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Effects of orotic and 5-aminoorotic acids on the free radicals accumulation in rat blood serum

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Orotic (HOA) and 5-aminoorotic (HAOA) acids are ligands in metal complexes with *in vitro* antioxidant and anticancer activities. Dietary orotic acid *in vivo* increases the free radicals formation in the liver by diminishing both activity and mRNA level of Cu,Zn-SOD. It seems that HOA may act as antioxidant by scavenging free radicals, but as prooxidant by diminishing the efficacy of Cu,Zn-SOD. The effect of orotic acid on the accumulation of free radicals (FRA) in the blood serum is still not assessed. In this investigation free radicals formation in rat blood serum was achieved by adding small amount of xanthine or Fe(II)/H_2O_2/ascorbate solution, thus superoxide or hydroxyl radical - induced free radicals formation was provoked. The effects of HOA and HAOA (within concentrations of 10^{-4} and 10^{-6} M) on the accumulation of free radicals in the blood serum were monitored using spectrophotometric method. FRA decreased with increasing of concentration of both compounds was observed, the effect being stronger for HAOA, and for the $OH^{\bullet}$ generating model system. The weaker antioxidant effect of HOA compared to HAOA on the free radicals accumulation in rat blood serum might be a result of negative influence on Cu,Zn-SOD, along with radicals scavenging activity of the molecule. It was assumed that at concentrations below 0.1mM, the antioxidant effect of HOA and HAOA in the blood serum prevailed. More detailed investigations are under way.

Keywords: Orotic acid, 5-Aminoorotic acid, antioxidant properties, Superoxide radical, Free radicals accumulation.

INTRODUCTION

Orotic acid is essential for the synthesis of the building blocks of the nucleic acids (RNA, DNA) for the transformations of the digestive lipids in the liver increases the utilization of fatty acids by the heart, increases the activity of lipoprotein lipases, the hepatic levels of uracil nucleotides, the expression of peroxisome proliferator- activated receptor α and its affected enzymes; metal orotates help the supplementation of the body with essential minerals [1]. Ca(II) orotate is used in the treatment of multiple sclerosis, while Mg(II) orotate reduces the severity of the chronic myocardial dysfunction and structural damage of cardiomyopathy in animal models. It also improved the exercise tolerance in patients with coronary artery disease and in trained athletes. The delicate balance within accumulation and elimination of orotic acid (HOA) is closely related with health status [2-5]. The deficiency of orotic acid leads to cell degeneration, heart problems, premature ageing, mental retardation, anemia, depressed immunity, crystals in the urine, skin problems and liver disorders. The excess of orotic acid was associated with Cu,Zn-SOD depletion, non-alcoholic fatty liver, liver steatosis, and in animal models - cancerogenesis. Hence, HOA may act as *in vivo* prooxidant *via* onset of diseases and pathologies due to its excess or deficiency [6-9], or by affecting the elements of antioxidant defense [10,11]. Orotic acid is synthesized in the body and supplied by exogenous sources (food, dietary supplements and medications) [12-15]. As *in vivo* source of free radicals, dietary HOA is potential hazard for onset of oxidative stress (OS) - induced cancer [16].

The use of metal complexes to disturb the redox-balance in cancer cells is promising approach in cancer treatment [17]. Fenton reaction [18] and superoxide accumulation [19] are among the major factors in development of ROS- induced oxidative stress in tissues and biological fluids. As the involvement of free radicals (especially of the reactive oxygen species, ROS) in cancerogenesis and cancer therapy is very complex and still not enough elucidated [20-23], the interactions with free radicals from the biological environment is important for the medicinal application of metal complexes with promising anticancer activity. Along with the anticancer action, possible involvement of a prospective drug in onset and development of OS in the living body is associated with manifestation of toxicity and undesirable side effects.

Metal complexes of both orotic (HOA) and 5-aminoorotic (HAOA) acids were found to be promising *in vitro* anticancer agents [24], some of them possessing antioxidant activity [25]. The effect of the ligands alone on the free radicals accumulation in the blood plasma was not enough

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elucidated. In this investigation, the influence of HOA and HAOA on the free radicals accumulation in biological environment was estimated, using rat blood serum as model system. The free radicals accumulation was initiated by introduction of small amount of xanthine or Fe(II)/H_2O_2/ascorbate solution, thus $O_2^{\bullet-}$ or $OH^{\bullet}$ - induced OS was provoked. The effects of HOA and HAOA on the accumulation of free radicals was monitored by measuring the transformation of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into MTT formazan.

MATERIALS AND METHODS

All chemicals were of finest grade (Sigma-Aldrich). Distilled water was used as solvent. The reaction medium was 50 mM Na,K-phosphate buffer (PBS) of pH=7.45. Stock 1 mM aqueous solutions of HOA and HAOA were prepared and diluted to 10^{-4}, 10^{-5}, and 10^{-6} molar concentrations, prior to experiment. For the model system generating 3mM aqueous solution of xanthine was prepared, to generate $O_2^{\bullet-}$ in the serum. Two solutions were used for the $OH^{\bullet}$ generating model system: aqueous solution of 3mM $FeCl_2$, 3 mM H_2O_2, and 0.4 mM EDTA, and aqueous solution of ascorbate (4 mg/ml). Ice-cold water was used in the preparation, and during the experiment they were kept in ice bath. The free radicals accumulation in the samples was monitored by measuring the transformation of the yellow MTT to purple MTT formazan (λ=576 nm in PBS) for 10 min, using Shimadzu 1600 spectrophotometer.

Blood serum was isolated as described elsewhere [26]. Briefly, the total blood was left at room temperature to coagulate and centrifuged at 4000 rpm in a refrigerated centrifuge (Zamezki K-24) for 10 min. The serum was transferred in plastic test tubes and kept at -25°C (freezer) to the next day, when the biochemical analysis was performed. Before the experiment, the amount of proteins in the serum was determined [27].

EXPERIMENTAL PART

Samples preparation: Serum with proteins content of 1mg/ml was allowed to interact with a solution of desired concentration for 15 min in ice bath, then conditioned to room temperature and used for determination of the free radicals accumulation. For the control measurement the HOA and HAOA solutions were omitted.

Xanthine/xanthine oxidase assay: The free radicals accumulation was initiated by a small amount of xanthine solution, which produced superoxide radicals by reacting with the xanthine oxidase in the blood serum. One ml of the sample cuvette contained blood serum corresponding to 1 mg proteins, 0.01 ml xanthine, 0.10 ml MTT, 0.10 ml sample solution, and PBS to 1 ml. For the control measurement, the sample solution was omitted, and for the blank measurement the serum was omitted. The relative change of the intensity at 576 nm was measured for 10 min.

Fe(II)/H_2O_2/EDTA/ascorbate assay: The free radicals accumulation was initiated by initiating Fenton reaction in the serum. For the sample measurement one ml of the cuvette contained serum corresponding to 1 mg proteins, 0.10 ml MTT, 0.10 ml of the sample solution, 0.025 ml Fe(II)/H_2O_2/EDTA, 0.025 ml ascorbate, and PBS to 1 ml. In the control measurement the sample solution was omitted, while for the blank measurement the serum was omitted.

Data management and processing: The free radicals accumulation (FRA) was calculated using the formula:

$$FRA = \frac{\Delta A_{sample} - \Delta A_{blank}}{\Delta A_{control} - \Delta A_{blank}} * 100 ,$$

where ΔA is the relative change of the absorption at 576 nm for the sample, control or blank measurement, as indicated in the subscript.

The effect on the FRA of each compound at each concentration was measured 5 times. Average values and standard deviations were used for the comparisons. The statistical verification of the concentration effect was performed using One Way ANOVA. The relative differences within solutions of HOA and HAOA at any given concentration were statistically evaluated by using non parametric *t*-test with Welch correction (two-tailed P and SDs belonging to different populations were assumed).

The options of ChemOffice were used to understand the solvent effect and pH on both molecules investigated. The effect of the basic PBS was presented by one $H_2PO_4^-$ anion, the effect of basicity itself was illustrated using one OH^-, while solvent effect was being simulated by introduction of one water molecule to each HOA, HAOA and $H_2PO_4^-$.

RESULTS AND DISCUSSION

It is seen that the free radicals accumulation in rat blood serum in presence of HOA and HAOA decreased in a concentration-dependent manner (Fig.1). For each compound investigated, if initiated by $O_2^{\bullet-}$, FRA was significantly higher (Fig.1,a) than if initiated by Fenton reaction (Fig. 1,b).

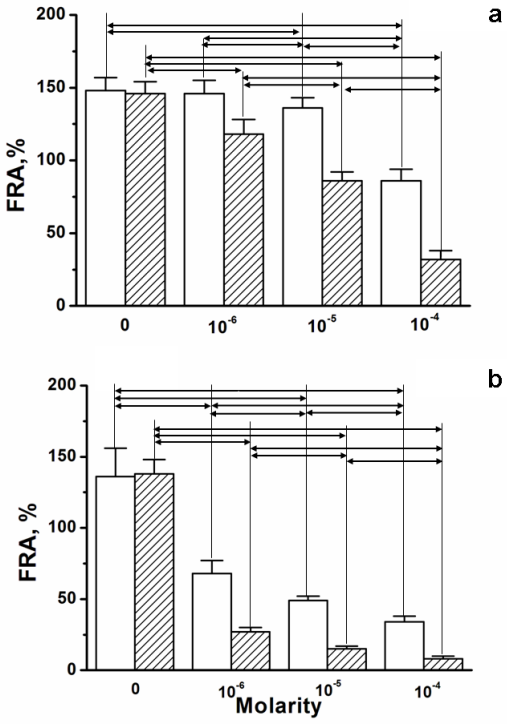
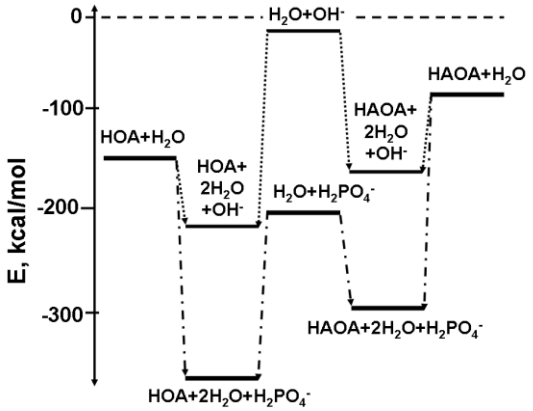


Figure 1. Free radicals accumulation (FRA, %) of HOA(□) and HAOA (▨) in rat blood serum, in the presence of model systems, creating $O_2^{-\bullet}$ (a) and $OH^{\bullet}$ (b). Statistically significant differences are marked with (↔). One Way ANOVA P<0.0001, all Bonferoni *p* are smaller than 0.05 (p<0.01, or p<0.001).

FRA in the presence of HAOA was lower than this in the presence of HOA (for each given concentration p<0.0001) in both ROS- generating model systems. It is well known that the higher the FRA, the lower the antioxidant activity is. Therefore, based on Fig 1 it was proposed that the molecules investigated were better antioxidants in the serum if the oxidative stress was initiated by Fenton reaction than if it was provoked by superoxide radical. This might be related with different types of interactions of HOA and HAOA with different model systems generating oxidative stress in blood serum. Large variety of interactions and/or reaction conditions might affect the potential energy of a molecule in a reaction medium, resulting in overall lower or higher minimum potential energy, this way either facilitating or obstructing the free radicals scavenging. In our experiment the reaction medium consisted of water as a solvent and PBS with physiological pH. Association of HOA and HAOA with solvent and influence of the pH on the potential energies of the solutes was possible. As lower than zero the potential energy, as stable the molecule in the reaction medium would be, as lower the probability to participate in free radical scavenging, and as low the antioxidant activity would be expected.

The possible effects of the pH if $H_2PO_4^-$ was carrier of the negative charge on the potential energy of aqueous associates of HOA and HAOA were estimated using a Molecular Mechanics modeling (Scheme 1). The effect of the solvent interaction with solutes on the minimum potential energy was illustrated by the effect of one water molecule associated with one HOA, HAOA, OH^- or $H_2PO_4^-$.



Scheme 1. Minimum potential energy (E, kcal/mol) of orotic (HOA) and 5-amino orotic (HAOA) acids in the presence of one OH^- or $H_2PO_4^-$, in aqueous medium.

The minimum potential energy of hydrated HAOA or HOA decreased if interacting with a hydrated hydroxyl anion. This indicated that the basicity of the medium and solvent interactions might affect the minimum potential energy of a molecule in a solution, this way affecting possible involvement in further chemical interactions. If hydroxyl anion was the carrier of basicity, the minimum potential energy level of the hydrated HAOA was higher than this of the hydrated HOA. If interacting with a free radical, the hydrated 5-aminoorotic acid might more easily undergo the energetic barrier than the hydrated orotic acid. If $H_2PO_4^-$ was carrier of basicity, the relative difference within minimum potential energy levels of hydrated HAOA and HOA was larger than this in basic environment created by OH^-. The relative difference within antioxidant activities of HOA and HAOA in PBS might be higher than the same relative difference in presence of OH^-. This proposition will be examined in future investigations. On the basis of this, higher reactivity of the HAOA than this of HOA might be expected in the PBS environment.

As the free radicals accumulation decreases in presence of antioxidants, the data in Fig. 1 suggested that both compounds investigated are antioxidants in rat blood serum, in PBS (pH 7.45), HAOA being better than HOA. The antioxidant behavior of these molecules might be a cumulative

result of their chemical properties and the effect of the environment. The relative difference in potential energies of both compounds investigated (Scheme 1) suggested that in PBS of homeostatic pH HAOA should be more reactive than HOA. This was in agreement with the data presented in Fig. 1. The relative difference within antioxidant activities in the presence of serum, in different ROS-producing model systems might be related with some interactions of HOA and HAOA with components of the antioxidant defense and/or with the components of the free radicals producing model systems. Orotic acid decreased *in vivo* the activity and gene expression of the Cu,Zn-SOD in rat liver [10,28]. The involvement of orotic and 5-aminoorotic acids in the redox homeostasis is still not enough elucidated. Possible interactions of HOA and HAOA with $O_2^{\bullet-}$ and $OH^{\bullet}$ might occur too. This hypothesis is under experimental evaluation right now.

On the basis of the present investigation and previous data [29-31] it may be proposed that both orotic and 5-aminoorotic acids may act as *in vitro* antioxidants in blood serum. This effect was stronger if OS was initiated by Fenton reaction than if it was provoked by superoxide resulting from enzymatic transformation of xanthine to uric acid *via* interaction with xanthine oxidase. The high antioxidant activity toward $OH^{\bullet}$- induced OS in blood serum is a favorable quality for a ligand in the metal complex that is prospective anticancer agent. To better understand the involvement of orotic and 5-aminoorotic acids in the free radicals homeostasis more investigations are needed.

CONCLUSIONS

1. Orotic and 5-aminoorotic acids diminished the free radicals formation in rat blood serum. It was proposed that both exhibited antioxidant properties at concentrations above 10^{-6} M.

2. The higher stability in PBS of pH 7.45 of the orotic acid compared with its 5-amino derivative may result in lower reactivity toward free radicals of HOA compared to this of HAOA.

3. The effect of the compounds investigated on the free radicals accumulation in rat blood serum might depend on the type of the free radical which initiated the OS.

4. In the case of orotic acid, the involvement in the free radicals homeostasis might be related with both chemical reactivity and influence on the Cu, Zn-SOD activity and expression.

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ЕФЕКТИ НА ОРОТОВАТА И 5-АМИНО ОРОТОВАТА КИСЕЛИНИ ВЪРХУ НАТРУПВАНЕТО НА СВОБОДНИ РАДИКАЛИ В КРЪВНА ПЛАЗМА НА ПЛЪХ

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(Резюме)

Оротовата (HOA) и 5-амино оротовата (HAOA) киселини са лиганди в метални комплекси с *in vitro* антиоксидантна и антиракова активност. Погълната с храната и лекарствата, оротовата киселина *in vivo* ускорява образуването на свободни радикали в черния дроб чрез понижаване едновременно на активността и m-РНК експресията на Cu, Zn-супероксид дисмутазата (Cu,Zn-SOD). Изглежда HOA може да се проявява като антиоксидант, но понижавайки активността и експресията на Cu,Zn-SOD, може да проявява прооксидантна активност. Ефектът на оротовата киселина върху натрупването на свободни радикали (FRA) в кръвен серум все още не е проучен. В това изследване натрупването на свободни радикали в кръвен серум на плъх бе постигнато чрез добавяне на малко количество ксантин, или в присъствие на моделна система Fe(II)/H_2O_2/аскорбат, при което оксидативният стрес се генерира от супероксиден или хидроксилен радикал. Ефектите на HOA и HAOA (в концентрационни граници между 10^{-4} и 10^{-6} M) върху натрупването на свободни радикали в кръвния серум бяха изследвани с помощта на спектрофотометрични методи. Натрупването на свободни радикали намаляваше с нарастване на концентрациите на двете изследвани съединения, като ефектът бе по-силен при HAOA отколкото при HOA и в присъствие на $OH^{\bullet}$-формиращата моделна система в сравнение с $O_2^{\bullet-}$-генериращата. По-слабият антиоксидантен ефект на HOA в сравнение с HAOA би могъл да бъде свързан с негативното влияние на първата молекула върху Cu,Zn-SOD, съпътстващо радикал-отнемащия ефект на съединението. Бе направено заключението, че при концентрации под 0.1 mM в кръвния серум антиоксидантният ефект на HOA и HAOA доминира. По-детайлни изследвания по тези въпроси са в ход.