# 5′-nukleotidaza, oksidacijski stres i antioksidacijski status kod konzumenata alkohola i bolesnika s cirozom jetre

# 5′-nucleotidase, oxidative stress and antioxidant status in alcohol consumers and cirrhotic patients

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## Sažetak

**Uvod:** Cilj istraživanja bio je izmjeriti aktivnost enzima 5′-nukleotidaza kod bolesnika s cirozom jetre i osoba koje uzimaju alkohol. U istraživanju se ispitivao i oksidacijski stres, antioksidansi te njihova povezanost s 5′-nukleotidazom.

**Materijali i metode:** Istraživanje je provedeno u tri skupine po 25 ispitanika jednake dobi i spola: I. skupina (kontrolni ispitanici), II. skupina (osobe koje uzimaju alkohol, tj. konzumenti alkohola) i III. skupina (bolesnici s cirozom jetre). Uzorci krvi prikupljeni od ispitanika centrifugirani su kako bi se odvojila plazma za analizu 5′-nukleotidaze. Odvojene stanice su tri puta isprane 0,9-postotnom hladnom fiziološkom otopinom i upotrebljene za analizu glutationa, malondialdehida i superoksid-dismutaze.

**Rezultati:** Aktivnost 5′-nukleotidaze u serumu bila je statistički značajno povišena kod skupine bolesnika s cirozom i skupine konzumenata alkohola. Koncentracije malondialdehida bile su također statistički značajno povišene kod bolesnika s cirozom jetre i konzumenata alkohola. Koncentracije glutationa i superoksid-dismutaze bile su statistički značajno snižene u obje skupine.

**Zaključak:** Iz ovih rezultata može se zaključiti da je aktivnost 5′-nukleotidaze u serumu dosljedno viša kod bolesnika s cirozom jetre i osoba koje uzimaju alkohol. Zapažena razlika mogla bi ukazivati na opseg oštećenja jetre, oštećenja hepatobilijarnog sustava i opstrukcije jetre.

**Ključne riječi:** konzumenti alkohola; učinci antioksidansa; 5′-nukleotidaza; oksidacijski stres

## Abstract

**Background:** The present study was undertaken to determine the 5′-nucleotidase enzyme activity in liver cirrhotic patients and alcohol consumers. Oxidative stress, antioxidants and their association with 5′-nucleotidase were also investigated.

**Methods:** The study included three groups of 25 age and sex matched subjects: group I (control), group II (alcohol consumers) and group III (cirrhotic patients). Blood samples were collected and centrifuged for separation of plasma for analysis of 5′-nucleotidase. Separated cells were washed thrice with 0.9% w/v cold normal saline and used for the analysis of glutathione, malondialdehyde and superoxide dismutase.

**Results:** The activity of serum 5′-nucleotidase was significantly increased in both cirrhotic patients and alcohol consumers. The levels of malondialdehyde were also significantly increased in both cirrhotic patients and alcohol consumers. The levels of glutathione and superoxide dismutase were significantly decreased in both cirrhotic patients and alcohol consumers.

**Conclusions:** Study results indicated the activity of serum 5′-nucleotidase to be consistently higher in cirrhotic patients and alcohol consumers. The difference recorded might be pointing to the extent of liver damage, hepatobiliary damage, and biliary stasis.

**Key words:** alcohol consumers; antioxidant effects; 5′-nucleotidase; oxidative stress

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## Uvod

5′-Nukleotidaza (5′-NT) je unutarstanični membranski glikoprotein koji je prisutan kao ektoenzim kod mnogih vrsta stanica sisavaca, a hidrolizira 5′-nukleotide do njihovih odgovarajućih nukleozida (1). Određuje ga se kao pokazatelja oštećenja jetre koje je primarno rezultat inteferencije s izlučivanjem žuči (2). Aktivnost enzima povećava se kod jetrenih bolesti uključujući cirozu jetre, kronični alkoholizam, novotvorine jetre i žučnih kanala, dobroćudnu bolest bilijarnog sustava, no svoju najvišu vrijednost doseže u jetrenoj opstrukcije (3,4). Dijagnostička vrijednost 5′-NT pokazala se boljom od one jetrenih enzima, naročito kod metastaza u jetri. Pojačana aktivnost 5′-NT izmjerena je kod 92% bolesnika s opstrukcijskom žuticom, 70% bolesnika s parenhimskom bolešću jetre i 81% bolesnika s metastazama u jetri (5,6). Također se izvještavalo da je aktivnost 5′-NT u serumu klinički korisna za postavljanje diferencijalne dijagnoze hepatobilijarnih i koštanih bolesti, budući da se aktivnost enzima pojačava samo kod hepatobilijarnih bolesti (7).

Iako određivanje samo oksidacijskih ili antioksidacijskih sastavnica može pružiti podatak o oksidacijskom stresu, određivanje oksidansa zajedno s antioksidansima je u ovom kontekstu korisnije (8,9). Istraživanja su ukazala na pojavu slobodnih radikala kisika u ranom stadiju fibroze i ciroze jetre (10). Kod bolesnika oboljelih od ciroze jetre uslijed uzroka koji nisu vezani uz alkohol ili zbog pretjeranog uzimanja alkohola bilježi se smanjena koncentracija antioksidansa kao što su glutation (GSH) i superoksid-dismutaza (SOD) i povećana koncentracija proizvoda lipidne peroksidacije kao što je malondialdehid (MDA) (11-19). Nastanak jetrene fibroze kod ciroze jetre izazvane alkoholom zamršen je proces koji, čini se, uključuje metaboličke proizvode oksidacije etanola; indukciju citokroma P450, pojačan oksidacijski stres, oslabljenu antioksidacijsku obranu, lipidna peroksidaciju, stvaranje aldehidnih proizvoda, učinke mitogenskih i fibrogenskih citokina i složene interakcije između jetrenih parenhimskih i ne-parenhimskih stanica s jetrenim zvjezdastim stanicama (Ito stanice, jetreni lipociti ili stanice koje pohranjuju masti) koje danas su prepoznate kao primarni izvori izvanstaničnog matriksa (20,21).

Postoje dokazi da je bolesna jetra kod bolesnika s kolestatskom bolešću jetre izložena oksidacijskom stresu povezanom s povećanom lipidnom peroksidacijom koja uključuje unutarorgansko stvaranje reaktivnih kisikovih spojeva (engl. *reactive oxygen species*, ROS), pričem je moguće da posreduju endotoksini, žučne kiseline i nakupljanje razgradnih produkata lipidne peroksidacije, kao što su lipidni peroksidi i MDA (20,22). Alkohol pospješuje stvaranje ROS i/ili utječe na normalan tjelesni obrambeni sustav koji brani organizam od tih spojeva (23). Istraživanja

## Introduction

5′-Nucleotidase (5′-NT) is an intrinsic membrane glycoprotein, present as an ectoenzyme in a wide variety of mammalian cells. 5′-NT hydrolyzes 5′-nucleotides to their corresponding nucleosides (1). It is measured as an indicator of liver damage resulting primarily from interference with the secretion of bile (2). Enzyme activity is elevated in liver diseases including liver cirrhosis, chronic alcoholism, neoplasms of the liver and bile ducts, benign biliary disease, but reaches its highest level in the presence of biliary stasis (3,4). The diagnostic value of 5′-NT has been shown to be superior to other liver enzymes, especially in liver metastasis. Raised levels of 5′-NT activities are found in 92% of patients with obstructive jaundice, 70% of patients with parenchymal liver disease and 81% of patients with hepatic metastasis (5,6). It is also reported that serum 5′-NT is clinically useful for differential diagnosis of hepatobiliary and osseous diseases, the enzyme activity being only increased in hepatobiliary disease (7).

Although determination of either oxidants or antioxidant components alone may give information about oxidative stress, determination of oxidants along with antioxidants is more useful in this context (8,9). Studies suggest that evidence of oxygen free radical is found early in the development of fibrosis and cirrhosis of the liver (10). Patients suffering from liver disease either due to non-alcohol or excessive alcohol intake show depletion of antioxidants such as glutathione (GSH) and superoxide dismutase (SOD), and increased concentration of the products of lipid peroxidation such as malondialdehyde (MDA) (11-19). Hepatic fibrogenesis in alcohol liver cirrhosis is an intricate process, which appears to involve metabolic products of ethanol oxidation, cytochrome P450 induction, enhanced oxidative stress, depletion of antioxidant defenses, lipid peroxidation, generation of aldehydic products, effects of mitogenic and fibrogenic cytokines, and complex interactions between liver parenchymal and non-parenchymal cells with hepatic stellate cells (Ito cells, hepatic lipocyte or fat storing cell) are now recognized as the primary source of extracellular matrix (20,21).

There is evidence that the diseased liver of patients with cholestatic liver disease is exposed to oxidative stress associated with increased lipid peroxidation involving intraorgan generation of reactive oxygen species (ROS), possibly mediated by endotoxins, bile acids and accumulation of degradation products of lipid peroxidation, such as lipid peroxides and MDA (20,22). Alcohol promotes the generation of ROS and/or interferes with the body's normal defense mechanism against these compounds (23). Studies suggest that evidence of oxygen free radicals is also found early in the development of fibrosis and cirrhosis of the liver (10).

pokazuju da su slobodni kisikovi radikali nađeni i u ranom stadiju razvoja fibroze i ciroze jetre (10).
Ovo je istraživanje provedeno kako bi se izmjerila aktivnost enzima 5′-NT kod bolesnika s cirozom jetre i osoba koje uzimaju alkohol. U istraživanje su uključeni i oksidacijski stres, antioksidansi i njihova povezanost s 5′-NT.

## Materijali i metode

### Ispitanici

Istraživanje je provedeno u 3 skupine od po 25 ispitanika u dobi od 35-70 godina. U svakoj je skupini bilo 13 muškaraca i 12 žena:
I. skupina (kontrolna skupina): zdravi ispitanici, raspon dobi od 35 do 70 godina (medijan: 55, interkvartilni raspon (*engl. interquartile range*, IQR): 45-60) bez ikakve bolesti jetre u anamnezi, nisu uzimali alkohol niti ga uzimaju sada;
II. skupina (skupina konzumenata alkohola): raspon dobi od 35 do 70 godina (medijan: 55, IQR: 45-60) s povijesti uzimanja alkohola kroz 10 godina ili više, ali bez jetrenih ili hepatobilijarnih poremećaja u anamnezi. Ispitanici ove skupine su tijekom istraživanja uzimali 6250-7500 mg alkohola na dan. Ispitanici iz ove skupine s poviješću alkoholizma odabrani su osobnim kontaktom preko poznatih osoba koje uzimaju alkohol.
III. skupina (skupina bolesnika s cirozom jetre): raspon dobi od 35 do 70 godina (medijan: 55, IQR: 45-60) s dijagnozom ciroze jetre s ili sa žuticom i uzimanjem alkohola ili hepatotoksičnih lijekova u anamnezi. Bolesnici s cirozom jetre su se liječili u slijedećim bolnicama u Mangaloreu: Okružna bolnica Wenlock; Bolnica K.M.C, Attavar; Bolnica K.M.C., okrug Ambedkar; A.J. Institut za medicinske znanosti, Kuntikana; Bolnica Yenepoya, Kodialbail.
Pri izboru ispitanika za istraživanje vodilo se računa o tome da se iz istraživanja isključe pušači, osobe s navikom žvakanja duhana, osobe s upalnim bolestima, kao što su tuberkuloza, reumatoidni artritis, šećerna bolest i maligne bolesti, jer sve ove bolesti u velikoj mjeri doprinose oštećenju uslijed oksidativnog stresa. Iz istraživanja su isključeni i ispitanici s bilo kojom bolešću kostiju ili s bilo kojim kliničkim stanjem koje bi moglo biti povezano s pojačanjem aktivnosti 5′-nukleotidaze.
Institucionalni klinički etički odbor Medicinskog koledža u Kastrubi, Mangalore, Indija odobrio je ovo istraživanje te su tijekom istraživanja poštovane etičke smjernice Odbora.

### Uzorci

Uzorci od 2 mL venske krvi sakupljeni su u obične bočice kako bi se odvojio serum za analizu 5′-NT. Iz medijalne kubitalne vene ili bazilične vene uzeto je 5 mL krvi i stavljeno u odvojenu epruvetu s EDTA pod strogim aseptičnim uvjetima. Od toga se 0,2 mL pune krvi rabilo za određiva-

The present study was therefore undertaken to measure the 5′-NT enzyme activity in liver cirrhosis and alcohol consumers. Oxidative stress, antioxidants and their association with 5′-NT were also investigated.

## Materials and methods

### Subjects

This study was conducted in three groups of 25 subjects each, age range 35-70 years. There were 13 males and 12 females in each group.
Group I (control group): healthy individuals, age range 35-70 years (median 55, IQR 45-60) having no history of liver disease or alcohol intake in the past or present.
Group II (alcohol consumer group): age range 35-70 years (median 55, IQR 45-60) with a history of 10 years or more of alcohol intake without any history of liver disease or other hepatobiliary disorders. Alcohol consumers were taking 6250-7500 mg of alcohol/day during the study. Alcohol consumers were recruited by personal contact from known alcohol consumers.
Group III (cirrhotic patient group): age range 35-70 years (median 55, IQR 45-60) diagnosed with liver cirrhosis and with a history of jaundice, alcohol intake or hepatotoxic drug intake. Patients were recruited from the following hospitals in Mangalore: District Wenlock Hospital; K.M.C Hospital, Attavar; K.M.C. Hospital, Ambedkar Circle; A.J. Institute of Medical Sciences, Kuntikana; and Yenepoya Hospital, Kodialbail.
On selecting subjects for test and control groups, care was taken to eliminate subjects with habits like smoking, tobacco chewing, and those with chronic inflammatory diseases like tuberculosis, rheumatoid arthritis, diabetes mellitus and malignancy, all of which play a vital role in contributing to oxidative stress injury. Exclusion criteria were any bone diseases or any other clinical conditions that might be involved in raised 5′-NT activity.
Approval to carry out these studies in humans was obtained from the Institutional Clinical Ethics Committee of Kasturba Medical College, Mangalore, India and their guidelines were followed during the study.

### Sample collection

Two mL of venous blood was collected in a plain vial for serum separation for the analysis of 5′-NT. Five mL of venous blood was collected in separate EDTA containers from the median cubital vein or basilic vein under strictly aseptic precautions. Of these, 0.2 mL of whole blood was used for glutathione estimation; the rest of the sample was centrifuged at 3000 rpm for 10 minutes within 3 h of collection. Plasma was discarded. The sediment was used to prepare erythrocyte suspension and used for the assay of MDA and SOD.

nje glutationa; ostatak uzorka se 10 minuta centrifugirao na 3000 okr./min unutar 3 sata od uzimanja krvi. Plazma se odbacila. Sediment se rabio za pripremu suspenzije eritrocita i za određivanje koncentracije MDA i aktivnosti SOD.

**Metode**

*Priprema suspenzije eritrocita*

Odvojeni sediment tri puta je ispran hladnom 0,9-postotnom fiziološkom otopinom, nakon čega je suspendiran u jednakom volumenu iste fiziološke otopine. Otopina je pohranjena kao 50% suspenzija stanica na 4-5 °C tijekom 24 sata, a nakon toga se rabila u testovima za određivanje koncentracije MDA i aktivnosti SOD.

*Određivanje aktivnosti 5′-nukleotidaze*

Aktivnost enzima 5′-NT u serumu određena je slijedećom metodom (5). U dvjema epruvetama mjerilo se slijedeće:

- Ukupna aktivnost: dodano je 0,2 mL seruma, 0,1 mL 0,02 M manganova sulfata i 1,5 mL 40 mM barbiton pufera, pH 7,5.
- Aktivnost tkivno nespecifične alkalne fosfataze: dodano je 0,2 mL seruma, 0,1 mL 0,02 M manganova sulfata, 1,3 mL 40 mM barbiton pufera, pH 7,5 i 0,2 mL 0,1 M niklovog klorida.
- Obje epruvete su zagrijane na 37 °C te je u svaku dodano 0,2 mL 10 mM adenozin 5′-fosfata te su 30 minuta inkubirane na 37 °C. Nakon toga je dodano 2 mL 10-postotne triklorooctene kiseline (TCA), dobro promiješano, ostavljeno kratko da odstoji i onda centrifugirano. Uzeto je 2 mL nadtaloga (0,1 mL seruma) kako bi se odredila koncentracija anorganskog fosfora. Kao slijepa proba rabio se 1 mL vode, a kao standard 1 mL fosfatnog standarda (*stock* otopina sa 6 mmol/L), svakome je dodan 1 mL TCA. U sve četiri epruvete dodano je 2M octenog pufera, pH 4,0; 0,5 mL 5-postotnog amonijevog molibdata i 0,5 mL metola (2 g metola i 10 g natrijevog sulfita u vodi otopljenog do 100 mL), promiješano, ostavljeno da odstoji 10 minuta i tada se vrijednost očitala crvenim filtrom ili na 680 nm. Koeficijent varijacije (% CV) ove metode bio je 1,96.

*Malondialdehid (MDA)*

Lipidna peroksidacija eritrocita ispitana je putem određivanja reakcijskih proizvoda tiobarbiturne kiseline (TBA). Slijedili smo metodu prema Stocku i Dormandyju s određenim izmjenama (24). Jedan mL suspenzije eritrocita dodan je u 8,5 mL 0,9-postotne fiziološke otopine i dobro promiješano. Nakon toga je dodano 0,44 M $H_2O_2$. Iz smjese je odmah izdvojeno 2,5 mL alikvota, čemu je dodan 1 mL 28-postotne TCA u 0,1 M natrijevog metaarsenita. To je dobro promiješano i ostavljeno da stoji 10 minuta, nakon čega je centrifugirano. Uzeto je 3 mL nadtaloga, čemu je dodan 1 mL jednopostotne TBA u 50 mM NaoH.

**Methods**

*Preparation of erythrocyte suspension*

The sediment separated was washed thrice with 0.9% cold normal saline and then suspended in an equal volume of the same saline solution. It was stored as 50% cell suspension at 4-5 °C for 24 h and then used for the assay of MDA and SOD.

*5′-nucleotidase determination*

Serum 5′-NT enzyme was estimated using the method reported elsewhere (5). Two test tubes were set up as follows:

- Total activity: 0.2 mL of serum were added, 0.1 mL of 0.02M manganous sulfate and 1.5 mL of 40 mM, pH 7.5 barbitone buffer.
- Non-specific alkaline phosphatase activity: 0.2 mL of serum were added, 0.1 mL of 0.02 M manganous sulfate, 1.3 mL of 40 mM, pH 7.5 barbitone buffer and 0.2 mL of 0.1M nickel chloride.
- Both test tubes warmed to 37 °C, then 0.2 mL 10mM adenosine 5′-phosphate were added to each test tube and incubated at 37 °C for 30 minutes. Then 2 mL of 10% trichloroacetic acid (TCA) were added, mixed well and left to stand briefly, then centrifuged. Two mLof supernatant were taken (=0.1 mL serum) for estimation of inorganic phosphorus. For the blank and standard 1 mL of water and 1 mL of the phosphate standard (stock solution containing 6 mmol/L) were taken for use, each with 1 mL of TCA added. To all four tubes 3 mL 2M, pH 4.0 acetate buffer, 0.5 mL of 5% ammonium molybdate and 0.5 mL metol (2 g metol and 10g sodium sulfite in water made up to 100 mL) mixed, allowed to stand for 10 minutes and then read using a red filter or at 680 nm. Percent coefficient of variation (% CV) of this method was 1.96.

*Malondialdehyde (MDA)*

Red cell lipid peroxidation was studied as thiobarbituric acid (TBA) reaction products. The method of Stocks and Dormandy was followed with certain modifications (24). One mL of erythrocyte suspension was added to 8.5 mL of 0.9% normal saline and mixed well. Then 0.5 mL of 0.44 M $H_2O_2$ was added. From the above mixture, 2.5 mL of aliquot was immediately taken, to which 1 mL of 28% TCA in 0.1 M sodium metaarsenite was added. This was mixed well and allowed to stand for 10 minutes, after which it was centrifuged. Three mL of the supernatant were then taken to which 1 mL of 1% TBA in 50 mM NaoH was added. This was then kept in a boiling water bath for 15 minutes and immediately cooled under tap water. The pink chromogen was read at 535 nm in a spectrophotometer. Values were expressed as nanomoles of MDA formed *per* dL of RBC, taking the molar extinction coefficient as $1.56 \times 10^5$. The % CV of this method was 1.88.

To je tada stavljeno na 15 minuta u kipuću vodenu kupku te nakon toga odmah ohlađeno pod mlazom tekuće vode. Apsorbancija ružičastog kromogena očitana je na spektrofotometru na 535 nm. Vrijednosti su izražene kao nmol/dL eritrocita, uzimajući koeficijent molarne apsorbancije kao 1,56 x $10^5$. CV ove metode bio je 1,88%.

*Glutation (GSH)*

Koncentracija glutationa u punoj krvi izmjerena je metodom prema Beutleru i sur. (25). 1,8 mL destilirane vode dodano je u 0,2 mL pune krvi i promiješano; dodano je 3 mL precipitirane otopine, pomiješano i ostavljeno da stoji 10 minuta na sobnoj temperaturi. Ta je smjesa tada centrifugirana. U 2 mL nadtaloga dodano je 8 mL fosfatne otopine (0,4 M) i 1 mL DTNB reagensa. Apsorbancija je očitana na 412 mn. CV za tu metodu iznosio je 1,91%.

*Superoksid-dismutaza (SOD)*

Za određivanje aktivnosti SOD slijedili smo metodu prema Fridovichu (26). Metoda se temelji na inhibiciji redukcije NBT (engl. *nitroblue tetrazolium*) radikalima superoksida nastalih iluminacijom riboflavina u prisutnosti kisika i donora elektrona. Kao temelj za određivanje aktivnosti SOD rabio se metionin. CV za ovu metodu iznosio je 1,75%.

*Priprema hemolizata*

Hemolizat je pripremljen prema metodi McCorda i Fridovicha (27). U 1 mL eritrocita (ispranih 0,9-postotnom fiziološkom otopinom) dodan je 1 mL deionizirane vode kako bi se lizirale stanice. Tome se dodalo 0,5 mL destiliranog etanola i 0,3 mL kloroforma te se dobro promiješalo i ostavilo 15 min da odstoji. Nakon toga je dodano 0,2 mL $H_2O$ i centrifugirano na 4 °C. Aktivnost SOD je vidljiva u nadtalogu, te se on rabio za test određivanja aktivnosti SOD, nakon što je razrijeđen kalij-fosfat puferom (pH 7,8; 0,05 M). 0,1 mL hemolizata razrjeđen je s 1,9 mL kalij-fosfat pufera. Tako konačno razrijeđeni hemolizat rabio se u niže opisanom postupku.

Uzete su četiri epruvete i svaka je označena jednom naljepnicom: *test*, *kontrola*, *slijepa proba*, *slijepa kontrola*. U epruvetu *test* je dodano 2,9 mL reakcijske smjese s NTB koja je sadržavala 149 mg metionina, 4,93 mL NTB (1 mg/mL), 0,63 mL riboflavina (1 mg/mL); ova smjesa se pomiješala s kalij-fosfat puferom (pH 7,8/0,05 M) do količine od 100 mL te je dodano 0,1 mL razrijeđenog hemolizata.

U epruvetu *slijepa proba* dodano je 2,9 mL iste reakcijske smjese bez NTB i 0,1 mL razrijeđenog hemolizata.

U epruvetu *kontrola* dodano je 2,9 mL iste reakcijske smjese s NTB i 0,1 mL kalij-fosfat pufera (pH 7,8/0,05 M).

U epruvetu *slijepa kontrola* dodano je 2,9 mL iste reakcijske smjese bez NTB i 0,1 mL kalij-fosfat pufera (pH 7,8/0,05 M). Svaka od ovih smjesa tada je stavljena u odgovarajuću kivetu od 10 mL. Kivete su stavljene na 10 minuta u kutiju obloženu aluminijskom folijom s fluorescentnom lam-

*Glutathione (GSH)*

Whole blood glutathione level was measured by the method of Beutler *et al*. (25). To 0.2 mL of whole blood, 1.8 mL of distilled water was added and mixed; 3 mL of precipitating solution was added, mixed and allowed to stand for 10 min at room temperature. This mixture was then centrifuged. To 2 mL of the supernatant, 8 mL of phosphate solution (0.4M) and 1 mL of DTNB reagent were added. The absorbance was read at 412 nm. The % CV of this method was 1.91.

*Superoxide dismutase (SOD)*

The method of Fridovich was followed on estimation of SOD (26). This method is based on the inhibition of nitroblue tetrazolium (NBT) reduction by superoxide radicals, generated by the illumination of riboflavin in the presence of oxygen and electron donor. Methionine was used as a basis for the assay of SOD. The % CV of this method was 1.75.

*Preparation of hemolysate*

It was done by the method of McCord and Fridovich (27). To 1 mL of erythrocytes (washed with 0.9% normal saline), 1 mL of deionized water was added to lyse the cells. To this 0.5 mL of distilled ethanol were added, followed by 0.3 mL of chloroform, mixed well and allowed to stand for 15 min. Now 0.2 mL of $H_2O$ were added and centrifuged at 4 °C. The supernatant contained SOD activity and was used for the assay of SOD; after dilution with potassium phosphate buffer (pH 7.8, 0.05 M) 0.1 mL of hemolysate was diluted with 1.9 mL of potassium phosphate buffer. It was the final diluted hemolysate that was used in the procedure described below.

Four test tubes were taken and labeled as *Test*, *Control*, *Test blank* and *Control blank*. To the *Test* tube, 2.9 mL of reaction mixture with NBT containing 149 mg of methionine, 4.93 mL of NBT (1 mg/mL), 0.63 mL of riboflavin (1 mg/mL) were added and made up to 100 mL with potassium phosphate buffer (pH 7.8/0.05M), and 0.1 mL of diluted hemolysate was added. To the 'Test blank' tube, 2.9 mL of the same reaction mixture without NBT and 0.1 mL of diluted hemolysate were added.

To the *Control test* tube, 2.9 mL of the same reaction mixture with NBT and 0.1 mL of potassium phosphate buffer (pH 7.8/0.05M) were added.

To the *Control blank* tube, 2.9 mL of the same reaction mixture without NBT and 0.1 mL of potassium phosphate buffer (pH 7.8/0.05M) were added. Each of these mixtures was now put into a 10-mL beaker. The beakers were kept in an aluminum foil lined box fitted with a 15 W fluorescent lamp for 10 minutes. The absorbance was read at 560 nm in a spectrophotometer for all the four beakers.

pom od 15W. Apsorbancija za sve četiri kivete izmjerena je u spektrofotometrom na 560 nm.

*Određivanje koncentracije hemoglobina*

Koncentracija hemoglobina u eritrocitima određena je cijanmethemoglobin metodom (28). Hemoglobin je tretiran reagensom koji sadrži kalijev heksacijanoferat, kalijev cijanid i kalijev dihidrogenfosfat (Drabkinsov reagens). Heksacijanoferat oksidira hemoglobin do methemoglobina koji se uz cijanid pretvara u cijanmethemoglobin. Apsorbancija je izmjerena u spektorfotometru na 540 nm. CV ove metode bio je 1,65%.

**Statistička analiza**

Svi biokemijski parametri među skupinama uspoređeni su Kruskal Wallisovim testom, a podaci su obrađeni statističkim programom SPSS Version 11 (statistički programski paket za društvene znanosti). Vrijednost P < 0,05 smatrala se statistički značajnom.

## Rezultati

Aktivnost 5′-NT u serumu u ovom je istraživanju bila najveća kod skupine bolesnika oboljelih od ciroze jetre, nešto niža kod skupine konzumenata alkohola, a najniža kod kontrolne skupine (tablica 1.). Pokazana je statistički značajna razlika u koncentraciji parametara između sve tri skupine (P < 0,05) (tablica 1.). Izgleda da su najviše vrijednosti aktivnosti 5′-NT u serumu kod skupine bolesnika s cirozom jetre ukazivale na veću osjetljivost testa na jetrenu opstrukciju ili oštećenje jetrenih stanica (2). Budući da je 5′-NT enzim kojega ima u obilju prvenstveno u jetri, moguće je da na njega utječu i manja područja opstrukcije.

Koncentracija MDA u eritrocitima u ovom istraživanju bila je najviša kod skupine bolesnika s cirozom jetre. Koncentracija MDA kod skupine konzumenata alkohola bila je relativno niža, a najniža je bila kod kontrolne skupine. Pokazana je statistički značajna razlika u koncentraciji parametara između sve tri skupine (P < 0,05).

*Determination of hemoglobin*

The hemoglobin content of erythrocytes was determined by the cyanmethemoglobin method (28). Hemoglobin was treated with a reagent containing potassium ferricyanide, potassium cyanide and potassium dihydrogen phosphate (Drabkins Reagent). The ferricyanide oxidizes hemoglobin to methemoglobin, which is converted to cyanmethemoglobin by cyanide. The absorbance was measured at 540 nm in a spectrophotometer. The % CV of this method was 1.65.

**Statistical analysis**

All biochemical parameters were compared between different groups using Kruskal Wallis test. The statistical software SPSS Version 11 (statistical package for social sciences) was used for this purpose. The value of P<0.05 was considered as significant.

## Results

In this study, serum 5′-NT activity was highest in the cirrhotic group, relatively high but less than cirrhotic and more than control in alcohol consumer group, and lowest in control group (Table 1). There was statistically significant difference in 5′-NT activity between three groups (P < 0.05). The highest 5′-NT activity recorded in the group of cirrhotic patients appeared to be indicative of greater sensitivity for intrahepatic obstruction or liver cell damage (2). Because it is a plentiful primary liver enzyme, 5′-NT may be more readily influenced by minute areas of obstruction.

The RBC MDA levels were highest in the group of cirrhotic patients, as shown in Table 1. MDA level was relatively lower in the group of alcohol consumers and was lowest in controls. There was statistically significant difference in MDA level between three groups (P < 0.05).

Blood glutathione levels were highest in the control group, relatively lower in alcohol consumer group and lowest in cirrhotic patient group, as shown in Table 1.

**Tablica 1.** Aktivnost 5′-nukleotidaze (5′NT), koncentracija malondialdehida (MDA) i glutationa te aktivnost superoksid dismutaze (SOD) kod kontrolne skupine, skupine konzumenata alkohola i skupine bolesnika s cirozom jetre.

**Table 1.** Concentrations of 5′-nucleotidase (5′-NT), malondialdehyde (MDA), glutathione (GSH) and superoxide dismutase (SOD) in control, alcohol consumer and cirrhotic patient groups.

| Parameters | Control (I) | Alcohol consumers (II) | Cirrhotic patients (III) | I *vs*. II (P; Kruskal Wallis) | I *vs*. III (P; Kruskal Wallis) |
|---|---|---|---|---|---|
| 5′-NT (nmol/L)[a] | 8.12 (8–10) | 12.14 (10–14) | 16.20 (13–18) | 0.045 | 0.032 |
| MDA (nmol/mL)[a] | 4.10 (4–6) | 5.22 (5–7) | 6.32 (6–8) | 0.025 | 0.015 |
| Glutathion (ng/mL)[a] | 550 (500–550) | 420 (400–450) | 200 (200–250) | 0.044 | 0.012 |
| SOD (ng/mL)[a] | 7541 (7000–7500) | 5630 (5500–6500) | 4231 (4000–5000) | 0.031 | 0.019 |

[a] median (interquartile range); N = 25; Kruskal Wallis test, 5′-NT – 5′-Nucleotidase; MDA – malanodialdehyde; SOD – superoxide dismutase

Koncentracija glutationa u krvi u ovom je istraživanju bila najviša kod kontrolne skupine, relativno niža kod skupine konzumenata alkohola, a najniža kod skupine bolesnika s cirozom jetre, kako je prikazano u tablici 1. Pokazana je statistički značajna razlika u koncentraciji parametara između sve tri skupine ($P < 0,05$).
Aktivnost SOD u eritrocitima bila je također najviša kod kontrolne skupine, relativno niža kod skupine konzumenata alkohola, a najniža kod skupine bolesnika s cirozom jetre, kako je prikazano u tablici 1. Pokazana je statistički značajna razlika u koncentraciji parametara između sve tri skupine ($P < 0,05$).

## Rasprava

U ovom su istraživanju uspoređene vrijednosti aktivnosti 5′-NT u serumu, koncentracije proizvoda lipidne peroksidacije i koncentracije antioksidansa između tri skupine ispitanika: skupine bolesnika s cirozom jetre, skupine osoba koje uzimaju alkohol i skupine zdravih ispitanika koji su predstavljali kontrolnu skupinu, ujednačene prema dobi.
Visoka aktivnost 5′-NT u serumu kod skupine bolesnika s cirozom jetre ukazuje na unutarjetrenu opstrukciju žučnih kanalića uslijed fibroze kao rezultata oštećenja jetrenih stanica. Relativno visoka aktivnost kod skupine osoba koje konzumiraju alkohol može se objasniti prethodnom tvrdnjom da katabolizam viših nukleinskih kiselina kod kroničnog trovanja alkoholom dovodi do povećanja aktivnosti ovoga enzima, budući da je on povezan s razgradnjom nukleinskih kiselina, poglavito mRNA. Kronični alkoholizam uzrokuje čitav niz bolesti jetre i drugih organa, što ovisi o količini unesenog alkohola i trajanju unosa alkohola. Učinak alkohola na jetru proteže se od masnih promjena do hepatitisa i ciroze, te će stoga i aktivnost 5′-NT imati raspon koji odgovara oštećenju jetrenih stanica i membrana žučnih vodova, što rezultira umjerenom opstrukcijom jetre. Kod alkoholizma je aktivnost enzima 5′-NT povišena.
Važna je i činjenica da pojačane aktivnosti 5′-NT u serumu, povezane s visokom vjerojatnošću prisutnosti bolesti jetre, potvrđuju kako je svako pojačanje aktivnosti 5′-NT visoko specifično za hepatobilijarne bolesti (1-2). Više koncentracije MDA kod bolesnika s cirozom i osoba koje uzimaju alkohol ukazuju na viši oksidacijski stres kod bolesnika s cirozom jetre i osoba koje uzimaju alkohol (23). Ovi su rezultati sukladni rezultatima nekoliko istraživanja koja su potvrdila uključenost slobodnih radikala u patogenezu oštećenja jetre u slučajevima ciroze jetre i kroničnog alkoholizma (29-31). Glutation ima veliko značenje u redukciji vodikovog peroksida i organskih peroksida (npr. lipidnih peroksida), u reakciji koju katalizira selen koji sadrži GHS-peroksidazu te drugi proteini koji također pokazuju aktivnost GSH-S-tranferaze (32). To je značajan endogeni antioksidans koji proizvodi stanica, a koji izravno sudjeluje u

There was statistically significant difference in glutathione concentration between three groups ($P < 0.05$).
The RBC SOD values were also highest in the control group, relatively lower in alcohol consumer group and lowest in cirrhotic patient group, as shown in Table 1. There was statistically significant difference in glutathione concentration between three groups ($P < 0.05$).

## Discussion

In this study, serum 5′-NT level, the product of lipid peroxidation and antioxidant level were compared among cirrhotic patients, alcohol consumers and normal age matched individuals as controls. The high level of 5′-NT in the cirrhotic group was suggestive of intrahepatic obstruction of bile canaliculi due to fibrosis as the result of liver cell injury. Its relatively higher activity in the alcohol consumer group found support in a previous report stating that higher nucleic acid catabolism in chronic alcohol toxicity leading to its elevation as the enzyme is related to the breakdown of nucleic acid, especially mRNA. Chronic alcoholism produces a wide spectrum of liver and other organ diseases depending on the amount and duration of alcohol intake. Hepatic effects range from fatty lesion to hepatitis and cirrhosis, and the activity of 5′-NT will also be ranging as *per* the damage done to the liver cells and bile canalicular membranes, resulting in mild to moderate biliary stasis. The 5′-NT enzyme is induced in alcoholism.
Of importance is the fact that higher 5′-NT activity in the serum of patients identified with a high probability for the presence of liver disease confirms that any increase in 5′-NT activity is highly specific for hepatobiliary disease (1,2). Higher levels of MDA in both cirrhotic patient and alcohol consumer groups were suggestive of higher oxidative stress in patients with liver cirrhosis as well as in alcohol consumers (23). These findings are consistent with the reports from several studies that confirmed the involvement of free radicals in the pathogenesis of liver injury in case of liver cirrhosis and chronic alcoholism (29-31). Glutathione is of major importance in the reduction of hydrogen peroxide and organic peroxides (e.g., lipid peroxides) in a reaction that is catalyzed by selenium containing GSH peroxidase and by other proteins that also exhibit GSH-S-transferase activity (32). It is the major endogenous antioxidant produced by the cell. It participates directly in the neutralization of free radicals, reactive oxygen compounds and maintains exogenous antioxidants such as vitamins C and E, and also plays a role in detoxification of many xenobiotics (23).
SOD plays a role in the removal of hydrogen peroxide ($H_2O_2$) formed in red cells and because hemoglobin and SOD have been shown to be in close association in red cells.

neutralizaciji slobodnih radikala, reaktivnih kisikovih spojeva i sadrži egzogene antioksidanse kao što su vitamini C i E, te ima ulogu u detoksikaciji mnogih ksenobiotika (23). SOD ima ulogu u uklanjanju vodikovog peroksida ($H_2O_2$) koji se stvara u eritrocitima i zbog toga je dokazano da su hemoglobin i SOD u uskoj vezi u eritrocitima. Smanjena koncentracija GSH i slaba aktivnost SOD u staničnim i izvanstaničnim tekućinama smanjuje njen kapacitet hvatanja slobodnih radikala kisika (engl. *oxygen derived free radical*, ODFR) te čini tkiva podložnijima oštećenjima što ih uzrokuju ODFR (33). Slaba aktivnost GSH i SOD kod bolesnika s cirozom jetre i osoba koje uzimaju alkohol ukazuje na viši oksidacijski stres kod ovih skupina (30).

Viša koncentracija lipidnih peroksida (MDA) i niža koncentracija antioksidansa (GSH i SOD) u eritrocitima bolesnika s cirozom ukazuje na promijenjen oksidacijski i antioksidacijski status (30). Smanjena koncentracija GSH čini stanice podložnijima oksidacijskom stresu.

Naši rezultati pokazuju da je antioksidacijska obrana kod ciroze jetre smanjena, što je povezano sa smanjenjem koncentracije glutationa i aktivnošću antioksidacijskog enzima SOD (18,19). Poznato je da alkohol smanjuje koncentraciju GSH, osobito u mitohondrijima kojima je obično svojstvena visoka koncentracija GSH, potrebna kako bi se uklonili reaktivni kisikovi spojevi proizvedeni tijekom aktivnosti u respiracijskom lancu.

Snižena koncentracija glutationa kod bolesnika s cirozom i u skupini konzumenata alkohola u skladu je s ostalim izvješćima (19). Do smanjenja koncentracije glutationa može doći uslijed (i) njegove funkcije u hvatanju slobodnih radikala, (ii) njegovog sudjelovanja u održavanju ne-GSH proteina sulfhidrila u reduciranom stanju, (iii) njegovog djelovanja kao kofaktora za glutation-S-transferazu (GST) tijekom detoksikacije ksenobiotika uključujući alkohol, (iv) oksidacije glutationa pomoću glutation peroksidaze za detoksikaciju hidrogen peroksida i/ili lipidnih peroksida, (v) supresije sinteze glutationa etanolom. Veći stupanj sniženja koncentracije GSH kod bolesnika s cirozom jetre koji uzimaju alkohol može biti rezultat sinergijskog djelovanja alkohola i ciroze jetre.

Najniža koncentracija glutationa izmjerena je kod skupine bolesnika s cirozom jetre, što ukazuje na to da je glutationski antioksidacijski sustav neuravnotežen kod ciroze jetre, a ti podaci podupiru hipotezu da oksidacijski stres ima važnu ulogu u razvoju ciroze jetre (19). Slaba aktivnost SOD izmjerena kod bolesnika s cirozom jetre i konzumenata alkohola ukazuju na oksidacijski stres koji bi mogao biti odgovoran za najveće uništenje arhitekture jetre. Znatno slabija aktivnost SOD izmjerena kod bolesnika s cirozom jetre ukazuje na to da je oksidacijski stres snažniji kod bolesnika s cirozom jetre nego kod osoba koje uzimaju alkohol, budući da je ciroza jetre čest ishod čitavog niza kroničnih bolesti jetre (22).

Značajno povišenje koncentracije MDA kod bolesnika s cirozom jetre i konzumenata alkohola ukazuje na to da

The low levels of GSH and SOD in cellular and extracellular fluids reduce their oxygen derived free radical scavenging capacity making the tissues more vulnerable to oxygen derived free radical (ODFR) damage (33). The low level of GSH and SOD in the cirrhotic patient and alcohol consumer groups was indicative of higher oxidative stress in patients with liver cirrhosis as well as in alcohol consumers (30).

The higher lipid peroxide levels (MDA) and lower antioxidant levels (GSH and SOD) in RBC of cirrhotic patients showed an altered oxidant and antioxidant status (30). Depletion of GSH renders the cell more susceptible to oxidative stress.

Our results demonstrated the antioxidant barrier to be impaired in liver cirrhosis, and to be associated with a decrease of glutathione level and the activity of the antioxidant enzyme SOD (18,19). Alcohol has been shown to deplete GSH levels, particularly in the mitochondria, which normally are characterized by high levels of GSH needed to eliminate the ROS generated during the respiratory chain activity.

Reduced glutathione in cirrhotic patient and alcohol consumer groups was consistent with other reports (19). This observation may be explained on the basis of (i) its utilization in scavenging free radicals, (ii) its involvement in maintaining non-GSH critical protein sulfhydryls in reduced state, (iii) acting as a co-factor for glutathione-S-transferase (GST) during detoxification of xenobiotics including alcohol, (iv) oxidation of glutathione to its oxidized form by glutathione peroxidase in detoxification of hydrogen peroxide and/or lipid peroxides, and (v) suppression of glutathione synthesis by ethanol. The more profound GSH reduction in alcohol cirrhotic patients may be due to the synergistic action of alcohol and liver cirrhosis.

The lowest level of glutathione found in the cirrhotic patient group indicated imbalance of the glutathione antioxidant system in cirrhosis, supporting the hypothesis that oxidative stress plays an important role in the development of liver cirrhosis (19). The low levels of SOD activity observed in the cirrhotic patient and alcohol consumer groups were indicative of oxidative stress that may be responsible for maximal destruction in liver architecture. A markedly lower SOD activity observed in the cirrhotic patient group indicated that oxidative stress was much more profound in liver cirrhosis patients than in alcohol consumers, as liver cirrhosis is a common outcome of a variety of chronic liver diseases (22).

A significant increase in MDA levels in the cirrhotic patient and alcohol consumer groups suggested that cirrhotic patients and alcohol consumers were at higher exposure to oxidative stress. The decreased levels of both GSH and SOD in the present study suggested that the increase in oxidative stress was associated with a proportionate

su ove skupine podložnije oksidacijskom stresu. Snižena koncentracija GSH i smanjena aktivnost SOD u ovom istraživanju ukazuju na to da s povišenjem oksidacijskog stresa dolazi do proporcionalnog slabljenja antioksidacijskog obrambenog sustava kod bolesnika s cirozom jetre i osoba koje uzimaju alkohol.

Iz ovih rezultata možemo zaključiti da aktivnost 5′-NT dosljedno raste kod bolesnika s cirozom jetre i osoba koje uzimaju alkohol ovisno o veličini oštećenja na jetri te da se kod bolesnika s cirozom jetre i osoba koje uzimaju alkohol povećava oksidacijski stres, a smanjuje antioksidacijski status, no da je opseg oksidacijskog stresa veći kod bolesnika s cirozom jetre nego kod osoba koje uzimaju alkohol.

## Zaključak

U ovom smo istraživanju pokušali odrediti opseg aktivnosti 5′-NT u serumu skupine bolesnika s cirozom jetra i skupine osoba koje uzimaju alkohol u usporedbi s kontrolnom skupinom zdravih ispitanika. Ovim smo istraživanjem također pokušali odrediti opseg oksidacijskog stresa i antioksidacijskog statusa kod bolesnika s cirozom jetre i osoba koje uzimaju alkohol.

Iz naših se rezultata može zaključiti da je aktivnost 5′-NT u serumu dosljedno viša kod skupine bolesnika s cirozom jetre i osoba koje uzimaju alkohol, sukladno veličini oštećenja jetre, oštećenju hepatobilijarnog sustava i opstrukciji jetre. Također možemo zaključiti da se povećao oksidacijski stres i smanjio antioksidacijski status kod obje promatrane skupine, bolesnika s cirozom jetre i osoba koje uzimaju alkohol, no opseg oksidacijskog stresa je veći kod bolesnika s cirozom jetre nego u osoba koje uzimaju alkohol s ili bez hepatobilijarnih bolesti u anamnezi. Parametri lipidne peroksidacije i antioksidacijske obrane mogli bi biti korisni biljezi kod promatranja bolesnika s hepatobilijarnim bolesima.

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decrease in the antioxidant defense system in cirrhotic patients and alcohol consumers.

From these findings, we can conclude that the activity of 5′-NT rises consistently in cirrhotic patients and alcohol consumers according to the extent of damage to the liver and that there is an increase in oxidative stress and a decrease of antioxidant status in both liver cirrhosis patients and alcohol consumers; however, the extent of oxidative stress is more profound in cirrhotic patients than in alcohol consumers.

## Conclusion

This study attempted to establish the extent of serum 5′-NT activity in cirrhotic patients and alcohol consumers as compared with normal individuals (controls). This study also attempted to establish the extent of oxidative stress and antioxidant status in cirrhotic patients and alcohol consumers.

Based on study results, it is concluded that the activity of serum 5′-NT is consistently higher in cirrhotic patients and alcohol consumers, according to the extent of liver damage, hepatobiliary damage, and biliary stasis. We can also conclude that there is an increase in oxidative stress and a decrease in antioxidant status in both liver cirrhosis patients and alcohol consumers, whereby the extent of oxidative stress is more profound in cirrhotic patients than in alcohol consumers with or without a history of hepatobiliary disease. The parameters of lipid peroxidation and antioxidant defense may be useful markers for monitoring patients with hepatobiliary disorders.

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