In Vitro and In Vivo Comparison of Different Grades of Chitosan with Common Surgical Hemostats

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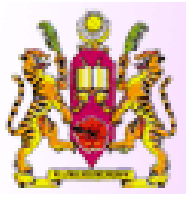

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III PREFACE

Bleeding secondary to civilian injuries and trauma and war injuries remain a significant cause of mortality in a pre-hospital setting. Intra-operative bleeding also cause significant mortality and prolong hospital stay. Several hematological conditions such as thrombocytopenia and disseminated intravascular coagulation further complicate patients condition and prolong bleeding and clotting time.

Chitosan has been shown through several previous studies to be superior comparative to common surgical hemostats currently available. Chitosan molecular weight and deacetylation degree affects its haemostatic potential. The greater the degree of deacetylation the better its haemostatic potential. It has been shown through several studies to induce hemostasis through its action on platelet aggregation and red cell aggregation. In addition, chitosan has been also shown to induce vasoconstriction and hence also contribute to hemostasis.

In this study we compared several different grades of chitosan prepared by SIRIM® Malaysia, with the currently present surgical haemostatic agents in the operation theatre or the surgical wards and emergency department. We also wanted to examine the effects of these haemostatic agents on platelet aggregation and red cell aggregation in vitro. It is hoped that this study could serve as a guide for proper selection of haemostatic agents for each clinical scenario. It is expected that it would prove that chitosan based dressings as superior if not equal to the commonly present hemostats.

IV TABLE OF CONTENTS

V ABBREVIATIONS

Abbreviations

%	Percent
XII	Twelve
CO	Chitooligosaccharides
DA	Deacetylation
ESR	Erythrocyte Sedimentation Rate
H bonds	Hydrogen bonds
IR	Infrared
LMWC	Low Molecular Weight Chitosan
m	Meter
mg	Milligram
µm	Micrometer
NMR	Nuclear Magnetic Resonance
n	Number (sample number)
pH	A measure of the acidity or basicity of a solution. It approximates p[H], the negative logarithm (base 10) of the molar concentration of dissolved hydronium ions
p value	is the probability of obtaining the same or more extreme data assuming the null hypothesis of no effect; p-values are generally (but arbitrarily) considered significant if $p < 0.05$
PBS	Phosphate Buffered Saline

p-G1cNAc	Poly N acetyl Glucosamine
pNAGC	Poly N acetyl Glucosamine
PRP	Platelet Rich Plasma
®	Registered trademark
RCT	Randomized Controlled Trials
RDH	Rapid Deployment Hemostat
SD	Standard deviation
SIRIM	Standards and Industrial Research Institute of Malaysia
SPSS	Statistical Package for the Social Sciences
TF	Tissue Factor
Vs	Versus

VI LIST OF FIGURES

VII LIST OF TABLES

VIII ABSTRAK

Kegagalan untuk memberhentikan pendarahan dengan sempurna adalah punca paling utama pendarahan intraoperatif . Hampir semua tinjauan pendarahan intraoperatif dan awal post-operatif menunjukan 75% hingga 90% dari pendarahan semua disebabkan punca teknikal. Pendarahan yang tidak terkawal menjadi punca kombinasi beberapa faktor yang boleh menyebabkan pendarahan yang berlanjutan. Pendarahan yang berterusan boleh juga disebabkan oleh masalah kekurangan sel platelet atau fungsi pembekuan darah yang terganggu. Ini merumitkan lagi usaha memberhentikan pendarahan. Penyelidikan kami adalah bertujuan untuk membandingkan keberkesanan produk kitosan dengan produk hemostat lain, yang biasa digunakan di dewan bedah. Hipotesis kami adalah bahawa hemostat berasaskan kitosan lebih unggul berbanding hemostat bedah yang lain dalam menyebabkan agregasi platelet dan sel-sel darah merah dan juga dalam mempengaruhi Prothrombin Time dan Partial Thromboplastin Time.

Kajian kami dibahagikan kepada dua bahagian. Dalam kajian in-vitro, sampel darah yang diperolehi dari bank darah diproses menggunakan sistem CP2D/AS-3 dengan larutan aditif (AS-3, Nutricel ®). Darah yang dikumpul telah dipisahkan kepada empat komponen iaitu darah utuh, darah utuh dicampur heparin, plasma kaya platelet, dan plasma yang mempunyai jumlah platelet yang sedikit. Dalam ujian platelet aggregation, PRP sitrat dicampurkan dengan 3.5 mg agen hemostatik yang dilembabkan dengan 100 liter mikro phosphate buffered saline (PBS) dalam tabung uji. 100 mikroliter supernatan (100 liter mikro) telah diambil setiap 5 minit untuk kesuluruhan 15 minit dan jumlah platelet disukat menggunakan counter sel elektronik (XT2000i Sysmax Analyzer, Sysmex America Inc, Mundelein, Illinois). Jumlah platelet dari setiap percubaan dinormalisasi menggunakan jumlah platelet dari PRP kawalan . Untuk setiap agen hemostatik, tiga set kajian dilakukan. Ujian ini telah diulang untuk setiap agen hemostatik yang dikaji. Dalam ujian PT/PTT agen-agen hemostatik ditindak balaskan dengan plasma kaya platelet dan plasma kurang platelet. Serum tersebut telah disentrifugasi untuk menghilangkan deposit yang

terkandung dalam plasma tersebut.. Percubaan ini telah diulang sebanyak enam kali dan kajian telah dilakukan keatas Prothrombin Time and Partial Thromboplastin Time menggunakan mesin penganalisis hemostasis (Stago STA Compact hemostatik Analyzer, Diamond Diagnostik, MA. USA)

Dalam ujian agregasi sel darah merah, setiap agen hemostatik telah ditindak balaskan dengan utuh, darah dicampur heparin dan darah kurang platelet. Darah dengan agen hemostatik dibiarkan berinteraksi dan tahap sedimentasi sel-sel darah merah diukur dengan mesin penganalisis sedimentasi Sedy400.

Dalam experiment invivo pula, 36 ekor tikus Sprague-Dawley telah digunakan. Tikus tikus tersebut telah dibius sebelum melakukan pembedahan. Pembedahan laporotomi telah dilakukan untuk mendedahkan kedua-dua ginjal tikus tersebut. Heminephrectomies dilakukan dan agen hemostat diterapkan ke permukaan yang dipotong. Masa yang diperlukan setiap agen hemostat untuk memberhentikan pendarahan tersebut diukur.

Dalam ujian agregasi platelet, didapati tidak ada trend yang jelas dalam fungsi agen-agen hemostatik tersebut dalam mengumpul atau mengagregasi platelet. No CMC 36 3% menunjukkan jumlah platelet terendah berbanding agen-agen hemostatik yang lain dalam 5 minit yang pertama ujian tersebut. Lyostypt ® dan Surgicel ® lebih unggul berbanding dengan agen hemostat kitosan pada masa 10 minit kajian tersebut.

Dalam ujian PT / PTT, prothrombin time untuk Chitosan (NoCMC 8%) adalah yang terpendek dalam plasma kaya dengan platelet. Dalam plasma kurang platelet pula, masa protrombin terpendek dilihat pada kedua-dua hemostat Chitosan (NoCMC 3% dan NoCMC8%). Partial thromboplastin time didapati terpendek untuk agen Chitosan (NoCMC 3%) .

Dalam ujian agregasi sel darah merah, agen hemostat Chitosan (NoCMC 3%) menunjukkan nisbah tertinggi sedimentasi sel-sel darah merah dalam darah kurang platelet serta spesimen darah dicampur heparin. Agen hemostat Chitosan (NoCMC 8%)

menunjukkan nisbah tertinggi sedimentasi sel-sel darah merah dalam darah utuh di campur heparin.

Dalam eksperimen invivo pula, didapati tidak ada perbezaan statistik yang ketara antara kesemua agen-agen hemostatik dalam mengurangkan pendarahan dari spesimen heminephrectomy. Agen hemostat Chitosan (NoCMC 36 3%) bagaimanapun berjaya memberhentikan pendarahan pada ginjal tikus dalam tempoh masa yang terpendek dibandingkan dengan agen-agen hemostatik yang lain.

Dari kajian-kajian yang telah dijalankan kami berpendapat bahawa agen hemostatik Chitosan hemostat menyebabkan agregasi platelet lebih awal, memendekkan Prothrombin dan Partial Thromboplastin Time lebih awal berbanding agen hemostatik yang lain. Mereka juga lebih baik berbanding agen hemostatik yang lain dalam menyebabkan agregasi sel-sel darah merah dalam proses pembekuan darah.

IX ABSTRACT

The most common cause of significant intra-operative bleeding is inadequate surgical hemostasis. Nearly all reviews of intra-operative and early post-operative bleeding point that 75% to 90% of all bleeding is technical in nature. Whatever the cause, uncontrolled bleeding can lead to a combination of factors which may further exacerbate the problem of a vicious bloody circle. Dilutional thrombocytopenia, platelet dysfunction and consumption of clotting factors present a difficult problem to address as continual blood loss continue to compound the problem while blood component replacement therapy attempts to correct the deficiency. Our study aims to compare haemostatic efficacy of different grades of chitosan compared to the common haemostatic surgical hemostats. We hypothesize that chitosan based hemostats are superior to the common surgical hemostats in inducing platelet aggregation, affecting Prothrombin Time and Partial Thromboplastin Time and red cell aggregation.

There were two parts to the study. In the in vitro study blood sample was obtained from the blood bank. Blood sample was collected utilizing the CP2D/AS-3 systems with additive solution (AS-3,Nutricel®) to maintain red blood cell viability. Collected whole blood was separated into four separate components (whole blood, heparinized whole blood, platelet rich and platelet poor plasma) . In the platelet aggregation test, stirred citrated PRP was contacted with 3.5 mg of each haemostatic agent premoistened with 100 micro liter of phosphate-buffered saline (PBS) in a test tube (as would be used in traditional platelet aggregometry). Aliquots of supernatant (100 micro liter) were removed every 5 minute for a total of 15 minutes and the platelet count was measured in triplicate utilizing an

electronic cell counter (XT2000i Sysmax Analyzer, Sysmex America Inc., Mundelein, Illinois); platelet counts from each experimental aliquot were normalized using counts from unreacted PRP. For each haemostatic agent three independent sets of experiments were performed. A similar set of platelet aggregation experiments was performed using the haemostatic sponge agents (3 Different grades of Chitosan, Lyostypt® , and Surgicel®) premoistened with PBS. In the PT/PTT test, each haemostatic agent was reacted with platelet rich and platelet poor plasma. The serum was centrifuged to remove possible deposition. Six parallel experiments were conducted to measure PT and APTT of the serum using a hemostasis analyzer (Stago STA Compact Haemostatic Analyzer, Diamond Diagnostics, MA. USA) In the red cell aggregation test, each haemostatic agent was reacted with whole blood, heparinized blood and platelet poor blood. The blood with haemostatic agents were left to stand and the erythrocyte sedimentation rate was measured with the Sedy400 sedimentation analyzer. In the animal experiment, 36 Sprague-Dawley rats were utilized. Under general anesthesia, via a midline laparotomy the right and left kidneys were isolated. Heminephrectomies were carried out and hemostats were applied to the cut surface and time taken to hemostasis was tabulated.

In the platelet aggregation test, no definite trend in platelet aggregation was observed. No CMC 36 3% showed the lowest platelet count of all haemostatic agents at 5 minutes. Lyostypt® and Surgicel® were superior compared to chitosan hemostats at 10 minutes of contact.

In the coagulation test (PT/PTT) mean prothrombin time for Chitosan (NoCMC 8%) was the shortest in platelet rich plasma. Mean partial thromboplastin time was the shortest for

Chitosan (NoCMC 3%) in platelet rich plasma. In platelet poor plasma, the shortest prothrombin time was seen in both the Chitosan hemostats(NoCMC 3% and NoCMC8%). Partial thromboplastin time was shortest for Chitosan (NoCMC 3%) hemostat. In the red cell aggregation test, Chitosan hemostat(NoCMC 3%) demonstrated the highest erythrocyte sedimentation ratio in platelet poor blood as well as heparinized blood specimens. Chitosan hemostat (NoCMC 8%) demonstrated the highest erythrocyte sedimentation ratio in heparinized whole blood.

In the animal experiment, there was no statistical difference between the hemostats in arresting bleeding from heminephrectomy specimens. The Chitosan hemostat(NoCMC 36 3%) however demonstrated the shortest time to hemostasis compared to the other hemostats.

From the study we concluded that Chitosan hemostats causes platelet to aggregate the earliest compared to other hemostats. They shorten prothrombin and partial thromboplastin time. They have been also found to aggregate red blood cells the most compared to other haemostatic agents.

INTRODUCTION

AND

LITERATURE REVIEW

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 Research background

Bleeding, technically known as hemorrhaging, is the loss of blood or blood escape from the circulatory system. It is a likely complication of any surgical procedure or trauma and introduces a challenge to the surgical and anesthetic teams. The grimness of bleeding can vary from Class I to a Class IV (15% to 40% and more of blood volume lost) .Mortality from bleeding in surgery can be increased from 1% to 20% when severe, unexpected and uncontrollable bleeding occurs. Liver and cardiac surgeries are associated with profuse hemorrhage and clinical experience and observational studies have shown that massive blood loss is linked to adverse outcomes and that 75% to 90% of intra-operative and early postoperative bleeding is affected by surgical technique(Marietta et al., 2006).

Uncontrollable hemorrhage also accounts for almost 50% of combat fatalities and up to 80% of civilian trauma fatalities within the United States. In settings such as these, around 30-40% of trauma related deaths(Sauaia et al., 1995) and more than 90% of combat deaths take place in the pre-hospital setting(Champion et al., 2003). Extremity injuries can be sufficiently treated with tourniquet placement. In a proximal limb injury a tourniquet can be hard to apply. Other areas in the body require manual compression which requires both personnel time and commitment(Gustafson et al., 2007) . The use of these maneuvers in a pre-hospital setting can substantially increase the survival of trauma patients with uncontrolled hemorrhage(Pusateri et al., 2006).

1.2 Hemostasis

1.2.1 History of hemostasis

Hemostasis is traced from the primitive man's realization that unchecked bleeding meant death up to Lister's experiments developing the present day absorbable ligature. The early Greeks and Romans used vegetable and mineral styptics on wounds received in battle; it was with these that Macheon ministered to Menelaus before the walls of Troy. Then the early surgeon began to make intentional incisions, but avoiding the blood vessels as they were uncovered. Not content with their own domain, the Greeks invaded Egypt and became familiar with the dissection known to the Egyptians.

Herophilus described the vessels as veins filled with blood and the arteries filled with blood and "pneuma", and he noted the difference in the walls of the two kinds of vessels. At the time of Celsus, ligature was advised as a last resort, only after the cautery and styptics had failed. Galen, during the decline and fall of the Roman empire, wrote a description of the circulation, remarkably close to the modern conception, and favored the cautery for hemostasis(Atterbury, 1976).

In the West, little progress was made until the fifteenth century. At that time, gun powder was invented and the incidence of battle wounds increased for the army surgeon. The printing-press launched forth medical, surgical, and scientific texts, and for the first time there was a chance for the operator to study the notes of others, and knowledge was more

widely disseminated. There were two differing schools of thoughts regarding healing. One, by first intention and the other by suppuration. Ambrose Pare, by accident, discovered that wounds left to “autodigest” healed better than wounds that were cauterized. He modestly described his first cure by saying that, "I dressed him and God healed him". Pare also described the bec de corbin, a pinching instrument for holding blood vessels. Morel is accredited with the invention, in I674, of the twisted stick tourniquet(Atterbury, 1976). A spring was added to the bec de corbin and rolls of bandage were placed under the tourniquet to press on the great vessels. Amputation became more frequent and coagulation of the blood was described by Jones and Lawson. Physick and Nathan Smith first had the courage to cut short the ends of their buckskin ligatures, contrary to the advice and teachings of Liston(Atterbury, 1976).

The forceps were gradually improved and Kocher and Halstead gave their names to their designs. Soon it was realized that more than one or two clamps were necessary for an operation, and the technique improved. Then Lord Lister introduced the aseptic technique and perfected the ligature by sterilization in dilute carbolic acid; the theory of asepsis having been supported by Pasteur and Koch(Lidwell, 1987). His experiments were thorough and the results were satisfactory as is evidenced by our modern operative procedures. Revealing these and many other facts, this text affords an opportunity for a pleasant and profitable adventure within the field of medical history(Atterbury, 1976).

1.2.2 The coagulation cascade

The modern concept of coagulation was presented in 1964 as the Waterfall/Cascade model, which might overwhelm many nonhematologists with its complexity. Coagulation proceeds through a series of proteolytic reactions involving trypsin-like enzymes that form a biochemical amplifier, culminating in generation of sufficient thrombin to form a fibrin clot. Initiation of fibrin formation through the “extrinsic pathway” occurs when plasma factor VIIa forms a complex with the integral membrane protein tissue factor. Tissue factor is not normally found at high concentrations in blood, but is present on cell membranes in sub endothelial layers of blood vessels and is exposed to factor VIIa when the endothelium is injured.

Alternatively, coagulation may be initiated through the “intrinsic pathway” when factor XII is activated on a charged surface by a process called contact activation. Activation of factor XII is followed sequentially by activation of factor XI and factor IX. The intrinsic and extrinsic pathways converge at the level of factor X activation. Factor Xa activates prothrombin to thrombin in the presence of the cofactor factor Va, and thrombin subsequently converts fibrinogen to fibrin(Romney and Glick, 2009).

1.2.2.1 Role of the extrinsic pathway

The TF:FVIIa complex is traditionally referred to as the extrinsic pathway and is proposed to be the primary activator of the coagulation protease cascade in vivo. Subsequently, propagation of the thrombus involves recruitment of additional platelets and amplification of the coagulation cascade by the intrinsic pathway of blood coagulation, which includes the hemophilia factors FVIII and FIX. Importantly, platelets play a critical role in the amplification of the coagulation cascade by providing a thrombogenic surface. Finally, fibrin stabilizes the platelet-rich thrombus(Mackman et al., 2007).

1.2.2.2 Role of the intrinsic pathway

The intrinsic pathway is typically depicted as a sequence of proteolytic reactions culminating in factor IX activation. However, the hemorrhagic phenotypes of patients deficient in components of this pathway suggest more complex interactions. Deficiency of factor IX or its cofactor, factor VIII, cause hemophilia B and hemophilia A respectively. The severe forms of hemophilia A and B are associated with crippling hemorrhage into joints and muscles, and soft tissue bleeding that can be life threatening. Factor XI deficiency; in contrast, is associated with a distinctly different, and usually milder disorder characterized by trauma or soft tissue-related hemorrhage, primarily involving tissues with high fibrinolytic activity. Finally, factor XII– deficient patients do

not exhibit an abnormal bleeding tendency, even with surgery, despite having markedly prolonged PTT clotting times. These observations argue strongly against the waterfall/cascade model in which proteases are exclusively activated in a linear sequential fashion.(Gailani and Renne, 2007)

1.2.3 Mechanism of platelet aggregation

Platelets adhere to exposed sub endothelial structures in response to alterations in the vessel wall, and become rapidly activated by interaction with thrombogenic substrates and agonists released or generated locally. In this manner, they acquire the ability to bind soluble adhesive molecules that become the reactive surface for continuing platelet deposition. Initial platelet tethering to a surface and subsequent platelet-platelet cohesion are typically identified as two separate stages of thrombus formation, respectively known as adhesion and aggregation. Most of the current concepts of aggregation mechanisms derive from experimental models in which platelets are studied in a stirred suspension and induced to interact with one another by appropriate stimuli in the absence of a reactive surface. In reality, thrombus growth involves the progressive accrual of individual platelets that attach to others already adhering at a site of injury. Therefore, adhesion and aggregation are related processes, because the initial platelet monolayer anchored to the vessel wall plays a key role in determining the progression of thrombus growth. This review focuses on recent developments in elucidating the mechanisms that regulate platelet aggregation. Central to this process are bidirectional signals induced by the binding of one or more agonist ligands to platelet membrane receptors, leading to the

modulation of integrin _IIb_3 into a high affinity/avidity binding site for soluble adhesive proteins(Savage et al., 2001).

1.2.4 Mechanism of red cell aggregation

Red cell aggregates may form when the cells come into close proximity. One explanation often advanced for aggregation is the bridging hypothesis proposed by Chien and Jan which postulates that long-chain macromolecules such as fibrinogen or dextrans of high molecular weight may be adsorbed onto the surface of more than one cell, leading to a bridging effect between cells(Chien and Jan, 1973). It has been proposed by other investigators that the reduced concentration of macromolecules in the vicinity of red cells lowers the osmotic forces in the vicinity, causing fluid to move away and increasing the tendency for adjacent red cells to come together. According to both the bridging and the depletion theories, the total adherent force between two cells is maximal when the cells are oriented en face, thus it is not uncommon to observe cells arranged in rouleaux. The shear stress required to separate two cells in this orientation suspended in 4% dextran 70 was determined by Chien and coworkers to be less than 1 dyn/cm2(Chien and Jan, 1973). The exact configuration of a group of cells depends on local conditions such as shear rate and cell concentration (haematocrit) and a variety of complex forms ranging from single rouleaux to a branching network of rouleaux to more compact spheroids may be seen. Two separate models exist for RBC aggregation: bridging and depletion. In the bridging theory, bridging forces due to adhesion of macromolecules to red blood cell surfaces overcome repulsive forces such as electrostatic repulsion, membrane strain and

membrane shearing. On the other hand the depletion force theory states that a depletion of macromolecules around the surface of the red cell creates an osmotic gradient and thus creating a depletion interaction.

1.2.5 Hemostats in surgical practice

A fundamental principle of good surgical technique is minimization of blood loss, and present day surgeons have a wide variety of agents and tools to aid them in this endeavor. Although no aid in hemostasis can negate the importance of good surgical technique, even the most talented surgeon has encountered persistent bleeding which has required focused attention.

i) Collagen-based agents trigger platelet aggregation resulting in clot formation when in contact with a bleeding surface. They are often combined with a procoagulant substance such as thrombin to enhance the haemostatic effect. A positive haemostatic effect has been shown in several human studies(Tomizawa, 2005) .

ii) Gelatin-based products can be used alone or in combination with a procoagulant substance(Seyednejad et al., 2008) . Swelling of the gelatin in contact with blood reduces the blood flow and, in combination with a thrombin-based component, enhances haemostasis. A similar or superior haemostatic effect has been observed with collagen-based agents.

iii) The effect of cellulose-based haemostatic agents on bleeding has been not well studied and only case reports that support their use are available.

iv) Fibrin and synthetic glues or adhesives have both haemostatic and sealant properties and their significant effect on haemostasis have been shown in several human RCTs involving vascular, bone, skin and visceral surgery(Wheat and Wolf, 2009).

v) Polysaccharide-based haemostatic can be divided into two broad categories]: N-acetyl-glucosamine containing glycosaminoglycans purified from microalgae and diatoms and micro porous polysaccharide haemospheres produced from potato starch. The mechanism of action is complex and depends on the purity or combination with other substances such as cellulose or fibrin. A number of different products are currently available and have been shown to be efficient for external use. An observational study showed that hemorrhage control was achieved using an N-acetylglucosamine-based bandage applied to 10 patients with severe hepatic and abdominal injuries, acidosis and clinical coagulopathy(King et al., 2006).

vi) The inorganic haemostatic based on minerals such as zeolite or smectite have been used and studied mainly on external bleeding (Achneck et al., 2010).

1.2.5.1 Chitosan

Chitosan is a copolymer consisting of β-(1→4)-2-acetamido-D-glucose and β-(1→4)-2-amino-D-glucose units, with the latter usually exceeding 80%. The proportion of the two monosaccharide units in chitosan depends on the alkaline treatment. Generally, the individual chains assume an essentially linear structure, which undergoes one full twist every 10.1–10.5 Å along the chain axis. Since each monosaccharide unit is chiral, the rotations of polymer chains show evident left or right. Accordingly, chitosan could be

divided into three crystal types: α, β and γ type, which could be identified by X-ray model and NMR spectra. Among these, α-type is the most common type obtained from crust of shrimp and crab. Chitosan has three types of reactive functional groups, an amino/acetamido group as well as both primary and secondary hydroxyl groups at the C-2, C-3 and C-6 positions, respectively. The amino contents are the main factors contributing to differences in their structures and physico-chemical properties, and its distribution is random, which make it easy to generate intra- and inter-molecular hydrogen bonds.

Many novel chitosan derivatives have been obtained by chemical modification using the reactive activities of hydroxyl- and amino groups. In particular, the amino group has nucleophilic property, allowing easy formation of imine by reaction with aldehyde or corresponding amide derivatives in acylating reagents; in acidic solution, the amino groups showed alkaline properties and receive protons to generate salts, presenting cationic polymer properties. Besides, the amino functional group has also been correlated with chelation, flocculation and biological functions. The characterization of a chitosan sample requires the determination of its average Degree of acetylation (DA).

The distribution of acetyl groups along the chain (random or block wise) may influence the solubility of the polymer and also the inter-chain interactions due to H-bonds and the hydrophobic character of the acetyl group. This distribution has been evaluated by various techniques such as IR, elemental analysis, enzymatic reaction, UV, ^{1}H liquid-

state NMR and solid-state ^{13}C NMR. Diad and triad frequencies were determined for homogeneous and heterogeneous chitosan with different values of DA .

Another important characteristic to consider for these polymers is the molecular weight and its distribution. The first difficulty encountered in this respect concerns the solubility of the samples and dissociation of aggregates often present in polysaccharide solutions. As to choice of a solvent for chitosan characterization, various systems have been proposed, including an acid at a given concentration for protonation together with a salt to screen the electrostatic interaction. The solvent is important also when molecular weight has to be calculated from intrinsic viscosity using the Mark-Houwink relation. High molecular weight chitosan is estimated to be 10^6. High molecular weight chitosans (HMWC) form solutions with higher viscosities than chitosans of lower molecular weight. Polymeric chitosan is soluble in acidic conditions and is insoluble at pH values above 6.3 (the pKa of chitosan). However, chitosan oligomer has a low viscosity, and is freely soluble at neutral pH.

Production of low molecular weight chitosan (LMWC) and chit oligosaccharides (COS) from the hydrolysis of chitosan can be achieved either by chemical or enzymatic methods. The chemical method needs high energy and is hard to control; hence, the enzymatic hydrolysis of chitosan offers many advantages. During preparation of different molecular weight chitosans, viscosity is used as a parameter to determine the molecular weight. Unlike most polysaccharides, chitosan, LMWC and COS have positive charges,

which allows them to bind strongly to negatively charged surfaces; this property is responsible for many of the observed biological activities.

However, it is important to mention that chitosans with different structures show different biological activities, and not all biological activities are found in one kind of chitosan. Each special type of bioactive chitosan has been developed by chemical modification and enzymatic hydrolysis for its potential pharmaceutical and medical application(Zhang et al., 2010). It is commercially available in the US as CELOXTM (Med trade, Newport, OR) or HemCon® (HemCon Medical Technologies, Inc., Portland, OR). At this time, they are only approved by the FDA for external usage. While the haemostatic mechanism of this agent has not yet been fully elucidated, it has been proposed that its primary activity occurs via the promotion of both platelet activation and electrophysiological interactions between red blood cells (RBCs) and the tissue surface.

Schwaitzberg et al. have found, in multiple animal protocols, that Chitosan may be beneficial in achieving hemostasis even in the presence of acquired or congenital coagulopathic disorders(Schwaitzberg et al., 2004). Klokkevold and colleagues have, likewise, demonstrated its effectiveness in a heparinized rabbit model(Klokkevold et al., 1992). In that study, the investigators found that, after application of Chitosan to a lingual laceration, the use of this agent resulted in 43% less blood loss when compared to controls ($p=0.001$). The authors hypothesized that these results were facilitated through an interaction between the erythrocytes and Chitosan that resulted in a cellular haemostatic plug. Despite the promising results of these initial studies, further

investigations are needed to define the role of this agent under coagulopathic conditions, especially for internal usage(Recinos et al., 2008).

1.2.5.2 Oxidized cellulose

Oxidized cellulose is commercially available in the US as Surgicel© and Nu-Knit© (Ethicon, Inc., Cincinnati, OH). This class of agent is intended for use as an adjunct to gauze packing, with their hemostatic properties primarily based on their ability to locally activate the coagulation cascade. While they do not contain any intrinsic coagulation components, they are designed to stimulate clot formation and to provide a favorable three-dimensional structure for clot organization. In order for these products to function, therefore, a functional coagulation system must be present (Table 1).

It should not be moistened before use since it exerts a greater haemostatic effect when applied dry. The material offers superior handling characteristics as compared with gelatin foams and the knitted fabric can be trimmed to fit any size. It does not stick to instruments and can easily be held firmly against bleeding tissue until hemostasis occurs. Surgicel Fibrillar® resembles cotton in consistency and remains pliable when laid into a wound. Either form of oxidized cellulose decreases the pH of its surroundings. This low pH causes red cell lyses, which explains the brown discoloration of the agent after contact with blood. The released hemoglobin reacts with acid to form acid hematin. One of the theoretical advantages of this low pH is an antimicrobial effect seen against a variety of pathogenic organisms(Spangler et al., 2003). Furthermore, the low pH acts as a caustic agent bringing about hemostasis by generating an artificial clot. Oxidized

cellulose disadvantage lies in the fact that the low pH inactivates other biologically active topical agents, such as thrombin, which limits its ability to be used in conjunction with other agents. Moreover, the acidic nature of Surgicel® may also increase inflammation of surrounding tissue and delay wound healing. The dissolution of oxidized cellulose depends on the amount used and ranges from 2 to 6 weeks. However, there are reports describing histological evidence of oxidized cellulose fibers several years after cardiac surgery. Moreover, cases have been reported where Surgicel® used for hemorrhage control during thoracotomy had passed through the intervertebral foramen and caused spinal cord compression(Brodbelt et al., 2002). Therefore, the smallest amount necessary should be used and any excess should be removed once hemostasis has been achieved.

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1.2.5.3 Lyostypt (collagen) hemostat

Lyostypt® is a wet-stable collagen hemostat. Collagen leads to thrombocyte adhesion and to activation of coagulation factor XII. Therefore, collagen is very effective in hemostasis. Advantages of this hemostat are rapid achievement of hemostasis, that it can be removed easily, applied endoscopically and that it can be combined with fibrin glue and antibiotics. It also can be removed easily. Collaged hemostats also do not affect cell growth. Collagen should be applied to the bleeding surface with dry instruments rather than with the surgeon's hands since it tends to stick to gloves. It has been used successfully to control wide-area parenchyma oozing(Sirlak et al., 2003).

In laparoscopic procedures, Endo-Avitene®, which is a rolled sheet of Collagen, is available with an applicator capable of placement through standard laparoscopic trocar. As with oxidized cellulose, we recommend removing excess Collagen from the surgical site after adequate hemostasis has been achieved, since it may bind to neural structures and cause pain or numbness. Since Collagen may pass through filters of blood scavenging systems, blood from operative sites where Collagen was used should not be returned to the patient.(Achneck et al., 2010).

The purpose of this study was to compare chitosan with other commonly used haemostatic agents in use in surgical practice. We hypothesized that chitosan would bring about clotting similar or better to other haemostatic agents. In addition to that we hypothesized that chitosan would induce substantial platelet and rbc aggregation in comparison to the other haemostatic agents in a heparinized model.

OBJECTIVES

OF

STUDY

2.0 OBJECTIVES OF STUDY

2.1 General

To asses the efficacy of different grades of chitosan in comparison to other common surgical hemostats in practice(Surgicel® and Lyostypt®).

2.2 Specific

i) To compare the effect of different grades of chitosan with other available, commonly used haemostatic agents (Lyostypt® and Surgicel® in inducing platelet aggregation.

ii) To compare the haemostatic efficacy of different grades of chitosan to commonly available haemostatic agents (Lyostypt® and Surgicel®) in altering Prothrombin and Partial Thromboplastin Time on contact with blood.

iii) To compare the effect of different grades of chitosan and common haemostatic agents (Lyostypt® and Surgicel®) on Red Blood Cell Aggregation

iv) To compare the effect of different grades of chitosan and common haemostatic agents (Lyostypt® and Surgicel®) in inducing hemostasis on induced laceration in rat kidney

2.3 Research hypothesis

i) Chitosan is more effective compared to other available, commonly used hemostatic agents (Lyostypt® and Surgicel®) in inducing platelet aggregation

ii) Chitosan in more effective compared to other available, commonly used hemostatic agents (Lyostypt® and Surgicel®) in altering Prothrombin time and Parial Thromboplastin Time on contact with blood.

iii) Chitosan in more effective compared to other available, commonly used hemostatic agents (Lyostypt® and Surgicel®) in aggregating Red Blood Cells.

iv) Chitosan in more effective compared to other available, commonly used hemostatic agents (Lyostypt® and Surgicel®) in inducing hemostasis on induced laceration in rat kidney.

2.4 Null hypothesis

i) Chitosan is not more effective compared to other available, commonly used hemostatic agents (Lyostypt® and Surgicel®) in inducing platelet aggregation

ii) Chitosan in not more effective compared to other available, commonly used hemostatic agents (Lyostypt® and Surgicel®) in altering Prothrombin time and Parial Thromboplastin Time on contact with blood.

iii) Chitosan in not more effective compared to other available, commonly used hemostatic agents (Lyostypt® and Surgicel®) in aggregating Red Blood Cells.

iv) Chitosan in not more effective compared to other available, commonly used hemostatic agents (Lyostypt® and Surgicel®) in inducing hemostasis on induced laceration in rat kidney.

MATERIAL

AND

METHODS

3.0 MATERIAL AND METHODS

3.1 Outline of laboratory experiment design

In this experimental study, blood sample for the study was obtained from the blood bank. A single healthy donor with no background medical illnesses and who was not on any medication for the past 6 months was chosen (Appendix 1). Blood sample was collected utilizing the CP2D/AS-3 systems with additive solution (AS-3, Nutricel®) to maintain red blood cell viability. For this study 3 types of blood products were required (Platelet rich plasma, Platelet poor plasma and heparinized whole blood.)

3.2 Preparation of platelet poor and platelet rich plasma

For platelet collection the blood sample was put under light spin at 900-1200Gs for 20 min which separates the blood into two separate components (A supernatant of Platelet Rich Plasma and a precipitate of Packed Cells component). To further obtain Platelet Poor Plasma the sample of Platelet Rich Plasma was spun a second time at 3000rpm to produce a supernatant of ***Platelet Poor Plasma*** and Sediment of Platelets

3.3 Preparation of heparinized whole blood

Heparinized whole blood test specimens were prepared by adding heparin to blood specimen until Partial thromboplastin time was 3x normal. .

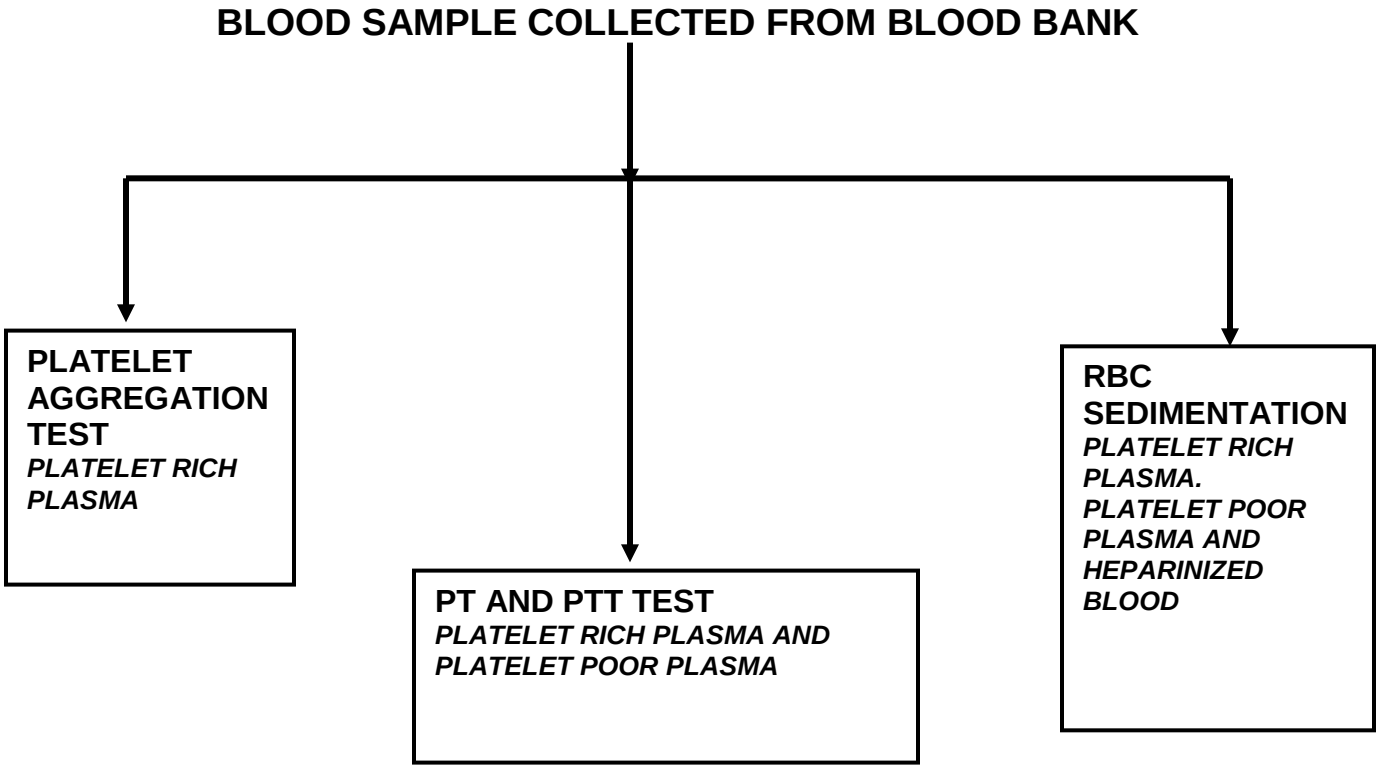

Figure 1: Flow chart of preparation of blood samples required for individual test designs

3.4 Haemostatic agents used.

In this experimental study, we used three different grades of chitosan (NoCMC 3%, NoCMC8% and OC52) . We also used Surgicel ® and Lyostypt ®.

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3.4.1 Chitosan samples

Chitosan haemostatic agents were used in this study was obtained from Industrial Biotechnology Research Centre, SIRIM Berhad (Standards and Industrial Research Institute of Malaysia) (Figure 5 -7).

3.4.2 Oxidized cellulose (Surgicel ®)

Surgicel ® was purchased from Johnson and Johnson's Ethicon subsidiary (Figure 8). It is a haemostatic agent made from oxidized cellulose polymer (polyhydroanglucoronic acid units. It was introduced into clinical practice in 1949. Surgicel® absorbable hemostat is a sterile absorbable knitted fabric prepared by the controlled oxidation of regenerated cellulose. The fabric is white with a pale yellow cast and has a faint caramel-like aroma. It is strong and can be sutured or cut without fraying. After Surgicel® has been saturated with blood; it swells into a brownish or black gelatinous mass which aids in the formation of a clot, thereby serving as a haemostatic adjunct in the control of local hemorrhage.