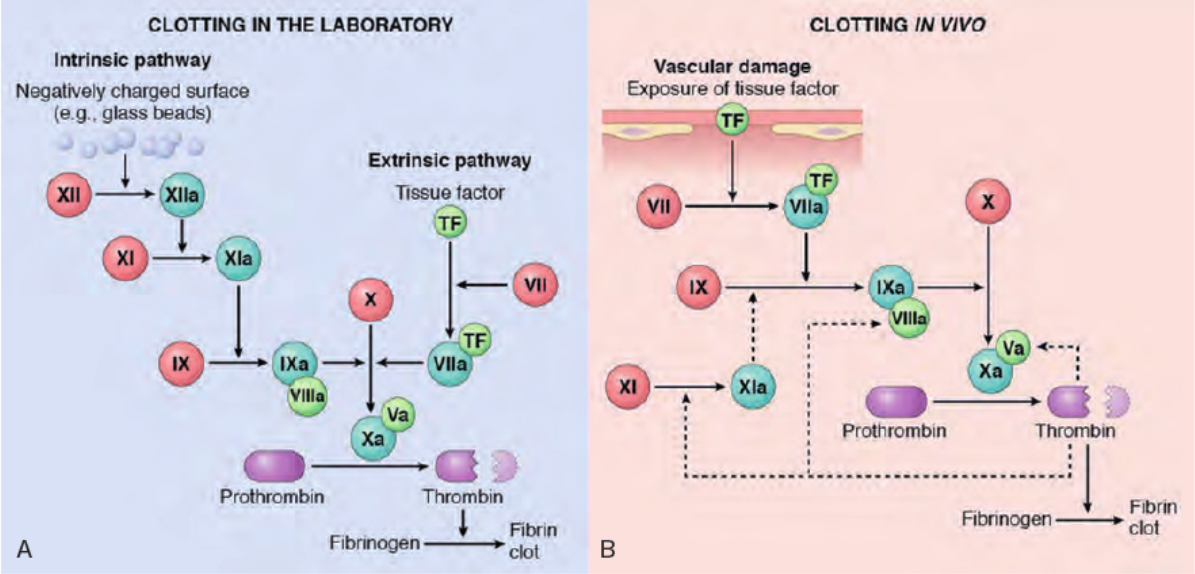


Figure 3: Coagulation cascade in the lab ad in vivo: Coagulation case is initiated in the lab with the addition of calcium, phospholiods and negatively charged materials such as glass beads or by using tissue factor. In vivo, tissue factor is the major initiator of coagulation, which is amplified by the feedback mechanism that are involved (showed by dotted lines).
Red polypeptides indicates the inactive factors, dark green polypeptides indicates active factors and light green polypeptides are the cofactors.
(Taken from: Kumar, V., Abbas, A. & Aster, J. *Robbins & Cotran Pathologic Basis of Disease*. (Elsevier Health Sciences, 2014).)

The most important step in the coagulation cascade is formation of thrombin. Exposure of tissue factor in the damaged area to circulating activated FVII is the initiation step in the coagulation cascade leading to thrombin generation which in turn leads to the activation of FIX in a feedback manner, leading to increased thrombin generation. Thrombin causes conversion of fibrinogen to fibrin which then polymerizes to form a stable clot[63]. Thrombin also plays a paramount role in the platelet activation process[70]. These effects are exercised only at the sites of damaged endothelial cell. When thrombin comes in contact with normal endothelium, it surprisingly behaves as an anticoagulant thus limiting the coagulation process to the area of damaged endothelium[67].

INHIBITORS OF COAGULATION

Natural inhibitors of coagulation prevent propagation of the clot. Continuous blood flow through the area washes the activated coagulation factors constantly from any given site[67] . A number of natural anticoagulants exist. Most important of these is Tissue factor pathway inhibitor (TFPI) that inhibits the factor VIIa-TF complex after forming a quaternary complex with Factor Xa[71]. Another important factor that plays a significant in this process is antithrombin that binds with thrombin to form thrombin-antithrombin complexes (TAT complexes) which are then removed from the circulation by liver. They also inactivate other activated clotting factors such as FXa, FIXa, FXIa, and FXIIa[48,72]. These help to reduce thrombin levels by about 60%. Further, when thrombin comes in contact with thrombomodulin that is present on the endothelial cells away from the site of vascular injury, it forms a thrombin-thrombomodulin complex that also has an anticoagulant effect as it leads to activation of Protein C and subsequently Protein S[73]. These eventually lead to inactivation of Factors V and VIII[51]. Antithombin

also plays a role in the inactivation of other coagulation factors such as factor X, IX, XI and XII[67].

FIBRINOLYTIC SYSTEM

Fibrinolytic system is in operation to ensure that the clot does not extend beyond the confines of the area where there has been a compromise in the vascular integrity. Plasminogen binds with fibrin and tissue plasminogen activator. This complex causes conversion of the proenzyme plasminogen to plasmin that plays an important role in fibrin breakdown and prevents fibrin polymerization[56,57]. Excess clot degradation can be assessed by measurement of fibrin derived d-dimers, which is used clinically as a marker of thrombotic state. Most important activator of plasminogen is tissue plasminogen activator (t-PA) that is synthesized and stored within the endothelial cells and is most effective when it binds to fibrin and hence is used clinically as a thrombolytic agent in case of extensive acute thrombosis. Plasmin activity is controlled by counter regulatory agents such as alpha2 antiplasmin that inhibits plasmin[74].

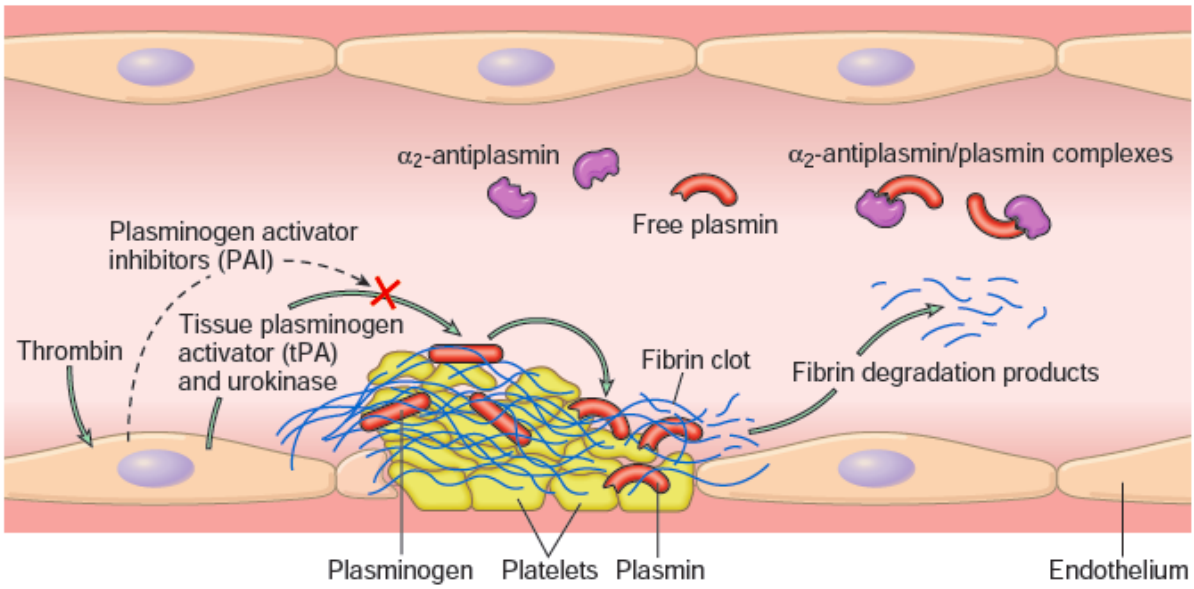


Figure 4: Fibrinolytic system: Tissue plasminogen activator activates plasminogen to plasmin, which forms a complex with alpha2-antiplasmin to form alpha2-antiplasmin-plasmin complex that causes fibrinolysis leading to degradation of a clot.
(Taken from: Kumar, V., Abbas, A. & Aster, J. *Robbins & Cotran Pathologic Basis of Disease*. (Elsevier Health Sciences, 2014).)

COAGULATION SCREEN TESTS

Coagulation tests to assess haemostasis include tests that check the integrity of the intrinsic and extrinsic coagulation pathways and platelet aggregation. Tests include Prothrombin time (PT), that assess the functionality of the various proteins in the extrinsic pathways, namely, factor VII, X, V, II and fibrinogen. In short, tissue factor, calcium and phospholipds are added to plasma and time to form a clot is measured and compared with the control[75,76].

Activated partial thromboplastin time (APTT) assesses the intrinsic pathway proteins, namely, Factor XII, XI, VIII, IX, X, II and fibrinogen. In this test, special factors to activate factor XII (such as ground glass, kaolin or silica) are added along with phospholipid and calcium and time to clot formation is measured and compared with the lab control[77].

Thrombin time (TT) assesses the final step in the common pathway with the addition of thrombin to the plasma and assesses the conversion of fibrinogen to fibrin. This is not affected by deficiency of any of the clotting factors in the intrinsic and extrinsic pathway[78].

Finally, platelet aggregation studies measures the degree of platelet aggregation in platelet rich plasma in the presence of different platelet activating agents (such as ADP, calcium etc)[79,80].

To summarize, there is a very tight balance between the factors that increase and decrease clot formation. Abnormal bleeding can result from either decreased clot formation or from enhanced clot lysis, while on the other hand, excess clot formation and decreased clot lysis leads to unnecessary thrombosis. As described above, an initial platelet-based haemostatic plug is formed at the site of vascular injury. With the activation of coagulation cascade, a fibrin clot is formed at the site. Termination of clot formation is dependent upon factors such as anti-thrombin, TFPI and protein C & S and finally, t-PA and plasmin play a significant role in clot lysis. Thus a carful interplay between the above factors helps in maintaining the homeostasis in the blood.

CHAPTER 4 HAEMOSTATIC TECHNIQUES IN CENTRAL NERVOUS SYSTEM

PREOPERATIVE PATIENT PREPARATION

Haemostatic abnormalities in a neurosurgical patient can be due to many reasons, either secondary to brain neoplasms[81-83], traumatic head injury[84,85], pre-existing blood abnormalities, use of anti-coagulant medications or complexity of the surgical procedure[6]. Pre-operative optimization of haemostatic parameters is important to prevent lethal complications.

Nature of Brain Pathology

Studies have shown variable expression of tissue plasminogen activator (tPA) in different neoplasms. tPA is a fibrinolytic agent and it has been shown that the concentration of tPA can be variable in patients with different brain pathologies, for example, there is an increased expression in meningioma compared to glioblastoma[86]. Another study showed that tPA content was three times higher in benign tumors compared to malignant tumors[87]. Also data suggests that there is an increased concentration of tissue factor pathway inhibitor (TFPI) in patients with glioblastoma[88]. Thus nature of the brain pathologies may have a direct effect on the haemostatic mechanism in the body and this has to be taken into account for perioperative management.

Traumatic brain injury v/s other brain injuries

Tissue factor is abundantly present in the normal brain parenchyma[89-91]. As discussed in our previous chapter, tissue factor in the main initiator of the extrinsic coagulation system. Release of this tissue factor from the injured neural tissue leads to activation of the clotting pathway and consumption coagulopathy and DIC[92-95]. Studies have also shown higher tissue factor expression in patients with severe head injury compared with

those with moderate head injury[96]. Hypothermia, shock, acidosis and anemia secondary to a trauma can also exacerbate this coagulopathy[97-101]. Aggressive management and meticulous monitoring of patient is essential to prevent secondary brain damage[84,102-106]. Treatment should be with cryoprecipitate, fresh frozen plasma, red cells and platelets based on the results of the coagulation parameters[107]. Recombinant FVIIa (rFVIIa) can be used for life threatening neurosurgical bleeds in children and adults[108,109].

Medications

It is well known that different blood thinning agents can affect haemostasis. It has been noted in a study, that nearly 47% of the patients with postoperative hematoma were on **antiplatelet agents**[10]. Thus information should be obtained from patients undergoing elective procedures regarding various medications they are on. Anti-platelet drugs should be stopped at least 7-10 days prior to the surgery. In cases of emergency procedures, platelet concentrates should be transfused peri-operatively as required. **Non-steroidal anti-inflammatory drugs (NSAIDs)** also cause reversible inhibition of platelet function. The duration of inhibition varies from 24 hours to 3 days for various agents[110]. Use of these medications should also be avoided in the perioperative period. Action of **oral anticoagulant** medications needs to be reversed prior to neurosurgical intervention. Use of Prothrombin complex concentrate has shown to be fast and effective in reversing the action of the anticoagulants[111-116]. Vitamin K should also be administered concurrently since the half-life of the Vitamin K is more than that of the Prothrombin concentrates[117]. For elective procedures, oral anticoagulants should be stopped prior to the procedure after consultation with the appropriate specialist. Bridging with low molecular weight heparin or unfractionated heparin should be

considered if anticoagulation is essential, and this should be withheld 12 hours prior to the surgery[118].

Pre-existing blood disorders:

Patients with any known pre-exiting bleeding/ other blood disorders should have their coagulation parameters and platelet count checked, and normalized before an elective intervention. A standardized questionnaire has been suggested to be used to assess bleeding tendency in a preoperative elective patient[7]. Routine coagulation tests are a part of the preoperative work up but they do not exclude all haemostatic abnormalities and hence further tests should be done based on the history and specialist advice[8,9].

Fluid replacement pre surgery:

Care should also be taken intraoperative with regards to volume replacement. Hydroxyethyl starch is commonly used in resuscitation in trauma patient and also to achieve hypertensive hypervolemic hemodilution therapy to manage cerebral vasospasm following SAH[119,120]. There are reports published showing association of this fluid with decrease in fibrin[121], low platelet count[122] and drop in von Willebrand factor and factor VIII[123,124], all of which lead to increased bleeding tendency. Patients with excessive intraoperative blood loss are at a higher risk of postoperative hematomas[125].

Thus, to conclude, pre-operative evaluation and management of neurosurgical patients is quite delicate since the homeostasis between procoagulant and fibrinolytic mechanisms can be affected by multiple factors.

INTRAOPERATIVE HAEMOSTASIS

Neuroanaesthesia

Goals of neuroanesthesia are a stable induction and intraoperative course, providing an optimal surgical field and rapid recovery for neurologic assessment[13]. There are different intravenous and inhalational agents available but the effect of drug on cerebral blood flow (CBF), cerebral metabolic rate (CMR), cerebral blood volume (CBV) and intracranial pressure (ICP) determine its use[12].

Volatile agents remain more popular in neurosurgery, in particular sevoflurane[13]. Animal model studies have shown sevoflurane to have the least effect on CBF and ICP when compared to desflurane and isoflurane[126]. Also clinical studies have shown sevoflurane to cause least vasodilatation and minimal effect on autoregulation[127-130].

Propofol an intravenous agent reduces CBV and ICP and does not affect autoregulation[131,132]. It is also is a potent vasoconstrictor and reduces CBF by decreasing metabolism[133,134].

Carbon Dioxide (CO2) responsiveness is also important since hypercarbia increases CBV and ICP by vasodilatation, while hyperventilation causes the opposite. CO2 reactivity is maintained by sevofluorane in both adults and children[135-138].

Steroids are routinely given in neurosurgery to decrease vasogenic cerebral edema[13]. But care should be taken with regards to high sugar levels caused by them, as it can be detrimental and lead to ischemia[139]. Other agents available to reduce edema and ICP are mannitol[140-143] and hypertonic saline[144,145].

Apart from medications/ drugs, intraoperative positioning also plays a role to help get a suitable operative field. Studies have shown that a ten-degree and 40-degree head up position helps decrease ICP but cerebral perfusion pressure remains unchanged[146-148]. There is published literature, on the effect of sitting position in reducing CBV[149,150].

Surgical Haemostasis

Bleeding control in surgery is of utmost importance. Effective haemostasis provides a good field for the surgeon to work in. It also minimizes blood loss thus preventing local and systemic complications for the patient.

Conventional methods to obtain haemostasis involve mechanical pressure, suture ligature or application of tourniquet. However these involve identification of the bleeder under direct vision and hence adds to operative time[151]. Thermal methods such as electro cautery is also commonly used to control bleeding, but this has its side effects of causing heat damage to nearby structures and increases chances of infection[152]. The above techniques are not effective for diffuse bleeding and in difficult to reach areas.
With a rising preference for minimally invasive surgical procedures (endoscopic, robotic, laparoscopic) use of topical haemostats is increasing[15]. Topical agents provide ease of use for the surgeon in different situations and currently there is a lot of active research in this direction[17].

As described in literature, an ideal haemostat is an agent that fulfills five important performance criteria[153], namely safety, efficacy, usability, cost and approvability. Till date this ideal agent has not been developed and there is continued research in this direction.

In this chapter we shall discuss briefly various topical haemostatic agents, their advantages, disadvantages and mechanism of action.

TOPICAL HAEMOSTATIC AGENTS

Based on literature review, topical haemostats are classified into the following different types based on their structure[15-17,19,154]:

1. Gelatin based haemostats
2. Collagen based haemostats
3. Cellulose based haemostats
4. Albumin derived haemostats
5. Polysaccharide based haemostats
6. Inorganic agents
7. Fibrin based agents
8. Antifibrinolytics
9. Vasoconstrictors
10. Bone wax
11. Polyethylene glycol polymers
12. Cyanoacrylates
13. Autologous human tissue- muscle patch
14. Others

GELATIN BASED HAEMOSTATS

These agents help to control bleeding predominantly by a mechanical action. When applied to a bleeding surface, the porous gelatin absorbs the blood and swells up. This aids in haemostasis by tamponade action at the hemorrhagic area[16,155-157]. They are available in the form of sponges, powders and paste, available commercially as Gelfoam or Surgifoam[15].

The advantages of gelatin-based agent are the simplicity of use and easy storage at room temperature. These agents once removed from the packs cannot be reused. They are also cost effective when compared to other topical haemostats[158].

Since the mode of action of haemostasis is increase in weight by virtue of the product swelling up, care must be exercised while using it in closed cavities[159]. Pressure effects on nearby structures such as nerves can lead to local injury, hence only a minimal amount of the agent should be used and any excess material should be removed[160-163]. Possible foreign body and allergic reaction to porcine and bovine gelatin should be kept in mind in patients who have known allergies[164]. Gelatin based products should not be used intravascular, because of the risk of embolization and are inappropriate to be used in conjunction with blood salvage equipment[15,165].

Gelatin can also be combined with thrombin to increase its efficacy. Thrombin acts by converting fibrinogen at the site of bleeding to fibrin and thus aids in clot formation[166]. Combination of gelatin with thrombin thus has dual advantage of mechanical pressure effect and chemical coagulation[166]. Thrombin available for use is currently available

from three main sources[167], namely, bovine thrombin, human pooled plasma derived thrombin and recombinant thrombin.

When used in combination with thrombin care should be taken in patients who have bovine allergies[168]. These can also lead to antibody stimulation in patients that can cause fatal coagulopathies[168]. Human pooled plasma thrombin has the risk of disease transmission and allergic reactions as well[167]. Care should also be taken while using blood salvage systems since thrombin can reverse the heparin anticoagulation effect[165].

There is published literature to support the safety and efficacy profile of the various gelatin-based products in neurosurgery and hence these are extensively used in different types of surgeries[155,169-183].

COLLAGEN BASED HAEMOSTATS

These agents have been around for more than 40 years[156,184-186]. Derived from a bovine source, these agents aid in bleeding control by increasing platelet aggregation, degranulation and eventual clot formation[187,188]. They are available either in the form of sheet, foam or powder[17]. They are also available in combination with thrombin[17]. The collagen-based products are easy to use and store (room temperature)[17]. They are more expensive than the gelatin haemostats but are more efficacious as well. Similar to gelatin-based products, these agents also tend to swell and hence pose the risk of tissue compression[189]. There are also studies showing granulomatous reactions at the site of use, adhesion formation and allergic reactions[190-198]. These agents can pass through filters and hence should not be used with blood salvage systems[199]. When used in combination with thrombin these agents are more effective[200-202, 203-206], but again

drawbacks and side effects of using thrombin (as mentioned above) should be kept in mind. Ultrafoam is a newer collagen product, which does not swell and is approved for use in all surgical specialties[15]. Commercially available products using collagen are- Avitene, D-stat, Instat, Integra, Vitagel.

CELLULOSE BASED HAEMOSTATS

Oxidized cellulose and oxidized regenerated cellulose are very commonly used in surgery for haemostatic purposes. In addition to their mechanical action they also aid in bleeding control by denaturing blood proteins, interaction with platelets and activation of the coagulation pathways[207]. Cellulose also provides a low pH, which is responsible for its bacteriostatic action[207,208]. However the acidic pH inactivates thrombin and hence should not be used in combination[155].

Surgicel is the most commonly used product in this group and is available in the form of sheets[15]. It provides high tensile strength and large coverage and its ease of use and storage makes it a very attractive option[15]. Like other agents, oxidized cellulose can also can cause pressure damage by virtue of swelling and hence should be avoided in closed cavities[209,210]. Reports of Surgicel granulomas[211,212], foreign body reactions[213] and neurological complications[214-216] are mentioned in literature.

ALBUMIN DERIVED HAEMOSTATS

These agents behave like tissue adhesives and aid in bleeding control. Most commonly used product in this group is BioGlue[17]. Components of BioGlue are albumin and gluteraldehyde, which are mixed just before use. Gluteraldehyde cross-links with

albumin and surface proteins in human tissue[217,218] and this crosslinking is independent of the patients coagulation system.

It is commonly used in neurosurgery to contain CSF leaks[219], in vascular surgery for aortic dissection[220] and anastomotic procedures[217] and in cardiovascular surgeries[221]. The advantage of this product is the strong adhesive it forms for sealing purpose that is independent of the physiological clotting mechanism[222]. However it is not without its drawbacks. Risks of using this product include embolization[223,224], strictures[225], pseudo aneurysm formation[226] and toxic effects of gluteraldehyde[227].

POLYSACCHARIDE BASED HAEMOSTATS

These agents are the recent entry into the topical haemostatic group. There are two types available, namely, Microporous polysaccharide haemospheres and N-acetyl glucosamine glycosaminoglycans compounds.

Microporous Polysaccharide Haemospheres

Derived from plant starch they work by dehydrating blood and thus aids in clot formation[228]. Reports have presented their efficacy to control capillary, venous and arteriolar bleeding[152,228-230]. They are quick to use and easy to store. However they are not without complications. Like other mechanical agents they too swell significantly and hence should be avoided in closed spaces[15]. Also care should be used in diabetic patients since they are starch based and can increase the glucose load[15].

N-Acetylglucosamine Glycosaminoglycans Compounds

There are of two types, those that are purified from diatoms and microalgae, poly-N-acetylglucosamine (p-GlcNAc) and those obtained from crustaceans, chitosan[17]. It is believed that the p-GlcNAc compounds aid in haemostasis by activation and aggregation of platelets, agglutination of RBC and release of vasoactive substances. It therefore can act independently of the patient's haemostatic mechanisms[31,231-233]. They are available as Syvek Patch and RDH (rapid deployment haemostat).

Chitosan derived agents are purified from crustaceans. It is a natural polymer with a good safety profile possessing haemostatic, anti-adhesion and wound healing, properties[32,234-237]. Exact mechanism by which Chitosan achieves haemostasis is unclear. Chitosan however is thought to work independent of the coagulation cascade and possibly due to the interaction between the positively charged chitosan and cell membrane of RBC[36,238]. It has been shown previously that Chitosan is an effective haemostatic agent both, in a heparinized model and in a platelet dysfunction model[33,35]. There are many commercially available preparations of chitosan- based haemostats.

In our research study we have used Chitosan Dextran gel, which has been patented and developed by our department with the University of Otago, NZ[239]. Chitosan Dextran gel is a combination of chitosan and dextran aldehyde and has been shown in various studies in the nose and sinuses to have significantly controlled bleeding, improved wound healing and reduced adhesion formation[37,234,239,240].

INORGANIC HAEMOSTATS

These haemostats were developed in conjunction with the US military for application in large traumatic hemorrhages and are not available for commercial use[241]. They consist of zeolite granules that act by absorbing water from the bleeding site, thus leading to concentration of platelets and clotting factors at the site[242]. This process releases heat at the site when it comes in contact with blood, leading to coagulation of the local bleeders[242-244]. It is also believed to activate the coagulation pathway[245]. However this exothermic reaction can at times raise the temperature too high causing tissue damage to the surrounding structures[243,246,247]. The efficacy of this product has been shown in a number of animal model studies[243,246,248] and only one human study [249].

FIBRIN BASED AGENTS

All fibrin-based products contain a fibrinogen source, thrombin, factor XIII and/or antifibrinolytic agent. When all the components are mixed a polymerized fibrin clot is formed which seals the bleeding site. This product eliminates the need for fibrinogen from the patient for bleeding control[250,251]. Concentration of thrombin and fibrinogen vary in different available commercial preparations. Higher concentration of fibrinogen tends to form stronger clots, while higher thrombin concentration form clots more quickly[251,252].

These agents have adhesive properties in addition to haemostatic and can also be used as a carrier of other substances to the bleeding site[253]. They have been reported to reduce adhesions[254] and improve wound healing[253]. As discussed previously the thrombin can be made available from three different sources[18]. Similarly, fibrinogen can also be obtained from either pooled human plasma or autologous plasma. Newer products are

thus available, which use autologous plasma for source of fibrinogen and/or thrombin. The side effects of using fibrin-based agents are similar to thrombin based agents, namely, risk of allergy[255], disease transmission[256], immunogenicity and antibody formation[257] and should not be used with blood salvage systems secondary to risk of clotting[15]. However there are many studies available in literatures that have shown that these agents are effective and safe [258-264].

ANTIFIBRINOLYTICS

Tranexamic acid, aprotonin and Epsilon Amino Caproic Acid (EACA) are the known antifibrinolytic agents. Antifibrinolytics are incorporated in some fibrin sealants to help increase the stability of the clot formed. They work by inhibiting plasminogen and thus increase the stability of the clot[265-268]. They are available as both systemic and topical agents for use in the form of mouthwash, tablets and solutions.

VASOCONSTRICTORS

Most commonly used agent in this category is epinephrine, commonly used in dentistry[269-271]. Its mechanism of action is to decrease bleeding by acting on the vessel wall receptors and producing vasoconstriction and stimulate platelet aggregation[271,272]. It is easy to use but the disadvantage of this agent is that the action is transient[270].

BONE WAX

Not very commonly used nowadays, it main use was to control bleeding from bony edges[160]. It causes mechanical obstruction of the bleeders and aided in haemostasis[160]. The disadvantage of this agent is that it inhibits bone healing and new bone formation[273].

POLYETHYLENE GLYCOL POLYMERS

Currently two products are available to be used as sealants in this category. Coseal and Duraseal are two synthetic agents that form a hydrogel that seals the tissues[15]. Coseal is used mainly in vascular surgery and Duraseal is mainly used to seal CSF leaks, both are used as adjuncts following primary repair. The safety of Coseal in vascular procedure has been studied in a multicenter trial showing 86% efficacy[274] and Duraseal showed 100% efficacy in CSF leak closure intra-operatively[275]. These agents also tend to swell up when they come in contact with human tissue and hence should not be advocated while use in closed cavities and delicate structures[153]. Other side effects include allergic reaction to the agents and their components, delayed wound healing and inflammatory reactions[153].

CYANOACRYLATES

These agents are for topically use only and act primarily as adhesives. Most common use is for skin closure where the edges can be approximated without tension and used as an adjunct for tissue closure[276-278]. They are simple to use and store. They are not approved for internal use though there is literature available, showing their use in gastric and esophageal varices[279]. They tend to produce heat through exothermic reaction when they come in contact with tissue, which may produce discomfort for some patients[280]. Care should also be taken to prevent eye injury.

MUSCLE PATCH

Use of skeletal muscle to control bleeding in surgery has been documented in literature as early as the 1900s[281,282]. However their use gradually diminished with

advent of newer topical agents. Recently however, there has been publications showing the use of muscle patch as a haemostat for controlling high-pressure arterial bleeds in endoscopic skull base surgery[38,283]. Literature on muscle patch in haemostasis is limited and a review of all available publications in this area is listed in Table 1.

Exact mechanism of action of muscle tissue as a haemostat is still unclear. Initially it was thought to be secondary to tissue factor activating the extrinsic pathway of coagulation[284]. However, studies have shown there is no tissue factor in skeletal muscle[285].

The benfits of using muscle is that it eliminates the need for other synthetic or animal derived agents thus decreasing side effects, such as immunogenicity, allergy and foreign body reaction. It is easily available and thus may prove to be cost effective as well. Since muscle is soft and pliable it can be used in all types of surgical fields, thus making its use versatile.

Table 1: A summary of literature on the use of muscle in haemostasis

Harvey Cushing	1911	Temporal muscle controlled venous bleeding from dura
Victor Horshley	1914	Haemostasis by a piece of muscle on exposed brain in a laboratory experiment
Edward Risley	1917	Compared haemostatic efficacy of fat, fascia and muscle to control bleeding in parenchymatous organs
Lawen	1917	Use of muscle grafts for haemostasis in two heart wounds
Ciminata	1924	Haemostasis in nephrotomy wounds
Joseph	1931	Haemostasis in nephrotomy wounds
Ockerblad	1934	Viable muscle grafts to control bleeding from kidney parenchyma
Howard Clute	1935	Muscle grafts for haemostasis in general surgery
Krakovskii *et al*	1974	Used canine models to demonstrate the haemostatic efficacy of muscle graft
Zolotorevskii *et al*	1976	
James AG	1984	Muscle Thromboplastin in haemostasis
Xu *et al*	1994	Control of presacral hemorrhage with electrocautery through a muscle fragment pressed on the bleeding vein
Reece *et al*	1995	Muscle tamponade to control venous bleeding around grafts to deeply intramyocardial coronary arteries.
Remzi *et al*	2002	Muscle tamponade to control presacral venous bleeding
Harrison *et al*	2003	Muscle fragment welding for control of massive presacral bleeding during rectal mobilization
Ayuste *et al*	2004	Rectus muscle fragment welding for presacral bleeding
Fehm *et al*	2005	Haemostasis in common carotid artery with fibrin sealant attached muscle pads in a rat model.
Weidenbecher *et al*	2005	Quadriceps muscle fascia graft for haemostasis in Internal Carotid artery injury during FESS.
Valentine *et al*	2011	Use of Sternomastoid muscle patch for haemostasis in a sheep model of carotid artery injury.
Padhye *et al*	2014	Efficacy of muscle patch on haemostasis, pseudoaneurysm formation and long term patency for different injury types in a sheep model of carotid bleeding

OTHERS

Self-Assembling Nano-Peptides

This is the latest in development of topical haemostats. These products have shown very promising results in the animal studies with bleeding control in less than 15 seconds[286]. They mainly consist of a four amino acid sequence that have opposite charges that self-assemble when in contact with physiological fluids to form a barrier and seal the leaking area. The advantage of this system is its ability to work independent of the coagulation cascade and later breakdown into amino acids, which can be used for wound repair[286,287]. Human trials are still pending to study the efficacy of these agents.

Thus to conclude, there are many different types of topical haemostats available in the market. Knowledge about these agents is essential so as to help use the best agent in a bleeding situation to get effective haemostasis with minimal complications. Table 2 shows the common commercially available products in the above-mentioned categories.

Table 2: Commercially available haemostats[15,17,154]

Gelatin based products	Gelfoam	Porcine gelatin
	Surgifoam	Porcine gelatin
	Floseal	Bovine gelatin +pooled human thrombin
	Surgiflo	Porcine (+/- thrombin)

Collagen based products	Avitene	Bovine collagen
	Helistat and Helitene	Bovine collagen
	Instat, Instat MCH, Ultrafoam	Bovine collagen
	Vitagel	Bovine collagen+bovine thrombin+ autologous plasma
	D-stat	Bovine collagen+bovine thrombin
Cellouse based products	Surgicel, Surgicel Fibrillar, Nu-knit	Oxidized regenerated cellulose
Albumin derived products	Bioglue	Bovine serum albumin and 10% glutaraldehyde
Polysaccharide based products	Syvek Patch	p-GlcNAc
	Rapid deployment haemostat (RDH)	p-GlcNAc
	HemCon	Chitosan
	CloSur PAD	Chitosan
	ChitoSeal PAD	Chitosan
	ChitoDex	Chitosan
	TraumaDex, Bleed-X, Hemaderm,Arista AH	MPH
Inorganic agents	Quickclot	Zeolite granules
Fibrin based agents	Tisseel	Human fibrinogen+human thrombin+ human Factor XIII+ bovine aprotonin
	Quixil	Human fibrinogen+human thrombin+Tranaxemic acid
	Evicel	Human fibrinogen+human thrombin
	TachoComb	Equine collagen+human

		fibrinogen+bovine thrombin+ bovine aprotonin
	TachoComb H	Equine collagen+Human fibrinogen+human thrombin +bovine aprotonin
	TachoSil	Equine collagen+human fibrinogen+human thrombin
	Vitagel	Individual human plasma+bovine collagen+bovine thrombin
	Artiss	Pooled Human Plasma
	Vivostat	Autologous
	Cryoseal	Individual human plasma
Polyethylene glycol polymers	Coseal	Two PEGs
	Duraseal	PEG+Trilysine Amine
Cyanoacrylates	Dermabond	Octyl Cyanoacrylate
	Indermil	Butyl Cyanoacrylate
	Histacryl, Histoacryl blue	Butyl Cyanoacrylate
Thrombin	Thrombin-JMI	Bovine thrombin
	Evithrom	Pooled human thrombin
	Recothrom	Recombinant thrombin

CHAPTER 5 WOUND HEALING

NORMAL WOUND HEALING

Wounds affect millions of people worldwide and many are hospitalised for wound management every year[288,289]. Wound healing is a well-organized process that goes through a set of sequential phases leading to the restoration of the integrity of the tissue. It involves interaction between the cells of different systems (such as immune cells, cells of the coagulation system and fibroblasts)[290-292].

Healing of skin wounds happens by either primary or secondary intention. When injury only involves the epithelial layer, such as those cause by surgical incision, healing is by primary intention and the phases of wound healing includes inflammation, epithelial proliferation and connective tissue maturation. When the injury involves more extensive tissue loss, such as those associated with necrosis, large ulcerations, abscess, there are four phases of wound healing, namely coagulation/ inflammatory phase, granulation tissue phase, matrix remodelling and scar tissue formation phase[291-293].

Coagulation/ Inflammatory Phase

After injury to an area/ break in the endothelial cell layer in the blood vessels, there is a sequence of events that takes place leading to the formation of a local haemostatic plug and activation of the coagulation cascade leading to clot formation[293] (Figure 5A). Platelets are amongst the first group of cells that reach bleeding site, get activated when they bind to the subendothelial Von Willebrand Factor (vWF) and release a number of platelet granules. There is also a release of a number of growth factors and cytokines. Important factors include platelet-derived growth factor (PDGF), Tissue Growth Factors (TGF-A1 and TGF-A2). These factors along with the other cytokines that are released attract the neutrophils and macrophages to the site. These inflammatory/ immune cells

release enzymes and proteases that play an important role in clearing the area of bacteria and other foreign bodies[291,294]. Gradually over a period of time there is resolution of this inflammatory response. The exact mechanisms that lead to the resolution of the inflammatory phase are not completely understood, but release of a number of anti-inflammatory cytokines such as interleukin-1 and TGF and other bioactive lipids play an important role in this process [295,296].

Proliferative/ Granulation Tissue Phase

An active proliferative phase begins as inflammation settles down. Growth factors and cytokines produced by the inflammatory cells act in an autocrine and paracrine manner to maintain fibroblast proliferation. These factors also play an important role in the proliferation of the basal layer of the skin leading to re-epithelisation[291,292]. In order to meet the increased metabolic demands, nutritional requirement needs to increase proportionally. This process drives the significant increase in the vascular supply to the region leading to marked angiogenic response (Figure 5A and 5B) [293].

Increased angiogenesis is a very important part of the proliferative phase. The process of angiogenesis starts immediately after the injury due to relative local hypoxia that occurs due to the disruption of blood vessels and vasoconstriction. A number of pro-angiogenic factors are released locally ranging from platelet derived growth factor (PDGF) that is released immediately by the platelets when they reach the site to Vascular endothelial Growth Factor (VGEF) and Fibroblast Growth Factor (FGF)[291,297,298]. More recently an important component that has been identified that plays a very significant role in this process are the Endothelial Progenitor Cells (EPCs), that are usually present in the bone marrow[299-301]. Due to the mediated effect of the cytokines (VEGF, Nitric Oxide and various metalloproteinase), the progenitor cells migrate

towards the area of injury and differentiate into new endothelial cells, thus playing an important role in angiogenesis[301]. There is further emerging evidence that in diabetic patients who often suffer from chronic wounds, there is a defect in the endothelial progenitor cell mobilization resulting in poor wound healing as a result of poor re-vascularization [300,302].

Remodelling/ Maturation Phase:

With increasing migration and proliferation of the epidermal cells as mentioned above, the integrity of the epidermal layer is reinstated. Proliferation of the fibroblasts ensues along with the synthesis of the extra cellular matrix leading to the formation of granulation tissue (Figure 5C). A provisional matrix is laid down that is composed of type III collagen, fibronectin, fibrin and hyaluronic acid[291,293]. This matrix is slowly replaced by type I collagen as the matrix matures. The tensile strength of the tissue increases with increasing type 1 collagen in the matrix[291]. The next phase in would healing is that of wound contraction that happens via a specialised type of fibroblast called myofibroblast that are differentiated fibroblasts that acquire smooth muscle actin fibres. There is associated continuous matrix reorganization[303,304]. This whole process is dynamic and goes through multiple cycles of matrix formation and degradation. Multiple matrix reorganization enzymes and matrix metalloproteinase play an important role in the remodelling process[304]. Final step in the wound healing process is apoptosis of the fibroblast, thus leaving behind a reasonably acellular scar tissue that is mainly composed of collagen and whose strength is similar to that of the surrounding tissue. Although multiple cytokines and growth factors have been implicated in this apoptosis process, exact mechanism remains unclear[305,306].

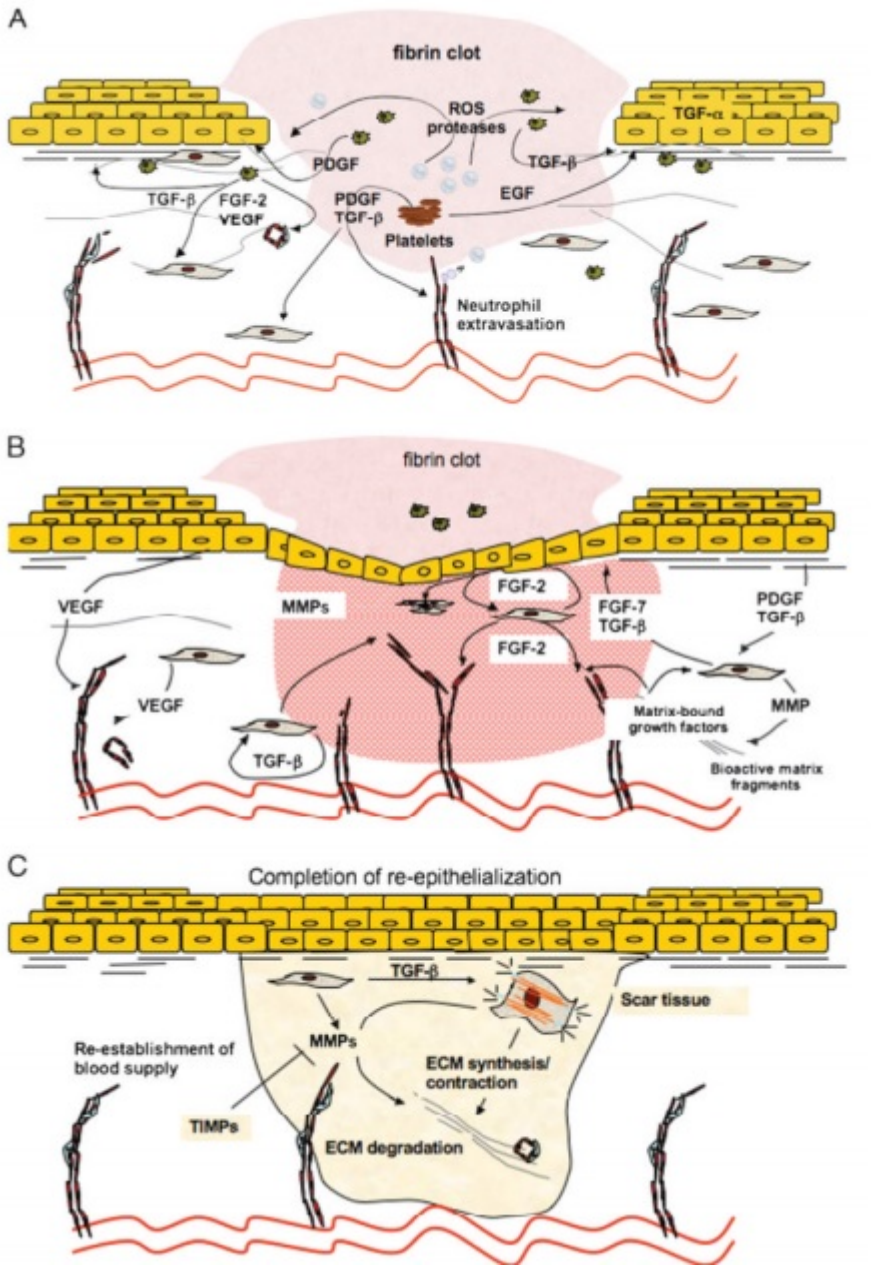


Figure 5: Phases of wound healing: Coagulation phase (described in previous chapters), Inflammatory phase (A), Proliferative/ granulation tissue phase (B), remodelling phase (C). In the coagulation and inflammatory phase (A), blood cells, namely, neutrophils, macrophages and monocytes play an important role in releasing the growth factors, cytokines, laying of the provisional matrix and initiation of epidermal migration. In the granulation/ proliferation phase, which starts in about 72 hours, there is fibroblast and keratinocyte proliferation, extracellular matrix synthesis and angiogenesis. In the remodelling phase (C), (at about 1 week post injury), there is remodelling of the

extracellular matrix, apoptosis of the cellular elements and leading to the formation of an acellular matrix.

(Taken from: Demidova-Rice, T.N., Hamblin, M.R. & Herman, I.M. Acute and impaired wound healing: pathophysiology and current methods for drug delivery, part 1: normal and chronic wounds: biology, causes, and approaches to care. *Advances in skin & wound care* **25**, 304 (2012).)

FACTORS THAT AFFECT WOUND HEALING

Several factors affect wound healing in the post-operative period and in general outpatient scenario. These can be classified as local and systemic factors and have been enlisted in Table 3.

Table 3: Factors affecting wound healing:

Local Factors	**Systemic Factors**
• Oxygenation • Infection • Foreign body • Venous sufficiency	• Age and gender • Sex hormones • Stress • Ischemia • Diseases: diabetes, keloids, fibrosis, hereditary healing disorders, jaundice, uremia • Obesity • Medications: glucocorticoid steroids, non-steroidal anti-inflammatory drugs, chemotherapy

	• Alcoholism and smoking • Immunocompromised conditions: cancer, radiation therapy, AIDS • Nutrition

Taken from : Guo S, DiPietro LA. Factors Affecting Wound Healing. J Dent Res 89(3) 2010: 219-229[307].

LOCAL FACTORS

Oxygenation

Oxygen plays a very important role in nearly all the previously mentioned steps of wound healing[308,309]. It has been seen in many studies that chronic wounds are usually associated with low oxygen tension[310]. Due to disruption in the blood flow in the initial phase nearly all the wounds are hypoxic in the acute phase[308,309]. Hypoxia acts as a strong stimulant to cause the release of cytokines and growth factors that play a significant role in epithelial migration, cellular proliferation, epithelisation and angiogenesis[309]. While prolonged hypoxia impedes wound healing, acute hypoxia triggers wound healing. Furthermore, release of various reactive oxygen species (such as peroxide and superoxide) plays an important role in the killing of microorganisms[309]. One of the therapeutic mechanisms to overcome hypoxia as a cause for poor wound healing is to provide hyperbaric oxygen therapy[309].

Infections

The most important factor that significantly affects wound healing is infection. Microorganisms on the skin surface can easily get access to wound site when there is a loss of continuity of the skin surface. This can lead to contamination, colonization or

spreading invasive infection[307,311]. The acute inflammatory process plays a very important role in removing the microorganisms from the surface of the wound. When there is a significant microbial infection, the inflammatory process becomes prolonged and this affects the subsequent stages of wound healing. Acute inflammation is associated with an excess of proteases and matrix metalloproteases that tend to degrade the extracellular matrix that is formed and hence affects the wound healing leading to the wound becoming a chronic non-healing wound[311,312]. Also, when bacteria remain on the wound surface for a prolonged period of time they tend to form biofilms around themselves that makes them resistant to usual antibiotics. Organisms that are particularly notorious to form such biofilms are P. aeruginosa and Staphylococcus aureus[311,313]. Thus decontamination of any wound surface is of paramount importance as early as possible to enhance wound healing[307].

Venous Insufficiency

Venous ulcers are a significant cause of ulcers in the lower limbs[314]. Faulty valves and failure of the venous pump in the lower limb often leads to misdirected flow of blood from the deep to the superficial venous system leading to venous congestion in the superficial veins leading to venous hypertension and affecting the draining of blood and supply of nutrients to tissue thus affecting the integrity of the skin surface leading to venous ulcers[315].

SYSTEMIC FACTORS

Age

Aging has a significant effect on wound healing. Blood supply to the skin and dermis decreases with age. Also there is a loss of collagen and a decrease in the ability to

produce collagen during the process of wound healing. These factors cumulatively affects wound healing in the elderly[316,317].

Peripheral Vascular Disease (PVD)

PVD affects blood supply to the tissue beyond the site of obstruction. This can affect a single region or can affect multiple sites. Blood supply to provide oxygen and nutrients to any tissue is very important especially to an area that is healing. Limb ischemia is unable to keep up with the metabolic demand of any tissue and this will significantly affect the wound healing process[318-320].

Diabetes

Diabetes causes vasculopathy, neuropathy and immunopathy[320]. Neuropathy predisposes diabetics to develop ulcers in the extremities[321]. These patients are at an increased risk of atherosclerosis and PVD which leads to increased non healing ulcers[322,323]. It is documented that there is decreased or impaired secretion of over a 100 cytokines and growth factors that play an important role in the process of granulation tissue formation, epithelisation, collagenisation and angiogenesis[314].

Stress

Both animal and human models have demonstrated that stress significantly delays wound healing[324,325]. There is an increased release of glucocorticoids during periods of stress. Steroids have anti-inflammatory effect and inhibit wound healing[326,327]. Furthermore, there is evidence that there is a reduction in the levels of various pro inflammatory cytokines such as IL-1, IL-6 and TNF-beta[327,328] during stressful periods.

Obesity

Several factors affect wound healing in obesity. Most important causes includes decreased vascularity in adipose tissue, friction caused by skin-to-skin contact impedes wound healing, increased wound tension, increased tissue pressure and venous hypertension[307].

Chemotherapeutic Agents

Most chemotherapeutic agents decrease cellular metabolism, cell division and have anti-angiogenic effect, resulting in poor wound healing[329,330]. These agents also affect the immune system of the body, which makes the patient immunopathic, which causes cancer patients to often have chronic ulcers[307]. A new group of chemotherapeutic agents is the angiogenic inhibitors such as bevacizumab which has specific anti VEGF properties and thus directly inhibit angiogenesis[331]. It is generally advised that any patient undergoing a surgery should not be on any angiogenic inhibitor or chemotherapy prior to surgery[307].

Malnutrition

Although there is insufficient data to suggest that nutritional supplementation improves wound healing[332,333], there is data to say that poor nutritional status causes delayed wound healing. It is imperative to get the blood albumin levels in all patient who have chronic non healing ulcers[334].

Smoking

Smoking is composed of exposure to nicotine and a mixture of several other hydrocarbons that together have a deleterious effect on wound healing[335,336]. This is

related to the vasoconstrictve effect of smoke leading relative local ischemia, decreased inflammatory response, poor collagen deposition and impaired bactericidal response, thus leading to poor wound healing[337-339].

WOUND HEALING IN CENTRAL NERVOUS SYSTEM

There are certain differences in the structure of skin and central nervous tissue. First being the presence of blood brain barrier (BBB) that plays a significant role in regulating the concentration of various substances in the vessels and in the extra cellular spaces within the nervous tissue[340]. Secondly, the endothelial cells lining the blood vessels form specialised tight junctions in certain sites such as in the kidneys and in the blood brain barrier that very selectively regulate the flow of substances[341]. BBB also protects the neural tissue from any harmful effects of the immune system[342]. This is important because neurological tissue lacks the ability to proliferate and replace if there is a toxic insult[343]. Other cells that play an important role in the nervous system is the supportive connective tissue cells such as oligodendrocytes, astrocytes and microglia.

The process of wound healing essentially involves the same steps, i.e haemostasis, inflammation (early and delayed inflammation) and repair (early and late repair).

Haemostasis

There is a break in the blood brain barrier whenever there is an injury at a particular site, leading to the exposure of the sub endothelial collagen[344] and movement of platelets to the site of injury culminating with the formation of a platelet plug[345]. There is then release of factors that activates the coagulation cascade[346]. In essence, the haemostatic mechanism is not different from that which happens elsewhere in the body.

Inflammation

Inflammation plays an important role in containing and neutralizing the extent of tissue injury that is cause by any insult[347]. Initial phase of the inflammation is caused by the neutrophils that reach the site as cytokines are released. These cytokines then act as chemoattractant that recruit more inflammatory cells to the site[348-351]. This phase of inflammation is also similar to that seen elsewhere in the body.

Subsequent phase of inflammation is mediated by monocytes that move from the blood to the tissue and are activated to become macrophages[347]. They play an important role in the phagocytosis of the harmful agents and other debris at the site. They often continue to exist for a few months depending on the nature of the injury. Macrophages thus play a more important role in the resolution of the inflammatory process[351-353].

When the blood brain barrier gets restored during the initial phase of inflammation, differences start emerging in the healing process in CNS and non CNS tissue. There is decreased leucocyte infiltration and hence reduced damage from acute inflammation. The phagocytosis process is taken over by the resident cells of CNS that have macrophage like function, i.e the microglial cells[343]. These cells at the site of injury can also release pro-inflammatory cytokines that play an important role in phagocytosis[354-356]. However, paradoxically, these cells can also secrete anti-inflammatory cytokines and thus protect the CNS cells from any undue damage by the inflammatory process[357-359].

Astrocytes are the other group of cells in the CNS that get activated post any injury. They normally play an important role in providing the supportive connective tissue

structure in the CNS[344]. However, post injury, like the microglial cells, they also secrete both pro and anti-inflammatory cytokines and thus regulate inflammation and healing[360]. It is seen that in about three days post injury, activated astrocytes are present in the periphery and macrophages and microglial cells are present in the centre of the injury site[361].

Finally, activated lymphocytes are able to cross the blood brain barrier and enter the site of injury by direct extravasation through an endothelial cell leaving the surrounding tight junctions intact. This process takes place slowly as against the same taking place within minutes in peripheral blood[362,363]. T cells undergo apoptosis in the CNS as they find the milieu antagonistic and hence the number of lymphocytes in the region always remain low making CNS an immunologically privileged site[364,365]. The inflammatory phase resolves over a period of time by the release of different anti-inflammatory cytokines[366], leading to the repair phase.

Early Repair Phase

The damaged tissue does not have significant tensile strength during the inflammatory phase. It is during the repair phase that excess of extracellular matrix gets deposited along with cellular proliferation and angiogenesis that helps the tissue get its strength back.

CNS repair differs from the repair mechanism at other sites as there is no regeneration of neurons[367]. As soon as there is an injury there is activation of astrocytes in the region, that start migrating towards the lesion and form a boundary of activated astrocytes. This is referred to as a glial response[368,369]. The centre of this region consists of microglia and

macrophages that play an important role in cleaning up the area of any foreign objects and debris. There is total loss of neural elements within three days[361,370]. Smaller lesions are essentially filled up by reactive astrocytes, while the larger lesions, a central cavity is left behind, surrounded by a boundary of reactive astrocytes. Such a lesion can be easily identified by imaging studies[361,371]. There is functional loss corresponding to the area where the neurons have been lost as there is no regeneration[361].

If an injury involves penetration from the exterior involving the meninges, then the external part of the wound comes in contact with the meningeal fibroblasts, leading to fibroblast proliferation and deposition of an extracellular matrix with associated fibrosis (due to collagen deposition)[370,372-374]. Similar mechanism ensues with injuries of the spinal cord as well, when the meninges have been injured. This is described in greater detail in the subsequent chapter (chapter 6).

In addition to astrocytes, the glial scar tissue has other cells such as endothelial cells and mesenchymal cells. Although the endothelial cells can attempt new blood vessel formation, mesenchymal cells deposit the basal lamina which inhibits the growth of neurons. Thus it appears that glial scar tissue may have a direct role in preventing neuronal regeneration[360,375]. Animal spinal model studies have been conducted to see the effect of removal of activated astrocytes in the areas of spinal injury. Although the glial scar formation is prevented, removal of the astrocytes resulted in the spread of the inflammatory cells to the surrounding region, resulting in areas around the injury getting directly infiltrated by the inflammatory cells thus leading to increased neuronal degeneration and increased loss of functionality[360,369,374]. Thus the glial scar helps in protecting the surrounding neural tissue from the effect of the inflammatory cells and

also prevents a disorganised proliferation of neurons which may result in abnormal neural signals[360,376,377].

Late Repair Phase/ Remodelling Phase

Skin wound that heals by secondary intention undergoes reorganisation or remodelling. Collagen rich scars do not have a very specific organised cellular and matrix architecture, and although the tissue gets back a reasonable amount of tensile strength, it does not come back to the same strength of the surrounding uninjured tissue[378,379]. This is the phase where there are significant differences between CNS and non-CNS tissue, as there is virtually no regeneration or proliferation of the neurons in the affected region[367]. On the contrary, during the phase of remodelling, the scar tissue within the CNS becomes denser as the astrocytic process get more intertwined. Any cytokine activity remains confined to within the area of the glial tissue[367]. There is no reorganisation, which is the hallmark in skin wound (including wound contraction etc). When the process of removal of the necrotic neurons is complete within the affected region, there is apoptosis of the macrophages and microglial tissue, finally leaving behind an empty cyst-like cavity filled with CSF that is surrounded by a dense layer of astrocytes that separates this cavity from the surrounding normal neuronal tissue. Normal neurons can never grow back into the central cavity due to the persistence of inhibitory molecules within the scar and hence neural functionality is never regained[367,370].

In summary there is no significant difference in the initial phase of healing between skin and CNS tissue, i.e the sequence of events that happen at the cellular level in the haemostasis and inflammatory phases are quite similar. There are some differences in

the early repair phase, as neural tissue does not have any regenerative capacity in the central nervous system. Further the glial scar tissue forms a layer separating the normal neural tissue from the injured area. This also prevents any inflammation from spilling over from the injured area to the surrounding normal neural area. Finally, there is no reorganisation that takes place in the remodelling phase/ late repair phase in the CNS compared to skin where there are extensive reorganisation of structures that happens in this phase.

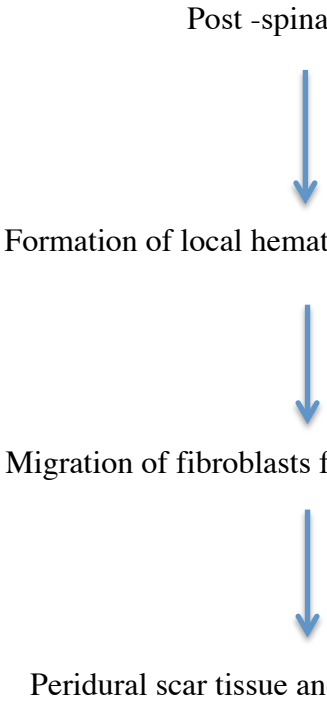
CHAPTER 6 EPIDURAL ADHESIONS: PATHOPHYSIOLOGY AND PREVENTION

PERIDURAL FIBROSIS- THE LAMINECTOMY MEMBRANE

The laminectomy membrane is defined as the dense fibrous scar tissue that fills the laminectomy surgical site and connects the dura to the overlying muscle[380]. LaRocca and Macnab were the first to describe the laminectomy membrane[28]. In their study, multilevel lumbar laminectomies were carried out in dogs and the surgical site was histologically examined at different time points to observe the healing process. They observed that in the early post-operative period, laminectomy defect area was filled with a hematoma. Later on, fibroblastic activity was seen from the undersurface of the erector spinae muscle, which gradually extended into the hematoma. In the last stage fibrous scar tissue replaced the blood clot that covered the whole defect from the muscle to the surface of dura and reaching up to the nerve roots. This laminectomy membrane formation was consistent in all laminectomies performed and the amount of peridural fibrosis increased with the size of the surgical defect[28].

Subsequent studies by others have also reported fibrosis post laminectomy and the potential role of platelet-derived growth factor (PDGF) in this process. PDGF derived from the aggregating platelets in the local hematoma is believed to be responsible for the migration and proliferation of the myofibroblasts[381-383]. Other possible causes mentioned in literature with regards to epidural fibrosis are foreign body reaction to the surgical materials and defect in the fibrinolytic pathway[28,384].

However the most common mechanism described repeatedly in literature is the one first mentioned by LaRocca[28]. A schematic diagram of the process of peridural scar tissue formation is shown below:



FAILED BACK SURGERY SYNDROME

Failed back surgery syndrome is defined as constant pain in the lower limbs or lower back of patients who have undergone back surgery (laminectomy or discectomy)[385]. There are many causes for this syndrome, but peridural fibrosis is one of the important causes of chronic post -operative pain[29,386 387-392].

Scarring is the natural byproduct of wound healing, and hence peridural fibrosis and adhesions seen post laminectomy/spine surgery is no surprise[26,393,394]. The mechanism of pain in this scenario has been explained[395]. In a normal human being movement of the back, neck and limbs leads to movement of the spinal cord, nerve roots and dura and this does not cause any pain. However, in an individual with peridural fibrosis, the dura and nerve roots are adhered to the scar tissue. Hence any motion of these structures causes more traction and tethering and results in pain.

It has also been reported that phospholipase A2 released secondary to the traction on the nerve roots and dura leads to chronic inflammation and pain[396,397]. Phospholipase A2 production leads to activation of the arachidonic acid pathway that culminates with the production of leucotrienes and prostaglandins, thus increasing inflammation[398]. Prostaglandins and leucotrienes are responsible for the explicit pain[399-404].

Removal of this scar tissue to ameliorate the pain by a repeat surgery often has poor outcomes and the rate of complications are higher because of the difficulty to identify the anatomy amidst the fibrotic tissue[30,405]. Hence the best way to treat Failed Back Surgery Syndrome is to prevent it in the first place i.e to prevent peridural fibrosis.

PREVENTION OF PERIDURAL FIBROSIS

As described previously, peridural scar formation is secondary to post operative wound healing. The extent of fibrosis is believed to be associated with the extent of the operation and haemostatic efficacy[27,395]. A 5-step method to decrease surgical adhesions has been suggested[14]. These include: (i) Control the initial inflammatory reaction (ii) preventing clotting of inflammatory exudates that have already formed (iii) removal of fibrin deposit (iv) barriers to mechanically separate dura and clot and (v) inhibition of fibroblastic reaction[14]. Thus meticulous surgical technique and effective haemostasis are the first steps to aid in reducing peridural fibrosis. We have discussed in the previous chapter the various topical haemostats available in the market to help bleeding control in surgery. We now discuss below the various physical barriers available to reduce post-operative adhesions in surgery.

PHYSICAL BARRIERS

An ideal agent in this category is described as being safe, biocompatible, hypoallergenic, easy to use and store, bio absorbable, retains its activity in the presence of physiological fluids and able to mechanically separate the raw surgical surfaces[406].

Hyskon

It was developed as a 32% dextran solution and its mechanism of action in preventing abdominal adhesions was by hydro floatation, i.e the agent when applied to the surfaces "floats" the organs in the abdominal cavity and separates them. It also has an anticoagulant effect and is easy to use[407,408]. Side effects of this agent includes pain, allergic reaction, edema and its high osmolarity can cause fluid and electrolyte imbalance[409,410]. With regards to the efficacy of the product, studies have shown inconsistent results and hence it is not commonly used[411-413].

Chitosan Dextran gel

This is polysaccharide gel developed by our department, The Department of ENT, University of Adelaide in conjunction with the University of Otago, New Zealand[239]. Its safety and efficacy as a haemostatic and anti adhesive agent in endoscopic sinus surgery has been studied in animal models and clinical studies[234,240]. Animal studies have also shown its anti adhesive efficacy in abdominal surgery[37]. In this research, we have used Chitosan Dextran gel to study its anti adhesion effect in a sheep model in post laminectomy spinal surgery. Its gel like consistency is easy to use and forms a mechanical barrier between the surfaces and prevents adhesions.

Oxiplex

Its components consist of carboxymethylcelluose, polyethylene glycol and calcium chloride. This biocompatible barrier gel has been shown to decrease adhesions[414-416] [417]and is approved for use in spinal surgery[418].

Oxidized Regenerated Cellulose

Another membrane barrier anti-adhesion agent, Interceed is clinically available for use. It has additional anti bacterial properties[419]. Its main drawback was reported that its efficacy reduced in the presence of blood[420,421]. Multiple studies have shown its anti-adhesion effects[422-424].

Hyaluronic Acid Based

This has been used in different forms, namely, solution (Incert or Sepracoat), hydrogel (Intergel) or as sheets (Seprafilm). Due to safety and efficacy concerns of Incert/Sepracoat it is currently not used in humans[425,426]. Seprafilm consists of a combination of hyaluronic acid and carboxymethylcellulose. Animal model and clinical trials have shown very good results with regards to adhesion reduction[427-430]. With regards to safety, the results are mixed and side effects like abscess formation and peritonitis have been reported[431-434]. Intergel is hyalruronic acid crosslinked with ferric chloride to get a gel like consistency. Due to safety issues, it is no longer used[435].

Polyethylene Glycol

FDA approved for human use, these agents are cleared through the kidney. **Spraygel** consists of two PEG solutions which forma a gel when it comes in contact with the tissue at the surgical site and thus acts as a mechanical separator. Animal model and

human trials have shown good antiadhesion results[436-438]. **Poloxamer 407** is another PEG-based antiadhesion agent[439], which too forms a gel at the surgical site[440], however it has reported to be less effective in the presence of blood[441].

Polytetrafluroethylene

Another barrier anti-adhesion agent that is flexible, easy to use and has a small pore size so prevents cell penetration[442]. Gore-Tex is the commercial product in this category[442]. The disadvantage of this product is the need for a second surgery to removed it since it non-absorbable[443].

ADCON-L

ADCON-L gel was introduced to prevent epidural fibrosis in the late nineties. It is a barrier antiadhesion agent that also has properties to prevent migration of fibroblasts and was bio absorbable in 4-6weeks[444,445]. It was widely used initially, however post operative complications like CSF leaks and hypotension were reported and the product is no longer used for this purpose[446-449].

Other than the above mentioned commercial products, other experimental agents like recombinant tissue plasminogen activator[450], liposome encapsulated hydroxycamptothecin[451], amniotic membrane[452], aceclofenac[453], mitomycin C, 5-fluorouracil, cyclosporine A[454], tenoxicam[455], urokinase[383], methylene blue[456], inhibition of pro-inflammatory cytokines with doxycycline[457], use of retinoic acid[458], calcium channel blockers[459] have also shown to reduce adhesions and peridural fibrosis in animal model studies. However till date no agent, which is completely safe and effective, has yet been developed for use to decrease epidural adhesion.

CHAPTER 7: THE EFFICACY AND SAFETY OF CHITOSAN DEXTRAN GEL IN A BURR HOLE NEUROSURGICAL SHEEP MODEL

Statement of Authorship

Title of Paper	The efficacy and safety of chitosan dextran gel in a burr hole neurosurgical sheep model
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Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author (Candidate)	Sukanya Rajiv		
Contribution to the Paper	Performed all background work, developed protocol for analysis, conducted the sheep trial and harvested surgical specimens, preliminary statistical analysis, wrote the manuscript, and made all necessary revisions for manuscript publication		
Signature		Date	3/3/15

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Contribution to the Paper	Performed histopathology of all the specimens		
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Contribution to the Paper	Assistance in development of study protocol and manuscript evaluation		
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Contribution to the Paper	Background polymer evaluation and manuscript evaluation		
Signature		Date	3/3/15

Name of Co-Author	Simon Robinson		
Contribution to the Paper	Manuscript evaluation		
Signature		Date	3/3/15

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Contribution to the Paper	Supervision and manuscript evaluation		
Signature		Date	3/3/15

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Acta neurochirurgica, v. 155 (7), pp. 1361-1366

CHAPTER 8: CHITOSAN DEXTRAN GEL AS AN ANTI ADHESION AGENT IN A POSTLAMINECTOMY SPINAL SHEEP MODEL

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Name of Co-Author	Amanda Drilling
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Name of Co-Author	Ahmed Bassiouni
Contribution to the Paper	Assisted in performing the experimental work, detailed statistical analysis and manuscript evaluation
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Contribution to the Paper	Assisted in evaluation of the MRI scans
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Name of Co-Author	Steve Moratti
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Contribution to the Paper	Manuscript evaluation
Signature	Date: 3/3/15

CHITOSAN DEXTRAN GEL AS AN ANTI ADHESION AGENT IN A POSTLAMINECTOMY SPINAL SHEEP MODEL

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Short (Running) Title: Chitosan Dextran gel in laminectomy

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Conflict of Interest:
Peter-John Wormald, Simon Robinson and Steve Moratti hold a patent for the chitosan dextran gel and PJW receives royalties from Medtronic ENT for instruments designed and is a consultant for Neilmed. No other conflicts of interest.

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ABSTRACT:

Introduction: Post-operative peridural adhesions are known to increase the morbidity after neurosurgical procedures. Aim of this study is to assess the safety and efficacy of Chitosan-Dextran (CD) gel as an anti–adhesion agent in a spinal laminectomy sheep model.

Methods: Eighteen sheep were used in the current study with 6 animals in each treatment arm (namely, CD gel, Gelfoam paste and normal saline control). Posterior lumbar laminectomy was performed in all animals and the dura was exposed and kept intact. CD gel or Gelfoam paste or normal saline was then applied over the exposed dura and the wound was closed in layers. Sheep were euthanized at the end of three months. MRI spine was performed immediately after euthanasia to assess epidural fibrosis. Extent of adhesion in the surgical spinal specimen was assessed by the Peel test and histopathological analysis was used to assess the safety of the agents.

Results: Average scores for the Peel test for CD gel, Gelfoam and normal saline control groups were 1.16 (95% CI, 0.5 – 1.7), 1.5 (95% CI, 0.6 – 2.3) and 3 (95% CI, 2.1 – 3.8) respectively. There was significant reduction in adhesions between treatment and normal saline treated groups (p=0.0292) but there was no difference between Gelfoam and CD gel groups (p=0.56). Average scores on the MRI for CD gel, Gelfoam and normal saline groups were 1.4 (95% CI, 0.9 – 1.8), 1.5 (95% CI, 1.2 – 1.8) and 1.6 (95% CI, 1.3 – 1.8) respectively. There was no difference in the overall epidural fibrosis in MRI scans among the three groups (p=0.2992). Histopathological analysis showed similar results in all the groups with none showing any adverse diagnosis.

Conclusion: CD gel is an effective agent to reduce epidural adhesions with a good safety profile in neural tissue.

INTRODUCTION:

Lumbosacral surgery is a common treatment for spinal disorders[1-3]. Adhesions and scar tissue formation seen following surgery leads to a number of complications and spinal surgery is no exception. The incidence of postoperative pain following laminectomy has been reported to be between 5 and 30%[4]. Adhesions between the dura/nerve roots with the peridural fibrous tissue may manifest clinically in the post procedure period as a recurrent disabling radicular pain and weakness in lower limbs and is referred to as Failed Back Surgery syndrome[5-9]. Epidural adhesions also increase the complication rate for repeat surgeries[10,11].

There have been a number of studies using different agents to prevent epidural adhesions. This includes use of modified surgical techniques[12,13], anti-inflammatory agents[14,15], biological[16,17] and synthetic barriers[18-22].

Chitosan Dextran (CD) gel is a haemostatic polysaccharide gel obtained from crustaceans[23]. Anti adhesion efficacy of this gel has been reported in animal and human studies of Endoscopic Sinus Surgery[24,25]. The gel has also been used in animal models of abdominal peritoneal adhesions with some success[26].

The aim of this study was to investigate the effect of Chitosan Dextran gel on epidural adhesions and wound healing in a post laminectomy sheep model.

MATERIALS AND METHODS:

Eighteen sheep were (merino cross wethers) used in this study. Ethics approval was obtained from the IMVS and University of Adelaide Animal Ethics Committees.

Test agents

Chitosan Dextran gel (CD gel) (University of Otago, New Zealand) was the primary test agent in this study and was prepared immediately prior to use by mixing the chitosan and dextran. The second treatment agent was Gelfoam® which is commonly used in neurological surgery for haemostasis[27]. Gelfoam® powder (Geli Putty, Gelita Medical BV Amsterdam) was mixed with a small volume of 0.9% normal saline until a paste like consistency was achieved. Normal saline (0.9%) flush was used as control.

Surgical procedure

Following acclimatization, sheep were fasted overnight prior to the administration of general anesthesia. General anesthesia was induced with Diazepam and Ketamine injection into the jugular vein. Following intubation, anesthesia was maintained with inhalational halothane.

The laminectomy procedure (at levels L1-L3) was done in the sheep as described previously in literature[19,22]. Briefly, a T13 vertebra was identified using image intensifier. A 20 cm long vertical skin incision was made in the midline lower back below the T13 spinous process. The paraspinal muscles were dissected from the spinous process and laminae with the help of electro cautery and blunt dissection. The dorsal spine processes of L1-L3 were removed by drilling and use of rongeurs. Dorsal spinal laminectomies of 3 cm x 8mm were performed medial to the articular facet joint. Intact dura was exposed and CD gel or Gel Foam paste was applied over the laminectomy site. In control sheep the dura was flushed with 0.9% normal saline. The wound was then closed in layers and the sheep were extubated and returned to the pens for 12 weeks.

Post-operative monitoring

The general condition of the sheep was monitored post-operatively in terms of food intake, temperature, teeth grinding, ground pawing and bleeding at the local wound site. Neurological assessment was done by measuring the tone and power of the hind limbs.

Bupivacaine with adrenalin 0.5% was administered locally at the surgical site in the immediate post-operative period for pain management. IM Clavulox was given before surgical intervention and on Day 1 and Day 2 post-operative. All animals received NSAIDS (Carbofen IM) pre operatively and post operatively for 2 days.

MRI

All animals underwent non-contrast MRI of the spine at the end of 12 weeks. The imaging was done immediately after euthanizing the sheep. Sagittal and transverse spin echo T1 and fast spin echo T2 were done from T13 to L4.

Fibrosis was scored on MRI by assessing the hypo intense area in the epidural region on the axial scans. Each level was divided into 15 slices and a scoring system was devised to look for epidural fibrosis as follows- Grade I – no epidural abnormality, Grade II - <8/15 slices with abnormalities, Grade III - >8/15 slices with epidural abnormalities that was <1mm, Grade IV- > 8/15 with epidural abnormalities that was >1mm. The assessment was done by a neurosurgeon who was blinded to the treatment.

Peel Test

At the end of 12 weeks the sheep were euthanized and following MRI the surgical site spinal canal specimen was harvested en bloc. The cranial half of the specimen was used for Peel test.

The muscles from the lateral and ventral aspects of the vertebra were removed. The bony vertebra, ventral to the spinal cord was removed with help of an electric saw and rongeurs.

A blinded independent observer performed the test. The spinal cord was slowly pulled ventrally and adhesion to the posterior scar tissue was then evaluated by using a scoring system described previously in literature[19] (0-No adhesions, 1- thin membranous threads easily detachable, 2- slight adhesion requiring only minimal blunt dissection, 3- moderate adhesions requiring some sharp dissection and 4- severe adhesions requiring extensive sharp dissection).

Histopathology

The caudal half of the spinal laminectomy specimen was sent for histopathological studies to assess the safety of CD gel in the spinal canal.

The specimen was fixed en bloc in 10% formalin. Following which, each specimen was cut into two pieces transversely with the help of a band saw and put into a decalcifying solution. Prior to decalcification the cut specimens were x-rayed. After approximately a week of decalcification the specimens were again x-rayed to confirm complete decalcification and then embedded into paraffin wax. One transverse section from each tissue block (two sections per laminectomy specimen) was cut and stained with Hemotoxylin and Eosin stain and submitted to the pathologist for assessment.

Inflammation in the scar tissue, the type of fibrosis, foreign body material and new bone formation was compared with the control agents. The pathologist was blinded to the treatment.

STATISTICAL ANALYSIS:

Kruskal-Wallis tests were used to test for the mean differences in scores between the three groups, with post-hoc pairwise t-tests with the Benjamini-Hochberg p-value correction.

RESULTS:

MRI: Average MRI scores for control, Gelfoam and CD gel groups were 1.6 (95% CI, 1.3 - 1.8), 1.5 (95% CI, 1.2 - 1.8) and 1.4 (95% CI, 0.9 - 1.8) respectively. There was no significant difference in the overall epidural fibrosis seen between the controls, Gelfoam and CD gel groups ($p=0.2992$) as shown in Figure 1.

Peel Test: There was significant difference ($p=0.029$) between the controls and the treatment groups (Gelfoam and CD Gel). The control group had an average score of 3 (95% CI, 2.1 – 3.8) and Gelfoam and CD gel had adhesion scores of 1.5 (95% CI, 0.6 – 2.3) and 1.16 (95% CI, 0.5 – 1.7), respectively. The scores of each group are depicted in Figure 2. Post-hoc tests showed that scores of both the Gelfoam and CD Gel were significantly lower than the control group. ($p < 0.05$)

Histopathology: Dense hyaline fibrotic scar tissue was observed in all specimens with no inflammation. Although a foreign body giant cell reaction was seen in five sheep, this was secondary to the presence of foreign suture material in all. All specimens also revealed evidence of new bone formation.

DISCUSSION:

In this study we investigated the anti-adhesion efficacy and wound healing profile of CD gel and compared it to Gelfoam and normal saline. Our study revealed that CD gel performed much better than the saline group in reducing adhesions. However, there was no difference between CD gel and Gelfoam. Overall, fibrosis in the epidural area assessed using MRI imaging was similar in all the three groups, however, the Peel test adhesion scores upon autopsy indicated a lower grade of adhesion formation for the Gelfoam and CD gel.

The pathophysiology of epidural adhesion formation involves post-operative local hematoma at the surgical site being replaced with fibrous tissue by migration of fibroblasts from the paraspinal musculature[28]. This contact between the fibroblasts and dura is believed to lead to post laminectomy adhesions[28]. Holtz et al described a 5-phase method to prevent post- operative adhesions. This included reduction of initial inflammatory reaction and exudate release, inhibiting the coagulation of the exudate, promoting fibrinolysis, mechanical separation of the dura and fibrous tissue and to inhibit fibroblasts proliferation[29].

CD gel is an effective haemostatic agent and biocompatible with neural tissue[27]. Its haemostatic efficacy is believed to work independent of the clotting mechanism[30]. It is also known to improve wound healing and decrease adhesions[24-26]. Studies have shown that chitosan decreased surgical adhesions by inhibiting fibroblast adherence[31] and that chitosan inhibited fibroblast proliferation without affecting the growth of epithelial cells[32]. It also acts as a mechanical barrier when applied over the dura, till it dissolves[25]. The antiadhesion efficacy of Gelfoam reported in literature is controversial. While it has

been reported by some that Gelfoam did not contribute to fibrosis and was effective[28], others have reported it to have equivocal effect[33] or increase adhesions[34,35].

Gelfoam is widely used in neurosurgery for bleeding control[36], however it is not without complications. Cauda Equina syndrome, spinal stenosis, meningitis, and arachnoiditis are some reported in literature[37] secondary to its pressure effect on the delicate neural structures. Other haemostatic agents like thrombin have been reported to increase adhesion formation[38,39]. Chandra et.al showed that Floseal (gelatin+thrombin) increased adhesion formation when used to control bleeding in endoscopic sinus surgery[40]. In addition, these agents carry the risk of disease transmission and allergic reactions secondary to antibody formation[41-43].

The safety profile of various preparations of chitosan has been studied previously with no reported toxic effects[30,44,45]. In this study, healing in the epidural space histologically was similar in all three groups with no adverse effects seen in the CD gel group. Similar results were also seen with the use of CD gel in the brain[27]. It has also been described that purified chitin is non-allergenic[46].

CD gel is currently being used in humans in endoscopic sinus surgery to control bleeding and to reduce adhesions[24]. These properties, haemostasis and anti-adhesion efficacy make it a very viable option to extend its use into neurosurgery.

CONCLUSION:

CD gel was similar to Gelfoam in anti-adhesion action when compared to no-treatment control in a sheep laminectomy model. This suggests that CD gel is an effective agent to reduce epidural adhesions with a good safety profile in neural tissue.

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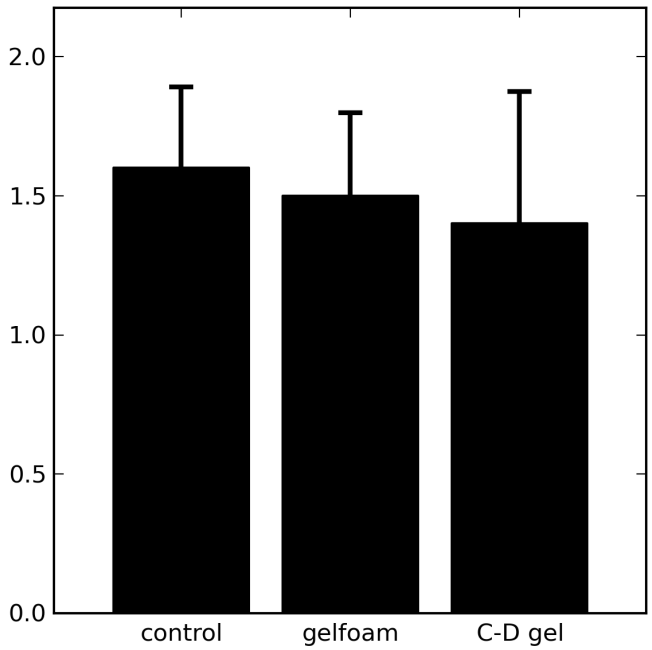

Figure 1- MRI scores of control (1.6), Gelfoam (1.5) and CD (1.4) gel treated sheep (p=0.2992). Vertical axis shows the MRI scores.

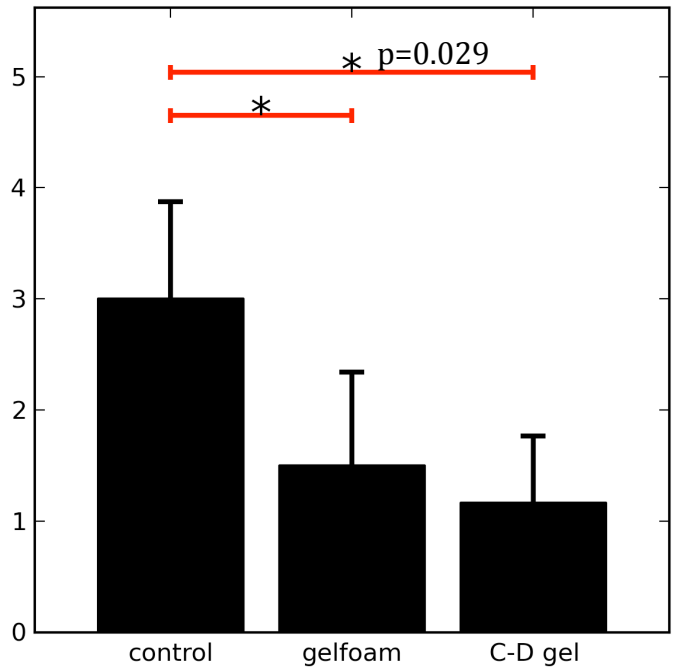

Figure 2- Peel test score of control (3), Gelfoam (1.16) and CD gel (1.15) treated sheep. Vertical axis shows the Peel test scores.

CHAPTER 9: ROLE OF CRUSHED SKELETAL MUSCLE EXTRACT IN HAEMOSTASIS

Statement of Authorship

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Contribution to the Paper	Performed all background work, developed protocol for analysis, conducted the in vitro work, preliminary statistical analysis, wrote the manuscript, and made all necessary revisions for manuscript publication		
Signature		Date	3/3/15

Name of Co-Author	Susan Rodgers		
Contribution to the Paper	Assisted in developing the protocol, supervision of in vitro work and manuscript evaluation		
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Contribution to the Paper	Assisted in performing the in vitro work, detailed statistical analysis and manuscript evaluation		
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Contribution to the Paper	Performed all background work, developed protocol for analysis, conducted the in vitro work, preliminary statistical analysis, wrote the manuscript, and made all necessary revisions for manuscript publication		
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Signature		Date	3/3/15

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ROLE OF CRUSHED SKELETAL MUSCLE EXTRACT IN HAEMOSTASIS

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Short (Running) Title: Muscle in haemostasis

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Peter-John Wormald receives royalties from Medtronic ENT for instruments designed and is a consultant for Neilmed. These have no influence on this paper. No other conflicts of interest.

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Keywords: skeletal muscle, crushed, haemostasis, platelet aggregation, coagulation

ABSTRACT:

Introduction: Use of muscle grafts for haemostasis during surgery has re-emerged with recent animal model studies showing effective bleeding control with their use. However, the mechanism of action is unknown. The aim of this study is to evaluate the action of muscle extracts on the coagulation pathways and platelet aggregation.

Methods: Muscle extracts were prepared by dissolving crushed snap-frozen muscle tissue (0.04mg-0.8mg) in 1ml saline. Saline was used as control. Prothrombin time, activated partial thromboplastin time (APTT), thrombin time and platelet aggregation studies were performed on both muscle extract and saline. Prothrombin time and APTT were repeated using Factor VII deficient plasma, Factor X deficient plasma, Lupus plasma and contact pathway inhibited plasma. Mean readings in the muscle group and control group were compared using non-parametric Mann-Whitney U test (Wilcoxon ranked sum test with continuity correction).

Results: Amongst the various coagulation parameters, there was no significant difference between saline and muscle ($p > 0.05$), except in the APTT using factor X-deficient plasma (mean APTT 133.89s and 185.10s for muscle and saline respectively; $p < 0.0001$). Higher concentrations of the muscle extract (>0.5mg/ml) increased platelet aggregation from 23.9% to 85.5% ($p=0.0001$).

Conclusion: Platelet aggregation plays a role in the haemostatic efficacy of muscle grafts. Even though action on the coagulation pathway via APTT is statistically significant, clinical significance may be low.

INTRODUCTION:

Achievement and maintenance of bleeding control in all surgical procedures is of utmost importance. There are many haemostatic agents available today[1-5]; nevertheless, a review of literature shows that autologous human tissue (such as fat, fascia, dura, clots and muscle tissue) is commonly used to achieve bleeding control[6,7]. Particularly, the use of muscle auto grafts is re-emerging for the management of major hemorrhage, with multiple studies reporting its effectiveness in achieving haemostasis[8-12].

Earliest reports on the use of muscle grafts in haemostasis were by Cushing[13] and Horsley[7]. They used muscle grafts to control bleeding from dura and brain tissue. Cushing concluded that a piece of muscle when held on the bleeding point by a smooth instrument adhered promptly and controlled bleeding better than gauze or cotton.
Following these reports, Clute[14] described effective use of both free and viable muscle grafts in haemostasis in various organs with no late complications. Comparing muscle with fat and fascia, it was seen that muscle had better haemostatic potential[6].

Muscle tissue has been successfully used to control carotid artery bleeding in both animal and human models[10,11,15,16]. It has been shown in a rat model[15] and in patients undergoing Functional Endoscopic Sinus Surgery (FESS)[10] that muscle tissue, if used in an appropriate manner, controls high-flow and high-pressure carotid artery bleeding effectively. Further, in a sheep model of endoscopic surgical carotid artery injury, Valentine *et al*[11] showed that the topical muscle patch and the U-clip anastomotic device were most effective for controlling high-flow and high-pressure vascular injuries.

We have thus hypothesized that muscle tissue may contain thrombogenic factor(s) that enhances coagulation. The aim of the study was to analyze the effect of crushed skeletal muscle on various coagulation parameters and platelet aggregation. We also briefly discuss potential mechanisms of action and advantages for its use in surgery.

METHODS:

Human muscle tissue was obtained from the viable region of the stump from patients who underwent below-knee lower limb amputation surgery at the Department of Vascular Surgery, Queen Elizabeth Hospital, Adelaide. The Human Ethics Committee approved the study protocol. Informed written consent was taken from all patients. Muscle specimens obtained were collected in PBS and immediately taken to the laboratory.

Tissue was washed in PBS and muscle was dissected away from any loose areolar tissue/fat or fascia attached to it and then stored at -80°C to halt any proteinase activity until further use. Later, muscle tissue was cut into small pieces weighing 0.04mg (minimum amount required to do adequate protein analysis, estimated using protein quantification assay) and were snap-frozen in liquid nitrogen, and then crushed with a mortar and pestle to obtain a fine powder. This was dissolved in 1ml of normal saline. These samples were then centrifuged at 4°C for 20 minutes at 13000 rpm (16060g). After centrifugation, the supernatant (crushed muscle extract) was stored as 100µl aliquots at -80°C to be later used in various coagulation tests.

The clotting tests were done using DIAGNOSTICA STAGO ST4 BIO in vitro analyzer (Diagnostica Stago, Asnières sur Seine, France) at the Haemostasis and Thrombosis

Laboratory, SA Pathology, Adelaide, South Australia. Tests of coagulation were performed with 50 µl of plasma (prepared in house) added to either the muscle extract or saline (used here as negative control). Following coagulation tests were performed- (a) Prothrombin time (PT): 50µl plasma was mixed with equal volume of the test agent (crushed muscle extract or saline). This was incubated for 1 minute. Following incubation 100µl Neoplastin (Diagnostica Stago S.A.S) was added and time for clot (fibrin) formation was measured; (b) Activated Partial Thromboplastin Time (APTT): 50µl plasma was mixed with 50µl test agent and 50 µl of Triniclot reagent (Diagnostica Stago S.A.S) and incubated for 5 minutes. After incubation 50 µl Calcium Chloride (Diagnostica Stago S.A.S) was added and time for clot formation was measured; (c) Thrombin time (TT): 50 µl plasma and 50 µl of the test agent was incubated for 1 minute and then 50 µl of 1.5 NIH thrombin (Diagnostica Stago S.A.S) was added to the mixture. Time to clot formation was measured. PT and APTT were also repeated using Factor VII deficient plasma (Diagnostica Stago S.A.S), Factor X deficient plasma (Diagnostica Stago S.A.S), Lupus plasma (prepared in house) and contact pathway inhibited plasma (Tubes containing trisodium citrate and corn trypsin inhibitor SCAT-27-4.5/5, Haematologic Technologies Inc, Essex Junction, VT, USA)-to find out if the crushed muscle extract helps in correcting the prolonged hemostatic parameters that is observed with these. At least four readings for each muscle or control sample were taken.

Helena Aggram Platelet aggregometer (Helena Laboratories, Beaumont, Texas, USA) was used to study the effect of crushed muscle extracts on platelet aggregation at 37°C. The test was performed within 2 hours of blood collection, as per standard protocol. Agents tested in the platelet aggregation assay were: Crushed muscle solutions using

0.04mg/ml, 0.2mg/mL, 0.3mg/mL, 0.4mg/mL, 0.6mg/mL, 0.7mg/mL and 0.8mg/ml normal saline (prepared as described for coagulation tests), Adenosine diphosphate (ADP) 1x 10^{-3}M stock solution (ADP Sodium salt, Sigma-Aldrich, NSW, Australia) as positive control agent and 0.9% Normal Saline as negative control. Blood was collected from a healthy volunteer, using a 19G butterfly needle, into one tenth volume 0.1M trisodium citrate anticoagulant (Greiner Bio-one, pH 6.5). The samples were centrifuged at 18°C for 10 minutes at 1000rpm (140g). Platelet Rich Plasma (PRP) obtained from the previous step was adjusted to a platelet count of 300 x 10^9/L with autologous platelet-poor plasma. The diluted PRP was then drawn up into a 20ml plastic syringe, excluding air from the syringe and then capped[17]. Platelet aggregation was measured using the platelet aggregometer using the test agents at the above-mentioned concentrations and ADP and saline as control agents.

STATISTICAL ANALYSIS:

The mean readings in the muscle group and the saline group were compared using non-parametric Mann-Whitney U test (Wilcoxon ranked sum test with continuity correction) from the *R computer language for statistics* (R Foundation for Statistical Computing, Vienna, Austria). Statistical significance was taken at $p<0.05$.

RESULTS:

Coagulation Studies: No statistically significant difference between saline and muscle was seen, except in APTT using factor X-deficient plasma, which was significant ($p < 0.001$). However, this result is thought to be of little clinical significance because the corrected APTT time was almost 3 times the normal value of humans[18,19].

Platelet aggregation studies: We found extensive aggregation (74-89%) with higher concentrations of muscle (0.6mg/mL, 0.7mg/mL, 0.8mg/mL). Lower concentrations of the muscle solution did not show significant platelet aggregation (5-44%). Results are shown in Table 1.

DISCUSSION:

In this study, we investigated the haemostatic potential of muscle tissue through various coagulation and platelet aggregation tests. The coagulation test results revealed no significant difference between the test agent (muscle) and control (saline) except in the APTT when using Factor X deficient plasma. This difference was thought not to be clinically significant. There was increased platelet aggregation noted with increasing muscle concentrations.

There have been several published reports on the efficacy of muscle as a topical haemostatic agent, but the exact mechanism of action remains elusive. Proposed hypotheses include pressure effect, release of muscle thromboplastin or tissue factor and action as a welding agent.

Application of direct pressure to a bleeding surface is a mechanical method to aid in haemostasis[20-22]. Remzi *et al*[23] reported two cases of use of muscle tamponade to control presacral hemorrhage. In both cases, two untied sutures were applied to the presacral tissues on either side of the bleeding point. Rectus abdominus muscle was harvested and the free muscle flap was then placed over that point and the ties were completed to provide a tamponade effect. There was no further bleeding, necrosis or abscess formation[23].

It was first hypothesized by James *et al*[12] that muscle thromboplastin, or tissue factor, could play a role in the haemostatic efficacy of muscle tissue through activating the extrinsic coagulation pathway leading to clot formation. However, there are also contradictory reports[24]. Drake et al used epitope-defined monoclonal antibodies to human tissue factor for immunohistochemical localization of tissue factor in normal human tissues. Amongst the muscular tissue, tissue factor was expressed strongly in cardiac muscle, variable expression was noted in smooth muscle and was nearly undetectable in skeletal muscle[24]. However, nearly all published literature, have used skeletal muscle as the haemostatic agent. To date no research study has conclusively proved, that muscle thromboplastin or tissue factor is responsible for the haemostatic potential of muscle tissue and hence it still remains speculation.

The third main hypothesis is the use of muscle as a welding agent. Xu and Lin[25] had described the use of rectus abdominus muscle in this manner to control presacral hemorrhage. In this method a piece of rectus abdominus muscle was harvested and then held in place with a forceps to occlude the bleeding site. Electro cautery was then applied to the forceps, which then eventually welded the bleeding site. The muscle fragment served as an electrode for indirect coagulation. Its high water content heated to the boiling point and the bleeding vessel was indirectly coagulated. They demonstrated the technique in 11 patients with a successful outcome in all. Others have since used this technique with positive results [26,27].

PT evaluates the extrinsic and common coagulation pathway, while APTT evaluates the intrinsic and common pathways[28,29]. In our study, the mean APTT (using factor X deficient plasma) was 133.9 seconds with the muscle extract compared to 185 seconds

with saline. Even though this particular result was statistically significant, since normal APTT range in humans is 22-40 seconds[18,19], its clinical significance can be considered to be negligible.

The platelet aggregation studies however show increased platelet aggregation with increasing muscle concentration. Up to 89% platelet aggregation was noticed in our study with the muscle. One limitation of our study is that we did not use increasing concentration of muscle in the coagulation tests. Although a detailed analysis of the stability of proteins post freezing and crushing was not evaluated in this study, there is data to suggest that freezing does not have a significant effect on the activity of various coagulation factors[30].

In this study we found that platelet aggregation appears to be the major mechanism by which muscle patch causes haemostasis. The normal bleeding control mechanism in our body involves three basic steps, namely, vascular wall constriction, platelet plug formation (adhesion and aggregation), and coagulation to form fibrin clot[28]. Studies have shown that skeletal muscle actin and myosin induce platelet aggregation and that these effects are possibly due to ADP associated with the contractile proteins[31]. Along similar lines, it has also been shown that filamentous actin (F-actin) has bound ADP and this helps to improve platelet aggregation by providing multiple interaction sites for platelets[32]. The results of these studies also showed that bound ADP (to F-actin) is more powerful in platelet aggregation than free ADP. It is possible that muscle grafts when applied to the bleeding site may provide a similar scaffold to aid with platelet activation. The major structural proteins in skeletal muscle are actin and myosin and the

bound ADP in these may help in bleeding control by promotion of platelet plug formation.

There are also many other advantages to the use of muscle grafts for the purpose of haemostasis. Muscle graft can be harvested from a nearby surgical area or from another site. Muscle being soft and pliable can conform into irregular bleeding sites and pressure can be applied effectively and uniformly over a large area. It can thus be used to control both pinpoint and diffuse bleeding. It is reabsorbed from the body and therefore does not pose any risk of infection or secondary complication unlike foreign agents. Also during our study we noticed that muscle tissue adheres well to blood vessels which was also previously reported[7].

CONCLUSION:

Reports from published literature provide evidence for the effectiveness of muscle tissue in controlling bleeding during surgery. However, the exact mechanism of action still remains unclear. Our coagulation studies suggest that, although the muscle tissue may have a role in the mechanism of action via the coagulation pathway, the contribution of this particular mechanism on the overall clinical effect is low. Platelet aggregation appears to play a significant role in the haemostatic effect of muscle tissue. This, in addition to the adhesive, aseptic and pliable properties of muscle, makes it a highly suitable agent for bleeding control. Further laboratory and in vivo work is still needed to delineate each of these proposed mechanisms of action.

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Table 1: Results of Platelet Aggregation with Muscle

	Platelet Aggregation (%)	
Muscle Concentration (mg/ml)	**Muscle Sample1**	**Muscle Sample 2**
0.04/0.08	<1	<1
0.1	2.2	4.6
0.2	5.1	4.7
0.3	31.6	26.2
0.4	44.4	31.4
0.6	74.6	89.0
0.7	84.9	89.1
0.8	86.2	89.2
0.9% Normal Saline (negative control)	5.0	
ADP 1 x 10^{-3}M (positive control)	96.7	

CHAPTER 10: EFFECT OF LIMB POSITION DURING SURGERY ON POST -OPERATIVE RECOVERY

Statement of Authorship

Title of Paper	Effect of limb position during surgery on post -operative recovery
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Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author (Candidate)	Sukanya Rajiv
Contribution to the Paper	Performed all background work, developed protocol for analysis, performed the sheep trial, wrote the manuscript.
Signature	Date: 3/3/15

Name of Co-Author	Amanda Drilling
Contribution to the Paper	Assisted in performing the experimental work and manuscript evaluation
Signature	Date: 3/3/15

Name of Co-Author	Tim Kuchel
Contribution to the Paper	Assisted in developing protocol for sheep trial and manuscript evaluation
Signature	Date: 3/3/15

Name of Co-Author	Prof. Peter-John Wormald
Contribution to the Paper	Supervision and manuscript evaluation
Signature	Date: 3/3/15

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Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author (Candidate)	Sukanya Rajiv		
Contribution to the Paper	Performed all background work, developed protocol for analysis, performed the sheep trial, wrote the manuscript.		
Signature		Date	3/3/15

Name of Co-Author	Loren Mathews		
Contribution to the Paper	Assisted in performing the experimental work		
Signature		Date	3 MAR 15

Name of Co-Author			
Contribution to the Paper			
Signature		Date	

Name of Co-Author			
Contribution to the Paper			
Signature		Date	

EFFECT OF LIMB POSITION DURING SURGERY ON POST -OPERATIVE RECOVERY

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Conflict of Interest:
Peter-John Wormald, Simon Robinson and Steve Moratti hold a patent for the chitosan dextran gel and PJW receives royalties from Medtronic ENT for instruments designed and is a consultant for Neilmed. No other conflicts of interest.

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Abstract:

Background: Proper intra operative positioning is essential to maintain blood flow and prevent venous congestion in the dependent areas of the body. This is particularly important in procedures that are prolonged to prevent post-operative ischemic complications.

Case: We report a complication encountered by us while performing laminectomy procedures in a sheep model as a part of our research work. Following posterior spinal lumbar laminectomy procedure and application of different treatment agents over the intact dura in sheep, knuckling of one of the hind limbs was seen in the immediate post-operative period. It was thought to be related to the test agent that was applied over the dura that was affecting the nerves as they were exiting the spinal canal. However similar complication was also seen with saline controls. Post mortem examination of the affected hind limbs of the animals revealed ischemic features compatible with pressure myopathy. The experiment was then carried out with the animals positioned on a beanbag to prevent pressure on the hind limbs. No knuckling was then seen in any of the subsequent animals.

Key words: limb, position, compartment, syndrome, knuckling, sheep, pressure, myopathy

INTRODUCTION:

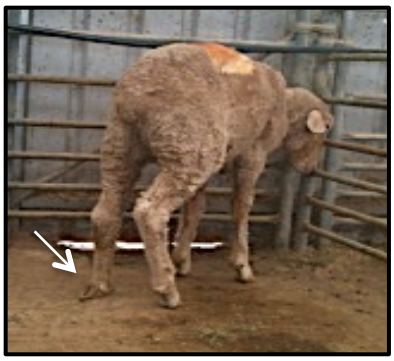
Optimal positioning of the animal during surgery is essential for a smooth post-operative recovery. Pressure myopathy and neuropathy are the main complications seen in large animals such as horses and cattle that have undergone a long procedure under general anesthesia[1]. These complications are related to the position of the animal during surgery and anesthesia. The clinical case presented here explains the similar problem in sheep – its clinical aspects and also management and prevention.

CASE REPORT:

In March 2013, dorsal spinal laminectomy procedures were performed on sheep as a part of a research study. The sheep was placed in a sternal recumbency position and posterior laminectomy at L1, L2 and L3 levels was carried out with exposure of the dura. Control and test agents were subsequently applied to the exposed dura and spinal cord following which the skin and subcutaneous tissue was closed over the area. The overall operating time ranged between four and five hours.

Immediately following the procedure it was noticed that five of the sheep showed knuckling of the fetlock of one of the hind limbs. The first sheep that showed evidence of knuckling in its right hind limb recovered well over a period of few weeks without any residual deficit. The fifth sheep also recovered well following splinting of the affected limb. Systemically the sheep did not show any signs of infection such as fever, tachycardia or tachypnoea and the wound site did not show any clinical picture of local inflammation or infection.

Following a deterioration of their conditions, three of the five knuckling sheep were euthanized and spinal cord specimens were harvested. In two animals, the necropsy included examination of the affected and unaffected hind limbs.



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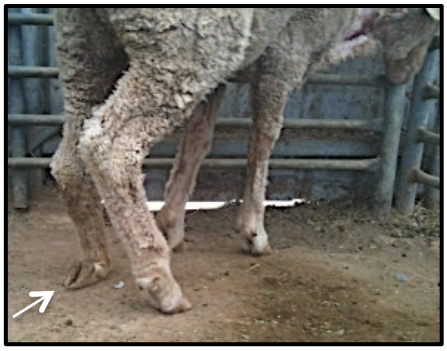

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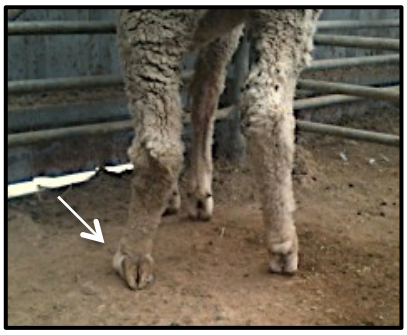

1(c)

Figure 1(a), (b), (c): Knuckling of left hind limb fetlock seen post-operatively (white arrow). The sheep recovered completely from the complication after few weeks without any intervention.

Post Mortem Findings:

Necropsy findings of two of the sheep in which the hind limbs were examined revealed congestion, hemorrhage and swelling of the muscle groups of the affected side. The unaffected limb was pristine. Grossly the spinal cord specimen looked unremarkable. The local skin incision and wound site did not reveal any pus or signs of infection.

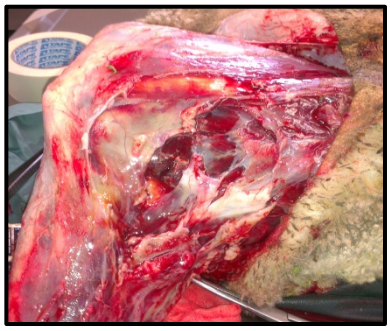

Figure 2: Congested muscle of affected limb

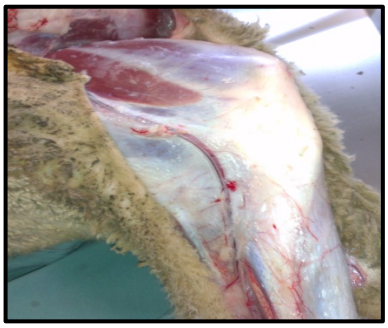

Figure 3: Opposite unaffected limb showing normal looking muscle

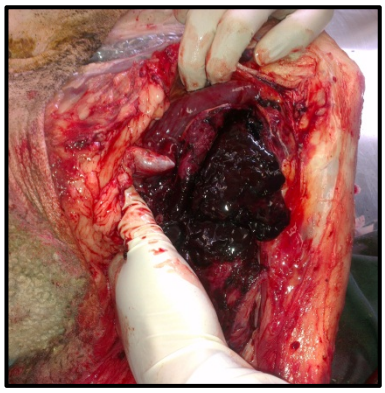

Figure 4: Haemorrhage within the muscle

DISCUSSION:

Clinical and necropsy findings suggested ischemic necrosis or compartment syndrome as the most likely diagnosis. Pressure myopathy is usually seen in large animals following a long procedure with the animal in the recumbency position. This is more commonly seen in the down/ hind limbs due to the weight of the animal pressing the dependent limb against the hard surface of the operating table and this in turn leading to decreased circulation and tissue hypoxia. Swelling of the involved muscle groups leads to further increase in pressure under the tight fascia and hence decreased circulation and muscle degeneration. Furthermore, the induced hypotension during surgery adds on to the decreased circulation[1]. This pressure myopathy is manifested as knuckling in the

animals. Review of the literature revealed that a similar complication had been previously experienced, also in a sheep model[2].

The four hour long surgery to perform with the sheep in the sternal recumbency position most likely has led to a compartment syndrome in the hind limbs of the sheep which is confirmed by the classic necropsy findings. Initially the surgery was thought to be the cause, however similar clinical signs would be expected in all sheep, which was not the case. Out of 13 sheep that underwent the initial surgical procedure only five developed post-operative knuckling. It was also considered whether application of the test agent was leading to the knuckling complication. However, both test agent and control sheep (where no agent was applied to the dura) were seen to experience knuckling, suggesting application of the test agent was not be the cause.

We then thought that possibly the position of the sheep during surgery under general anesthesia might have caused compression of the hind limb muscles. Owing to this, the consequence of fetlock knuckling was seen in the animal after the operation.

Hence to prevent and solve this problem a trial was done to obtain the optimal position of the sheep during the surgery. The animal was placed on a soft beanbag so that the hind limbs hung down in their natural position and the pressure effect on the legs were eliminated and then the sheep was anaesthetized for five hours with no procedure performed. The animal recovered well with no myopathy or neuropathy seen post-operatively.

The research study was then continued using this beanbag padding under the animal during the posterior laminectomy procedure with proper positioning of the hind limbs. Nine more procedures were performed and all the animals recovered well with no signs of knuckling. Mean arterial blood pressure during the surgery was maintained above 70 mm Hg, intravenous fluids were administered during the procedure and operating time was reduced by one hour.

The animals, which showed paresis initially, were all treated with NSAIDS and splinting. One sheep that was splinted immediately upon signs of knuckling recovered completely, but the other sheep in which splinting was delayed (by one week) still shows knuckling but with no further decline in the motor function.

Figure 5A: Beanbag

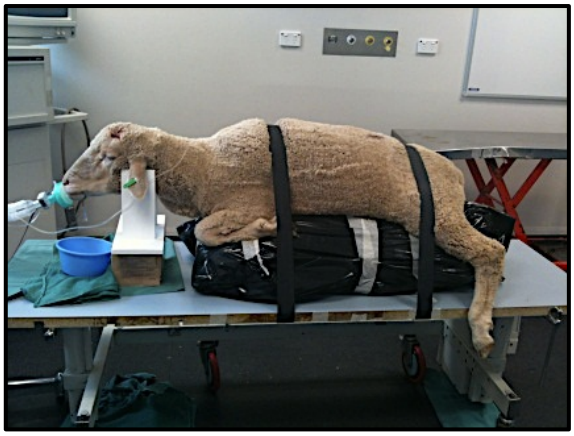

Figure 5B- Sheep positioned on the Beanbag

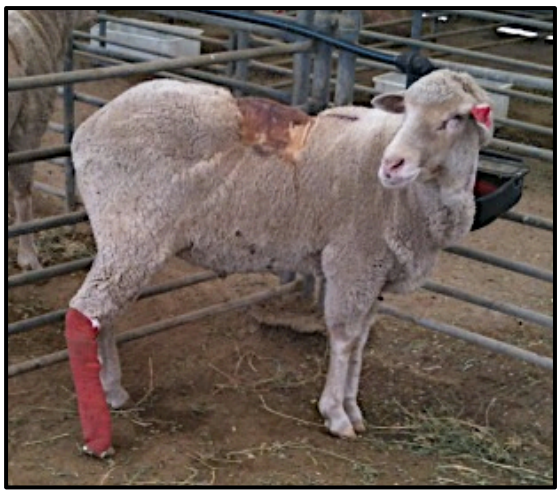

Figure 6: Splinting of the sheep hind limb done immediately when knuckling was evident. The sheep recovered completely.

CONLCUSION:

Proper positioning of the animal during surgery is essential to avoid complications of pressure myopathy. It is clear from our case report that the beanbag padding and decrease in surgery time prevented the compartment syndrome.

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SUMMARY AND CONCLUSION

Bleeding and adhesion are two common problems that increase morbidity post neurosurgical procedures. There are many agents available in the market to overcome these problems. However, none are fully effective in achieving surgical hemostasis and minimizing post-operative adhesions with minimal or no side effects[17,460].

In our research work we have tested Chitosan Dextran gel for its safety, haemostatic efficacy and anti adhesion property in sheep models (burr-hole and spinal laminectomy models). We have also looked at the mechanisms of action of muscle tissue as a haemostatic agent by in-vitro studies.

CHITOSAN IN HAEMOSTASIS

Chitosan is a polysaccharide obtained from crustaceans[36] and its haemostatic properties have been shown in numerous studies[33,35,461]. The bleeding control action of chitosan is believed to be independent of the clotting mechanism in the body. Experimental studies have shown that it causes agglutination of the red blood cells secondary to its cationic properties[238].

Only a single study has been published so far that has demonstrated the use of chitosan to control bleeding in brain tissue. They had used liquid chitosan in cat brain to effectively control diffuse bleeding with no cytotoxic effects[236]. Chitosan-Dextran gel is a chitosan variant that has been shown to be an effective haemostatic agent in endoscopic sinus surgeries and is presently used in human post sinus surgery[240].

In our neurosurgical burr hole sheep study we showed that Chitosan-Dextran gel was safe without toxic effects on neurological tissue and that it effectively controlled bleeding in the animal brain tissue. It was as effective as Gelfoam, however Gelfoam has the disadvantage of pressure effects on delicate structures if not used with care. There have been previous reports of cauda equina syndrome, headaches and paraesthesias caused by Gelfoam[160].

Studies have also shown comparable haemostatic potential of chitosan with respect to fibrin and thrombin agents[462,463]. However fibrin and thrombin products have the risk of increasing adhesions by activation of the coagulation cascade, antibody formation, transmission of diseases and allergic reactions[464-466]. The safety of chitosan has been studied and it is shown to be non- toxic[238]. Though it is obtained from crustaceans its purified form is hypoallergenic[34].

CHITOSAN AS AN ANTI ADHESION AGENT

Post-operative adhesion formation leads to many complications especially in the central nervous system following spinal surgery. Best treatment of post -operative adhesions is to prevent their formation in the first place[14]. Most effective way to prevent surgical adhesions in spinal surgeries is by the control of bleeding and the use of barrier agents that prevent contact between the dura and para-spinal muscles/ fibroblasts.

Organization of the surgical site hematoma by migration of fibroblasts from the nearby paraspinal muscles leading to the formation of fibrous tissue forms the basis of the pathophysiology of epidural adhesions[28].

Chitosan has been shown in vitro to inhibit fibroblast proliferation[467-469]. It is believed that the attachment of chitosan to injured surface prevents cell adhesion molecules like fibronectin and vitronectin from attaching[467]. It is also reported that chitosan selectively inhibits fibroblasts but does not affect the growth of epithelial cells[470].

Animal model and human endoscopic sinus surgery studies have shown Chitosan-Dextran gel to be effective in decreasing post-operative endonasal adhesions[234,240]. Animal studies have also shown the anti-adhesion efficacy of Chitosan Dextran gel in abdominal surgeries to reduce intraperitoneal adhesions[37].

In our work we studied the anti adhesion efficacy of Chitosan Dextran gel in a sheep model of spinal laminectomy. Chitosan Dextran gel significantly reduced adhesion formation compared to normal saline. However its efficacy in reducing adhesions was similar to Gelfoam. Reports of Gelfoam reducing epidural adhesions have been variable[28,471-473]. As mentioned previously Gelfoam is known to cause pressure effects on delicate neural structures and hence needs to be used very cautiously in closed cavities[160].

Thus, Chitosan Dextran gel has shown to be an effective haemostatic agent in the brain tissue and also decrease epidural adhesions following spinal laminectomy surgery.

MUSCLE TISSUE AS A HAEMOSTATIC AGENT

There has been a recent re-emergence of interest in the use of autologous human tissue for bleeding control. Muscle patch has been shown to be effective in high-pressure carotid artery bleeding in animal studies[38,283]. Muscle tissue gives the advantage of being

soft and pliable and hence ability to contour any bleeding area. It can be harvested from any site and since it is an autologous tissue, risks of infection and allergy are minimized. Good adhesion of muscle to blood vessel has been described previously[281]. All these properties make it a desirable agent for bleeding control. It has been mentioned in literature that tissue factor or thromboplastin in muscle tissue was responsible for haemostasis[284]. However studies have shown that there is very minimal expression of tissue factor in skeletal muscle tissue, which is the most common muscle that is used as a haemostatic agent[285].

We assessed the potential haemostatic factors in the skeletal muscle tissue in vitro and found that increased platelet aggregation appeared to be the most likely cause for the haemostatic properties of muscle tissue.

CONCLUSION

Work presented in our thesis provides a platform for furthering the use of Chitosan-Dextran gel from endonasal sinus surgery into neurosurgery. Further trials are needed to evaluate the safety and efficacy of CD gel in humans.

Role of platelet aggregation in the haemostatic efficacy of muscle patch has not been reported before. In fact there have been no previous studies exploring as to how muscle patch aids in bleeding control. This is the first study to evaluate the detailed haemostatic profile of muscle tissue.

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