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A different insight into blood coagulation in vitro

İnvitro pıhtılaşmaya farklı bir bakış açısı

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ABSTRACT

Objectives: The known model of blood coagulation involves a series of zymogen activation reaction sequences. At each stage, a zymogen is converted to an active protease by cleavage of one or more peptide bonds in the precursor molecule. The aim of this study was to investigate amino acid profiles during coagulation process in different conditions in vitro.

Methods: Samples of serum and plasma (treated by EDTA or citrate) were obtained from healthy donors and from patients with Phenylketonuria (PKU). Amino acid profiles analyzed with reverse phase HPLC column.

Results: There were no differences between two plasma amino acid levels which were obtained by EDTA and acid citrate ($p>0.05$). Serum aspartate (asp), glutamate (glu), serine (ser), histidine (his) and phenylalanine (phe) levels were significantly higher in serum than plasma ($p<0.05$). This significant difference was not observed in patients with PKU.

Conclusion: As a result the enzymatic reactions of coagulation process generate some aminoacids and these reactions take place in an appropriate chemical microenvironment. This microenvironment can be used to clarify the stages of coagulation cascades with further studies. *J Clin Exp Invest 2010; 1(3): 173-176*

Key words: Coagulation, amino acids, Phenylketonuria, in vitro

ÖZET

Amaç: Koagülasyon bilindiği üzere bir dizi zimojenin aktivasyon serisinden oluşur. Her basamakta öncül molekül bir veya daha fazla peptit bağının kopmasıyla aktif proteaza dönüşür. Bu çalışmanın amacı in vitro farklı durumlarda pıhtılaşma sırasında amino asit profilini araştırmaktır.

Yöntem: Sağlıklı yetişkinlerden ve fenilketonürili çocuklardan alınan örnekler serum, ETA'lı plazma ve sitratlı plazma olarak ayrıldı. Örneklerin aminoasit analizleri ters faz HPLC kolonuyla yapıldı.

Bulgular: EDTA'lı ve sitratlı plazmalar arasında anlamlı bir fark görülmedi ($p>0.05$). Serum aspartat (asp), glutamate (glu), serin (ser), histidin (his) ve fenilalanin (phe) seviyelerinde plazmaya göre anlamlı bir artış kaydedildi ($p<0.05$). Bu anlamlı artış fenilketonürili hastalarda gözlenmedi.

Sonuç: Sonuç olarak koagülasyon kaskadının enzimatik reaksiyonları sonucu bazı aminoasitlerin açığa çıktığını ve bu reaksiyonların uygun kimyasal mikroçevre içerisinde gerçekleştiğini söyleyebiliriz. Bu mikroçevrenin rolünün ileri çalışmalarla aydınlatılmasına ihtiyaç vardır. *Klin Den Ar Derg 2010; 1(3): 173-176*

Anahtar kelimeler: Pıhtılaşma, amino asit, Fenilketonüri, vücut dışı

INTRODUCTION

Blood coagulation is a complex physiological process which leads to the formation of fibrin clot through the proteolytic action of thrombin on fibrinogen. When blood is collected from the body and placed in a test tube, it clots quickly. The formation of thrombin generation during blood coagulation has become the most reliable approach to assess the global integrity of this complex process. Although all cellular and soluble plasma participants to this reaction have to be present to give the full picture of the physiology of this multifactorial reaction, in their natural whole blood environment[1], in vitro conditions may give some important clues to this reaction.

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The procoagulant process participates in the generation of thrombin that activates the plasma procofactors, factor V and factor VIII; aggregates platelets, cleaves fibrinogen, activates factor XIII, and serves numerous other physiological processes.[2] Other cells in blood and the vessel wall may also contribute to procoagulant and anticoagulant activities.[3] In vitro, different plasma coagulation pathways lead to the generation of thrombin. Known models are the intrinsic pathway and the extrinsic pathway.[4] Intrinsic pathway is initiated by contact action which takes place generally in vitro. Tissue factor pathway has been considered to be central to thrombin generation in normal hemorrhage control in vivo.[5] In all blood coagulation models a cascade of zymogen activation reactions are take place. At each stage a precursor protein is converted to an active protease by cleavage of one or more peptide bonds in the precursor molecule. During each stage a protease (from the preceding stage), a zymogen, a non-enzymatic protein cofactor and calcium ions play basic role. An organizing surface provided by a phospholipid emulsion in vitro or by platelets in vivo is also essential factor to clotting. The final protease generated is thrombin. Thrombin converts the soluble protein fibrinogen into an insoluble fibrin gel, which is strengthened further by covalent cross-linking catalyzed by factor XIIIa.

The protease zymogens involved in coagulation are secreted by hepatocytes into the bloodstream. Amino acid residues at the C-terminal end of each zymogen are homologous to trypsin and contain the active site Ser, Asp, and His residues of the protease (catalytic domain). These domains appear to be involved in specific interactions between the proteases and their substrates, cofactors, and inhibitors.[6] So we hypothesised that these amino acids may give some clues to determine the physiologic process of coagulation.

Phenylketonuria (PKU) is a disorder in which the aromatic amino acid Phe cannot be converted to Tyr.[7] In PKU patients, excess Phe level is the most prominent feature in laboratory. Amino acid imbalance in PKU patients prepares a distinct molecular environment for coagulation process. Thus to clarify the difference of produced amino acids in coagulation in vitro, we monitored the Phe and Tyr levels in PKU patients and healthy donors.

MATERIALS AND METHODS

The study population consisted of 5 PKU patients (mean: 1 year, 1 month-3 years) and 17 healthy volunteers (mean:29 years, 19-52 years). The participants had not been taking any medication, in particular, cyclooxygenase inhibitors or aspirin, within the 4 wk before blood withdrawal. Exclusion criteria were known coagulation disorders, renal disease, liver disease or increased plasma concentrations of aspartate aminotransferase and alanine aminotransferase (50> IU/L). Phe concentrations of selected PKU patients were increased (maximum 32 mg/dL).

All PKU patients were admitted to Pediatrics Clinics of Dicle University. All blood samples were collected from an antecubital vein at the same time of day. Blood samples (2.0 mL) were collected during which time the participants fasted. Whole blood was obtained by collecting into EDTA and citrate containing blood tubes. Serum was obtained by collecting into tubes without anticoagulant. The plasma and serum were then separated by centrifugation for 15 minutes. The activated partial thromboplastin time (aPTT) test and the prothrombin time (PT) tests were performed by manual methods depending on fibrin clot formation. Determined amino acids were Alanine (Ala), Arginine (Arg), Aspartate (Asp), Cysteine (Cys), Glutamate (Glu), Glycine (Gly), Histidine (His), Isoleucine (Ile), Leucine (Leu), Lysine (Lys), Methionine (Met), Phenylalanine (Phe), Serine (Ser), Threonine (Thr), and Tyrosine (Tyr). Amino acid analysis were performed by a well-recognized high-performance liquid chromatography (HPLC) method on Agilent 1100 HPLC described by manufacturer with flourosence detection and reverse phase HPLC column.

Data were analyzed by Mann Whitney U and Spearman correlation tests. All analyses were performed with the SPSS 10.0 statistical package. P less than 0.05 was accepted as significant.

RESULTS

The PKU patients studied were 3 girls and 2 boys with a mean age of 2±2 years (0-4 years). Healthy volunteers were 10 women and 7 men with a mean age of 29±7 years (22-36 years). No significant differences were found between obtained plasma amino acid levels in EDTA compared with acid cit-

rate. The amino acid parameters were not different between serum and plasma in healthy group with five exceptions including serum levels of Asp, Ser, His, Glu and Phe. Serum levels of these aminoacids were significantly higher than their plasma levels in healthy group ($p<0.001$) (Figure 1).

However, in PKU patients there were no significantly difference between serum and plasma levels of determined amino acid parameters. No differences were found in aPTT and PTT tests between PKU patients and healthy volunteers (Table 1).

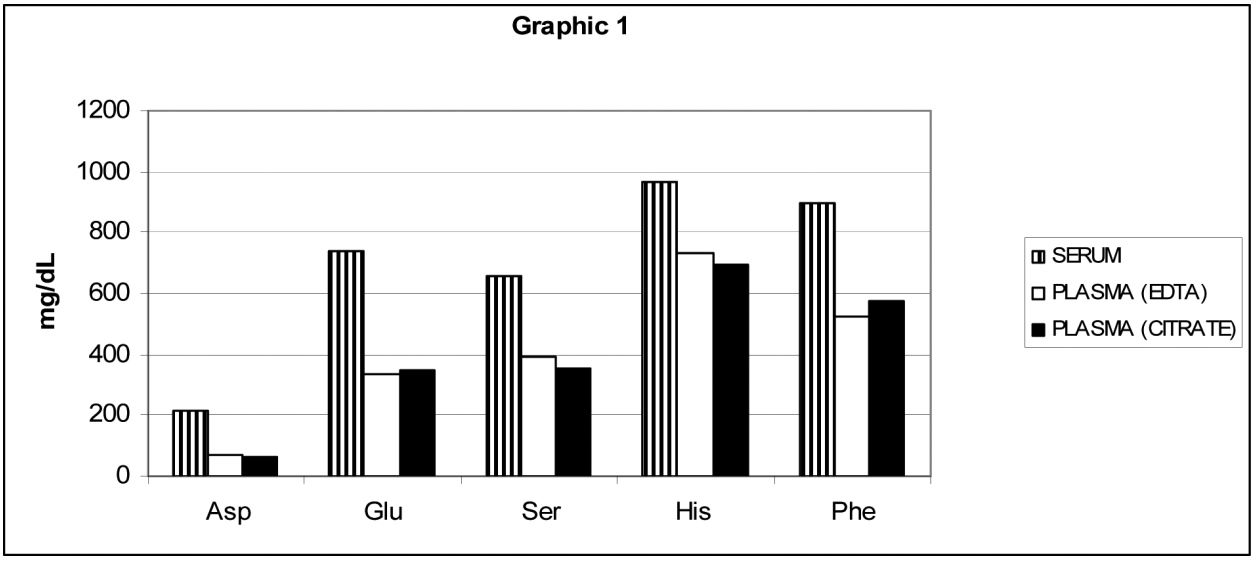

Figure 1. Serum aminoacid levels in healthy subjects.

Table 1. Comparison of coagulation test results between phenylketonuria (PKU) and healthy groups ($p>0.05$)

	Healthy Group (n=17)	PKU Group (n=5)
aPTT (mean, seconds)	29.2	32.4
PTT (mean, seconds)	12.3	12.1

DISCUSSION

Coagulation and generation of thrombin necessitate main enzymes responsible for clot formation as well as other procoagulant and anticoagulant functions during the blood coagulation process occurs in a nonlinear fashion.[8] Thus micro environment of coagulation process has great importance to determine the interaction of molecules that take part in process. Among this molecules serine protease enzymes are having important role because all of the factors that participate in coagulation cascade are produced and destroyed by proteolytic events. Serine proteases comprise nearly one-third of all known proteases identified to date and play crucial roles in a wide variety of cellular as well as extracellular functions, including the process of blood clotting. Their hallmark is that they contain the so-called "classical" catalytic Ser/His/Asp triad.[9]

In our present study, these three amino acids were increased after clotting, possibly shedding of destroyed ones after catalysis. This irreversible cascade may generate Ser, His, Asp. Additionally, post-translational modifications of some coagulation factors phosphorylation takes place on Serine.[10,11] This amino acid may have important role in enzymatic reactions of clotting.

The source of Glu, that was found increased in our study, may be generated in catalytic pathway. Because Factors II, VII, IX, and X are homologous to each other at their N-terminal ends. After removal of the signal peptide, a carboxylase converts ~10-12 Glu residues to g-carboxyglutamate (Gla). The propeptide is removed from the carboxylated polypeptide prior to secretion. The Gla residues bind calcium ions and are necessary for the activity of these coagulation factors.

According to our best of the knowledge, no study was found on role of Phe in blood coagulation

in the literature. But the elevation of Phe levels after clotting is important to identify the molecular basis of process and clarify the consequential effects. In the further study it is planned to determine the Phe related elements (dopamine, epinephrine, serotonin etc.) in clotting area.

Phenylketonuria, an inborn error of metabolism in which phenylalanine accumulates in patients secondary to lack of the enzyme phenylalanine hydroxylase, which converts dietary phenylalanine to tyrosine. High Phe concentrations in the plasma of PKU patients may lead to alter the normal physiology of coagulation. However, according to our results this alteration did not enough to affect aPTT and PTT test results.[12]

There was no linearity in the amino acid parameters of healthy persons with PKU patients. Theoretically high Phe levels may disturb the amino acid balance in the clotting area in PKU patients. Hydrolysis of a polypeptide chain requires proper recognition, orientation and binding of the substrate polypeptide backbone[13] and serine proteases require proper ion balance for optimal activity[14] and specificity.[15] However alteration of balance in amino acids in the area of clotting showed no effect to final result based on fibrin formation time. It appears that this result may be due to a new compensation mechanism or a different way of clotting that product of the clotting was showing different metabolites. Increased blood coagulation in vitro by hemodilution with crystalloids[16] is evidence of some other unknown mechanisms in coagulation cascades.

Studies on blood-plasma coagulation strategies reveal serious inconsistencies that cannot be resolved by individual studies. Further studies are needed to explain molecular mechanisms of blood coagulation cascade.

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