The Effect of Green Tea Consumption on Thrombin Time among Adult Healthy Sudanese Volunteers

تأثير استهلاك الشاي الأخضر على مستوئٍ وقت الثرومبين بين المتطوعين السودانيين الأصحاء

A dissertation submitted in partial fulfillment for the requirements of M.Sc. in Medical Laboratory Science (haematology & immunohaematology)

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«بِسْمِ اللّهِ الرَّحْمَنِ الرَّحِيمِ

قال تعالى:

أوَلَمْ يَرَ الَّذِينَ كَفَرُوا أَنَّ السَّمَاوَاتِ وَالأَرْضَ كَانَتَا رَتْقًا فَفَتَقْنَاهُمَا وَجَعَلْنَا مِنَ الْمَاء كُلَّ شَيْءٍ حيٍّ أَفَلا يُؤْمِنُونَ

صدق الله العظيم

سورة الأنبياء الآية 30»
Dedication

I dedicated this work to my family, my mother, who supported me and to all my friends and colleagues.
Acknowledgement

Praises and thanks to almighty Allah

I would like to gratefully thank Dr. Ibrahim Khider Ibrahim my supervisor, for his guidance throughout the study and his permanent support and encouragement.

I would like to express my gratitude to all those who helped me during my work.

Finally my thanks extent to all my colleagues and friends who participated in this study.
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<tr>
<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
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<tr>
<td>ADPase</td>
<td>Adenosine diphosphatase</td>
</tr>
<tr>
<td>APTT</td>
<td>Activated partial thromboplastin time</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BMD</td>
<td>Bone mineral density</td>
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<tr>
<td>Ca</td>
<td>Calcium</td>
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<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
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<tr>
<td>Da</td>
<td>Dalton</td>
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<tr>
<td>EGCG</td>
<td>Epigallocatechin-3-gallate</td>
</tr>
<tr>
<td>EPCR</td>
<td>Endothelial protein C receptor</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetra acetic acid</td>
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<tr>
<td>FDP</td>
<td>Fibrinogen degradation products</td>
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<tr>
<td>GPI</td>
<td>Glycophosphoinositol</td>
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<tr>
<td>HMWK</td>
<td>High molecular weight kininogen</td>
</tr>
<tr>
<td>KDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
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<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>PPs</td>
<td>Polyphenols</td>
</tr>
<tr>
<td>P.P.P</td>
<td>Platelet poor plasma</td>
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<tr>
<td>PA</td>
<td>Plasminogen activator</td>
</tr>
<tr>
<td>PAI</td>
<td>Plasminogen activator inhibitor</td>
</tr>
<tr>
<td>PPAI-1</td>
<td>Plasmingen activator inhibitor-1</td>
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<tr>
<td>PK</td>
<td>Prekallikerin</td>
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<tr>
<td>SPSS</td>
<td>Statistical package for the social science</td>
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<tr>
<td>TFs</td>
<td>Theaflavins</td>
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<tr>
<td>TF</td>
<td>Tissue factor</td>
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<tr>
<td>TFPI</td>
<td>Tissue factor pathway inhibitor</td>
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<tr>
<td>t-PA</td>
<td>Tissue plasminogen activator</td>
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<tr>
<td>TX A2</td>
<td>Thromboxane A2</td>
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<tr>
<td>TXB₂</td>
<td>Thromboxane B₂</td>
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<tr>
<td>TAFI</td>
<td>Thrombin-activated fibrinolysis inhibitor</td>
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<tr>
<td>u-PA</td>
<td>Urikinase plasminogen activator</td>
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<td>VWF</td>
<td>Von willebrand factor</td>
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Abstracts

Background
Thrombin is the central protease in the cascade of blood coagulation and having many biologically important functions such as the activation of platelets, conversion of fibrinogen to a fibrin network, and feedback amplification of coagulation.

Objective
This study aimed to examine the effect of the consumption of green tea(*Camellia sinensis*) on thrombin time level among healthy adult Sudanese volunteers.

Materials and Methods
Forty (40) healthy adult volunteers in this study; 21 of them were females and 19 were males; age ranged between 20 and 45 years. Baseline thrombin time was measured for all the participants, then they were instructed to drink two cup of green tea per day for 30 days. Venous Blood samples were collected from all subjects in 3.2% tri- sodium citrate anticoagulant before and after consumption of green tea and thrombin time was measured for each sample by using fully-automated coagulometer (stago compact max)

Results
The mean thrombin time level was found significantly lower in the samples collected after green tea consumption than that of samples collected before green tea consumption (Mean±SD: 15.9±1.4 and 16.9±16.8 respectively, *P* value 0.000). No statistically significant difference was found in the mean of thrombin level between females and males in both pre- and post-green tea consumption samples (*p* value=0.09 and 0.05 respectively).

Conclusion
In summary we conclude that regular consumption of two cup per day green tea will reduce the time of thrombin among healthy Sudanese.
المستخلص

خلفية
الثرومبين هو البروتين الرئيسي في سلسلة تجلط الدم، وله العديد من الوظائف، عملياً بفضل تنشيط الصفائح الدموية، وتحويل الفيبرينوجين إلى شبكة الفيبرين، وتضخيم ردود الفعل للتحجر.

الهدف

هذه الدراسة هدفها تأثير استهلاك الشاي الأخضر على مستوى وقت الثرومبين بين المتطوعين السودانيين الأصحاء.

المواد والطرق

أربعون (40) من المتطوعين البالغين الأصحاء في هذه الدراسة، 21 منهم من الذكور، 19 من الإناث. تتراوح أعمارهم ما بين 20 و 65 سنة. تم قياس زمن الثرومبين الأساسي لجميع المشاركين، ثم تم إرشادهم لشرب كوبين من الشاي الأخضر يومياً لمدة 30 يومًا. تم جمع عينات الدم الوريدي من جميع المشاركين في 3.2٪ من مضادات تأثير سترات الصوديوم قبل وبعد استهلاك الشاي الأخضر، وتم قياس زمن الثرومبين لكل عينة باستخدام مقياس تجلط الدم الاتماتيكي الكامل.

النتائج

توصلت الدراسة وجود دلالة إحصائية في تقلص مستوى الثرومبين في العينات التي تم جمعها بعد استهلاك الشاي الأخضر (0.99٪) من العينات التي تم جمعها قبل استهلاك الشاي الأخضر (1.99٪) (ق: 0.0000).

لم يتم العثور على فروق ذات دلالة إحصائية في متوسط الثرومبين بين الإناث والذكور في جميع العينات ما قبل وبعد استهلاك الشاي الأخضر.

الخاتمة

الاستهلاك المنتظم لكيشان من الشاي الأخضر يوميا يقلل من وقت الثرومبين بين السودانيين الأصحاء.
CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW
1.1 Introduction

The coagulation of blood is the initial phase of the biological repair process that responds to perforating trauma to the vasculature; its function is to stop blood loss from the circulatory system by establishing a temporary barrier between the intra- and extra-vascular compartments. The chief product of the coagulation pathways is thrombin, which cleaves fibrinogen to produce fibrin and thus the fibrin clot (Mann and Lorand, 1993).

Thrombin is the central protease of the vertebrate blood coagulation cascade. Prior to the advent of modern biochemistry, thrombin was identified as a substance capable of promoting the formation of a fibrous blood clot (fibrin), and it accordingly derives its name from thrombos, the Greek word for clot. Thrombin is synthesized in the liver and secreted into the general circulation in an inactive zymogen form (prothrombin), a complex multi-domain glycoprotein that is activated to yield thrombin at sites of vascular injury by limited proteolysis following upstream activation of the coagulation cascade (Drake TA et al., 1989).

The tea plant, *Camellia sinensis*, is a member of the Thecae family, black, and green tea are produced from its leaves. It is an evergreen shrub can grow to heights of 30 feet but is usually pruned to 2-5 feet for cultivation. The leaves are dark green, alternate and oval, with serrated edges, and the blossoms are white, fragrant, and appear in clusters or singly (Mukhtar and Ahmad, 2000).
1.2 Literature review

1.2.1 Tea

Tea is one of the most widely consumed beverages in the world (Jun, 2009). Tea holds second position in consumption among all beverages. Tea has been obtained from leaves of plant Camellia sinensis for almost 50 centuries ago. The plant of tea was originated from Southeast Asia and is now being cultivated in more than 30 countries. About three billion kilograms of tea are produced and consumed yearly (Mukhtar and Ahmad, 2000). Tea has been categorized into three main types on the basis of processing during manufacturing. Of the tea produced worldwide, 78% is black tea, which is usually consumed in the Western countries, 20% is green tea, which is commonly consumed in Asian countries, and 2% is oolong tea which is produced (by partial fermentation) mainly in southern China (Cabrera et al., 2006).

Three main types of tea i.e. green, black and oolong tea differ in manufacturing processes. For the production of green tea, freshly harvested leaves are rapidly steamed or panfried to inactivate enzyme polyphenol oxidase, thereby preventing fermentation and producing a dry, stable product. To produce black and oolong teas, the fresh leaves are allowed to wither until their moisture content is reduced to <55% of the original leaf weight, which results in the concentration of polyphenols (PPs) in the leaves. The withered leaves are then rolled and crushed, initiating fermentation of the PPs. During these processes the catechins (a group of natural polyphenols in green tea, accounting for its characteristic color and flavor) are converted to polymeric compounds, theaflavins (TFs) and thearubigins, consequently decreasing the catechin content. Oolong tea is prepared by firing the leaves shortly after rolling to terminate the oxidation and drying the leaves. Normal oolong tea is considered to be nearly half as fermented as black tea. The
fermentation process causes oxidation of simple polyphenols to more complex condensed polyphenols which give black and oolong teas their characteristic colors and flavors. The extended fermentation lowers the polyphenol content and elevates the caffeine content. Black tea has 2-3 times more caffeine as compared to the green tea (Adak and Gabar, 2011).

1.2.2 Green Tea (Camellia sinensis)

is produced by steaming (Japan) or panning (China) to prevent catechin oxidation by polyphenol oxidase. With no fermentation, green tea leaves retain their green color and almost all of their original polyphenol content (Eric W.C. Chan et al., 2011). There is hardly any other food or drink reported to have as many health benefits, including its anti-cancer, anti-obesity, anti-diabetes, anti-inflammatory, and anti-neurodegenerative effects (V. R. SINIJA, & H. N. MISHRA, 2008). The antioxidant properties of catechins are mainly related to the number and position of hydroxyl group in the molecules and consequently binding and neutralization of free radicals by these hydroxyl groups (Milić et al., 1998).

1.2.2.1 Green Tea Composition

Green tea is produced from steaming fresh leaves at high temperatures, thereby inactivating the oxidizing enzymes and leaving the polyphenol content intact. The polyphenols found in tea are more commonly known as flavanols or catechins. The main catechins in green tea are epicatechin, epicatechin-3gallate, epigallocatechin, and epigallocatechin-3-gallate (EGCG), with the latter being the highest in concentration. Other compounds are alkaloids (caffeine, theophylline and theobromine), amino acids, carbohydrates, proteins, chlorophyll, volatile organic compounds (chemicals that readily produce vapors and contribute to the odor of tea), fluoride, plant aluminum, minerals and trace elements (GRAHAM, 1992).
1.2.2.2 Green Tea Processing

The production of green tea is characterized by an initial heating process, which kills the enzyme polyphenol oxidase, which is responsible for the conversion of the flavanols in the leaf into the dark polyphenolic compounds that colour black tea. The other important process is rolling, in which leaves are cut and twisted. The final form of green tea depends on the particular variant being produced. The rolling stage is very similar to the operation with the same name in black tea production. Green tea production is restricted mainly to China and Japan Green Tea Processing: Steaming/Roasting → Cooling → 1st Rolling → 1st Drying (110°C/70°C) → Final Rolling → Final Drying (120°C/80°C) (McKay DL, 2002).

1.2.2.3 Green Tea Extracts and Standard Preparation

Green tea extracts were prepared by infusing green tea leaves in 100 volumes (v/w) of 50% aqueous methanol for 12 h at 25 °C in a shaking incubator. Catechins and Trolox standards for evaluation of antioxidant activity were dissolved in 20% aqueous methanol and methanol, respectively, at a concentration of 1 mM and stored at −20°C. After that, stock solutions of each standard and the green tea extracts were diluted within the linear range of the assays (Lan-Sook Lee et al., 2014).

1.2.2.4 Beneficial Effects of Green Tea

1.2.2.4.1 Antioxidant Activity

Green tea is rich in antioxidants; the later are known as scavengers of free hydroxyl radicals, peroxyl radicals, superoxide ions, etc. Polyphenols, mainly flavonoids present in the tea are a well-documented antioxidant agent. The imbalance between reactive oxygen species and antioxidants result in oxidative stress, responsible for cellular damage. Catechins present in green tea increase the
serum superoxide dismutase activity and aorta catalase enzyme and have a protective role against reactive oxygen stress. Its uptake also reduces the malondialdehyde level, an oxidative stress marker (S.M. Chack et al., 2010). Aromatic rings and hydroxyl groups of the polyphenols mainly contribute to the antioxidant action by neutralizing the lipid free radicals (A. Michalak, 2011).

**1.2.2.4.2 Anti-carcinogenic**
Experimental evidence points to the potential of green tea to protect against cancer at several stages of carcinogenesis, including cancer prevention, endogenous carcinogen activation, DNA damage and destabilization, cell proliferation, neoplastic growth and metastasis (Weisburger & J. H, 1999). Green tea polyphenols, particularly EGCG, may be effective in preventing cancer of the prostate and reduced the incidence of cancers of the stomach, small intestine, pancreas, lung, breast, skin, urinary bladder, prostate, oesophagus and mouth. Also, it has been shown to reduce tumour size and growth in cancer-bearing animals (Komori et al., 1993).

**1.2.2.4.3 Antithrombogenic effects**
Platelet aggregation contributes to both the development of atherosclerosis and acute platelet thrombus formation, followed by embolization of stenosed arteries. Activated platelets adhering to vascular endothelium generate lipid peroxides and oxygen free radicals, which inhibit the endothelial formation of prostacyclin and nitrous oxide. It was shown in the 1960s that tea pigment can reduce blood coagulability, increase fibrinolysis and prevent platelet adhesion and aggregation (Lou FQ et al., 1989). Flavonols are particularly antithrombotic because they directly scavenge free radicals, thereby maintaining proper concentrations of endothelial prostacyclin and nitric oxide (Gryglewski RJ et al., 1987).
1.2.2.4 Anti-Arthritis Effect
Green tea protects against rheumatoid arthritis by modulating arthritis-related immune responses. It suppresses both cytokine IL-17 (an inflammatory substance) and antibodies to Bhsp65 (arthritis inducing protein) and increases cytokine IL-10 (an anti-inflammatory substance) (Kim et al., 2008).

Bone mineral density (BMD) may be influenced by chemical compounds in tea such as caffeine, fluoride and phytoestrogens. Black tea consumption had a moderately positive effect on BMD, particularly in older women. There was a significant increase in BMD with higher levels of tea consumption (four or more cups per day) (Chen et al., 2003).

1.2.2.5 Body Weight Control
Green tea it has recently become the latest weapon in fighting over weight conditions. It appears to fight obesity by increasing the rate of calories burning, reducing body fate level and preventing excess weight gain. The consumption of the green tea extract is associated with statistically significant reduction in total and low density lipoprotein cholesterol level (Kim et al., 2011). Green tea catechin enhance exercise induced abdominal fat loss in overweight and obese adult (Maki Kevin C et al., 2009). Green tea catechins epigallocatechin gallate (EGCG) have been shown to reduce adipocytes differentiation and proliferation lipogenesis i.e birth of new fat cells; fat mass, body weight, fat oxidation plasma level of triglyceride, free fatty acid, cholesterol, glucose, insulin and leptin and increased Beta-oxidation thermogenesis (Wolfram S et al., 2006).

1.2.2.6 Dosage and Toxicity
Based on current literature there does not appear to be any significant side-effects or toxicity associated with green tea consumption. However, overconsumption of caffeine containing substances may cause intoxication but not clinical dependence. Effects include insomnia, restlessness, flushing, diuresis, twitches, nervousness,
rambling thoughts and speech, tachycardia, and psychomotor agitation, with symptoms lasting six to 16 hours. Caffeine consumption is contraindicated during pregnancy. Lactating women should also limit caffeine intake to avoid sleep disorders in infants. In general, the stimulatory effect from green tea is considerably less than that of coffee. On average, a cup of green tea contains less than 50 mg of caffeine, whereas coffee may contain up to 150 mg per cup (DerMarderosian A, 1999).

The average infusion of green tea varies in its phenolic content, ranging from 50-400 mg of polyphenols per cup. Depending on species, harvesting variables, and brewing methods, recent human epidemiological studies suggest a total daily intake of approximately 10 cups of green tea per day has a chemopreventive effect (Imai K, 1997).

1.2.2 Hemostasis

Hemostasis is a dynamic process whereby blood coagulation is initiated and terminated in a rapid and tightly regulated fashion (Orkin, S. H et al., 2003). Haemostasis is the process that maintains the integrity of a closed, high pressure circulatory system after vascular damage. Vessel wall injury and the extravascular of blood from the circulatory rapidly initiate events in the vessel wall and in the blood that seal the breach. Circulatory platelet are recruited to the site of injury, where they become a major component of the developing thrombus; blood coagulation, initiated by tissue factor, culminate in the generation of thrombin and fibrin. These event occur concomitantly, and under normal condition, regulatory mechanism contain thrombus formation temporally and spatially (Black S et al., 2005).
1.2.2.1 Component of normal haemostasis (primary)

Hemostasis is regulated by three basic components namely, the vascular wall, platelets, and the coagulation cascade. The haemostatic mechanism have several important function to maintain blood in a fluid state while circulating within the vascular system, to arrest bleeding at the site of injury or blood loss by formation of haemostatic plug; and to ensure eventual removal of the plug when healing is complete. Under normal conditions, the formation and dissolution of thrombi is maintained in a delicate balance. An individual experience either excessive bleeding (conditions associated with excessive bleeding referred to as hypocoagulable state) or thrombosis (condition there is uncontrolled thrombosis referred to hypercoagulable) (Lotstpeich-steininger et al., 1998).

1.2.2.1.1 The Blood Vessels

The blood vessel wall has three layers: Tunica intima, Tunica media, and adventitia. The intima consists of endothelium connective tissue and subendothelial connective tissue connective tissue and is separated from the media by the elastic lamina interna. Endothelial cells form a continuous monolayer lining all blood vessels. The structure and the function of the endothelial cells vary according to their location in the vascular tree, but in their resting state they all share three important characteristics: they are “nonthrombogenic” (i.e., they promote maintenance of blood in its fluid state), they play an active role in supplying nutrients to the sub endothelial structures, and they act as a barrier to macromolecules and particulate matter circulating in the bloodstream. The permeability of the endothelium may vary under different conditions to allow various molecules and cells to pass (Bain B et al., 2006).
1.2.2.1.2 Endothelial cell function

The luminal surface of the endothelial cell is covered by the glycocalyx, a proteoglycan coat. It contains heparin sulphate and other glycosaminoglycans, which are capable of activating antithrombin, an important inhibitor of coagulation enzymes. Tissue factor pathway inhibitor (TFPI) is present on endothelial cell surfaces, bound to these heparins but also tethered to a glycophosphoinositol (GPI) anchor. Endothelial cells express a number of coagulation-active proteins that play an important regulatory role, such as thrombomodulin and the endothelial protein C (PC) receptor. Thrombin generated at the site of injury is rapidly bound to thrombomodulin (a specific product of endothelial cell). When bound, which can activate PC (activated protein C which cleavage factors Va and VIIIa) bound to the endothelial protein C receptor (EPCR) and a carboxypeptidase which inhibits fibrinolysis. Thrombin also stimulates the endothelial cell to generate tissue plasminogen activator (tPA). The endothelium can also synthesis protein S, the cofactor for PC. Finally, endothelium produces von Willebrand factor (VWF), which is essential for platelet adhesion to the subendothelium and stabilises factor VIII (FVIII) within the circulation. VWF is both stored in specific granules called Weibel Palade bodies and secreted constitutively, partly into the circulation and partly toward the subendothelium where it binds directly to collagen and other matrix proteins. The lipid bilayer membrane also contains ADPase (adenosine diphosphatase), an enzyme that degrades adenosine diphosphate (ADP), which is a potent platelet agonist. The endothelial cell participates in vasoregulation by producing and metabolizing numerous vaso-active substances. On one hand it metabolises and inactivates vasoactive peptides such as bradykinin, on the other hand, it can also generate angiotensin II, a local vasoconstrictor, from circulating angiotensin I. Under appropriate stimulation the endothelial cell can produce
vasodilators such as nitric oxide (NO) and prostacyclin or vasoconstrictors such as endothelin and thromboxane. These substances have their principal vasoregulatory effect via the smooth muscle but also have some effect on platelets. The subendothelium consists of connective tissues composed of collagen (principally types I, III and VI), elastic tissues, proteoglycans and non-collagenous glycoproteins, including fibronectin and VWF. After vessel wall damage has occurred, these components are exposed and are then responsible for platelet adherence. At low shear platelets can bind to collagen but in practice this appears to be largely mediated by VWF binding to collagen. VWF bound to collagen undergoes a conformational change and platelets are captured via their surface membrane glycoprotein Ib binding to VWF. Platelet activation follows, and a conformational change in glycoprotein IIbIIIa allows further, more secure, binding to VWF via this receptor as well as to fibrinogen (Bain B et al., 2006).

1.2.2.1.3 Vasoconstriction

Vasoconstriction vessels with muscular coats contract following injury thus helping to arrest blood loss. Although not all coagulation reactions are enhanced by reduced flow, this probably assist in the formation of a stable fibrin plug. Vasoconstriction also occurs in the microcirculation in vessels without smooth muscle cells. Endothelial cells themselves can produce vasoconstriction such as angiotensin II. In addition, activated platelets produce thromboxane A2 (TXA2), which is a potent vasoconstriction (Hoffbrand et al., 2002).
1.2.2.2 Platelets

1.2.2.2.1 Platelet production

Platelets are produced by the bone marrow megakaryocytes as a result of fragments of the cytoplasmic membrane. Megakaryocytes are derived from the haemopoetic stem cell, which is stimulated to differentiate to mature megakaryocytes under the influence of various cytokines, including thrombopoietin. Once released from the bone marrow young platelets are trapped in the spleen for up to 36 hours before entering the circulation, where they have a primary haemostatic role. The normal lifespan of platelets is 7-10 days and the normal platelet count range from 150,000-450,000/μL. The mean platelet diameter is 1-2 μm and the normal range for cell volume (MPV) is 8-11 fl. Although platelets are non-nucleated cells, those that have recently been released from the bone marrow contain RNA and are known as reticulated platelets (Lefkowitz J, 2009).

1.2.2.2.2 Platelet adhesion and activation

Primary hemostasis and arterial thrombosis are the results of a complex series of cell–cell, cell–protein and protein–protein reactions that involve platelets, leukocytes, subendothelial matrix and plasma proteins, such as fibrinogen, von Willebrand factor (vWF) and others. When blood vessel injury, platelets adhere to Exposed subendothelial matrix proteins via special adhesive glycoproteins (GP). Under condition high shear, e.g. arterioles, the exposed subendothelial matrix is initially coated with VWF multimer. Then platelets contact with VWF via the GP1b-XI-V complex on platelets. This initiates plate rolling in the direction of blood flow over the exposed VWF with activation of GP1b/IIa receptor. From adhesion is established by the slower bind stronger interaction of other glycoproteins including activated GPIIb/IIa with VWF and GPVIα to integrin α 1/
β2 with collagen and other component of the subendothelial matrix. Under static shear conditions, platelets adhere predominant to collagen of the subendothelium. Collagen bind initially to GPIa/IIa, cross-links many of these integrin molecules, and in these way activates platelets. This ligand receptor binding results in a complete cascade of signals which result in platelet activation. The events that follow are shape change and spreading, activation of GPIIb/IIIa and granule secretion Platelets become more spherical and extrude low pseudopodia which enhance platelet vessel and platelet-platelet interaction, The end result of spreading is a flattened spread out platelet with granules and organelles in the centre, resulting in a characteristic fried egg appearance. These changes are brought about by the actin cytoskeleton. The granules are secreted from the Centre of the cell (Hoffbrand et al., 2002).

1.2.2.2.3 Platelet aggregation

Platelet aggregation may occur by at least two independent but closely linked pathways. The first pathway involves arachidonic acid (AA) metabolism. Activation of phospholipase enzymes (PLA₂) releases free arachidonic acid from membrane phospholipids (phosphatidyl choline). About 50% of free arachidonic acid is converted by a lipo-oxygenase enzyme to a series of products including leukotrienes, which are important chemoattractants of white cells. The remaining 50% of arachidonic acid is converted by the enzyme cyclooxygenase into labile cyclic endoperoxides, most of which are in turn converted by thromboxane synthetase into TXA₂. TXA₂ has profound biological effects, causing secondary platelet granule release and local vasoconstriction, as well as further local platelet aggregation via the second pathway below. It exerts these effects by raising intracellular cytoplasmic free calcium concentration and binding to specific granule receptors. TXA₂ is very labile with a half-life of less than 1 min before it is degraded into the inactive thromboxane B₂ (TXB₂) and malonyldialdehyde.
The second pathway of activation and aggregation can proceed completely independently from the first one: various platelet agonists, including thrombin, TXA$_2$, and collagen, bind to receptors and, via a G-protein mechanism, activate phospholipase C. This generates diacylglycerol and inositol triphosphate, which in turn activate protein kinase C and elevate intracellular calcium, respectively. Calcium is released from the dense tubular system to form complexes with calmodulin; this complex and the free calcium act as coenzymes for the release reaction, for the activation of different regulatory proteins and of actin and myosin and the contractile system, and also for the liberation of arachidonic acid (AA) from membrane phospholipids and the generation of TXA$_2$. The aggregating platelets join together into loose reversible aggregates, but after the release reaction of the platelet granules, a larger, firmer aggregate forms. Changes in the platelet membrane configuration now occur; “flip-flop” rearrangement of the surface brings the negatively charged phosphatidyl-serine and -inositol on to the outer leaflet, thus generating platelet factor 3 (procoagulant) activity. At the same time specific receptors for various coagulation factors are exposed on the platelet surface and help coordinate the assembly of the enzymatic complexes of the coagulation system. Local generation of thrombin will then further activate platelets (Bain B et al., 2006).

Platelets are not activated if in contact with healthy endothelial cells. The “nonthrombogenicity” of the endothelium is the result of a combination of control mechanisms exerted by the endothelial cell: synthesis of prostacyclin, capacity to bind thrombin and activate the PC system, ability to inactivate vasoactive substances, and so on. Prostacyclin released locally binds to specific platelet membrane receptors and then activates the membrane-bound adenylate cyclase (producing cyclic adenosine monophosphate, or cAMP). cAMP inhibits platelet
aggregation by inhibiting arachidonic acid metabolism and the release of free cytoplasmic calcium ions.

Thus platelets have at least three roles in haemostasis:
1. Adhesion and aggregation forming the primary haemostatic plug
2. Release of platelet activating and procoagulant molecules
3. Provision of a procoagulant surface for the reactions of the coagulation system. (Bain B et al., 2006).

1.2.2.2 Secondary Hemostasis

Secondary hemostasis includes the response of the coagulation process to vessel injury. The intrinsic coagulation system is activated in vivo by the contact of certain coagulation proteins with sub-endothelial connective tissue. In contrast with extrinsic coagulation pathway, is initiated with the release of tissue factor (TF) from injured vessel endothelial and sub-endothelial. TF, a high molecular weight lipoprotein, is found in most organ as well as in large blood vessels (such as the vena cava and aorta). Especially high concentration are found in lungs, brain, and placenta. Both intrinsic and extrinsic coagulation pathway lead to the activation of the common pathway and formation a stable fibrin clot (lotspeich-steininger et al., 1998).

1.2.2.2.1 The coagulation protein

The coagulation pathway a series of reactions that involve coagulation factors known as zymogen (enzyme precursors). non enzymatic cofactors, calcium(Ca$^{2+}$) the substrate protein fibrinogen (factor I), and phospholipid. All coagulation factors except tissue factor (TF) are normally present in the plasma and phospholipid is provided by platelets. The zymogen are factors II, VII, IX, X, XI, XII, XIII, and prekallikrein; the cofactors are V, VIII, TF, Ca$^{2+}$, and high molecular
weight kininogen (HMWK). Zymogen are substrates that have no biological activity until convert by enzymes to active enzymes. With the exception of factor XIII, the zymogen factors are converted to enzyme called serine proteases, which hydrolyze arginine or lysine containing peptide bonds of other zymogen, thus converting them to serine proteases. When converted to active enzyme form, factor XIII has an active enzyme containing cystein instead of serine. The haemostatic process simultaneously provides amplification of the control mechanisms that prevent excessive clotting and clot lysis (Iotstpeich-steininger et al., 1998).
Table 1.1: The characteristic of the coagulation factors, adapted from (Lefkowitz J, 2009)

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrinogen (factor I)</td>
<td>Molecular Weight (MW)= 340 kilodaltons (kDa); glycoprotein</td>
<td>Adhesive protein which aids in fibrin clot formation.</td>
</tr>
<tr>
<td>Prothrombin (factor II)</td>
<td>Vitamin K-dependent serine protease; MW= 72 kDa</td>
<td>Activated form is the main enzyme of coagulation</td>
</tr>
<tr>
<td>Tissue factor (factor III)</td>
<td>Known as thromboplastin; MW= 37 kDa</td>
<td>Lipoprotein initiator of the extrinsic pathway</td>
</tr>
<tr>
<td>Calcium ions (factor IV)</td>
<td>Required for coagulation factors to bind to phospholipid</td>
<td>Metal cation which is important in coagulation mechanisms</td>
</tr>
<tr>
<td>Factor V (labile factor)</td>
<td>MW = 330 kDa</td>
<td>Cofactor for the activation of prothrombin to thrombin</td>
</tr>
<tr>
<td>Factor VII (proconvertin)</td>
<td>MW = 50 kDa; vitamin K-dependent serine protease</td>
<td>With tissue factor, initiates extrinsic pathway</td>
</tr>
<tr>
<td>Factor VIII (antihaemophilic factor)</td>
<td>MW = 330 kDa</td>
<td>Cofactor for intrinsic activation of factor X</td>
</tr>
<tr>
<td>Factor IX (Christmas factor)</td>
<td>MW = 55 kDa; vitamin K-dependent serine protease</td>
<td>Activated form is enzyme for intrinsic activation of factor X</td>
</tr>
<tr>
<td>Factor X (Stuart–prower factor)</td>
<td>MW = 58.9 kDa; vitamin K-dependent serine protease</td>
<td>Activated form is the enzyme for final the common pathway activation of prothrombin</td>
</tr>
<tr>
<td>Factor XI (Plasma thromboplastin antecedent)</td>
<td>MW = 160 kDa; serine protease</td>
<td>Activates intrinsic activator of factor IX</td>
</tr>
<tr>
<td>Factor XII (Hegman(contact ) factor)</td>
<td>MW = 80 kDa; serine protease</td>
<td>Factor that nominally(aPTT) based intrinsic pathway</td>
</tr>
<tr>
<td>Factor XIII ( Fibrin stabilizing factor)</td>
<td>MW = 320 kDa; Cross links fibrin</td>
<td>Transamidase which cross-links fibrin clot</td>
</tr>
<tr>
<td>HMWK (fitzgerald factor or William factor)</td>
<td>MW=110kDa circulate in a complex with factor XI</td>
<td>Cofactor</td>
</tr>
<tr>
<td>Prekallikerin (Fletcher factor )</td>
<td>MW=85 kDa; serine protease</td>
<td>Activated from that participates at beginning aPTT-based intrinsic pathway</td>
</tr>
</tbody>
</table>

*APTT, activated partial thromboplastin time

*HMWK, high molecular weight kininogen
1.2.2.2 Contact activation pathway (intrinsic)

The intrinsic pathway consists of a cascade of protease reactions initiated by factors that are present within the blood. When in contact with a negatively charged surface such as glass or the membrane of an activated platelet, a plasma protein called FXII (Hageman factor) becomes FXIIa (the suffix “a” indicates that this is the activated form of FXII). A molecule called high molecular weight kininogen(HMWK), a product of platelets that may in fact be attached to the platelet membrane, helps anchor FXII to the charged surface and thus serves as a cofactor. However, this HMWK-assisted conversion of FXII to FXIIa is limited in speed. Once a small amount of FXIIa accumulates, this protease converts prekallikrein to kallikrein, with HMWK as an anchor. In turn, the newly produced kallikrein accelerates the conversion of FXII to FXIIa, an example of positive feedback. In addition to amplifying its own generation by forming kallikrein, FXIIa (together with HMWK) proteolytically cleaves FXI, forming FXIa. In turn, FXIa (also bound to the charged surface by HMWK) proteolytically cleaves FIX to FIXa, which is also a protease. FIXa and 2 downstream products of the cascade, FXa and thrombin, proteolytically cleave FVIII, forming FVIIIa, a cofactor in the next reaction. Finally, FIXa and FVIIIa together with Ca2+ (which may come largely from activated platelets) and negatively charged phospholipids (the major constituents of cell membranes) form a trimolecular complex termed tenase. Tenase then converts FX to FXa, yet another protease (Boron & Boulpaep, 2005).

1.2.2.3 Extrinsic coagulation Pathway

The extrinsic pathway also includes protein cofactors and enzymes. This pathway is initiated by the formation of a complex between TF on cell surfaces and FVIIa that is located outside the vascular system. Nonvascular cells constitutively express the integral membrane protein TF (variably known as FIII or tissue
thromboplastin), which is a receptor for the plasma protein FVII (Kumar et al., 2005). When an injury to the endothelium allows FVII to come into contact with TF, the TF non proteolytically activates FVII to FVIIa. The mechanism of the initial conversion of the zymogen FVII to FVIIa is still debated but is most likely due to autocatalytic activation and not a TF effect (M. Hoffman & Monroe, 2005). This binding of FVIIa to TF forms an enzyme complex that activates FX to FXa. The FVIIa/TF complex, similar in function to the tenase complex, converts FX to its active form (FXa), which binds to the cofactor FV and is bound on membrane surfaces in the presence of calcium ions to generate the prothrombinase complex. The prothrombinase complex converts prothrombin to thrombin, which converts fibrinogen to fibrin to generate the fibrin clot (Hoffman, R et al., 2005).

1.2.2.2.4 Common coagulation Pathway

The common pathway begins with the activation of FX within the intrinsic, and extrinsic pathway. FXa from either the intrinsic or extrinsic pathway is the first protease of the common pathway. FXa, in the presence of FV, Ca2+, and phospholipids, converts prothrombin to its active form, thrombin (Harmening, D, 2002). The main action of thrombin is to catalyze the proteolysis of the soluble plasma protein fibrinogen to form fibrin monomers that remain soluble. Fibrin monomers then polymerize to form a gel of fibrin polymers that trap blood cells. Thrombin also activates FXIII, which is converted to FXIIIa and mediates the covalent cross-linking of the fibrin polymers to form a mesh termed stable fibrin, which is less soluble than fibrin polymers (Boron & Boulpaep, 2005).

1.2.2.2.5 Conversion of fibrinogen to fibrin

The conversion of fibrinogen to fibrin involves three steps. The enzymatic step. First there is cleavage of tow A and B fibrinopeptides from fibrinogen molecule by enzymatic cleavage of thrombin. The polymerization step. Finbrinopeptide A & B
are negatively charged, and their result is a fibrin monomer with a significant decreased electronegativity, thus. Given the correct environment, including PH & ionic concentration the fibrin monomers aggregate spontaneously by forming weak bonds(primary hydrogen) between the fibrin monomers and the last step is stabilizing step provides for formation of insoluble fibrin by stabilization, which requires thrombin, factor VIIIa and Ca$^{2+}$ (lotstpeich-steininger et al., 1998).

1.2.2.6 The fibrinolytic system

The deposition of fibrin and its removal are regulated by the fibrinolytic system. Although this is a complex multicomponent system with many activators and inhibitors, it centres on the fibrinogen- and fibrin-cleaving enzyme plasmin. Plasmin circulates in its inactive precursor form, plasminogen, which is activated by proteolytic cleavage. The principal plasminogen activator (PA) in humans is tissue plasminogen activator(t-PA), which is another serine protease. tPA and plasminogen are both able to bind to fibrin via the amino acid lysine. When they are both the rate of plasminogen activation is markedly increased and thus plasmin is generated preferentially at its site of action and not free in plasma. The second important physiological PA in humans is urokinase (uPA). This single-chain molecule (scuPA or pro-urokinase) is activated by plasmin or kallikrein to a two-chain derivative (tcuPA), which is not fibrin-specific in its action.

However, the extent to which this is important in vivo is not clear, and the identification of cell surface receptors for uPA suggests that its primary role may be extravascular. The contact activation system also appears to generate some plasminogen activation via FXIIa and bradykinin-stimulated release of tPA. The cleavage products released by the action of plasmin on fibrin are of diagnostic use and are discussed later in this chapter. The activation of plasmin on fibrin is restricted by the action of a carboxypeptidase, which removes the amino terminal lysine residues to which plasminogen and tPA bind. This carboxypeptidase is
activated by thrombomodulin-bound thrombin and is referred to as thrombin-activated fibrinolysis inhibitor (TAFI) (Bain B et al., 2006).

PAI-1 (Plasminogen activator inhibitor-1) is a potent inhibitor of tPA, produced by endothelial cells, hepatocytes, platelets and placenta. Levels in plasma are highly variable. It is a member of the serpin family and is active against tPA and tcuPA. A second inhibitor, PAI-2, has also been identified, originally from human placenta, but its role and importance are not yet established.

The main physiological inhibitor of plasmin in plasma is plasmin inhibitor (α2-antiplasmin), which inhibits plasmin function by forming a 1:1 complex (plasmin–antiplasmin complex, PAP). This reaction in free solution is extremely rapid but depends on the availability of free lysine-binding sites on the plasmin. Thus, fibrin-bound plasmin in the clot is not accessible to the inhibitor. Deficiencies of the fibrinolytic system are rare but have sometimes been associated with a tendency to thrombosis or haemorrhage (Bain B et al., 2006).

1.3 Rationale

Although green tea is more frequently known to have beneficial effects on the human health, it is rarely used for daily consumption as a drink and there are no documented data on its beneficial effects on haemostasis at the national level. We, therefore, conducted this prospective, interventional study into the effect of daily green tea consumption on Thrombin Time.

1.4 objectives

1.4.1 General objectives

To study the effect of green tea intakes on thrombin time level among healthy Sudanese volunteers.
1.4.2 Specific objectives

- To determine the mean of thrombin time among healthy Sudanese volunteers pre and post consuming two cup of green tea per day for one month using Stago fully-automated coagulometer analyzer.
- To correlate the effect of green tea consumption on thrombin time level with the participant’s gender and age.
CHAPTER TWO

Materials and methods
2.1 Study design

This study was a prospective, interventional study (before – after).

2.2 Study area and duration

The study was conducted at Khartoum state in the period from March 2018 to April 2018.

2.3 Study population and sample size

This study was conducted on 40 apparently healthy Sudanese volunteers to study the effect of green tea consumption on thrombin time level; the volunteers consumed two cups of green tea per day for one month. Thrombin time was estimated for each participant before and after the consumption of green tea for the one month’s study period.

2.4 Study variables

Thrombin time level as dependent variable and the following are independent variables:

- Age
- Gender
- Green Tea

2.5 Sample collection

Venous Blood samples were collected from all subjects in 3.2% tri- sodium citrate anticoagulant before and after consumption of green tea and thrombin time was measured for each sample.
2.6 Preparation of Platelet Poor Plasma (P.P.P)

It was prepared PPP by centrifugation at 2000 g for 15 minutes (approximately 4000 rev / min in a standard bench). Then we count platelet by chamber which gave count below 10/ml, we stored the sample as batch at -30 ºc for several weeks, the stored sample then mixed after thawing and centrifuged before testing.

2.7 Determination of thrombin time

Determination of thrombin time (TT) was analyzed by fully-automated coulometer(stago compact max).

2.8 Data collection

Patient’s data collected using structural interview questionnaire.

2.9 Data Analysis

The data were analyzed using SPSS software version 20; the paired t-test was applied to analyze the changes in the thrombin time level. A P-value less than 0.05 was considered as significant.

2.10 Ethical consideration

Informed consent was taken from all subjects before sample collection.
CHAPER THREE

Results
Results

This study was done in Khartoum state at Sudan university of since and technology in the period from March to May 2018 to evaluate the effect of green tea consumption on thrombin time test. 40 healthy Sudanese volunteers aged between 20-45 years were enrolled to participate in this study twenty one (21) of them were females and nineteen (19) of them were males. The studied subjects who consumed two cups of green tea per day for one month, Then TT was measured from pre and post samples collected from them.
Figure 3.1: distribution of study population according to gender
**Table (3.1) Summary of Mean of TT before and after green tea consumption**

<table>
<thead>
<tr>
<th>Variables</th>
<th>Mean (±SD)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-Green Tea</td>
<td>Post -Green Tea</td>
</tr>
<tr>
<td>TT before and after green tea consumption</td>
<td>16.99 (±1.9)</td>
<td>15.90 (±1.4)</td>
</tr>
</tbody>
</table>

- Significant at <0.05

The statistical analysis of the results showed that there is a significant difference (p.value= 0.000) among participant’s TT between before 16.99 (±1.9) and after 15.90 (±1.4) green tea consumption samples (Table 3.1).

**Table 3.2 TT according to the Gender**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Gender</th>
<th>Mean (±SD)</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>pre-green tea consumption TT</td>
<td>Male</td>
<td>17.5 (±2.0)</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>16.5 (±1.5)</td>
<td></td>
</tr>
<tr>
<td>post-green tea consumption TT</td>
<td>Male</td>
<td>16.3 (±1.5)</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>15.4 (±1.1)</td>
<td></td>
</tr>
</tbody>
</table>

- Significant at <0.05

When we compared the thrombin time level in both males and females the mean of pre green tea consumption samples among male was 17.5(± 2.0) and among female was 16.5 (±1.5). While the mean of thrombin level in post green tea consumption samples collected from male was 16.3 mg/dl (SD±1.5) and it was 15.4(±1.1) in samples collected from females, the results showed there was no significant difference between males and females in the pre and post samples. (p.value= 0.09 and 0.05 respectively).
CHAPTER FOUR

Discussion, Conclusion and Recommendations
4.1 Discussion

Thrombin is the key effector enzyme of the coagulation system, having many biologically important functions such as the activation of platelets, conversion of fibrinogen to a fibrin network, and feedback amplification of coagulation (Mann and Lorand, 1993). This study was carried out to evaluate the effect of green tea consumption on thrombin time level in apparently healthy Sudanese people aged between 20-45 years.

In this study we found that the mean of TT in the post samples were significantly lower than the mean of pre-samples, our results agrees with findings of study done by Jalali et al (2008) reported that after regular consumptive of 4 g/d green tea for one month there was a small significant decrease in thrombin time level. Another study done by Hussam et al (2017) which agreed with our study and found that after regular consumption of two cup of green tea for one month there was a small significant decrease in coagulation profile tests. No statistically significant difference was found when we compared mean of TT between males and females.

Another study done by Kannan et al (2014) which study the effect of green tea on pharmacodynamics of warfarin and showed that after regular consumptive of green tea for one month there was a small significant reduction in the mean of pro thrombin time level after green tea consumption and patients who used green tea with warfarin were reduced in pro thrombin time level more than who used warfarin alone.
4.2 Conclusion

The study concludes that consumption of a two cup of green tea per day for 30 days significantly reduce the level of thrombin time among healthy Sudanese volunteers.

4.3 Recommendations

- Another study should be done with increase sample size, different number of cups per day and also different duration of consumption for more obvious finding.
- Further study should be conducted to evaluate the effect of green tea consumption on thrombin level among subjects with known higher thrombin level.
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Appendix
Appendix No (1): Green Tea Lipton
Appendix No (2): STA Compact MAX
Appendix No (3)