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# ANTIOXIDATIVE DEFENSE ENZYME INHIBITION IN ERYTHROCYTES OF PATIENTS WITH ACUTE MYOCARDIAL INFARCTION

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Increased amount of reactive oxygen and nitrogen species were detected during myocardial ischemia after acute myocardial infarction (AMI). Decreased activity of some antioxidative defense enzymes in erythrocytes creates conditions for oxidative stress and propagation of thus induced damages. In this study, we tried to detect changes in antioxidative defense enzyme activities in erythrocytes of patients with AMI and to determine a nature of this changes. Activity of copper zinc superoxide dismutase (CuZn SOD, EC 1.15.1.1.), catalase (CAT, EC 1.11.1.6.), glutathione peroxidase (GSH-Px, EC

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1.11.1.9.) and glutathione reductase (GR, EC 2.5.1.18.) were determined in AMI patiens and compared with controls. Activities of CuZn SOD ($p<0.005$) and CAT ($p<0.05$), as well as, GSH-Px ($p<0.02$) were significantly lower comparing with controls. Activity of GR was significantly higher ($p<0.005$) with respect to controls. Activity of CuZn SOD in patients with AMI were more inhibited ($47 \pm 5$ %) with 6 mM diethyldithiocarbamate (DDC) than corresponding controls ($32 \pm 3$ %). *In vitro* inhibition of control samples with hydrogen peroxide ($H_2O_2$) and electrophoretic analysis indicate $H_2O_2$ as potential inhibitor of CuZn SOD in patient with AMI.

*Key words*: acute myocardial infarction, antioxidative defense enzymes, diethyldithiocarbamate, electrophoresis, enzyme inhibition, oxidative stress

## INTRODUCTION

Previous examination on antioxidative defense system in human blood after acute myocardial infarction (AMI) have shown changes at the level of low molecular mass antioxidants, as well as, in the activity of antioxidative defense enzymes (MUZÁKOVÁ *et al.*, 2001; DUSINOVIĆ *et al.*, 1998; SIMOVIĆ *et al.*, 1995). Disturbed balance between production of reactive oxygen and nitrogen species and their elimination under such conditions were claimed to be responsible for oxidative stress conditions formed and as molecular cause for propagation of oxidative damage and reperfusion injury. Under chronic ishemic conditions it was found that protective protein such as CAT, Bcl 2 are induced (SHIMIZU *et al.*, 2001), but cause of decreased activity of these proteins in acute ishemic conditions such as AMI are not determined yet.

Examination of antioxidative defense enzyme activities *in vitro* have shown that CuZn SOD have constant real specific activity and may be inhibited with copper helators such as DDC, as well as, with strong oxidants such as $H_2O_2$. Activity of CAT can be reversible inhibited with nitric oxide (NO) and ireversible with superoxide anione and hydroxyl radical. GSH-Px can be inhibited with peroxinitrite due to tyrosine residue nitration, or can be lowered due to selenium deficit (PADMAJA *et al.*, 1998).

This determined us to examine the activities of antioxidative defense enzyme (CuZn SOD, CAT, GSH-Px and GR) in the blood of AMI patients and to compare them with corresponding controls, as well as, to induce *in vitro* conditions for enzyme inhibition in order to determine reactive species responsible for changes observed *in vivo*.

## MATERIAL AND METHODS

This study included 6 AMI patients indicated for streptokinase treatment after admission in Coronary Unit, Clinical Centre of Serbia. All patients were with typical symptoms of heart failure (Killip III) and were treated with streptokinase (1.500.000 units in 150 ml 5% glucose) as intravenous infusion, during 60-90 minutes. The blood samples were taken in the moment before starting streptokinase.

Control group was 36 healthy volunteers working in the Institute for biological research "Siniša Stanković", without signs of cardiovascular disease, as well as, other acute or chronical disaeses.

Blood samples from patients were collected in heparinized glass tubes. The erythrocytes and plasma were separated by centrifugation (10min, 5000 rpm, 4°C). The aliquots of three times washed erythrocytes with physiological saline were lysed with cold water. In isolated erythrocytes of all patients we measured CuZn SOD, CAT, GSH-Px and GR activities. CAT was measured by the procedure proposed by BEUTLER (1982) and the enzyme activity was expressed in U/g Hb. GSH-Px activity was measured by GSH recycling enzyme assay (PAGLIA and VALENTINE, 1967), modified by TAMURA *et al.* (1982), using t-butyl hydroperoxide as a substrate and activity was expressed in nmol NADPH oxidized/ min/mg Hb. GR activity was assayed as suggested by GLATZLE *et al.* (1974) and activity were expressed in mmol NADPH/min/mg Hb. The hemoglobin was removed from lysates by the method of TSUCHIHASHI (1923) and the supernatant was used for the analysis of CuZn SOD activity. Erythrocyte CuZn SOD activity was assayed by the xanthine oxidase / xanthine/ cytochrome c method suggested by McCORD and FRIDOVICH (1968). Inhibition of erythrocyte CuZn SOD activity with DDC and $H_2O_2$ was followed through determination of CuZn SOD activity assayed by the method of autooxidation of epinephrine (MISRA and FRIDOVICH, 1972).

Polyacrylamide gel electrophoresis (PAGE) was carried out on glass plate with 12 % running gel and 4 % stacking gel. CuZn SOD from blood erythrocytes were dissolved to 2 U/mL in solution, containing 12 % glycerol, 0,5 mM Tris-HCl (pH 6,8) and 0,2 M EDTA and 50mL was loaded per well.

Data are presented as mean ± SEM. Comparisons between groups of patients were performed by using the Students t-test. Values of $p<0.05$ were considered significant.

## RESULTS

As shown in Table 1., activities of CuZn SOD ($p<0.005$), CAT ($p<0.05$) and GSH-Px ($p<0.02$) were decreased in comparison with corresponding controls, while GR activity ($p<0.005$) were increased in respect to controls. *In vitro* inhibition of CuZn SOD activity with 6 mM DDC showed greater inhibition in AMI samples (47 ± 5 %) than control samples (32 ± 3%) indicated presence of oxidative damage in AMI samples probably induced by $H_2O_2$ (Table 2).

*Table 1. - Antioxidative defense enzyme activities in AMI patients and corresponding controls*

| ANTIOXIDATIVE DEFENSE ENZYMES | CONTROLS (n=36) | AMI (n=6) |
|---|---|---|
| CuZn SOD (U/mg Hb) | 1.98 ± 0.05 | 1.42 ± 0.16 *** |
| CAT (U/g Hb x $10^4$) | 20.84 ± 0.60 | 17.33 ± 1.39 * |
| GSH-Px (nmol NADPH/min/mg Hb) | 18.50 ± 0.43 | 16.02 ± 1.84 ** |
| GR (mmol NADPH/min/g Hb) | 4.27 ± 0.16 | 6.98 ± 1.46*** |

*p<0.05 ** p<0.02 *** p<0.005

*Table 2. - CuZn SOD activity in AMI patients and controls and inhibition of CuZn SOD activity in presence of DDC*

| | CuZn SOD (U/mg Hb) | CuZn SOD + DDC (U/mg Hb) | % inhibition |
|---|---|---|---|
| Control (n=5) | 3659 ± 495 | 2520 ± 396 | 32 ± 3 |
| AMI (n=3) | 2516 ± 342 | 1339 ± 214 | 47 ± 5 |

*In vitro* enzyme treatment with 9 mM $H_2O_2$ lead to similar inhibition in both, AMI and control samples, indicated that $H_2O_2$ is *in vivo* inhibiting agent for CuZn SOD in AMI samples.

Electroforetic pattern analysis of lysate of erythrocytes have shown differences among samples of patients with AMI and controls (Fig 1.). The controls have shown significantly less CuZn SOD inhibition with DDC. A major band is more visible in controls than in samples of AMI patients, probably because the samples of AMI have already been damaged by oxidative stress, so copper from CuZn SOD is more exposed to inhibition with DDC.

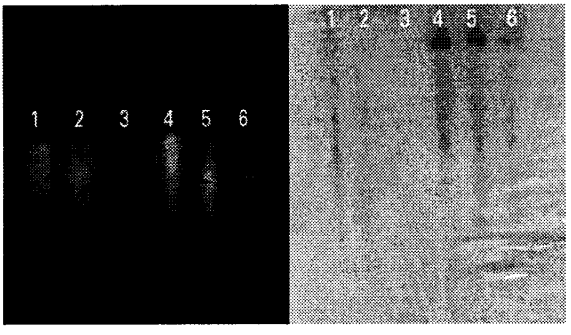


Fig. 1. - Polyacrylamide gel electrophoretic protein pattern - activity of CuZn SOD (left) and proteins (right): 1. control; 2. control + DDC; 3. control + $H_2O_2$ + DDC, 4. AMI; 5. AMI + DDC; 6. AMI + $H_2O_2$ + DDC

## DISCUSSION

Comparation of previous studies of antioxidative defense enzyme activities in the blood of AIM patients (DUSINOVIĆ *et al.*, 1998; MUZÁKOVÁ *et al.*, 2001) showed that activities are usualy lower than in controls, but not always statisticaly significant which may be caused by different type and intensity of AMI.

In our study activity of three antioxidative defense enzymes were lower among which CAT activity was significantly lower as in all published studies. It can be explained by reversible inhibition with NO whose production is increased under condition of acute ischemia. CuZn SOD activity is lower in this research indicated that increased amount of $H_2O_2$ due to CAT inhibition may oxidatively damage CuZn SOD. Hydrogen peroxide, or rather its conjugate base ($OH_2^-$), reacts with CuZn SOD, reducing Cu(II) to Cu(I), followed by the reaction of Cu(I) with a second $H_2O_2$ forming an active site oxidant, putatively described as copper-bound hydroxyl radical (HODGSON and FRIDOVICH, 1975a). This in turn leads to enzyme inactivation through 2-oxohistidine formation (UCHIDA and KAWAKISHI, 1994) and to the oxidation of various substrates in what is called the "peroxidative" activity of CuZn SOD (HODGSON and FRIDOVICH, 1975b; YIM *et al.*, 1990; JEWETT *et al.*, 2000). This effect is lower expressed on the activity than on structure of active center channel as it was shown with DDC inhibition results.

Our results indicate that CuZn SOD-DDC inhibition test may serve for determination of ROS production effect under AMI conditions and potentially can be very useful for prediction of possible reperfusion damage after thrombolytic therapy.

Activity of GR was higher in AMI patients than from corresponding controls indicated that increased forming of oxidized glutathione (GSSG) under condition of acute ischemia need to be eliminated to prevent deleterious intracelular effect of GSSG.

Examination of antioxidative defense enzymes changes under conditions of reperfusion induced by thrombolytic therapy is in progress in our laboratory and aim of that study is distinquish reversible and ireversible inhibition of antioxidative enzymes under conditions of reperfusion induced increased production of ROS.

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# INHIBICIJA AKTIVNOSTI ANTIOKSIDATIVNIH ENZIMA U ERITROCITIMA PACIJENATA SA AKUTNIM INFARKTOM MIOKARDA

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Izvod

Povećana količina reaktivnih vrsta kiseonika i azota i snižena aktivnost prirodnih odbrambenih sistema detektovana je tokom srčane ishemije. Enzimske komponente antioksidacione zaštite u eritrocitima smanjuju aktivnost stvarajući uslove za propagaciju oštećenja izazvanih oksidacionim stresom. U ovom radu, pokušali smo da detektujemo promene u aktivnosti antioksidacionih enzima u eritrocitima pacijenata obolelih od AIM-a i utvrdimo prirodu promena. Određivana je aktivnost bakar cink sadržavajuće superoksid dismutaze (CuZn SOD), katalaze (CAT), glutation peroksidaze (GSH-Px) i glutation reduktaze (GR) kod pacijenata obolelih od AIM-a i upoređivana sa aktivnošću kod kontrola. Aktivnosti SOD ($p<0.005$) i CAT ($p<0.05$), kao i GSH-Px ($p<0.02$) bile su značajno niže u odnosu na kontrole. Aktivnost GR ($p<0.005$) bila je značajno viša u odnosu na kontrolu. Aktivnost CuZn SOD bila je inhibirana 6 mM dietilditiokarbamatom (DDC-om) više kod pacijenata obolelih od akutnog infarkta miokarda ($47 \pm 5$ %) nego kod kontrola ($32 \pm 3$ %). *In vitro* inhibicija kontrolnih uzoraka sa vodonik peroksidom i elektroforetska analiza ukazuju na vodonik peroksid kao mogući inhibitor CuZn SOD kod AIM-a.

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