IN VITRO EFFECTS OF SOME ANTIBIOTICS ON ENZYME ACTIVITY OF CARBONIC ANHYDRASE FROM BOVINE ERYTHROCYTES

Mikail Arslan[1,*], Serap Beyaztas[2] and Mahmut Erzengin[3]

[1] Balıkesir University, Susurluk Technology Vocational School of Higher Education, Balıkesir, 10600 Turkey
[2] Balikesir University, Science and Art Faculty, Department of Chemistry/Biochemistry Section, 10145 Balikesir, Turkey
[3] Aksaray University, Science and Art Faculty, Department of Chemistry/Biochemistry Section, 68100 Aksaray, Turkey

ABSTRACT

The purpose of this study was to investigate the *in vitro* effects of six commonly used veterinary medical drugs (Killoxacin™, Gentavet™, Geosol™, Primoxal™, Lypectin™ and Penoksal-la™) on erythrocyte bovine carbonic anhydrase (CA, EC 4.2.1.1) activity. The enzyme was purified by affinity chromatography, and the purity was confirmed by SDS–PAGE. Inhibition or activation effects of six different antibiotics on the purified enzyme were determined using the CO_2-hydratase activity method. IC_{50} values of the drugs that caused inhibition were determined by means of Activity % *vs* [Inhibitor] diagrams. The concentrations of Killoxacin™, Gentavet™, Geosol™, Primoxal™ and Lypectin™ that inhibited 50% of the enzymatic activity were 9.25, 3.79, 0.72, 6.59 and 2.12 mM, respectively. On the other hand, the enzyme activity was increased by Penoksal-la™.

KEYWORDS: Bovine carbonic anhydrase, *in vitro*, inhibition, veterinary medical drugs, affinity chromatography, purification

1. INTRODUCTION

Carbonic anhydrases (CAs, EC 4.2.1.1) are widespread zinc-containing metalloenzymes in all living organisms [1-6]. Several CA isozymes are responsible for important physiological and pathological functions, such as pH and CO_2 homeostasis, respiration and transport of CO_2/HCO_3^- between metabolizing tissues and the lungs, electrolyte secretion in a variety of tissues/organs, tonic modulation of brain excitability through modulation of amino acid receptors and biosynthetic reactions (such as gluconeogenesis, lipogenesis and ureagenesis); prominent pathological effects include acceleration of plaque deposition in Alzheimer's disease and exacerbation of excitotoxic neuron injury [7].

Sixteen isozymes have been described so far, that differ in their subcellular localization, catalytic activity and susceptibility to different classes of inhibitors [8]. Five of these isozymes are cytosolic (CA I, CA II, CA III, CA VII and CA XIII), two are mitochondrial (CA VA and CA VB), one is secreted in saliva (CA VI), five are membrane bound (CA IV, CA IX, CA XII and CA XIV), and three are "acatalytic" (CA VIII, CA X and CA XI) [9]. It has been reported that CA XV isoform is not expressed in humans or in other primates, but it is abundant in rodents and other higher vertebrates [10]. The two major CA isozymes (CA I and CA II) are present at high concentrations in the cytosol of erythrocytes, and CA II has the highest turnover rate of all the CAs [7]. It has been reported that the activity levels of CA isozymes in erythrocytes vary considerably under certain pathological and physiological conditions. Changes in CA activity have been associated with metabolic diseases, such as diabetes mellitus and hypertension [11, 12].

CA enzymes purified from various organisms have been shown to be inhibited by several compounds. Sulfonamides and their derivatives are clinically used inhibitors for CAs [13]. Metal-complexing anions, such as cyanide, (thio)cyanate, hydrogen sulfide, azide, sulfate, perchlorate, as well as some antibiotics like ampicillin, sefazolin, gentamicin and pesticides including deltamethrin, diozinon, parathion-methyl and nuarimol were also shown to be inhibitors on CA enzyme activity [14-16]. Many chemical substances and synthesized compounds affect metabolisms by changing enzyme activities. Chemicals are generally known to activate or inhibit several body enzymes *in vitro* and *in vivo* [17-22], and affect the metabolic pathways. An antibiotic is a substance or a compound that kills or inhibits the growth of bacteria. Antibiotics belong to the broader group of antimicrobial compounds, used to treat infections caused by microorganisms, including fungi and protozoa. They are ineffective against viruses which either kill microorganisms or stop them from reproducing [23].

The great number of diverse antibiotics currently available can be classified in different ways, e.g., by their

* Corresponding author

chemical structure, their microbial origin, or their mode of action. They are also frequently designated by their effective range [24]. With recent progress in medicinal chemistry, most antibiotics are now semisynthetic that is, modified chemically from original compounds found in nature, as is the case with ß-lactams (which include the penicillins produced by *Penicillium,* the cephalosporins, and the carbapenems) [25]. Many antibiotics have been used in treatments. There are only a few literature reports related with changing of enzyme activities. It has been reported that some increasing or decreasing enzyme activity levels were found, such as aspartate aminotransferase (AST; SGOT), alanine amino transferase (ALT; SGPT) and alkaline phosphatase [26-30].

Although, the inhibitory effects of several different chemicals on CA have been studied in most tissues and red blood cells, no study has yet been reported on bovine erythrocyte CA related to *in vitro* effects of antibiotics, such as Killoxacin™, Gentavet™, Geosol™, Primoxal™ Lypectin™ and Penoksal-la™. Therefore, in this present study, we have purified carbonic anhydrase from bovine erythrocytes and examined the *in vitro* effects of these important antibiotics on purified enzyme. Since the changes in CA activity have been associated with metabolic diseases, therefore, the outcomes of this work may help with the control of animal diseases.

2. MATERIALS AND METHODS

2.1. Materials

Sepharose 4B, L-tyrosine, sulfonamide, protein assay reagents, phenol red and chemicals for electrophoresis were obtained from Sigma-Aldrich Co. All other chemicals were of analytical grade. Medical drugs were provided by local pharmacy.

2.2. Purification of carbonic anhydrase from bovine erythrocytes by affinity chromatography

Erythrocytes were purified from fresh bovine blood obtained from a slaughter-house at Balikesir. The blood samples were centrifuged at 3,000 rpm for 20 min, and then the plasma and buffy coat were removed. The red cells were isolated and washed twice with 0.9% NaCl, and hemolyzed with 1.5 volumes of ice-cold water. The ghost and intact cells were removed by centrifugation at 20,000 rpm for 30 min at 4 °C. The pH of hemolysate was adjusted to 8.7 with solid Tris, and applied to the prepared Sepharose 4B-L-tyrosine-sulfonamide affinity column equilibrated with 25 mM Tris–HCl/22 mM Na_2SO_4 (pH 8.7) [29]. The affinity gel was washed with the same buffer. The bovine CA was eluted with 0.1 M $NaCH_3COO$/0.5 M $NaClO_4$ (pH 5.6). The absorbance of the protein in the column effluents was determined spectrophotometrically at 280 nm. CO_2-hydratase activity in the eluates was determined, and the active fractions were collected. The purified enzyme was stored at 4 °C, in order to maintain activity.

2.3. Total protein determination

The absorbance at 280 nm was used to monitor the protein in the column effluents. Quantitative protein determination was achieved by absorbance measurements at 595 nm according to Bradford [32], with bovine serum albumin standard.

2.4. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) of the enzyme

SDS-PAGE was performed in order to verify the purified enzyme. It was carried out in 12 and 3% acrylamide concentrations for the running and the stacking gel, respectively, containing 0.1% SDS according to Laemmli [33]. Sample (20 mg) was applied to the electrophoresis medium. Gel was stained for 1.5 h in 0.1% Coomassie Brilliant Blue R-250 in 50% methanol and 10% acetic acid, and then destained with several changes of the same solvent without the dye.

2.5. CA Enzyme Activity Assay

The CA enzyme activity was assayed by following the hydration of CO_2 according to the method described by Wilbur and Anderson [34]. CO_2-hydratase activity of the enzyme was determined at room temperature in a 0.15 M Na_2CO_3/0.1 M $NaHCO_3$ (pH 10.0) buffer using phenol red (pH 8.6) as an indicator, and saturated carbon dioxide concentration as substrate in a final volume of 4.2 ml. Duration (in seconds) of the colour change from red to yellow in solution was measured in a 10-ml glass tube with 1 cm diameter. The enzyme unit (EU) was calculated using the equation (t_0–t_c/t_c), where t_0 and t_c are the times for pH changes of the non-enzymatic and enzymatic reactions, respectively.

2.6. *In vitro* studies for antibiotics

In this study, Killoxacin™, Gentavet™, Geosol™, Primoxal™, Lypectin™ and Penoksal-la™ were used as antibiotic drugs. Different concentrations of the antibiotics (for Killoxacin™ $9{,}27x10^{-4}$, $18{,}53x10^{-4}$, $27{,}8x10^{-4}$, $37{,}07x10^{-4}$, $46{,}33x10^{-4}$, $55{,}6x10^{-4}$, $64{,}87x10^{-4}$, $74{,}13\ x10^{-4}$, $83{,}4x10^{-4}$ and $92{,}67x10^{-4}$ M; for Gentavet™ $3{,}63x10^{-4}$, $7{,}27x10^{-4}$, $10{,}9x10^{-4}$, $14{,}53x10^{-4}$, $18{,}17x10^{-4}$, $21{,}8x10^{-4}$, $25{,}43x10^{-4}$, $29{,}07x10^{-4}$, $29{,}07x10^{-4}$, $32{,}7x10^{-4}$ and $36{,}3x10^{-4}$ M; for Geosol™ $3{,}37x10^{-4}$, $6{,}73x10^{-4}$, $10{,}1x10^{-4}$, $13{,}47x10^{-4}$, $16{,}83x10^{-4}$, $20{,}2x10^{-4}$, $23{,}57x10^{-4}$ and $26{,}93\ x10^{-4}$ M; for Primoxal™ $2{,}63x10^{-3}$, $5{,}27x10^{-3}$, $7{,}9x10^{-3}$, $10{,}53x10^{-3}$, $13{,}17x10^{-3}$, $15{,}8x10^{-3}$, $18{,}43x10^{-3}$, $21{,}07x10^{-3}$, $23{,}7x10^{-3}$ and $26{,}3x10^{-3}$M; for Lypectin™ $6{,}73x10^{-4}$, $13{,}47x10^{-4}$, $20{,}2x10^{-4}$, $26{,}93x10^{-4}$, $33{,}67x10^{-4}$, and $40{,}4\ x10^{-4}$M and for Penoksal-la™ $4{,}56\ x10^{-4}$, $9{,}12\ x10^{-4}$, $13{,}68\ x10^{-4}$, $18{,}25\ x10^{-4}$, $22{,}81\ x10^{-4}$ and $27{,}37\ x10^{-4}$M) were added to the enzyme activity determination medium in 4.2 ml of total volume. Duration (in seconds) of the colour change from red to yellow in solution was measured in a 10-ml glass tube with 1 cm diameter. Control cuvette activity in the absence of inhibitor was taken as 100%. All compounds were tested in triplicate at each concentration used. The enzyme unit (EU) was calculated using

the equation (t_0-t_c/t_c), where t_0 and t_c are the time periods for pH change of the nonenzymatic and enzymatic reactions, respectively. For each inhibitor, an Activity%- [Inhibitor] graph was drawn (Fig. 1).

3. RESULTS

In this study, bovine erythrocyte CA was purified, with a simple one step method, by using Sepharose 4B-L-tyrosine-sulfonamide affinity gel with the elution buffer 0.1 M $NaCH_3COO$/0.5 M $NaClO_4$ (pH 5.6). Purity of the enzyme was confirmed by SDS–PAGE (data not shown). Inhibiton or activation effects of drugs on enzyme activity were tested under *in vitro* conditions. Inhibition graphs, using the drugs with concentrations as described in Section 2.6, are shown in Fig. 1. IC_{50} values were calculated from Activity %- [I] graphs and are given in Table 1. Carbonic anhydrase activity in the absence of a drug was accepted as 100% activity. Lypectin™ has been shown to be the strongest inhibitor against CA activity (Fig. 1b). Conversely, Penoksal-la™ considerably stimulated the enzyme activity at the applied concentrations (Fig. 1f).

TABLE 1 - Concentrations of the drugs causing 50% inhibition of bovine erythrocyte carbonic anhydrase activity.

Antibiotics	IC_{50} (mM)
Killoxacin™	9.25
Gentavet™	3.79
Geosol™	0.72
Primoxal™	6.59
Lypectin™	2.12
Penoksal-la™	stimulated the activity

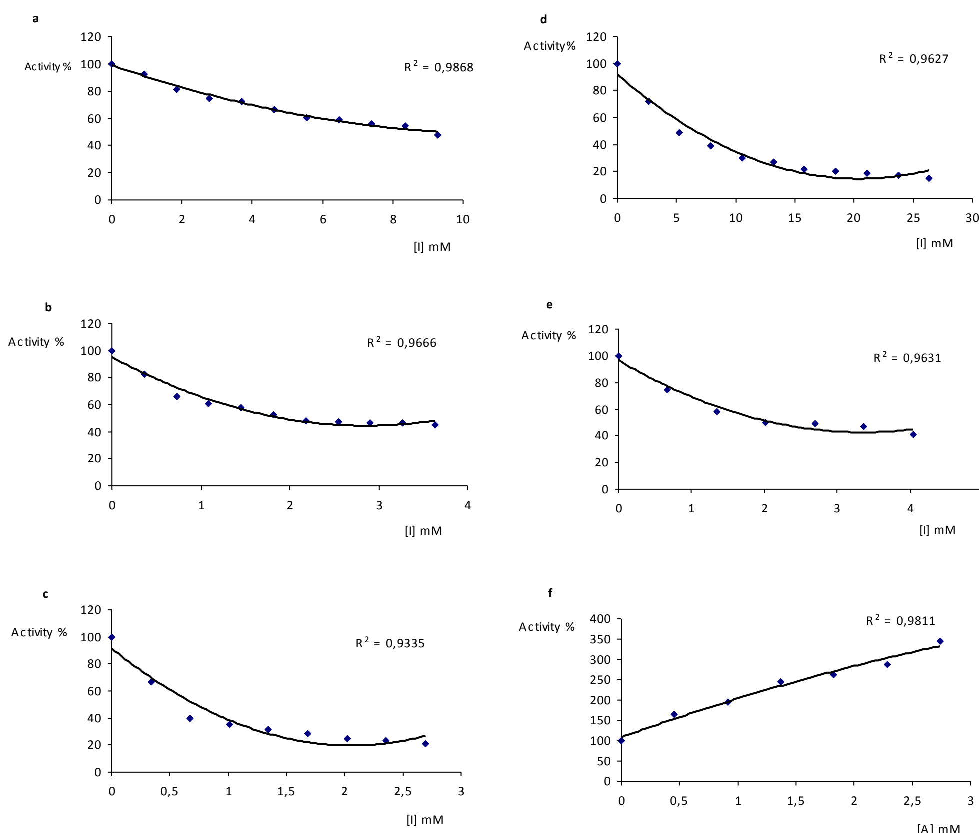
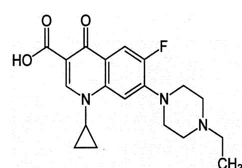


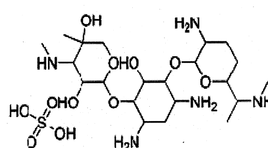
FIGURE 1 - Effect of Killoxacin™ (a), Gentavet™ (b), Geosol™ (c), Primoxal™ (d), Lypectin™ (e) and Penoksal-la™ (f) drugs on the enzyme activity of bovine. A purified carbonic anhydrase from bovine was assayed for hydratase activity in the presence of various concentrations of the above drugs. IC_{50} values were determined from these graphs.

4. DISCUSSION

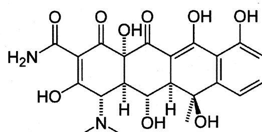
Most of the chemicals change the activity of an enzyme by combining within a way that influences the binding of substance and/or its turnover numbers. Chemicals that reduce an enzyme activity in this way are known as inhibitors. Many inhibitors are substances that structurally resemble their enzyme's substrate but either do not react, or react very slowly, compared with the substrate. Such inhibitors are commonly used to probe the chemical and conformational nature of a substrate-binding site as part of an effort to elucidate the enzyme's catalytic mechanism [24]. Many chemicals at relatively low dosages affect the metabolism of biota by altering normal enzyme activity, particularly inhibition of a specific enzyme [35]. The importance of CA isoenzymes for pH homeostasis, gas/fluid/ion exchange processes, digestion, bone resorption, renal function, fertilization and many other fundamental physiological processes have been reported [1, 3]. Carbonic anhydrase has been purified from different organisms and the effects of several metal ions, various chemicals, organic compounds and drugs on its activity have been investigated [14-20, 24, 31, 35]. It has been reported that the most common inhibitors for CA are several medical drugs [38, 39]. In addition, some sulfonamide derivatives, antiepileptic drugs and melatonin have been used for inhibition of CA activity *in vivo* and *in vitro* [24]. However, there was not much information available on inhibition of bovine carbonic anhydrase enzyme by antibiotics. In the present study, effect of certain medical drugs on bovine erythrocyte carbonic anhydrase was investigated. An antibiotic is a substance or compound that kills, or inhibits the growth of, bacteria. Antibiotics belong to the broader group of anti-microbial compounds, used to treat infections caused by microorganisms, including fungi and protozoa. Killoxacin™, Gentavet™, Geosol™, Primoxal™, Lypectin™ and Penoksal-la™ veterinary medical drugs were chosen for the investigation of inhibition or activation effects on bovine carbonic anhydrase. All six antibiotics contain active substances (enrofloxacin, gentamicin sulfate, oxytetracycline, sulfamethoxazol, lincomycin and dihydrostreptomycin, respectively. The structures of the active substances of the used antibiotics are shown in Fig. 2.



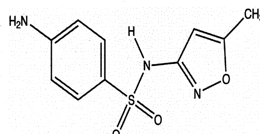
Enrofloxacin



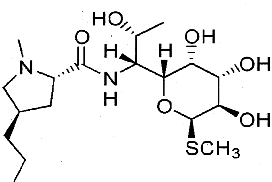
Gentamicin sulfate



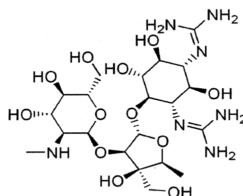
Oxytetracycline



Sulfamethoxazol



Lincomycin



Dihydrostreptomycin

FIGURE 2 - Structures of the active substances of the used antibiotics.

The results of the present study show for the first time that erythrocyte CA activity from bovine is inhibited by commonly used antibiotics (Killoxacin™, Gentavet™, Geosol™, Primoxal™ and Lypectin™) at different levels and stimulated by Penoksal-la™ (Fig. 2). Lypectin™ was the most effective inhibitor for bovine erythrocytes carbonic anhydrase. In literature, for any animal, there is no data available for these antibiotics effect on carbonic anhydrase. Ayik et al. [36] studied the *in vitro* inhibitory effects of some disinfectants on enzyme activity of carbonic anhydrase from rainbow trout (*Oncorhynchus mykiss*) gills. They reported that IC_{50} values of malachite green, methylene blue, potassium permanganate, chloramine-T and copper sulphate were determined as 0.05 mM, 0.023 mM, 0.15 mM, 0.32 mM and 5.39 mM. On the other hand, Sinan et al. [24] and Beydemir et al. [38] studied the effect of various antibiotics including gentamicin sulfate (Gentavet™) on human carbonic anhydrase (HCA-I and HCA-II). Sinan et al. [24] reported that IC_{50} values of sodium ampicillin were 0.00163 mM on HCA-I and 0.00114 mM on HCA-II. Beydemir et al. [38] also reported that HCA-I and HCA-II were inhibited by sodium ampicillin. Sinan et al. [24] also found that cefazolin sodium had minimum effect on HCA-I and HCA-II (0.00358 mM and 0.00308 mM, respectively) when comparing the other antibiotics.

In this study, IC_{50} value of gentamicin sulfate was found to be 0.00147 mM for bovine carbonic anhydrase. On the other hand, Sinan et al. [24] reported that gentamicin sulfate had inhibition effects on each enzyme (HCA-I and HCA-II), and IC_{50} values of gentamicin sulfate were found to be 0.00235 mM and 0.00253 mM. The importance of CA from several different sources has been reported. Especially, the necessity of CA activity in animals has been emphasized expressing its essential functions to remove excessive CO_2 from alveolar cells of mammary gland, to maintain intracellular pH [24]. In conclusion, the present study has revealed the serious inhibitory effects of some commonly used antibiotics against CA activity from bovine for the first time. CA is an important enzyme found both in erythrocytes and some other organs such as kidneys and eyes. Therefore, inappropriate usage of these antibiotics is undesirable for this enzyme.

Herein, a strategy for the purification of the CA enzyme was developed. Bovine carbonic anhydrase was purified by affinity chromatography, specifically designed for CA enzyme. Some antibiotics, investigated for their effects in our study, showed different degrees of inhibition on bovine CA. We have not come upon before a similar study. For the antibiotics that showed inhibition effects, the IC_{50} values of the chemicals causing inhibition were determined by means of activity percentage-[I] diagrams (Fig. 1). The concentrations of Killoxacin™, Gentavet™, Geosol™, Primoxal™ and Lypectin™ that inhibited 50% of the enzymatic activity were 9.25 mM, 3.79 mM, 0.72 mM, 6.59 mM and 2.12 mM, respectively. On the other hand, enzyme activity was increased by Penoksal-la™.

Most of the drugs affect the enzyme systems as an activator or inhibitor [40-42]. Many drugs exhibit the same effects both *in vivo* and *in vitro*, but some of them may not show the same effects on enzymes [43]. Many antibiotics are being used in therapies, but there are few reports related to changes in enzyme activities [42].

Chemical compounds showing inhibitory effects on bovine CA give rise to effects bound to the active center or other parts of enzymes. On the other hand, it can be said that the reason of IC_{50} values` changing, with respect to the enzyme, depend on the range, number and kind of enzyme amino acids.

ACKNOWLEDGEMENT

The authors would like to thank Balikesir University Research Center of Applied Sciences (BURCAS) for providing the research facilities.

REFERENCES

[1] Supuran, C.T., Scozzafava, A. and Conway, J. (2004) Carbonic Anhydrase - Its Inhibitors and Activators. CRC Press: Boca Raton, 14, 667-702.

[2] Ekinci, D., Beydemir, S. and Kufrevioglu, O.I. (2007) In vitro inhibitory effects of some heavy metals on human erythrocyte carbonic anhydrases. Journal of Enzyme Inhibition and Medicinal Chemistry 22 (6), 745-750.

[3] Supuran, C.T. and Scozzafava, A. (2007) Carbonic anhydrases as targets for medicinal chemistry. Bioorganic and Medicinal Chemistry 15, 4336-4350.

[4] Supuran, C.T., Scozzafava, A. and Casini, A. (2003) Carbonic anhydrase inhibitors. Medicinal Research Reviews 23, 146-189.

[5] Winum, J.Y., Scozzafava, A., Montero, J. L. and Supuran, C. T. (2006) The sulfamide motif in the design of enzyme inhibitors. Expert Opinion on Therapeutic Patents16, 27-47.

[6] Scozzafava, A., Mastrolorenzo, A. and Supuran, C. T. (2004) Modulation of carbonic anhydrase activity and its applications in therapy. Expert Opinion on Therapeutic Patents 14, 667-702.

[7] Ozensoy, O., Kockar, F., Arslan, O., Isik, S., Supuran, C.T. and Lyon, M. (2006) An evaluation of cytosolic erythrocyte carbonic anhydrase and catalase in carcinoma patients: An elevation of carbonic anhydrase activity. Clinical Biochemistry 39, 804-809.

[8] Bayram, E., Senturk, M., Kufrevioglu, O.I. and Supuran, C.T. (2008) In vitro inhibition of salicylic acid derivatives on human cytosolic carbonic anhydrase isozymes I and II. Bioorganic & Medicinal Chemistry 16, 9101-9105.

[9] Supuran, C.T. (2008) Nature. Reviews. Drug Discovery 7, 168.

[10] Hilvo, M., Innocenti, A., Montim, S.M., De Simone, G., Supuran, C.T. and Parkkila, S. (2008) Recent advances in research on the most novel carbonic anhydrases, CA XIII and XV. Current Pharmaceutical Design14, 672-678.

[11] Parui, R., Gambir, K.K. and Mehrotra, P.P. (1991) Changes in carbonic anhydrase may be the initial step of altered metabolism in hypertension. Biochemistry International 23, 779-789.

[12] Parui, R., Gambir, K.K., Cruz, I. and Hosten, A.O. (1992) Erythrocyte carbonic anhydrase: A major intracellular enzyme to regulate cellular sodium metabolism in chronic renal failure patients with diabetes and hypertension. Biochemistry International 26, 809-820.

[13] Borras, J., Scozzafava, A., Menabuoni, L., Mincione, F., Briganti, F., Mincione, G. and Supuran, C.T. (1999) Carbonic anhydrase inhibitors: synthesis of water-soluble, topically effective intraocular pressure lowering aromatic/heterocyclic sulfonamides containing 8-quinoline-sulfonyl moieties: is the tail more important than the ring?. Bioorganic & Medicinal Chemistry 7, 2397-2406.

[14] Doğan, S. (2006). The in vitro effects of some pesticides on Carbonic Anhydrase activity of Oncorhynchus mykiss and Cyprinus carpio carpio. Fish. Journal of Hazardous Materials 132 (2-3), 171–177.

[15] Isık, S., Kockar, F., Ozensoy, O. and Arslan, O. (2004) Differential in vitro effects of some pesticides on CA activities from some freshwater and seawater fish erythrocytes. Fresenius Environmental Bulletin 13(1), 25–29.

[16] Turan, Y., Arslan, O. and Kockar, F. (2002) The inhibitory effects of some pesticides on human erythrocyte CA activity (in vitro). Fresenius Environmental Bulletin 11 (1), 14-17.

[17] Bayram, E., Senturk, M., Kufrevioglu, O.I. and Supuran, C.T. (2008) In vitro inhibition of salicylic acid derivatives on human cytosolic carbonic anhydrase isozymes I and II. Bioorganic and Medicinal Chemistry 16(20), 9101-9105.

[18] Senturk, M., Gulcin, I., Dastan, A., Kufrevioglu, O.I. and Supuran, C.T. (2009) Carbonic anhydrase inhibitors. Inhibition of human erythrocyte isozymes I and II with a series of antioxidant phenols. Bioorganic & Medicinal Chemistry 17(8), 3207-3211.

[19] Senturk, M., Talaz, O., Ekinci, D., Cavdar, H. and Kufrevioglu, O.I. (2009) In vitro inhibition of human erythrocyte glutathione reductase by some new organic nitrates. Bioorganic & Medicinal Chemistry Letters 19, 3661-3663.

[20] Senturk, M., Kufrevioglu, O.I. and Ciftci, M. (2008) Effects of some antibiotics on human erythrocyte glutathione reductase: an in vitro study. Journal of Enzyme Inhibition and Medicinal Chemistry 23(1), 144-148.

[21] Omeroglu, E, E., Karaboz, I. and Sukatar, A. (2009) In vitro susceptibility of antibiotics against bioluminescent Vibrio harveyi TEMO5 and TEMS1 isolated from Holothuria tubulosa and seawater. Fresenius Environmental Bulletin 18(11), 2023-2028.

[22] Kucuk, C. and Kivanc, M. (2009) Effects of fungicides, antibiotics and NaCl in *Rhizobium* sp isolated from clover root nodules. Fresenius Environmental Bulletin 18, 744-748.

[23] Parui, R., Gambir, K.K., Cruz, I. and Hosten, A.O. (1992) Erythrocyte carbonic anhydrase: A major intracellular enzyme to regulate cellular sodium metabolism in chronic renal failure patients with diabetes and hypertension. Biochem International 26, 809-820.

[24] Sinan, S., Ozensoy, G.O. and Arslan, M. (2009) Effects of Some Antibiotics on Enzyme Activities of Carbonic Anhydrase from Erythrocytes in vivo and in vitro. Hacettepe Journal of Biology and Chemistry 37(2), 111-122.

[25] Nussbaum, F.V., Brands, M., Hinzen, B., Weigand, S. and Häbich, D. (2006) Antibacterial Natural Products in Medicinal Chemistry-Exodus or Revival?. Angewandte Chemie International Edition 45(31), 5072-5129.

[26] Pickering, L.K., O'Connor, D.M., Anderson, D., Bairan, A.C., Feigin, R.D. and Cherry, J.D. (1973) Clinical and pharmacologic evaluation of cefazolin in children. The Journal of Infectious Diseases 128, 407-414.

[27] Turck, M., Clark, R.A., Beaty, H.N., Holmes, K.K., Karney, W.W. and Reller, L.B. (1973) Cefazolin in the treatment of bacterial pneumonia. The Journal of Infectious Diseases 128, 382-385.

[28] Singhvi, S.M., Heald, A.F., Gadebusch, H.H., Resnick, M.E., Difazio, L.T. and Leitz, M.A. (1977) Human serum protein binding of cephalosporin antibiotic *in* vitro. Journal of Laboratory and Clinical Medicine 89, 414-420.

[29] Meder, B., Malmborg, A.S. and Wersall, J. (1974) A clinical and laboratory evaluation of the effects of cephradine on patients with upper respiratory tract infections. Experta Medica., 14-16.

[30] Honjo, T. and Watanabe, A. (1984) Clinical experience on sulbactam/cefoperazone in the pediatric field. The Japanese journal of antibiotics 37, 32-36.

[31] Sinan, S., Gencer, N., Turan, Y. and Arslan, O. (2007) In vitro inhibition of the carbonic anhydrase from saanen goat (Capra hircus) with pesticides. Pesticide Biochemistry and Physiology 88, 307-311.

[32] Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72, 248-251.

[33] Laemmli, D.K. (1970) Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 227, 680-683.

[34] Wilbur, K.M. and Anderson, N.G. (1976) Electrometric and colorometric determination of carbonic anhydrase. The Journal of Biological Chemistry 176, 147-151.

[35] Hochster, R.M., Kates, M. and Quastel, J.H.(1972) Metabolic Inhibitors, Academic Press, New York 3-4, 71-89.

[36] Ayik, O., Hisar, O., Bulbul, M., Hisar, S.O. and Yanik, T. (2005) The *in vitro* inhibitory effects of some disinfectants on enzyme activity of carbonic anhydrase from rainbow trout (*Oncorhynchus mykiss*) gills. J. Anim. Vet. Adv., 4, 645-650.

[37] Hisar, O., Hisar, S.O., Küfrevioglu, Ö.I. and Yanik, T. 2003, Inhibitory effects of some antibiotics on activity of carbonic anhydrase from rainbow trout erythrocytes in vitro and in vivo, J. Aquat. Anim. Health, 15,222-229.

[38] Beydemir, S., Cifci, M., Ozmen, I., Buyukokuroglu, M.E., Ozdemir, H. and Kufrevioglu, O.I. (2000) Effects of some medical drugs on enzyme activies of carbonic anhydrase from human erytrhrocytes *in vitro* and from rat erytrhrocytes *in vivo*. Pharmacological Research 42(2), 187-191.

[39] Beydemir, S., Cifci, M., Kufrevioglu, O.I. and Buyukokuroglu, M.E. (2002) Effects of gentamicine sulfate on enzyme activities of carbonic anhydrase from human erythrocytes *in vitro* and from rat erythrocytes *in vivo*. Biological and Phar-

maceutical Bulletin 25(8), 966-969.

[40] Edward, E. and Morse, M.D. (1988) Toxic effects of drugs on erythrocytes. Annals of Clinical Laboratory Science 18, 13-18.

[41] Jacobasch, G. and Rappoport, S.M. (1996) Hemolytic anemias due to erythrocyte enzyme deficiencies. Mol. Asp. Med. 17, 143-170.

[42] Çiftçi, M., Türkoğlu, V. and Aldemir, S. (2002) Effects of some antibiotics on glucose 6-phosphate dehydrogenase in sheep liver. Veterinarni Medicina 47(10), 283-288.

[43] Ciftci, M., Beydemir, S., Yilmaz, H. and Bakan, E. (2002), Effects of some drugs on rat erythrocyte 6-phosphogluconate dehydrogenase: an in vitro and in vivo study. Polish Journal of Pharmacology 54(3), 275-280.

Received: July 27, 2010
Revised: October 14, 2010
Accepted: October 22, 2010

CORRESPONDING AUTHOR

Mikail Arslan
Balıkesir University
Susurluk Technology Vocational School of Higher Education
Balıkesir, 10600
TURKEY

Phone: +90266 8657153
Fax: +902668657155
E-mail: marslan@balikesir.edu.tr

FEB/ Vol 20/ No 2a/ 2011 – pages 439 - 445

EFFECTS OF CADMIUM ON NITROGEN METABOLISM IN TILLERING STAGE OF *ORYZA SALIVA* L.

Fangming Yu[1,3,4], Kehui Liu[2,4,*], Mingshun Li[1,3,4], Zhenming Zhou[1,3,4], Hua Deng[1,3,4] and Bin Chen[1,3,4]

[1]College of Resource and Environment, Guangxi Normal University, Guilin 541004, China
[2]College of Life and Environmental Science, Guilin University of Electronic Technology, Guilin 541004, China
[3]Guangxi Key Laboratory of Environmental Engineering, Protection and Assessment, Guilin 541004, China
[4]Key Laboratory of Ecology of Rare and Endangered Species and Environmental Protection (Guangxi Normal University), Ministry of Education, China

ABSTRACT

The effects of 60-d Cd exposure on the growth of *Oryza saliva* L. seedlings at tillering stage were studied using soil culture experiments. Cd concentrations in the treatments are 0 (control), 5, 10, 20, 40, and 80 mg kg^{-1} $CdCl_2 \cdot 2.5H_2O$. Research findings showed that under 5, 10, 20, 40 and 80 mg kg^{-1} Cd exposure, tiller numbers decreased by -2.28%, 14.92%, 6.99%, 25.13% and 39.92%, respectively, compared with those of the control. The dry biomass of shoots decreased by 7.25-35.23% and, accordingly, that of roots by 6.67-45.00%. The concentrations of NO_3^-, NH_4^+ and soluble protein were normal at low Cd exposure (5 mg kg^{-1}), while all changed significantly at high Cd exposure (≥10 mg kg^{-1}). In 10, 20, 40 and 80 mg kg^{-1} Cd treatments, free proline concentrations in the root increased significantly (P <0.05) by 30.65, 63.07, 134.97 and 148.94%, respectively, and also in the leaf by 23.26%, 24.86%, 45.13% and 55.68% (P <0.05). At the same time, concentrations of nitrate reductase, glutamine synthetase and glutamate synthase linearly decreased in tissues of both leaves and roots (R^2 >0.92, n = 18). However, the activities of glutamate dehydrogenase in leaves and roots rose markedly (P < 0.05) with Cd treatments ≥10 mg kg^{-1}.

KEYWORDS:
Oryza saliva L., cadmium, plant growth, nitrogen metabolism

1. INTRODUCTION

Cadmium (Cd) was reported to be a wide-spread heavy metal pollutant in natural and artificial environment in China resulting from agriculture and industrial activities, such as pigments, mining, smelting and electroplating, etc [1, 2]. Cd is highly toxic to humans, animals and plants; it can enter the food chain through uptake of cadmium-polluted foods and, therefore, poses a serious hazard to human health and ecosystem [3]. In China, according to a recent report, agricultural field area polluted by Cd was above 2 million hm^2, and the agricultural products polluted by Cd were above 14.6 hundred million kg per year [4].

The stress of Cd would affect plant growth [5, 6] and plant metabolism, such as nitrogen metabolism [7], photosynthesis [8], carbohydrate metabolism [9], sulphate assimilation [10]; and the influences of Cd on plant metabolism is mainly through inactivating several key enzymes of nitrogen metabolism, including nitrate reductase (NR) [7, 11], glutamine synthetase (GS), glutamate synthase (GOGAT) [12, 13] and glutamate dehydrogenase (GDH) [14].

Especially, Cd pollution can reduce the rice production [16], which is the world's 2nd largest crop and the most popular food in China [15], and tillering stage is one of the most important stages during rice growth and also essential to the rice yield, population structure and photosynthesis. However, the effect of Cd on nitrogen metabolism in rice tillering period has been rarely studied. The purpose of this study was to examine the influences of Cd on the plant growth, the activities of nitrate reductase (NR), glutamine synthetase (GS), glutamate synthase (GOGAT), glutamate dehydrogenase (GDH) and NO_3^-, NH_4^+ , soluble protein content as well as proline content both in roots and leaves of *Oryza saliva* L. This study may provide new insight on the function of plant nitrogen metabolism under Cd stress in rice tillering period.

2. MATERIALS AND METHODS

2.1. Soil preparation and plant culture

The soil was collected from the 0-20 cm depth of a paddy soil in Guilin (25°16′ N, 110°17′ E), China. Selected properties of the air-dried soil are shown in Table 1. Soil samples were mixed thoroughly with basal fertilizers (100 mg N kg^{-1} dry weight (DW) soil as ammonium nitrate, and 80 mg P kg^{-1} and 100 mg K kg^{-1} as KH_2PO_4). Six

* Corresponding author