Investigation of Some Vitamin Type Inhibition on Human Cord Blood Carbonic Anhydrase I and II

Bazı Vitaminlerin İnsan Kordon Kanı Karbonik Anhidraz I ve II Üzerine İnhibisyon Tiplerinin İncelenmesi

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ABSTRACT

Objective: Human cord blood, a specialized blood tissue, contains many substances including several enzymes which act by different biochemical reactions. Carbonic anhydrase (CA) isoenzymes are one of the most important enzyme family which are crucial for living organisms to survive. However, there is limited data regarding the effect of vitamins on CA isoenzymes. The aim of this study is to test the inhibitory effect of some widely used vitamins on human cord blood CA I and II (hcbCA I and hcbCA II).

Methods: We purified hcbCA I and hcbCA II from human cord blood erythrocytes by Sepharose-4B-l-tyrosine-sulfanilamide affinity gel chromatography. The inhibitory effects of vitamin A (retinol), vitamin B12 (cyanocobalamin) and vitamin K (menadione sodium bisulfate, k3) on two isoenzyme were checked using IC_{50} values.

Result: IC_{50} values for vitamin A, vitamin B12 and vitamin K were found to be 44, 90, and 31 μM for hcbCA I and of 51, 62, and 21 μM for hcbCA II; respectively. All these substances were found to be non-competitive inhibitors.

Conclusion: Vitamins are vitally important for continuation of life and activity of some of enzymes. While the physicans recommend pregnant women to take vitamins during pregnancy, we suggest that the findings obtained in this study be taken into consideration. *(JAREM 2013; 3: 79-83)*

Key Words: Cord blood, vitamins, carbonic anhydrase, inhibition effect

ÖZET

Amaç: İnsan kordon kanı, farklı biyokimyasal reaksiyonların oluşumunda rol alan enzimler gibi birçok maddeyi içeren özeleşmiş bir kan dokusudur. Karbonik anhidraz (CA) izoenzimleri canlı organizmaların hayatta kalmaları için gerekli olan en önemli enzim ailelerden biridir. Fakat Vitaminlerin CA izoenzimleri üzerine etkileri hakkında yeterli bilgi bulunmamaktadır. Bu çalışmanın amacı, yaygın olarak kullanılan bazı vitaminlerin insan kordon kanından saflaştırılan CA I ve II üzerine *in vitro* etkilerini araştırmaktır.

Yöntemler: İnsan kordon kanı eritrositlerden hcbCA I ve II hcbCA izo enzimleri Sefaroz-4B-L-tirozin-sülfamid afinite jel kromatografisi kullanılarak saflaştırıldı. İki izoenzim üzerine A vitamini (retinol), B12 vitamini (Siyanokobalamin) ve K vitamini (menadion sodyum bisülfat, K3) inhibisyon etkileri IC_{50} değerleri kullanılarak kontrol edildi.

Bulgular: Sırasıyla, A vitamini, B12 vitamini ve K vitamini için IC50 değerleri hcbCA I için 21 μM, 90 μM, 44 μM ve hcbCA II için 51 μM, 62 μM, 31 μM olarak bulunmuştur. Çalışılan vitaminlerin, yarışmasız inhibisyon gösterdiği bulundu.

Sonuç: Vitaminler bazı enzimlerin aktivitleri ve hayatın devamı için oldukça önemlidir. Doktorlar, gebelikte vitamin desteği önerisinde bulunurken çalışmamızda elde ettiğimiz sonuçları da dikkate almalarını öneririz. *(JAREM 2013; 3: 79-83)*

Anahtar Sözcükler: Kordon kanı, vitaminler, karbonik anhidraz, inhibisyon etkisi

INTRODUCTION

Recently, cord blood has become one of the major areas of interest in science. For instance; cord blood stem cells are currently used in the treatment of several life-threatening diseases such as cancer, genetic diseases, and blood disorders. Determination of the kinetic properties of cord blood has become very important today (1, 2). Cord blood contains many enzyme and these enzymes are very important for the life attendance. One important family is the carbonic anhydrases, which catalyze the reversible reactions of CO_2 and water (3). CA is of broad interest because it is one of the fastest enzymes known; the turnover number or kcat of some CA isoforms exceeds (1×10^6 s^{-1}) (4). In addition, the reactions of CA isoenzymes are essential to several physiological processes such as calcification, photosynthesis, respiration, ionic, acid-base and fluid balance, metabolism and cell growth (5).

Classification of Vitamins is divided into two groups, such as according to biological and chemical activity, but not their structure. Vitamins analysed according to their biochemical function mainly are divided into two groups; first, some of them are organometallic compounds with important metabolic derivatives that act as cofactors faor certain enzymes. Those in the second group have an antioxidant activity. Both functions are important

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for fulfilling activities of enzymes and elimination of oxidant substances. A number of investigations have reported vitamin A (retinol), vitamin B12 (cyanocobalamin) and vitamin K (menadione, k3) (Fig. 1) as having exhibited beneficial effects on the regulation of metabolism (6-8).

Cyanocobalamin contributes to the optimal functioning of the central nervous system through its role as cofactor in numerous catalytic reactions in the human body, which are required for neurotransmitter synthesis and functioning and myelination of the spinal cord and brain (9, 10). Vitamin A is a vital nutrient for humans and is converted to the visual chromophore, 11-cis-retinal, and to the hormone, retinoic acid (11). Vitamin K is another important essential molecule in nutrients. This molecule is associated with the blood-clotting cascade. Vitamin K has three different molecules and one of them is menadione (Vitamin K3). Most of the researches are focused on menadione. It was used as an oxidative model for the explanation of anticancer effects both in vitro and *in vivo* (12). On the other hand, dosages of vitamins are important for regulation of metabolism. It was shown that vitamins have inhibitory effects on some enzymes. Menadione inhibited the lactoperoxidase activity. It demonstrated competitive inhibition. Also, milk xanthine oxidase was inhibited by folic and ascorbic acids. Also, it demonstrated non-competitive inhibition on adenylate kinase (13-15).

Human cord blood carbonic anhydrases I, and II are important for the reversible hydration of carbon dioxide (CO_2) to bicarbonate (HCO_3) with production of a proton (H^+). As known, all of these vitamins are commonly used during pregnancy. Vitamins show inhibitory effects on enzymes (13-15). However, their dosage in different organisms must be investigated carefully in the light of these inhibitory actions. It is important to identify the enzyme activities during pregnancy and characterize the factors increasing or decreasing those activities. Therefore, in an effort to provide beneficial data for further investigations, the present study aimed to evaluate the *in vitro* effects of commonly used vitamins on hcbCA I and hcbCA II isoenzymes.

METHODS

Chemicals

Sepharose 4B, protein assay reagents and 4-nitrophenylacetate were obtained from Sigma-Aldrich Co. (Sigma-Aldrich Chemie GmbH Export Department Eschenstrasse 5, 82024 Taufkirchen, Germany). All other chemicals were analytical grade and obtained from Merck (Merck KGaA Frankfurter strasse 250, D 64293 Darmstadt Germany).

Purification of carbonic anhydrase isozymes from human cord blood by affinity chromatography

The purification of the both hcbCA isoenzymes was performed in a single-step method by means of Sepharose-4B-L tyrosine-sulphanilamide affinity gel chromatography. Firstly, we obtained letters of consent from patients before cesarean section and normal delivery.Then we got fresh human cord blood obtained from the Blood Centre of Erzincan Hospital. Erythrocytes were purified from this fresh blood. The blood samples were centrifuged at 2250x*g* for 15 min and the plasma and buffy coat were removed. The red cells were isolated and then washed twice with NaCl (0.9%) and haemolysed with 1.5 volumes of ice-cold water. The ghost and intact cells were removed by centrifugation at 14500 x*g* for 30 min at 4°C. The pH of the haemolysate was adjusted to 8.7 with solid Tris. CNBr-activated-Sepharose 4B was filtered by a Buchner funnel and washed with cold $NaHCO_3$ buffer (0.1 M, pH 10.0). L-Tyrosine by using saturated L-tyrosine solution in the same buffer was coupled to Sepharose 4B-L-tyrosine activated with CNBr. The affinity gel was obtained by diazotization of sulphanilamide and coupling of this compound to the Sepharose 4B-L-Tyrosine. After 10 minutes of reaction, the diazotized sulphanilamide was poured into 40 mL of the Sepharose 4B-L-Tyrosine suspension. The pH was adjusted to 9.5 with 1 M NaOH. After gentle stirring for 3 hours at room temperature, the coupled red Sepharose derivative was washed with 1 L of water and then 200 mL of Tris-sulphate (0.05 M pH 7.5). The haemolysate was applied to the prepared Sepharose-4B-L tyrosine-sulphanilamide affinity column equilibrated with 25 mM Tris-HCl / 0.1M Na_2SO_4 (pH 8.7). The affinity gel was washed with 25 mM Tris-HCl/22 mM Na_2SO_4 (pH 8.7). The human cord blood carbonic anhydrase (hcbCA I and hcbCA II) isozymes were eluted with 1 M NaCl / 25 mM Na_2HPO_4 (pH 6.3) and 0.1 M CH_3COONa / 0.5 M $NaClO_4$ (pH 5.6), respectively. All procedures were performed at 4°C (16, 17).

Hydratase activity assay

CA activity was assayed by following the hydration of CO_2 according to the method described by Ozturk Sarikaya et al. (18). Enzyme unit (EU) of CO_2-hydratase activity was calculated by using the following equation (t_o-t_c/t_c) where t_o and t_c are the times for pH change of the non-enzymatic and the enzymatic reactions, respectively.

Esterase activity assay

CA activity was assayed according to the method described by Innocenti et al. (19, 20). CA activity was determined by following the change in absorbance at 348 nm of 4-nitrophenylacetate (NPA) to 4-nitrophenylate ions over a period of 3 min at 25°C using a spectrophotometer (CHEBIOS UV-VIS) according to the method described by Verpoorte et al. (21). The enzymatic reaction, in a total volume of 3.0 mL, contained 1.4 mL 0.05 M Tris-SO_4 buffer (pH 7.4), 4-nitrophenylacetate (1 mL, 3 mM), H_2O (0.5 mL) and enzyme solution (0.1 mL). A reference measurement was obtained by preparing the same cuvette without enzyme solution. The inhibitory effects of A (retinol), vitamin B12 (cyanocobalamin) and vitamin K (menadione sodium bisulfate, k3) were examined. All compounds were tested in triplicate at each concentration used. Different inhibitor concentrations were used. The activity of the control cuvette was accepted as 100%. For each inhibitor, activity (%)-[Vitamins] graphs were drawn. For determination of Ki values, three different vitamin concentrations were tested. In these experiments, 4-nitrophenylacetate was used as substrate at five different concentrations (0.15-0.75 mM). The Lineweaver–Burk curves were drawn (22).

Protein determination

Protein during the purification steps was determined spectrophotometrically at 595 nm according to the Bradford method (23), using bovine serum albumin as the standard.

SDS polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis was performed after purification of the cord blood isoenzymes. It was carried out in

Table 1. Purification scheme of CA I, and CA II from human cord blood erythrocytes

Step	Activity (EU/mL)	Total Volume (mL)	Protein (mg/mL)	Total Protein (mg)	Total Activity (EU)	Specific Activity (EU/mg)	Recovery (%)	Purification (Fold)
Haemolysate	138.00	50.00	17.70	885	6901.00	7.793	100	1.00
hcbCA I	417.60	12.50	0.3528	4.41	5220.00	1183.67	75.64	151.88
hcbCA II	747.12	6..60	0.1181	0.78	4931.00	6321.79	71.45	811.21

EU: Enzyme unit

Table 2. Inhibitory activities of vitamins

Compound Name	IC_{50} hcbCA I (μM)	K_i hcbCA I (μM)	Inhibition type	IC_{50} hcbCA II (μM)	K_i hcbCA II (μM)	Inhibition type
Vitamin A	42.6	27.0	Non-competitive	49.0	29.5	Non-competitive
Vitamin K	32.2	117.0	Non-competitive	21.8	8.6	Non-competitive
Vitamin B12	97.1	158.0	Non-competitive	51.4	30.0	Non-competitive

IC_{50}: The half maximal inhibitory concentration, K_i: The inhibitor constant, μM: micromole

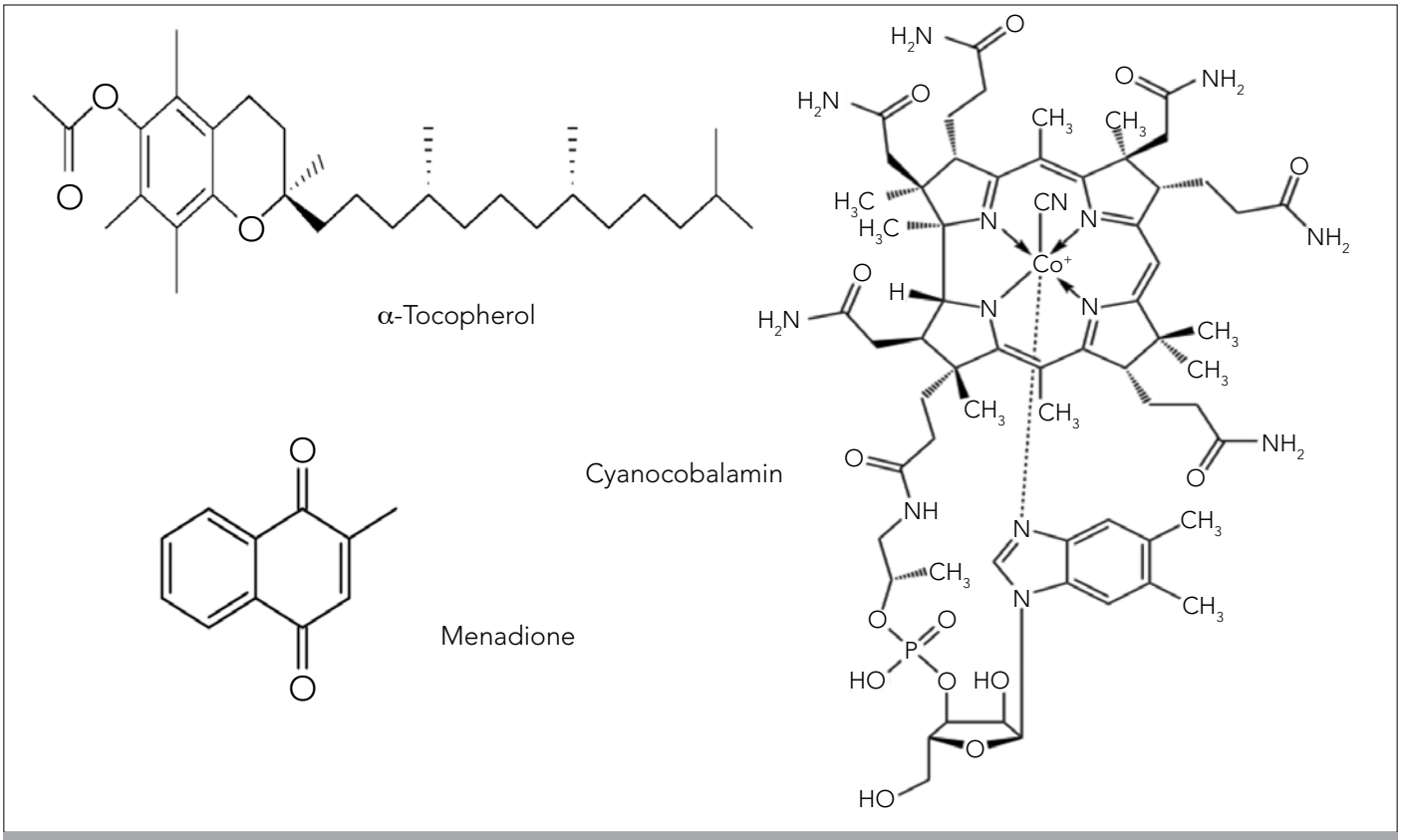

Figure 1. Chemical structures of tested vitamins

10% and 3% acrylamide for the running and the stacking gel, respectively, containing SDS (0.1%) according to the Laemmli procedure (24). A 20 mg sample was applied to the electrophoresis medium. Gels were stained for 1.5 h in Coomassie-Brilliant Blue R-250 (0.1%) in methanol (50%) and acetic acid (10%), and then destained with several changes of the same solvent without the dye. The electrophoretic pattern was photographed (Fig. 2).

Statistical evaluation

This study does not need any statistical hypothesis testing.

RESULTS

CA I, and II from human cord blood erythrocytes were purified by a simple step procedure using Sepharose 4B L-tyrosine sulphanilamide affinity column. As can be seen in Table 1, hcbCA I was

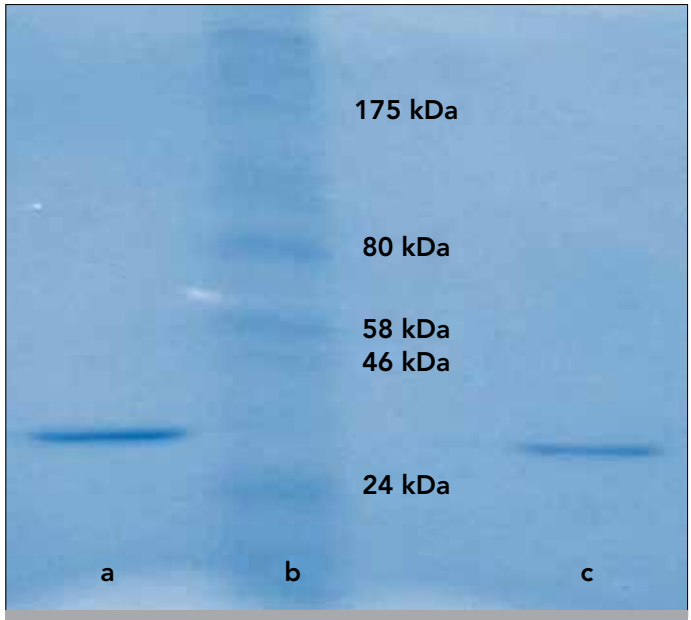

Figure 2. SDS-PAGE zymogram. Lane a, hcbCA II, line c, hcbCA I, line b, standard proteins, Standard proteins: 1) MBP (Maltose-binding protein-β- galactosidase, 175 kDa), 2) MBP (Maltose binding protein)-paramyosin (fusion of MBP and paramyosin, 80 kDa), 3) MBP-CBD (Chitin binding domain, fusion of MBP and chitin binding domain, 58 kDa), 4) CBD-Mxe Intein-2CBD (Fusion of the chitin binding domain and the Mxe Intein followed by two chitin binding domains, 46 kDa). 5) soybean trypsin inhibitor (24 kDa)

purified 151.88-fold with specific activity (1183.67 EU mg^{-1}) and yield (75.64%). On the other hand, hcbCA II was purified 811.21-fold with specific activity (6321.79 EU mg^{-1}) and yield (71.45%). SDS-PAGE zymograme of both isoenzymes showed a single band (Fig. 2). Three inhibitors [vitamin A (Retinol), vitamin B12 (cyanocobalamin) and vitamin K (menadione, k3)] were prepared and evaluated for the inhibitory effects on hcbCA I, and II. The inhibitory effects of these vitamins were tested in the range of 0.001-1000 mM. All vitamins showed inhibitory effects on hcbCA I, and hcbCA II, under *in vitro* conditions. IC_{50} values were calculated by Activity (%)-[Vitamins] graphs and are given in Table 2. K_I values were calculated from Lineweaver-Burk graphs (21, 22) and are given in Table 2.

DISCUSSION

Cord blood is an alternative stem cell source for treating cancer and genetic diseases. Therefore, analysing the enzyme property of cord blood is crucial for the organism. Umbilical cord blood contains different enzymes such as glucose 6-phosphate dehydrogenase, glutathione reductase and carbonic anhydrases (25, 26). CA included 16 different isoforms, which are found in different tissue and organisms. Most of the investigations focused on CA I, and II, because they were extracted from blood erythrocytes. CA I, and CA II were purified by affinity column chromatography. The effects of various chemicals, pesticides and drugs on its activity have been investigated (16-20).

Vitamins are organic compounds required by an organism as a vital nutrient in limited amounts (27). Thirteen vitamins are universally recognized at present. Some of them show hormone-like functions as regulators of mineral metabolism, or regulators of cell and tissue growth and differentiation (28). Vitamin A refers to the compounds retinal, retinol and its esters, whereas provitamin A refers to the carotenoids β-cryptoxanthin (29). Vitamin A is indispensable for cell differentiation, embryonic development and vision, besides many other roles (e.g. glycoprotein synthesis, carcinogenesis, growth hormone production) (30, 31). Vitamin B12 is an important water-soluble vitamin that regulates red blood cell, neural cell activity and displays antioxidant properties and modulates nucleic acid metabolism and gene regulation (32, 33). It is generally used as a therapeutic agent and supplement, because of its efficacy and stability (34, 35). All of these investigations exhibited vitamins important for continuation of life.

Vitamin A, vitamin B12, and vitamin K3 have many benefits for organisms, but some investigations have revealed that these three vitamins have inhibitory actions on some enzymes. Vitamin K3 showed competitive inhibition on lactoperoxsidase[14], another investigation reported, this vitamin has inhibitory effects on human mitochondria DNA polymerase gamma (pol gamma) (36). Vitamin K3 has inhibitory effects on aldehyde oxidase (37). Some of the researches exhibited the effects of some vitamins on carbonic anhydrase (CA). For example, Mraz and friends checked the effect of vitamin D on the activity of erythrocyte carbonic anhydrase isoenzymes (CA) *in vitro* and *in vivo*. Vitamin D inhibited CA I, and CA II (38, 39). There is not much detailed study regarding the effect of vitamins on CAs activity. In this study, vitamins which are important during life were investigated for their inhibitory effects on hcbCAI and hcbCAII *in vitro*, and their kinetic constants (K_I and IC_{50} values) were reported. This study showed that vitamins had strong inhibitory effects on hcbCAI and hcbCAII activity, which have not been reported previously. As evident from IC_{50} values, hcbCA I, and hcbCA II inhibition by vitamin K are higher than vitamin A and vitamin B12, respectively. IC_{50} values correlate with the Ki values of the vitamin as shown in Table 2.

CONCLUSION

We believe that the results of this study should draw attention to the use of vitamins throughout the whole life period, since CAs have a part in the interconversion of CO_2 and water to HCO_3 and H^+; use of vitamins may decrease hcbCA I and hcbCA II activity in human cord blood erythrocytes. If it is necessary to give these vitamins during pregnancy, their dosage should be very carefully considered to decrease the potential side effects. Investigations of the *in vitro* effects of these vitamins on hcbCA I and hcbCA II activity is more important from a clinical point of view and for this reason, detailed studies are required.

Conflict of Interest
No conflict of interest was declared by the authors.

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Author Contributions
Concept - T.A.Ç., M.Ç., U.N., A.Ç., M.K.; Design - T.A.Ç., M.Ç., U.N., A.Ç., M.K.; Supervision - T.A.Ç., M.Ç., U.N., A.Ç., M.K.; Funding - T.A.Ç., M.Ç., U.N., A.Ç., M.K.; Materials - T.A.Ç., U.N., A.Ç., M.K.; Data Collection and/or Processing - T.A.Ç., M.Ç., U.N., A.Ç., M.K.; Analysis and/or Interpretation - T.A.Ç., M.Ç., U.N.; Literature Review - T.A.Ç., M.Ç., U.N., A.Ç., M.K.; Writing - T.A.Ç., M.Ç., U.N.; Critical Review - T.A.Ç., M.Ç., U.N., A.Ç., M.K.

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