ORIGINAL ARTICLE

Post ERCP Changes in Oxidative Stress as a Possible Cause of Pancreatitis

Moshe Shiller, Dmetry Iarovinsky, Dikla Dror-Tzur, Anas Kadah, Wisam Sbeit

Department of Gastroenterology and ELIACHAR Research laboratory, Galilee Medical Center, Nahariya
Bar Ilan University, Faculty of Medicine In the Galilee, Safed, Israel

ABSTRACT

Oxidative Stress is caused by imbalance between the oxidative and anti-oxidative systems. Excessive production of reactive oxygen species has a recognized role in the pathophysiology of acute pancreatitis in the animal model. Pancreatitis is the most common unplanned event resulting from ERCP, with an incidence of about 2-9% of procedures. **Aim of the study** To examine whether ERCP causes oxidative stress, and to examine whether pre-procedure elevation of laboratory indicators of oxidative stress can predict pancreatitis following ERCP. **Materials and methods** A prospective study that was carried out over 6 months in 40 patients. We obtained markers of oxidative stress prior to ERCP, 24 and 48 hours postprocedure (MDA, AOPP- Advanced oxidation protein products, glutathione, inflammation markers (CRP, fibrinogen)). In addition, we measured serum amylase levels with the same timing. **Results** Five patients developed post-procedure pancreatitis, Ranson <3. Mean levels of markers for oxidative stress AOPP, MDA and levels of fibrinogen preprocedure were very elevated for the study population; these levels decreased in the first 24 hours, but not to normal levels. There was a marked reduction in glutathione levels after 24 hours. There was no difference in the pre- or post-procedure levels of fibrinogen, CRP, MDA or AOPP between the pancreatitis and non-pancreatitis groups. Patients with glutathione >2 micromol/liter before the procedure did not develop pancreatitis. **Conclusion** Most patients showed high oxidative stress prior to ERCP. The parameters of oxidative stress that we examined were not elevated following the procedure. With regard to the decrease observed in glutathione levels, we can infer indirectly that there was some increase in oxidative stress following the procedure.

INTRODUCTION

Free radical production occurs continuously and under various conditions, as part of normal cellular biochemical processes. Excess free radical production, originating from endogenous or exogenous sources, play an important role in the pathophysiology of many diseases. The free radicals superoxide (O_2^-) and hydroxyl radical ($^{\bullet}OH$) are partial reduction byproducts of the mitochondrial respiration process, created by the partial reduction of oxygen, together with hydrogen peroxide (H_2O_2). These metabolites, known as ROS (reactive oxygen species), react with cellular macromolecules and in many cases start chain reactions of free radicals triggering oxidative stress. Many radicals behave like oxidants or reductants. The result is that most of the radicals have short half-lives in the biological systems [1]. Superoxide may be formed by the oxidation of various molecules, including adrenalin, nuclear acids and sugar. These processes are stimulated by the presence of transition metals such as iron and copper. Enzymatic systems may also produce superoxide, for example cytochrome P450 in the liver, or enzymatic systems that participate in the synthesis of the adrenal hormones. Additional sources for the production of superoxide are phagocytic cells in the respiration processes and endothelial cells of the blood vessels [1].

Oxidative stress may be caused by external and environmental factors as well, such as the use of alcohol, medication, trauma, cold, air pollution, toxins, radiation and UV light. Today, there is no doubt that pulmonary infection caused by air pollution is a result of pulmonary oxidative stress [2]. Hydrogen peroxide is not a free radical; it belongs to the reactive oxygen species metabolites. It directly attacks proteins and enzymes that contain thiol groups.

Living cell had developed protection mechanisms against toxic oxygen metabolites. They may be divided into three main groups [1]:

Antioxidant enzymes, catalase is the first antioxidative enzyme described. It catalyses the decomposition of hydrogen peroxide to water and oxygen. Additional enzymes with antioxidative properties are glutathione peroxidase, glutathione reductase, ceruloplasmin and superoxide dismutase.

Metal binding proteins, proteins able to bind and carry transition metals' ions, mostly iron and copper (ferritin,

Received March 02nd, 2017 - Accepted March 28th, 2017
Keywords Glutathione; Oxidative Stress
Abbreviations AOPP advanced oxidation protein products; ERCP endoscopic retrograde cholangiopancreatography; ROS reactive oxygen species
Correspondence Wisam Sbeit
Institute of Gastroenterology
Galilee Medical Center
Israel
Tel +00972507887733
E-mail wisams@gmc.gov.il

transferrin, lactoferrin, ceruloplasmin). It prevents them from assisting the production of hydroxyl radicals.

Chain breaking antioxidants, when a free radical binds to another molecule, additional new radicals may be formed that may bind to other molecules to from new radicals. This is a chain reaction. A typical example of such a chain reaction is the peroxidation of lipids. This process will continue to propagate until two radicals combine to form a stable product, or until the radicals are neutralized by a chain breaking antioxidant. Antioxidants are small molecules able to accept an electron from a radical or to donate an electron to a radical with the formation of a stable product.

Antioxidants Form Two Groups

Lipid Phase Chain Breaking Antioxidants: Those processes occur in the membranes and lipoproteins. The main antioxidant in this group is Vitamin E that in addition of being an antioxidant plays a role in the membrane stabilization. α-Tocopherol is a very important human antioxidant in the tocopherols group (one of the natural forms of Vitamin E). An additional group of lipid breaking antioxidants are the carotenoids, the most important being β-carotene. Some of them are used as building stones for Vitamin A (retinal) that also has antioxidant properties, independent of the oxygen concentration [3]. A large group of antioxidants from food, fruits, beverages, such as wine and tee was identified. This group is called flavonoids and is composed of a great number of polyphenolic compounds [4, 5, 6]. Epidemiologic studies had found an inverse relation between the flavonoids intake and the frequency of chronic diseases of the coronary blood vessels [7, 8, 9].

Aqueous Phase Chain Breaking Antioxidants: These antioxidants act on radicals present in the aqueous compartment. Quantitatively, the most important antioxidant in this group is Vitamin C (ascorbate) that acts as a cofactor for several enzymes and as an antioxidant in the aqueous environment. In addition to Vitamin C, some other antioxidants may be found in plasma in high concentrations, for example uric acid and the bilirubin-albumin complex. Thiol groups present on plasma proteins are very important antioxidants. Sulfhydril groups present on plasma proteins can act as antioxidants by donating an electron to neutralize a free radical. Albumin is the main plasma antioxidant protein. It has 17 disulfide links and one cysteine residue able to neutralize peroxyl radicals [10]. This property is very important due to the albumin role as a blood carrier of lipid acids. An additional characteristic of albumin is its ability to bind copper ions thus inhibiting peroxidation processes dependent on copper presence. Albumin is destroyed by its activity as antioxidant but due to its high plasma concentration and its short half-life this has no biological importance.

In the reduced state, Glutathione is the main source of thiol within cells, but of minor importance outside the cells [11]. It is important to mention that antioxidants that interrupt the free radicals generation chain reactions in lipid or aqueous environments act in close coordination, therefore it is impossible to determine which one is more important. All depends on the conditions and circumstances present in the immediate environment and on the oxidation damage caused.

Another important characteristic of antioxidants of the chain breaking type is their ability to act as pro-oxidants. Sometimes the presence of antioxidants may enhance the oxidative damage. For example, it was reported that Vitamin C may sometimes cause a more severe oxidative damage if given together with iron [12].

Oxidative stress, caused by the imbalance between free radicals formation and the antioxidant protective mechanisms, causes damage to the lipid, protein and nuclear acids molecules. For example, LDL in the oxidized state has several characteristics that enable it to catalyze atherosclerosis processes. It is readily uptaken by macrophages, exhibits chemotactic properties towards blood macrophages and smooth muscle cells, catalyzes monocytes binding to the blood vessels endothelium and displays a cytotoxic effect on the endothelium cells [13]. Mitochondria play an important role in the life and death of the living cell. Evidence suggests that calcium accumulation in the mitochondria, accompanied by high oxidative stress, affects its permeability and depletes ATP reserves leading to the necrotic death of the cell or to the release of cytocrome C and apoptosis [14]. Antioxidants interrupt the chain reaction by removing free oxygen residues/ prevent their generation or by repairing the damage caused within the cell by free radicals [15].

Block et al. had examined in their work [16] two lipid peroxidation markers ((MDA) malonaldehyde, F_2-isoprostanes (F_2- isoP)) in a healthy population and found:

- A significant increase in the values of the markers in women and in a subgroup with high values of C-reactive protein.
- A decrease in the concentration of those markers in a subgroup with a fruits rich diet.
- High plasma values of ascorbic acid, carotene and transferrin.
- No age connection was found.
- A direct relationship between MDA level and smoking and plasma cholesterol concentration.
- A strong positive connection between F_2- isoP and BMW.

Alcohol enables the accumulation of ROS, thus affecting the body defense mechanisms against these products, mainly in the liver. Alcohol stimulates cytocrome P450 that enhances ROS production. In addition, alcohol is likely to affect the concentration of several metals thus promoting the production of ROS. Finally, alcohol is likely to lower the antioxidants levels resulting in oxidative stress and cell damage. ROS production and oxidative stress in the liver cells play a central role in the development of alcoholic liver diseases [17].

γ –tocopherol has several properties absent in α-tocopherol, the ability to reduce nitrogen dioxide to an antioxidant state and the ability to suppress the expression of the gene ras-p21 that is responsible for coding a protein with oncologic properties. It is possible that this is the connection between Vitamin E and its ability to prevent gastrointestinal tumors. Tocotrienols, the main components of Vitamin E in palm oil may play a role in the pathogenesis of breast cancer. These substances accumulate mainly in the fatty tissue that is the main component of the breast. It was found that oral administration of those substances to mice stopped breast cancer spreading. It is possible that in the future Vitamin E will be used for the treatment of the Alzheimer disease, preeclampsia and upper respiratory duct infections in adults [18].

ERCP, since the 70s pioneers in the domain of endoscopy attempted to investigate the papilla of Vater using optical fibers endoscopes [19]. Five years later, the first incisions of the papilla (sphincterotomy) were reported [20].

During the intervention, following the identification of the papilla of Vater opening, a contrast agent was injected in order to obtain the image of the bile duct and the pancreas.

There is an increase in the use of contrast agents for imaging tests in intrusive interventions. Due to this increase, nephropathy caused by contrast agents constitutes the third leading cause for acute in-hospital renal failure. This disease is accompanied by mortality and death [21]. Studies show that ROS play an important role in the pathogenesis of kidney damage caused by contrast agents and that it might be possible to reduce this damage by antioxidants such as MESNA [22].

MESNA (sodium-2-mercaptoethane sulphonate) is a small molecule whose sulfhydryl group (SH) provides it with the ability of neutralizing ROS particles. MESNA is administrated intravenously, undergoes spontaneous oxidation in the blood and is converted to its inactive form dimesna. This substance is taken up by the tubular cells of the kidney and is reduced by cell glutathione to mesna. Masna has a short half-life of 0.36 hours, therefore it is effective only a short time after its injection [23]. ROS, formed as a result of the use of contrast agents, can cause damage to the kidneys in several ways: the ionic contrast agent is hyperosmotic therefore can damage the tissue and lead to the accumulation of infected cells, polimorfonuclear leukocytes, at the injured place and to the release of ROS. Another possible mechanism is accumulation of ROS at the place affected by the contrast agent. The iodine attached to the benzoic acid ring can start the free radical generation process and the accumulation of ROS that can finally damage the glomerulus [24].

Pancreatitis is the most frequent complication of ERCP, and in most of the prospective studies was reported between 2% to 9%. It may appear as a mild disorder expressed as abdominal pain and abdominal unpleasant feeling requiring the extension of the hospitalization by one or two days or a very severe disorder accompanied by pancreas necrosis and even death [25]. The pathogenesis of this disorder is not known, technical factors, related to the procedure itself, may be involved such as: mechanical, chemical (injection of contrast agents to pancreatic tracts), hydrostatic (excess of contrast agent), enzymatic, microbiologic and thermal factors. Factors related to patients' characteristics may also play a role [26]. Attempts were made to identify in advance patients at risk of developing acute pancreatitis following ERCP intervention by using animal models. Evidence shows that activation of the gastric enzymes occurs in the acinar cells [27].

Oxidative stress may accompany various diseases and is expressed by oxidation of complex molecules including proteins. Protein glycoxidation processes, for example, are a result of the creation of covalent bonds between residues of glucose aldehydes and free amine groups on the proteins. These processes trigger unique changes in the proteins' properties such as structure and activity, ability to bind to other molecules, prone to lead to devastating results. The role played by ROS in the pathogenesis of acute pancreatitis in animal models is known. Not enough data on changes occurring in the oxidant- antioxidant systems in humans has yet been accumulated, in spite of a few works showing dependency between those changes and the severity of the disease [28]. Decrease in plasma ascorbate concentration in patients with acute pancreatitis, mainly patients with severe disease, and increase in the concentration of protein carbohydrates indicate the presence of oxidative stress. No increase in the concentration of lipids peroxidation products (MDA) was found [29]. Fibrinogen is a plasma protein, very sensitive to oxidation and defined as acute phase protein marker. Increase in its concentration increases the risk of cardiovascular disorders and causes blood coagulation problems. High concentrations of fibrinogen were found in patients with acute pancreatitis and in animal models. Attempts were made to use plasma concentration dynamics as a prognostic marker of acute pancreatitis [30]. Changes in the oxidative stress appear in an early stage of the disease and continue for a longer period of time than the clinical expression. Understanding the connection between disease severity and the intensity of oxidative stress changes may open alternative paths for the treatment of this disease [31].

The Purpose of the Work

Acute pancreatitis after ERCP intervention is the most frequent complication and its etiology is not clearly understood.

The purpose of this work is to investigate if the ERCP intervention causes a change in the oxidative stress, and if it does, in which group of patients. Oxidative stress may increase in all the patients that underwent ERCP, independently of their basic state of health, since it was proved that contrast agents have the ability to increase the oxidative stress in kidney cells. The oxidative stress may also increase as a result of the clinical state of the patient prior to the intervention and the complexity of the

intervention. Therefore we shall measure the oxidative stress markers prior and after the ERCP intervention so that each patient will constitute his own control. An additional object is to check if the levels of oxidative stress markers in plasma prior to the intervention may be used as early predictive markers of the development of acute pancreatitis as a result of the intervention.

If the results are positive, it will be possible, in phase two, in additional works, to check if early treatment with antioxidant drugs may prevent or mitigate the severity of acute pancreatitis.

Work Performance

In a prospective study, all the patients that underwent ERCP at the gastroenterology institute were assessed after receiving their informed consent to the participation in the medical study in humans as required by the Helsinki Committee procedure. The 6 months study, carried out on a population of 40 patients, was approved by the local Helsinki Committee. The following information was gathered on the patients: Demographic data, background diseases, coronary artery diseases, diabetes, general health condition and severity of disease. According to the recommendations of the American Anesthetists Society (ASA classification: anesthesia risk class) patients are classified as Class I (healthy patient) to Class V (severe systemic disease with tangible death risk). The instruction for ERCP intervention was classified according to the indications of the American Society for Gastrointestinal Endoscopy (ASGE approved indications for ERCP). In addition, the intervention process and its technical difficulty level were documented (ERCP degree of difficulty grades). The patient height and weight were measured and the BWI (body mass index) was calculated. The behavioral parameters alcohol intake and smoking were also recorded. In addition blood tests that included sugar level, amylase, cholesterol, liver and kidney functions, CRP level (Plasma C reactive protein) transferrin saturation were run. Oxidative stress markers in plasma were measured before ERCP and after 24 and 48 hours:

- MDA (malondialdehyde) plasma level (lipids oxidation product)
- AOPP (advanced oxidation protein products) level
- Glutathione plasma level (total, and reduced)

Several inflammation markers were measured before the ERCP and after 48 hours, in addition to the oxidative stress markers:

- Fibrinogen level
- CRP level
- Transferase saturation level

MDA Level Measurement

The MDA levels will be measured by a TBARS (Thiobarbituric acid reactive substances) assay using the spectrophotometric method [32].

A 250 microliter sample of plasma is diluted 1:1 with the same volume of PBS. To each sample, 1 ml of TBARS pH=7, 0.12 M solution is added. The samples are heated in a water bath at 100°C for 30 minutes and centrifuged at 800 g for 10 minutes. The absorption intensity of the supernatant liquid is measured by a spectrophotometer at a wavelength of 532 nm. The MDA levels are determined from the calibration curve (0-7.5 nmol MDA).

Fibrinogen levels will be measured in the plasma sample using a commercial kit based on the immunoturbidimetric assay, and the Cobas Mira analyzer.

Measurement of Glutathione and Oxidized Glutathione Levels

The Glutathione level in plasma was measured by the Adam & Griffith method, modified according to Kristal et al. [33] using Glutathione reductase. Two separate measurements were performed:

a. Measurement of total Glutathione (total GSH) that is equal to the reduced Glutathione and the oxidized Glutathione (GSSG).

b. Measurement of the oxidized Glutathione level only.

The reduced Glutathione level was calculated as the difference between the total Glutathione and the oxidized Glutathione levels. GSH=Total GSH – GSSG

Measurement of AOPP (Advanced Oxidation Protein Products) Level

Plasma samples were diluted 1:5 with PBS and acetic acid was added. AOPP were measured by the spectrophotometric method – the absorption was measured at a wavelength of 340 nm. AOPP concentration was calculated using a calibration curve of the solution [33, 34].

Statistical Analysis

Patients' characteristics were described using suitable location and dispersion measurements.

Quantitative variables (such as age) were described using average and standard deviation. Characteristics presented according to division into groups as well as qualitative variables (such as gender) were described using frequency and percents. The correlation between the patient's disease severity and the demographic characteristics presented as ordered variables or ration scale variables, the correlation between the disease severity, the intervention indication, the intervention difficulty level, patients' data before the intervention, and the products (changes in the oxidative stress and the frequency of the complications, were analyzed using the Spearman correlation coefficient. Non-parametric tests such as Wilcox rank sum test or Kruskal Wallis test were used for the analysis of differences in the changes scale of the oxidative stress , and in the severity of various diseases between groups of patients. When products were combined according to subjects, parametric tests such as t-test for independent samples were run.

RESULTS

The prospective study was performed for 6 months. 40 patients that underwent ERCP were tested. The patients signed an informed consent to participate in the study, according to the Helsinki Committee principles, and after the approval of the institutional committee, no patients hospitalized due to acute pancreatitis prior to the intervention were included in the research.

The average age was 74 years, the standard deviation was 12.8 years, the age range 39-96 years.

Most of the patients were male (N=24, 60%). In regard with their general health condition and the severity of their disease most of them were included in group ASA II or in group ASA III. No patient was included in group ASA V **(Table 1)**.

Most of the patients had normal weight (N=17, 42.5%) or suffered from overweight N=18, 45.0%), and a few suffered from class I or class II obesity. patients were included in the weight group according to BMI score.

80% of the patients did not smoke, 2.5% were passive smokers, 10% smoke less than 30 cigars a day, 7.5% smoke 30 cigars a day or more.

87.5% of the patients did not use to drink alcohol, 7.5% used to drink less than one drink a week, 2.5% used to drink between 1 – 2 drinks a day.

The causes for the ERCP intervention were classified according to the indications of the American Society for Gastrointestinal Endoscopy (ASGE): 37.5% were referred due to jaundice suspected of being caused by the obstruction of the biliary tract. 30.0% due to suspicion of disease of the pancreas or the biliary tract, 32.5% for papilla incision or cannulation and stent insertion.

Most of the interventions were defined as therapeutic (80%) and a few as diagnostic (20%).

The intervention process and its technical difficulty level were documented (ERCP degree of difficulty grades) in **Table 2**.

Almost all the activities performed on the bile duct during ERCP were of the lowest grade, Grade 1, (HN=32, 91.4%). The remaining activities were defined as Grade 2 (N=2, 5.7%) or 3 (N=1, 2.9%). No activities on the bile duct were performed on five patients. All the activities performed on the pancreatic ducts on 21 patients were defined as Grade 1.

The documentation includes, in addition to the intervention process and its technical difficulty grade, any unplanned events of the activity. Unplanned events were divided into two groups: unplanned events one hour after the intervention and unplanned events that occurred 24 hours after the intervention.

Eight patients suffered from abdominal pain within one hour from the intervention (N=8, 20%) and one patient suffered from vomiting after 24 hours, seven patients (17.5%) suffered from abdominal pain, one suffered had fever, five patients (12.5%) suffered from acute pancreatitis as a result of the intervention (the diagnosis was determined based on the clinical findings and the amylase blood test. For the diagnosis, results two times higher than the higher limit of the norm. Abdominal CT was not performed; the inflammation was defined as mild, Ranson's criteria <3. No bleeding or intestine perforation events occurred.

The biochemical profile of all the patients was checked (sugars, blood lipids, blood proteins, liver function) prior to the intervention. The average values of sugars, blood lipids and blood proteins were normal.

Amylase, transferrin saturation and oxidative stress markers values were measured at different time points. The average values are detailed in **Table 3**.

The average value of blood amylase before the intervention, T=0, was normal. A significant increase in its value was observed 24 hours after the intervention, and a significant decrease 48 hours after the intervention, as compared to its value after 24 hours (Paired T-test, 1 tailed, p=0.002, p=0.0005 respectively) **(Figure 1)**.

The average value of the oxidative stress marker AOPP prior to intervention was higher than the normal value, a signi9ficant decrease occurred after 24 hours, but it still was not normal. There was no change in the average value of the marker after 48 hours, as compared to its value after 24 hours (Paired T-test, 1 tailed, p=0.003, p=0.4775 respectively) **(Figure 2)**.

The antioxidant enzymatic system, glutathione, was investigated. A decrease in the average value of total GHS was observed at 24 hours after the intervention, then an increase in the average value at 48 hours after the intervention, compared with the average value after 24 hours, but these changes were not statistically significant. Similar changes, also not statistically significant, were observed in the average values of reduced glutathione and oxidized glutathione, except for the increase in the average value of oxidized glutathione after 48 hours when compared with its average value after 24 hours, which was significant. (Paired T-test, 1 tailed, p=0.0195) **(Figure 3)**.

The changes in the oxidative stress marker MDA were similar: decrease in the average value (but still above the normal value) after 24 hours when compared with its value at time zero (T=0). The decrease was statistically significant. (Paired T-test, 1 tailed, p=0.034). The increase

Table 1. ASA classification (anesthesia risk class) of the patients.

ASA CLASS	**Frequency**	**Percent**
I	**5**	**12.5**
II	**13**	**32.5**
*II*I	**15**	**37.5**
IV	**7**	**17.5**
Total	**40**	**100.0**

Table 2. ERCP degree of difficulty grade.

	Biliary procedures	Pancreatic procedures
Grade 1	Diagnostic Cholangiogram: a. Cytology b. Standard sphincterotomy and or removal of stones < 10 mm c. Stricture dilatation/ stent/ nasobiliar drainage due to extrahepatic stricture or bile leakage	Diagnostic pancreatogram Cytology
Grade 2	Diagnostic Cholangiogram with a. Anatomy B II b. Removal of stones > 10 mm c. Stricture dilatation/ stent/ nasobiliar drainage due to hilar tumors or intrahepatic stricture	Diagnostic pancreatogram with a. Anatomy B II b. Cannulation of minor papilla
Grade 3	Manometry of Oddi sphincter a. Cholangiogram b. Any therapy with B II anatomy c. Removal of intrahepatic stones or any other stone by litotripsia	Manometry of Oddi sphincter a. Pancreatoscopy b. Any pancreatic therapy including seudocyst drainage

Table 3. Average values of amylase, transferrin saturation and oxidative stress markers.

After 48 hours	After 24 hours	Prior to intervention (T=0)	Test name
165	440	95	Amylase (U/L)
124	124	139	AOPP (mmol/l)
1.07	0.94	1.36	(Glutathione) total GHS µmol/l
0.5	0.36	0.42	GSSG (Oxidized Glutathione) µmol/l
0.65	0.56	0.62	GSH (Reduced Glutathione (mmol/l)
11.9	11.5	12.64	MDA (nmol/ml)
787.5	736.5	780.15	Fibrinogen MG/DL
49.66		46.9	CRP (mg/l)
29.64		26.75	Transferrin saturation (%)

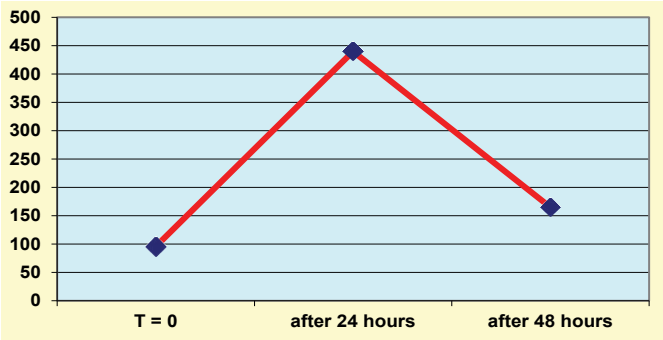

Figure 1. Changes in the average value of blood amylase.

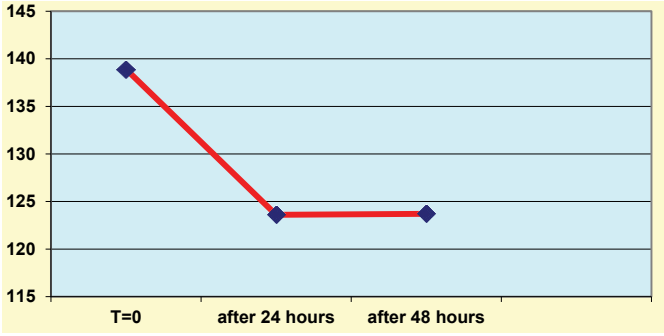

Figure 2. Changes in the average values of AOPP.

after 48 hours, to a value lower than that at T=0 was not statistically significant **(Figure 4)**.

The average value of Fibrinogen at T=0 was very high, almost twice more than the higher value of the normal range, after 24 hours there was a statistically significant decrease in the average value but it still remained above the normal range, (Paired T-test, 1 tailed, p=0.0305). After 48 hours another significant increase was observed when compared with the value after 24 hours (Paired T-test, 1 tailed, p=0.0175) **(Figure 5)**.

The average value of CRP prior to the intervention, at T=0, was very high, 9 times more than the normal range. 48 hours after the intervention, its value was still very elevated and no decrease trend was observed.

A small group of five patients suffered from mild acute pancreatitis. The average age in this group was 70, most of them were male (N=4/5, 80%). One patient was graded as Grade I of ASA Class, two patients were Grade II and two other patients were Grade III. Most of them suffered from excessive weight, none smoke or consumed alcoholic drinks. All of them underwent a therapeutic intervention. The degree of difficulty of the intervention on both the biliary and pancreatic tracts was Grade I **(Figure 6)**.

Changes in amylase levels at all times, when compared with T=0, and the change in the average value after 48 hours, when compared with the value after 24 hours, were significant (Wilcox Signed Ranks Test, 1 tailed, p=0.031). The drop in the average value of AOPP, from the value at T=0 to the value after 24 hours, was significant. The additional drop after 48 hours, when compared to the average value after 24 hours, was not significant. The changes in other parameters were not significant.

The pattern of change in the amylase blood values of the two groups was studied. One group did not develop acute

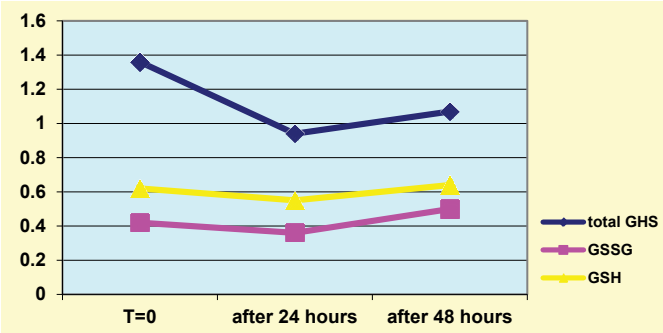

Figure 3. Changes in the average value of glutathione.

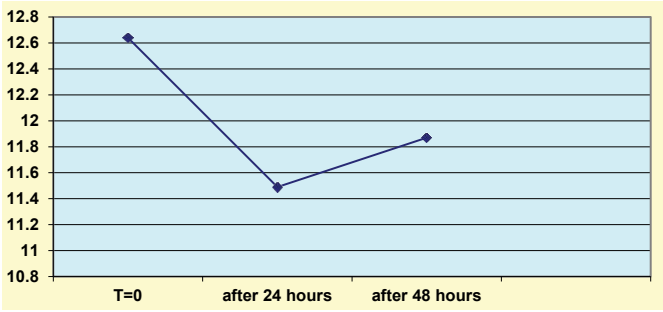

Figure 4. Changes in the average value of MDA.

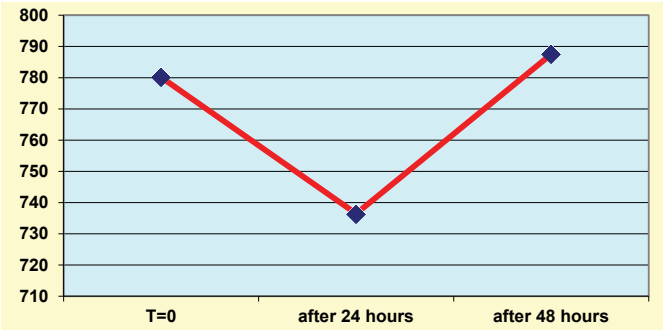

Figure 5. Changes in the average value of fibrinogen.

pancreatitis as a result of the ERCP intervention, the second one did. At T=0, prior to the beginning of the intervention, there was no statistically significant difference between the two groups (Wilcox rank sum test, 2 sided, p=0.774).

The Wilcox Signed Ranks Test was run in order to assess the differences after 24 and 48 hours: Both groups (that with and that without acute pancreatitis) showed statistically significant changes **(Table 4 and Figure 6)** at both times.

A statistical difference in amylase values was found at both times, between the group with and the group without acute pancreatitis.

A statistical difference was found in the amylase values at the same points in time between the group with and the group without acute pancreatitis, after the performance of the ERCP intervention.

At 24 hours after the intervention - Wilcoxon rank sum test 2 sided, p<<0.001.

At 48 hours after the intervention - Wilcoxon rank sum test 2 sided, p<<0.001.

As already mentioned, at T=0 there was no statistically significant difference.

In addition, in the same test, there were statistical differences in the magnitude of the changes in amylase levels of the two groups, between any two points in time: between T=0 and T=24 hours (1 sided, p<<0.001), between T=24 hours and T=48 hours (1 sided, p<<0.001), between T=0 and T=48 hours (1 sided, p<<0.005). In the group with acute pancreatitis, the changes were bigger **(Figure 7)**. No differences were found between the AOPP and MDA values in the two groups.

No difference was found in the fibrinogen concentration at the different points in time between the two groups, (with and without acute pancreatitis).

In the group of patients with acute pancreatitis (N=5) there were no statistical differences in the fibrinogen concentration at both points in time, on the other hand, in the group of patients without acute pancreatitis (N=34) statistical differences were found between T=24 hours and between T=24 hours and T=48 hours. No difference was found between T=0 and T=48 hours.

The magnitude of the change in the fibrinogen concentration between any two points in time was not different in patients with and without acute pancreatitis.

In the group of patients with acute pancreatitis (N=5) there was no significant difference in the CRP values between T=0 and T=48 hours. In the group of patients without acute pancreatitis no difference was found between T=0 and T=48 hours, but a difference was found in the increase of the values at the two points in time, in the

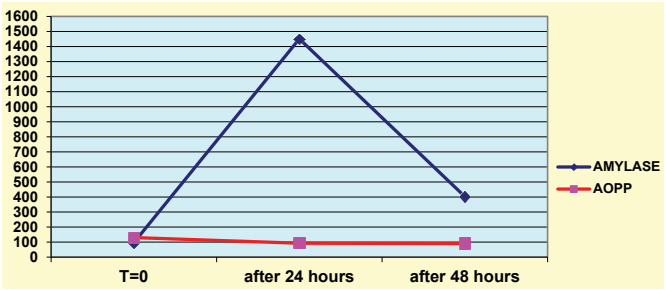

Figure 6. Changes in the average values of amylase and AOPP in a group of patients with acute pancreatitis.

Table 4. Changes in the amylase values in patients groups with and without acute pancreatitis.

Time: 48 hours - 0	Time: 48 h – 24 h	Time: 24 h - 0	
P=0.035	P=0.004	P=0.006	Without acute pancreatitis
P=0.031	P=0.031	P=0.031	With acute pancreatitis

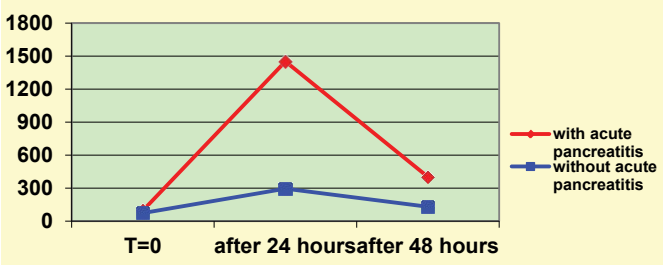

Figure 7. Changes in the amylase values in patients groups with and without acute pancreatitis.

group of patients with acute pancreatitis the change was bigger (Wilcoxon rank sum test 1 sided, p=0.014). there was no change in the CRP value at T=0 between the two groups, and at T=48 hours its value in the group with acute pancreatitis was two times higher (Wilcoxon rank sum test 1 sided, p=0.035).

Since it is known that the sugar levels and the renal function affect the oxidative stress, the correlation between the sugar levels and the renal function and the oxidative stress parameters at T=0 (prior to the intervention) was investigated: the percentage of diabetic patients and the percentage of patients with impaired renal function was similar in the group of patients with normal oxidative stress values and in the group with high values.

The value of glutathione in the patients that suffered from acute pancreatitis (N=5) was lower than 2 μmol/l. No cases of acute pancreatitis were observed. This difference was not statistically significant, most probably due to sample size.

No statistically significant correlation was found in the Nonparametric Correlations test (Spearman`s Correlation Coefficient test) between the levels of sugar, AOPP, MDA and glutathione at T=0.

The correlation between the general health status of the patients, as expressed by their ASA classification, and the oxidative stress parameters (Wilcoxon rank sum test, 1 sided**)** was evaluated: the group of patients with higher oxidative stress levels were not graded higher on the ASA scale than the group with normal values. Spearman's correlation test did not show a positive correlation between ASA grade and the levels of AOPP, MDA and the blood concentration of glutathione at T=0. The analysis of the correlation between the various oxidative stress parameters showed a negative correlation between glutathione and MDA (Spearman`s Correlation Coefficient test, Rs=-0.280, 1 sided, p-0.044) and between glutathione and AOPP (Spearman`s Correlation Coefficient test, Rs=-0.341,1 sided, p-0.020) and a positive correlation between the blood concentrations of MDA and AOPP at T=0 **(**Rs=0.551, 1 sided, p<<0.001). No correlation was found between gender and oxidative stress parameters (AOPP, MDA and glutathione level), or between inflammation parameters (CRP) and fibrinogen. No correlation was found between age and oxidative stress parameters (AOPP, MDA and glutathione level) or between BMI values and these parameters.

Due to the small number of smokers, the correlation between smoking and oxidative stress parameters was not evaluated, neither was the correlation between alcohol and oxidative stress parameters, due to the low consumption of alcohol reported.

DISCUSSION

The number of studies related to the damage to cells and tissues caused by oxidative stress had significantly increased during the last years. The living cells had developed protection mechanisms against the toxic oxidation metabolites, but these mechanisms do not ensure complete prevention of the damage. The cells are constantly under a certain level of oxidative stress, accompanied by diseases. It must be emphasized that it is not always clear whether the oxidative stress is the cause or the result of the disease.

Acute pancreatitis is the most frequent non planned event that occurs after ERCP interventions. Most of the prospective studies showed an incidence of 2% to 9% or more. The severity of the disease ranged from a mild disorder expressed as abdominal pain and abdominal unpleasant feeling requiring the extension of the hospitalization by one or two days, to a very severe disorder accompanied by pancreas necrosis and even death. The pathogenesis of this disorder is not known. Independently of the mechanism that triggers the onset, the disease follows the regular path of acute pancreatitis. Oxidative stress may play a role in the pathogenesis of acute pancreatitis [35]. The role played by surplus ROS in the pathogenesis of acute pancreatitis in animal models is known. Not enough data on changes occurring in the balance between oxidant and antioxidant systems in humans has yet been accumulated. At present, there is insufficient clinical data to support the administration of antioxidant drugs, alone or in combination with conventional therapy, for the treatment of acute pancreatitis [36].

In addition, it was proved that the contrast agents used in the ERCP intervention are able to intensify the oxidative stress. Therefore it was expected to observe an increase in the oxidative stress as a result of the ERCP intervention. On the other hand, the probability that candidates to the ERCP intervention may present high values of oxidative stress prior to the intervention, as part of their condition, could not be rejected.

In our study, we found high average values of oxidative stress markers: AOPP and MDA at time zero, that is prior to the intervention. Our conclusion is that oxidative stress accompanied the patients prior to the ERCP intervention. No increase in the concentrations of the measured oxidative stress markers occurred as a result of the intervention. On the contrary, there was a statistically significant decrease in their values at 24 hours after the intervention, compared with time zero. This decrease didn't result in normal values. The conclusion is that high oxidative stress was present in the candidates to ERCP intervention and that the intervention itself does not trigger an increase in the values of the evaluated parameters, but on the contrary – a decrease in their values.

Most of the ERCP interventions were defined therapeutic. This may be the reason why the treatment of the basic problem of the patients caused a significant decrease in the values of the oxidative stress markers. If this is true, then why the values did not reach normal values? And why 48 hours after the intervention the values were still above normal? Is it possible that this is a hidden expression of the fact that the intervention triggers oxidative stress, therefore between 24 to 48 hours after

the intervention there was no additional decrease in the oxidative stress values, but on the contrary the MDA values after 48 hours were higher than those after 24 hours. It is possible that the expected changed could have been identified should we have measured additional oxidative stress markers. It may be concluded indirectly, from the decrease in the glutathione values, that the oxidative stress may have been intensified as a result of the intervention, thus triggering a response of the protective system in the form of a decrease in the glutathione values (antioxidative system).

The average values of the inflammation markers fibrinogen and CRP were also very high at time zero. 48 hours after the intervention their values were still very high and no decrease trend was observed in their values. No positive correlation was found between the general health condition of the patients (ASA Classification), gender, age, BMI (body mass index) values and the oxidative stress markers.

As mentioned above, there was a small group of five patients that suffered from acute pancreatitis following the ERCP intervention. No changes in the oxidative stress level and in the concentration of the inflammation markers were found in this group at the various time points. Statistically significant differences were found in the magnitude of the change in the amylase blood concentration between the group with and the group without acute pancreatitis. In the group without acute pancreatitis the changes were bigger. No difference was found in the fibrinogen concentration at the different points in time between the two groups. The magnitude of the change in the fibrinogen concentration between any two points in time was not different in patients with and without acute pancreatitis. The increase in the value of the inflammation marker CRP was borderline significant from time zero to 48 hours after the intervention in the group with acute pancreatitis.

The value of glutathione in all the patients that suffered from acute pancreatitis was lower than 2 μmol/l at time zero. No cases of acute pancreatitis were observed in patients with glutathione levels above 2 μmol/l. This difference was not statistically significant, most probably due to sample size.

Acute pancreatitis is the most frequent non planned event that occurs after ERCP interventions. Since the incidence of this event is not low, and may sometimes reach 10% and more, the large number of studies on this matter is aimed at a better understanding of its pathogenesis and at establishing a system that may protect the patients against it.

Although oxidative stress may play an important role in the pathogenesis of post ERCP acute pancreatitis, a meta-analysis that include 11 studies (3010 patients) could not prove that the administration of antioxidants leads to a change the incidence and the severity of acute pancreatitis [37, 38].

In the present work we did not succeed in proving directly, and based on the parameters evaluated, that the ERCP intervention triggers by itself an increase in the oxidative stress. We couldn't prove that the oxidative stress was higher in the group that suffered from post ERCP acute pancreatitis. We could not define the group that suffered from acute pancreatitis and could not build a unique and characteristic model for this group. We found in literature a work showing that protein carbonyls could be useful markers of oxidative stress in patients with acute pancreatitis caused by different factors. These findings differ from ours. We did not find similar changes in the AOPP values [29]. That work did not report either higher MDA values in patients with acute pancreatitis, compared with the control group.

This result was unexpected, is it possible that the sensitivity of the test was affected during plasma dilution. On the other hand the test was sensitive enough when run on pancreatic tissue and pancreatic liquid in animal models. Most of the data on changes in the MDA levels come from works on animal models.

It is also possible that lipids oxidation processes are transient and quick, therefore if samples are not taken early enough and close to the event, the early changes in MDA concentration cannot be identified.

Indeed there are works that show that the peak concentration of MDA occurs within 4 hours from the onset of the disease in the animal [29].

It is possible that we did not succeed to show in our work and increase in the oxidative stress because the MDA level was measured only 24 hours after the ERP intervention. It is technically difficult to take plasma samples for the MDA level tests at such short periods of time therefore the work must be repeated in order to measure the oxidative stress markers protein carbonyls, whose half-life is much longer thus offering the possibility to identify changes in their levels at different points in time.

Many other studies conducted in the past that aimed at a better understanding of the pathology of acute pancreatitis offered several explanations: technical reasons such as multiple papilla catheterization attempts, injection of contrast agents, local heat produced during papilla incision, infection and patients' characteristics.

The ability to develop a predictive tool for the development of acute post ERCP pancreatitis is very important in the process of informed consent for better clarifying the patient's risk to contract this disease and for considering the suitability of this procedure based on this risk.

Many attempts were made since the development of the ERCP procedure to identify in advance the patient's risk of contracting the disease. The present assumption is that past history of acute post ERCP pancreatitis, sphincter of Oddi dysfunction, women, normal serum bilirubine levels are factors related to patient's characteristics.

Fibrinogen is a plasma protein, very sensitive to oxidation and defined as a marker of the type acute phase protein. An increase in its concentration accelerates

and enhances the risk for cardiovascular diseases and adversely affects the function of the coagulation system. A high level of fibrinogen was also found in patients with acute pancreatitis and in the animal model. Attempts were made to use the dynamics of plasma concentrations as a prognostic marker for acute pancreatitis [30]. Changes in the oxidative stress probably appear in an early stage of the disease and continue for a longer time than the clinical expression. The correlation between the severity of the disease and the intensity of the change in oxidative stress may open new options for additional therapy for this disease [31].

In our work it was not proved that the oxidative stress markers measured and the inflammation markers (including fibrinogen) may be used as early predictive markers for the development of acute post ERCP pancreatitis. On the other hand it is possible that the glutathione level prior to the intervention may constitute an additional early predictive marker for the development of acute post ERCP pancreatitis. Patients with levels above 2 μmol/ l did not develop acute pancreatitis. This assumption must be further examined in large samples.

CONCLUSION

Most of the patients suffered from high oxidative stress prior to the ERCP intervention. No direct increase in the oxidative stress was identified based on the measured oxidative stress parameters. It may be concluded indirectly, based on the decrease in the glutathione levels that the oxidative stress increased as a result of the intervention. The oxidative stress parameters and inflammation markers measured are not good predictive markers for the development of acute pancreatitis. It is possible that glutathione (oxidative system) may constitute an early predictive marker. One limitation of this study is a small number of sample size. Further studies with larger samples are needed in order to reinforce this assumption.

Conflict of Interest

The authors have declared that no competing interests exist.

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