Oxidative Stress Status in Patients with Choledocholithiasis: Before and After Endoscopic Sphincterotomy and Biliary Clearance

CATALIN VICTOR SFARTI[1], ALIN CIOBICA[2,3,4]*, CAROL STANCIU[3], GHEORGHE G. BALAN[1], IRINA GARLEANU[1], ANCA TRIFAN[1]

[1]Grigore T. Popa University of Medicine and Pharmacy, 16, Universitatii Str., 700115, Iasi, Romania

[2] Alexandru Ioan Cuza University, Faculty of Biology, Department of Research, 11 Carol I, Blvd., 700506, Iasi, Romania

[3] Center of Biomedical Research, Romanian Academy, 8 Carol I Blvd.,700505, Iasi, Romania

[4] Academy of Romanian Scientists, 54 Splaiul Independentei, 050094, Bucharest, Romania

Choledocholithiasis may cause biliary obstruction which leads to hepatocellular injury. Oxidative stress has been proposed as a possible mechanism involved in this disorder. This study evaluates the oxidative stress burden in patients with choledocholithiasis and secondary cholestasis, before and after endoscopic sphincterotomy. Experimental part: Patients diagnosed with choledocholithiasis and secondary extrahepatic cholestasis were included in the study between January 1st 2016 and October 31st 2016. In all patients oxidative stress markers were collected within 2 hours before and 48 hours after therapeutic ERCP. Selected markers were superoxide dismutase (SOD), glutathione peroxidase (GPX) and malondialdehyde (MDA). The results were compared to those from a group of 40 healthy subjects. Significantly lower concentrations of SOD ($p = 0.03$) and GPX ($p < 0.0001$) activities, associated with an increased level of MDA level ($p < 0.0001$) were shown in patients before biliary clearance compared with the healthy control group. After ERCP the only oxidative stress parameter which showed improvement was the SOD specific activity ($p = 0.037$). This study shows that extrahepatic cholestasis secondary to choledocholithiasis is associated with increased oxidative stress status. After biliary clearance one oxidative stress marker was significantly improved (SOD), suggesting a possible antioxidant effect of such procedure.

Keywords: elderly, endoscopic retrograde cholangiopancreatography, superoxide dismutase, gallstones

Choledocholithiasis is a very common clinical condition, with a prevalence of about 10% to 20% of patients diagnosed with symptomatic gallstones [1, 2]. Nevertheless, up to 25% of the bile duct stones could be discovered incidentally during bile duct surgery [3]. Bile duct stones should be removed because of their increased risk for development of complications such as cholangitis, acute pancreatitis or bile duct obstruction [4]. Consequently, obstructive disease triggers extrahepatic cholestasis and jaundice, which leads to hepatocellular injury and inflammation. Extensive research is dedicated to understanding how extrahepatic cholestasis is linked to hepatocellular injury and systemic inflammation [5]. Pathogenically, besides the hepato-billiary transport of toxic organic compounds [6] or the decrease in cannalicular aquaporin-8 expression, recently it has been suggested that oxidative stress may also determine changes in the nature and function of membrane lipids and embedded proteins of the liver [7, 8] in cholestatic patients.

Oxidative stress is the condition arising from an imbalance between toxic reactive oxygen species (ROS) and antioxidant systems. Such disturbance may lead to cellular damage either when there is a lack of antioxidant capacity secondary to impaired production and distribution of antioxidant molecules [9, 10], or when reactive oxygen species are overabundant secondary to various pathogenic causes [11-15]. To date, oxidative stress has been reported to be implicated in the development and progression of various conditions [16] including many gastrointestinal disorders as irritable bowel syndrome, cirrhosis and hepatic encephalopathy [17, 18], spontaneous bacterial peritonitis [19], and inflammatory bowel diseases [20].

Although there are previous studies that have aimed to link oxidative stress to choledocholithiasis [5, 9, 21, 22], even to date there is still no clear evidence regarding such pathogenic relationship. It seems that acute biliary obstruction and secondary cholestasis determines increased concentrations of bile acids inducing peroxidation of lipids by stimulation of phagocytes' activity in inflammatory cells present after biliary tract obstruction [23]. This process is associated with production of oxygen-derived free radicals and impaired cellular antioxidant mechanisms [24-26]. At an enzymatic level, cholestasis has been linked to a decreased activity of antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPX) or catalase [22, 27, 28].

In addition to the current studies on patients, similar aspects were demonstrated also in animal models [5, 29] which showed that cholestatic liver disease is associated with increased lipid peroxidation in the kidney, brain and heart, as demonstrated by the decreased levels of glutathione or SOD and increased malondialdehyde (MDA) concentrations.

The aim of our study was to evaluate the oxidative stress status in patients with choledocholithiasis and secondary cholestasis, before and after endoscopic sphincterotomy (ES) with biliary stone clearance.

Experimental part

Materials and method

Patients. The study was performed between January 1st 2016 and October 31st 2016. A total of 35 patients were included in the study, of which 19 females and 16 males, aged between 32 and 56 years old, diagnosed with choledocholithiasis and secondary extrahepatic cholestasis, who were referred to the Endoscopy Unit of the Institute of Gastroenterology and Hepatology, Iasi, Romania for therapeutic endoscopic retrograde cholangiopancreatography (ERCP). Inclusion criteria were positive diagnosis of choledocholithiasis, age over 18, and

* email: alin.ciobica@uaic.ro

the absence of any concomitant evolutive chronic diseases. Exclusion criteria were represented by history of smoking and alcohol abuse, or history consistent of active restrictive diet. Diagnosis and management plan of choledocholithiasis were previously established clinically, by abdominal ultrasound, and by magnetic resonance cholangiography according to the criteria developed by Maple et al. [30]. The results were compared to those from a homogenous group of 40 healthy subjects aged between 35 and 60 years old, of which 21 females and 19 males. All patients signed an informed consent for both ERCP and study inclusion. The Ethical Committee of the Institute approved the study which was performed within the rigors of The Code of Ethics of the World Medical Association, and taking into account some published models and guidelines [31 - 33].

Biochemical studies. Blood samples for biochemical evidence of cholestasis and of oxidative stress markers were collected within 2 h before and 48 h after therapeutic ERCP. All blood samples were collected after a minimal period of 12 h fasting, and immediate centrifugation was performed. Serum was aliquot into Eppendorf tubes and stored at -40 degrees centigrade prior to measurement. All samples were measured in duplicate and average values were calculated. Oxidative stress markers selected were SOD and GPX specific activities and MDA levels.

Determination of SOD specific activity

SOD activity was measured by the percentage reaction inhibition rate of enzyme with a water soluble tetrazolium dye (WST-1) substrate and xanthine oxidase using a SOD Assay Kit (FLUKA, 19160) according to the manufacturer's instruction. Each endpoint assay was monitored by absorbance at 450 nm (the absorbance wavelength for the colored product of WST-1 reaction with superoxide anions) after 20 minutes of reaction time at 37 degrees centigrade. The percent inhibition was normalized by mg protein and presented as SOD activity units.

Determination of GPX specific activity

GPX activity was measured using the GPX cellular activity assay kit GCP-1 (SIGMA). The kit uses an indirect method based on the oxidation of glutathione (GSH) to oxidized glutathione (GSSG) catalyzed by GPX, which is then coupled with recycling GSSG back to GSH utilizing glutathione reductase and NADPH. The decrease in NADPH at 350nm during oxidation of NADPH to NADP is indicative for GPX activity

Determination of MDA levels

MDA levels were determined by thiobarbituric acid reactive substances (TBARs) assay as described by Atamer et al, 2016 [15]. 200 microliters of serum were added and briefly mixed with 1 mL of trichloroacetic acid at 50%, 0.9 mL of TRIS-HCl (*p*H 7.4) and 1 mL of thiobarbituric acid 0,.73%. After vortex mixing, samples were maintained at 100 degrees centigrade for 20 min; the samples were then centrifuged at 3000 rpm for 10 min and the supernatant read at 532 nm. The signal was read against an MDA standard curve and the results were expressed as nmol/mL.

Statistics. From each sample, multiple determinations were performed, the systematized data representing the mean of these replicas ± the standard deviation. Statistical analyses were performed using the t-Student test. The differences between the control and the exposed samples being considered significant at $p < 0.05$ (***$p < 0.001$ - very significant; ** $0.001 < p < 0.005$ - significant; *$0.01 < p < 0.05$ - less significant; $0.05 < p < 0.5$ - not significant).

Results and discussions

Clearance of bile duct stones was achieved in all patients. Regarding the specific activity of SOD, firstly it has been observed a significant group difference ($F(2,39) = 6$, $p = 0.006$), as shown in Figure 1. Post-hoc comparisons also showed a significant decrease in SOD specific activity in patients before ES versus controls ($p = 0.003$). Furthermore, there was a significant increase of the SOD activity in patients after ES when compared to the results before ES ($p = 0.047$). No significant differences were observed between the patients after ES and the control group ($p = 0.13$), suggesting that important SOD activity modifications may occur as a result of ES and biliary clearance.

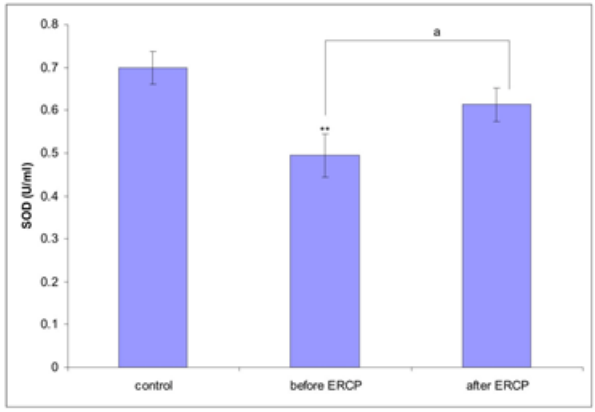

Fig. 1. Superoxide dismutase specific activity in the serum of control subjects and ERCP patients. The values are mean ± SEM (n = 40 in control, n = 35 before-ERCP and n = 35 post-ERCP). For post-hoc analysis **p = 0.003 for patients before ERCP versus the control group; p = 0.047 for patients after ERCP versus before ERCP

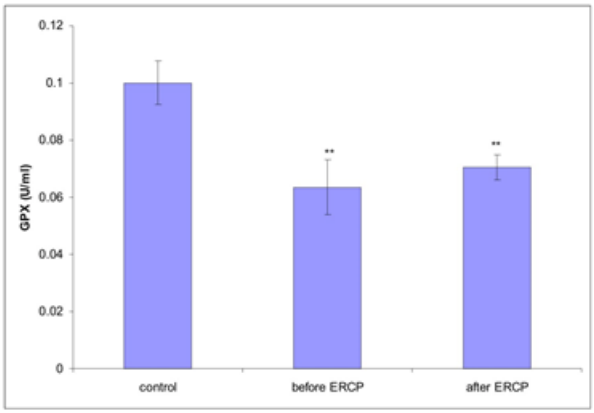

Fig. 2. Glutathione peroxidase specific activity in the serum of control subjects and ERCP patients. The values are mean ± SEM (n = 40 in control, n = 35 before-ERCP and n = 35post-ERCP groups). For post-hoc analysis **p < 0.006 versus the control group

In what GPX specific activity is concerned, we found significant differences between our groups ($F(2,39) = 6$, $p = 0.0063$), as shown in Figure 2. Moreover, when we performed the post-hoc analysis, we observed a significant decrease in GPX specific activity in both before ES ($p = 0.006$) and after ES ($p = 0.002$), as compared to the control patients. However, no significant differences were observed in terms of GPX specific activity when we compared just the study group, before and after ES ($p = 0.5$).

In the case of the lipid peroxidation marker MDA, we demonstrated very significant differences between the study groups and the controls ($F(2,39) = 29$, $p<0.0001$), as shown in Figure 3. Additionally, post-hoc analysis showed

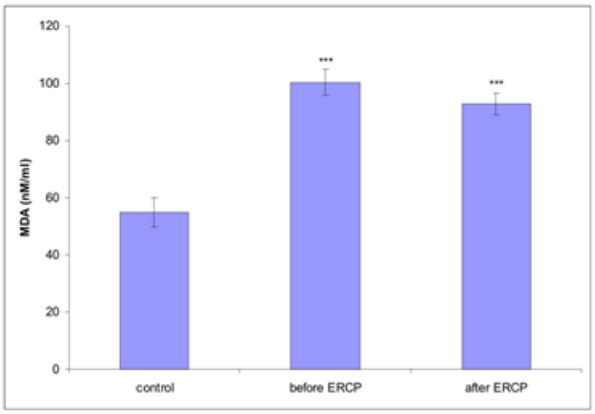

Fig. 3. Malondiadehyde levels in the serum of control subjects and ERCP patients. The values are mean ± SEM (n = 40 in control, before-ERCP and post-ERCP groups). For post-hoc analysis ***p < 0.0001 versus the control group

a significant increase for the MDA levels in both patients before ES ($p<0.0001$) and after ES ($p<0.0001$) when compared to the control patients. No significant differences were observed within the study group before and after ES in terms of MDA levels ($p = 0.45$).

Our study showed evidence of increased oxidative stress parameters in patients with choledocholithiasis and extrahepatic cholestasis before and after ES and biliary tract clearance, as expressed by decreased serum SOD and GPX antioxidant activities and increased levels of MDA as a marker of lipid peroxidation. Furthermore, we demonstrated a significant increase in the specific activity of SOD in patients after ES as compared to the results obtained before ES, suggesting a possible protective effect of the endoscopic biliary clearance when it comes to the oxidative stress status of such patients.

A potential correlation between the oxidative stress in patients with cholestasis and the subsequent inflammatory process of these patients has been previously suspected by Copple et al. [5]. Many in vitro and ex vivo studies showed significant results in this respect, especially in relationship to the increased lipid peroxidation in the affected tissues. Jungst et al. [34] has demonstrated that MDA stimulates secretion of mucin in a concentration dependent manner in gallbladder epithelial cell cultures. Moreover, Geetha et al. [35] found clear markers of increased cellular oxidative stress in gallbladder mucosal cells from patients who underwent cholecystectomy for symptomatic gallstones.

MDA levels have clearly been linked to biliary diseases causing extrahepatic cholestasis [9, 36]. As the end product of lipid peroxidation, MDA is considered by some authors the most important marker for oxidative stress [22], and a positive dynamics of the marker was proved by some in vivo animal studies with extrahepatic cholestasis through bile duct ligation (BDL) [36-38]. Such effect is considered to be a systemic one, as elevated levels of MDA were found in the kidney, brain and heart in ex vivo models after previous BLD [29]. Furthermore, elevated levels of MDA together with reduced total glutathione concentration were afterwards found also in patients with obstructive cholestasis [5, 9, 34]. Although our study shows similar results, it is thought that maximal MDA levels are achieved after 72 hours as lipid peroxidation is a late event [22, 40], therefore greater levels of such markers could be showed even after ERCP and biliary desobstruction [22]. Nevertheless, MDA levels were characterized by a negative dynamic towards normal range after resolution of cholestasis in patients, as a case-control study shows [41]. Such results are consistent also with our findings which showed a significant increase for the MDA levels in both patients before and after ES when compared to the control patients. There are studies assessing some other thiobarbituric acid reactive substances besides MDA, like the paraoxonaze-1 (PON-1) activity [21, 42, 43] which were found to be significantly lower in patients with cholelithiasis [21].

The results obtained in the case of SOD specific enzymatic activity could be in a way explained by the fact that SOD is the first line of defense against oxidative stress development. Thus, regarding the markers determined by us, SOD is an essential antioxidant enzyme which detoxifies the superoxide anion (O_2) generated by activated neutrophils and macrophages, by converting it to hydrogen peroxide (H_2O_2). GPX acts further in the extracellular environment to transform H_2O_2 into O_2 and H_2O. Along with catalase, a cellular active enzyme, SOD and GPX represent the main markers of antioxidant defense [44]. Not only SOD and GPX, but also the vitamin E and selenium levels show similar dynamics secondary to oxidative stress [44-46]. Nevertheless, there are some animal studies which found no difference in GPX activities before and after BDL [28, 39]. The results of our study are consistent with those of other clinical studies in both the dynamics of SOD and GPX [22, 47], the difference being that the other studies assessed Cu and Zn SOD activities which were afterwards correlated also with the catalase levels. Therefore, the increase of SOD could represent a compensatory process or could suggest a protective effect expressed through a decrease in the oxidative stress status after ES and biliary drainage. In this way, the results shown by us could be more relevant, especially considering the fact that currently the exact mechanisms of oxidative stress resulting from cholestasis and the impact of applied therapy are not fully understood. Nevertheless, given the large volume of data regarding the oxidative stress implication in cholestasis, recently there is also an increased interest towards the possible relevance of antioxidant therapy such as N-Acetylcysteine (NAC) to be administered in cholestasis [48-51].

Given the fact that our study has shown dynamics of biochemical parameters suggestive for an increased oxidative stress status in such patients, this suggests that antioxidants could be useful in therapy. Thus, there are recent studies on rats with BDL showing some encouraging results in this respect, in which substances like clofibrate, curcumin, thymoquinone or NAC were significantly associated with improvement of oxidative stress parameters in such animal models with acute cholestasis. Clofibrate treatment was proven to restore also protein levels and consequently to improve hepatic function while it's effect on reducing hepatic lipid peroxidation was potentially connected with the activity of liver fatty acid binding protein [52, 53]. On the other hand, the protective effect of curcumin on the systemic cholestasis-induced injury was primary related to the decrease in tumor necrosis factor-alpha levels [54]. NAC has been shown to improve both biochemical parameters like AST or alkaline phosphatase and oxidative stress parameters like malondialdehyde, luminol or glutathione in animal models with acute cholestasis similary to it's effects proven in patients [51, 55], so that NAC is to date used off-label in patients with cholestasis-induced hepatotoxicity. Not the least, also thymoquinone was proven to maintain the activity of antioxidant pathways reducing liver oxidative damage by inhibition of ductular proliferation, in this way

being potentially useful in conservation of liver function in acute cholestasis [56]. Similar antioxidant effects were also obtained related to hyperbaric oxygen aplication which has been shown to attenuate cholestasis-induced oxidative injury, liver damage, bile duct proliferation, and fibrosis in animal models [57-60]. A more complex process secondary to reducing oxidative stress was shown in a study on Ganoderma lucidum which has been proven to protect against nucleic acid damage in BDL rats with obstructive jaundice [57]. Furthermore, sulfasalazine has been linked with decreased neutrophil accumulation and subsequent lipid peroxidation in BDL rats with lipopolysaccharide-induced sepsis [61-63], showing promising perspectives for future studies on the infectious complications in acute cholestasis.

As shown both in animal models and clinical studies, patients with extrahepatic cholestasis are exposed to elevated levels of oxidative stress and the increased inflammatory status may play an important role in the progression of the oxidative processes [64-66]. Consequently, biliary clearance after therapeutic ERCP leads to a decrease in this enzymatic burden and subsequently, as our findings suggest, to a milder oxidative exposure. Clearer results on a potential normalization of the oxidative stress parameters after therapeutic ERCP could be obtained with a longer enzymatic follow-up period. Our study showed several strengths of which the prospective and controlled methodology with thorough exclusion criteria are the most consistent, and certain limitations as the relatively small number of patients included and the short follow-up period.

Conclusions

Our study indicated that extrahepatic cholestasis secondary to choledocholithiasis is associated with increased oxidative stress status. A significant increase in the specific activity of SOD in patients after ES as compared to the results obtained before ES, suggests a possible antioxidant effect of such procedure. Nevertheless, we showed evidence of increased oxidative stress parameters in patients with extrahepatic cholestasis before biliary clearance expressed by decreased serum SOD and GPX antioxidant activities and increased levels of MDA as a marker of lipid peroxidation.

References

1.LAMONT, J.T, AFDHAL, N.H., Curr. Opin. Gastroenterol., **10,** 1994, p. 523.

2.THE SOUTHERN SURGEONS CLUB, New Engl. J. Med., **325**, 1991, p. 1517.

3.DENIS, B, BAS, V, GOUDOT, C., Gastroenterology, **104**, 1993, A315.

4.LAMBERT, M. E., BETTS, C. D., HILL, J., FARAGHER, E. B., MARTIN, D. F., TWEEDLE, D. E. F., British journal of surgery, **78**, no. 4, 1991, p. 473

5.COPPLE, B.L., JAESCHKE, H., KLAASSEN, C.D., Sem. Liv. Dis., **30**, 2010, p.195.

6.BILZER, M., LAUTERBURG, B.H., J. Hepatol., **13**, 1991, p. 84.

7.CHOJKIER M., Prog. Liver Dis., **13**, 1995, p. 37.

8.MURIEL, P., SUAREZ, O.R., J. Appl. Toxicol., **14**, 1994, p. 423.

9.RIDHA, N.M., TAHER, M.A., J. Fac. Med. Baghdad, **55**, 2013, p. 73.

10.SHANI, K.H., ZUBAIR, M., RIZWAN, K., RASOOL, N., ERCISLI, S., MAHMOOD, A., ZIA-UL-HAQ, M., DIMA, L., PASCU, A.M., Oxid. Commun., **38**, nr. 3, 2015, p. 1252-1264.

11.HA, H. L., SHIN, H. J., FEITELSON, M. A., YU, D. Y., World journal of gastroenterology: WJG, **16**, no. 48, 2010, p. 6035.

12.TIMOFTE, D., TOARBA, C., HOGAS, S., COVIC, A., CIOBICA, A., CHIRITA, R., LEFTER, R., ARHIRE, L., ARCAN, O., ALEXINSCHI, O., SERBAN, D., GRAUR, M., POROCH, V., Romanian Biotechological Letters, **21**, nr. 1, 2016, p. 11246.

13.HALICIU, A.M., FOLESCU, R., ZUGUN, F., STRAT, L., POROCH, V., ZAMFIR, C.L., Rev. Chim. (Bucharest), **68**, nr. 3, 2017, p. 624.

14. TEODORESCU, A., IFTENI, P., PETRIC, P., TOMA, S., BARACAN, A., GAVRIS, C., BALAN, G.G., POROCH, V., PASCU, A.M., Rev. Chim. (Bucharest), **68**, no. 12, p. 2952.

15. GAVRIS, C., POROCH, V., SIMION, L., BARACAN, A., TOADER, E., PASCU, A.M., Rev. Chim. (Bucharest), **68**, no. 7, 2017, p. 1586.

16.HALLIWELL, B., GUTTERIDGE, J.M.C., Free radicals in biology and medicine, Oxford, UK: Clarendon; 2007.

17.IRIMIA, R., CIOBICA, A., STANCIU, C., TRIFAN, A., Arch. Biol. Sci., **65**, 2013, p. 1245.

18.IFTENI, P., TEODORESCU, A., MOGA, M.A., PASCU, A.M., MICLAUS, R.S., Neuropsychiatric Disease and Treatment, **13**, 2017, p. 201, DOI: 10.2147/NDT.S122367

19.MUNTEANU DANULESCU, R.S., CIOBICA, A., STANCIU, C., TRIFAN, A., Arch. Biol. Sci., **65**, 2013, p. 1593.

20.ACHITEI, D., CIOBICA, A., BALAN, G., GOLOGAN, E., STANCIU, C., STEFANESCU, G., Digestive diseases and sciences, **58**, no. 5, 2013, p. 1244.

21.ATAMER, A., KURDAS-OVUNC, A.O., YESIL, A., ATAMER, Y., Saudi J. Gastroenterol., **20**, 2014, p. 66.

22.ERCIN, C. N., BAGCI, S., YESILOVA, Z., AYDIN, A., SAYAL, A., ERDEM, G., AJCI, **2**, no. 4, 2008, p. 150.

23.KOUNTOURAS, J., BILLING, B.H., SCHEUER, P.J., Brit. J. Exp. Pathol., 65, 1984, p. 305.

24.GONZALEZ CORREA, J. A., DE LA CRUZ, J. P., MARTIN AURIOLES, E., LOPEZ EGEA, M. A., ORTIZ, P., DE LA CUESTA, F. S., Hepatology, 26, no. 1, 1997, p. 121.

25.MURIEL, P., SUAREZ, O.R., GONZALEZ, P., ZUNIGA, L., J. Hepatol., 21, 1994, p. 95.

26.SINGH, S., SHACKLETON, G., AH-SING, E., CHAKRABORTY, J., BAILEY, M.E., Gastroenterology, 103, 1992, p. 1625.

27.HALLIWELL, B., GUTTERIDGE, J.M., CROSS, C.E., J. Lab. Clin. Med., **119**, 1992, p. 598.

28.PASTOR, A., COLLADO, P. S., ALMAR, M., GONZALEZ-GALLEGO, J., Journal of Hepatology, **27,** no. 2, 1997, p. 363.

29.LJUBUNCIC, P., TANNE, Z., BOMZON, A., Gut, **47**, 2000, p. 710.

30.MAPLE, J. T., IKENBERRY, S. O., ANDERSON, M. A., APPALANENI, V., DECKER, G. A., EARLY, D., FUKAMI, N., Gastrointest. Endosc., **74**, no. 4, 2011, p. 731.

31.AGHEORGHIESEI CORODEANU, D.T., POROCH, V., 6th LUMEN International Conference on Rethinking Social Action Core Values, 16-19 April 2015, Iasi, Romania, Rethinking Social Action. Core Values, p. 33.

32.ROGOZEA, L., REPANOVICI, A., CRISTEA, L., BARITZ, M., MICLĂU‘, R., PASCU, A., Proceedings of the 4th WSEAS/IASME International Conference on Educational Technologies (Edute’08), Book Series: Recent Advances in Computer Engineering, Corfu, Greece, 2008, Oct. 26-28, pp. 87-90.

33.POROCH, V., AGHEORGHIESEI, D.T., Postmodern Openings, 9, no. 2, 2018, p. 225.

34.Jüngst, C., Sreejayan, N., Eder, M. I., Von Stillfried, N., Zündt, B., Spelsberg, F. W., Von Ritter, C., European journal of clinical investigation, **37**, no. 9, 2007, p. 731.

35.GEETHA, A., JBMBB, **6**, 2002, p. 427.

36.CONTE, D., FRAQUELLI, M., GIUNTA, M., CONTI, C. B., J. Gastrointestin. Liver. Dis., **20**, no. 1, p. 9.

37.ALPTEKIN N, MEHMETCIK G, UYSAL M, AYKAC-TOKER G., Pharmacol. Res., **36**, 1997, p. 243.

38. MONTILLA, P., CRUZ, A., PADILLO, F. J., TUNEZ, I., GASCON, F., MUNOZ, M. C., PERA, C., Journal of pineal research, **31,** no. 2, p. 138.

39.ORELLANA, M., RODRIGO, R., THIELEMANN, L., GUAJARDO, V., Comp. Biochem. Phys. C., **126**, 2000, p. 105.

40.PAROLA, M., LEONARDUZZI, G., ROBINO, G., ALBANO, E., POLI, G., DIANZANI, M. U., Free Radical Biology and Medicine, **20**, no. 3, p. 351.

41.VENDEMIALE, G., GRATTAGLIANO, I., LUPO, L., MEMEO, V., ALTOMARE, E., J. Hepatol., **37,** 2002, p. 601.
42.FURLONG, C.E., MARSILLACH, J., JARVIK, G.P., COSTA, L.G., Chem-Biol. Interact., **259**, 2016, p. 51.
43.FURLONG, C.E., RICHTER, R.J., SEIDEL, S.L., COSTA, L.G., MOTULSKY, A.G., Anal. Biochem., **180**, 1989, p. 242.
44.BLUM, J., FRIDOVICH, I., Arch. Biochem. Biophys., **240**, 1985, p. 500.
45.HODGSON, E.K., FRIDOVICH, I., Biochemistry, **14**, 1975, p. 5294.
46.JI, L.L., STRATMAN, F.W., LARDY, H.A., Arch. Biochem. Biophys., **263**, 1988, p. 150.
47.TOGASHI, H., SHINZAWA, H., WAKABAYASHI, H., NAKAMURA, T., YAMADA, N., TAKAHASHI, T., ISHIKAWA, M., Journal of hepatology, **11**, no. 2, 1990, p. 200.
48.AYVAZ, S., AKSU, B., INAN, M., UZUN, H., AYDIN, S., BILGI, S., PUL, M. Saudi medical journal, **30**, no. 1, 2009, p. 24.
49.OZDIL, B., KECE, C., COSAR, A., AKKIZ, H., SANDIKCI, M., J. Clin. Pharmacol., **50**, 2010, p. 1414.
50.JIN, F., CHENG, D., TAO, J. Y., ZHANG, S. L., PANG, R., GUO, Y. J., ZHAO, L., BMC gastroenterology, **13,** no. 1, 2013, p. 79.
51.TAHAN, G., TARCIN, O., TAHAN, V., EREN, F., GEDIK, N., SAHAN, E., YUCEL, O., Digestive diseases and sciences, **52**, no. 12, 2007, p. 3348.
52.WANG, G., SHEN, H., RAJARAMAN, G., ROBERTS, M. S., GONG, Y., JIANG, P., BURCZYNSKI, F., European journal of pharmacology, **560**, no. 1, 2007, p. 61.
53.OLTEANU, A.V., GILCA BLANARIU, G.E., BALAN, G.G., MITRICA, D.E., GOLOGAN, E., TIMOFTE, O., TOADER, E., STATESCU, C., DIACONESCU, S., STEFANESCU, G., Rev. Chim. (Bucharest), **69**, no. 4, 2018, p. 961.
54.TOKAÇ, M., TANER, G., AYDIN, S., ÖZKARDEª, A. B., DÜNDAR, H. Z., TAªLIPINAR, M. Y., BASARAN, N., Food and Chemical Toxicology, **61**, 2013, p. 28.
55.LUNCA, S., PERTEA, M., BOURAS, G., DUMITRU, L., HANTJISSALATAS, S.G., Rom J Gastroenterol, **14**, nr. 2, 2005; p. 151.
56.OGUZ, S., KANTER, M., ERBOGA, M., ERENOGLU, C., Journal of molecular histology, **43**, no. 2, 2012, p. 151-159.
57.AYVAZ, S., KANTER, M., AKSU, B., SAHIN, S. H., UZUN, H., ERBOGA, M., PUL, M., Journal of Surgical Research, **183**, no. 1, 2013, p. 146.
58.BOANCA, M., MITITELU-TARTAU, L., LUPUSORU, R. V., POROCH, V., BIBIRE, N., LUPUSORU, C. E., Farmacia, 63, no. 3, 2015, p. 362.
59. CIUCA, I.M., POP, L.L., ROGOBETE, A.F., ONET, D.I., et. al., Clin. Lab. 2016; 62(9):1725-1730. doi: 10.7754 / Clin. Lab.2016.160141
60. CIUCA, I.M., POP, L., RANETTI, A.E., et. al., Farmacia. 2015; 63(4)
61.AYDIN, S., AYTAC, E., UZUN, H., ALTUG, T., MANSUR, B., SAYGILI, S., SARIYAR, M., Asian Journal of Surgery, **33,** no. 4, 2010, p. 173.
62.DIRLIK, M., KARAHAN, A., CANBAZ, H., CAGLIKULEKCI, M., POLAT, A., TAMER, L., AYDIN, S., Current Therapeutic Research, **70**, no. 4, 2009, p. 299.
63.CALIN, A., LOGHIN, I., DOROBAT, C.M., et al., Mat. Plast., **53**, no. 4, 2016, p. 787.
64.DIMA, N., REZUS, E., SINGEAP, A.M., TRIFAN, A., REZUS, C., Rev. Chim. (Bucharest), **67,** no. 5, 2016, p. 948.
65.GANCEANU-RUSU, R., MITITELU-TARTAU, L., STATESCU, C., BOANCA, M., LUPUSORU, R.V., DIMA, N., REZUS, E., REZUS, C., LUPUSORU, C.E., Medical-Surgical Journal-Revista Medico-Chirurgicala, **121,** no. 3, 2017, p. 638.
66.REZUS, E., CONSTANTIN, M.M.L., REZUS, C., Rev. Chim. (Bucharest), **66,** no. 7, 2015, p. 1015.

Manuscript received: 14.01.2018