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Time-related alterations of superoxide radical levels in diverse organs of bile duct-ligated rats

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Abstract
The time-related alterations of superoxide radical measured *in vivo* by employing an ultrasensitive fluorescent assay in the liver, intestine, kidney and brain of rats with experimentally induced obstructive jaundice was investigated. Eighteen rats were randomly divided into Group A, rats subjected to sham operation, and Group B, rats subjected to bile duct ligation (BDL). Three rats from each group were subsequently killed at different time points post-operatively (1, 5 and 10 days). As compared to sham-operated, BDL rats showed a gradual increase with time of superoxide radical in the intestine, liver, kidney and brain: for animals sacrificed on the 1st, 5th and 10th day the increase was 45%, 50% and 96% in the liver, 76%, 81% and 118% in the intestine, 64%, 71% and 110% in the kidney and 76%, 95% and 142% in the brain, respectively. This study provides direct evidence of an early appearance of oxidative stress in diverse organs, implying a uniform systemic response to biliary obstruction and emphasizing the need of early bile flow restoration.

Keywords: *Oxidative stress, obstructive jaundice, superoxide radical, dihydroethidine, reactive oxygen species, free radicals*

Introduction

Experimental and clinical studies have shown that the pathophysiological sequelae of events that follow the extrahepatic biliary obstruction may cause multiple organs failure due to the accumulation in the systemic circulation of (i) bile acids and in particular their hydrophobic types, which are well known pro-oxidants and cellular toxins [1,2] and (ii) endotoxins which activate a systemic inflammatory response [3]. The exploration of these systemic effects of obstructive jaundice has demonstrated the presence of high oxidative stress levels in the liver and the intestine, but also in remote organs such as the kidneys, heart, blood and even brain [4–7].

Specifically, we have previously shown that obstructive jaundice of 10 days' duration in rats induces significant alterations of intestinal and hepatic lipid peroxidation, protein oxidation and thiol redox state (with oxidation of non-protein and protein thiols) [8–10]. Additionally, in a previous study we found that, despite brain homeostasis and the protection of the blood–brain barrier, experimental jaundice caused changes of brain oxidative state, assessed by a battery of biochemical markers, as early as 5 days after the remote incident of bile duct ligation (BDL) in rats [6]. Investigating in more depth systemic oxidative stress alterations in obstructive jaundice, we directly assessed the formation rate

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of superoxide radical ($O_2^{\bullet -}$), which is the primary parameter of oxidative stress, and found that it is significantly increased in the intestine, liver, kidneys and brain after 10 days of obstructive jaundice in rats [7,11].

The present study was undertaken to investigate the time-related alterations of $O_2^{\bullet -}$ production rate in organs functionally affected by obstructive jaundice, e.g. the intestine, liver, kidneys and brain. The potential parallel evolution and severity of oxidative stress in these organs with progression of cholestasis in time (from the 1st to the 5th and 10th day after BDL) is of great pathophysiological interest and would provide more convincing evidence for the causal relation of cholestasis with systemic oxidative stress.

Materials and methods

Animal model and experimental design

Eighteen male albino Wistar rats, weighing between 250–350 g, were used. The animals were housed in stainless-steel cages (two rats per cage) under controlled temperature and humidity conditions, with 12-h dark/light cycles. They were maintained on standard laboratory diet with tap water *ad libitum* throughout the experiment, except for an overnight fast the night before surgery. Animals were randomly divided on day 0, into two groups; Group A ($n = 9$): rats subjected to sham operation and Group B ($n = 9$): rats subjected to bile duct ligation (BDL). Three rats from each group were subsequently killed at different time points: on the 1st, 5th and 10th day after surgical procedure.

Surgical procedure

All surgical procedures were performed under strict sterile conditions using light ether anaesthesia and at the same time of the day (between 14:00 and 15:00). All experimental animals underwent laparotomy on day 0. Before laparotomy, the incision site was prepared with shaving and topical application (three times) of an antiseptic solution of Povidone Iodine 10% and was infiltrated with lidocaine 1% for preemptive analgesia. Via a 1 cm upper midline incision, the gastroduodenal ligament was isolated and the common bile duct was mobilized. In the animals of group B, the common bile duct was further doubly ligated with a 4-0 silk suture and was transected between the ligatures. The abdominal incision was closed in two layers with 4-0 and 2-0 polyglycolic acid and nylon sutures, respectively. All animals had an almost immediate and uneventful recovery from surgery and were returned to clean cages with free access to water and laboratory diet. Thereafter, their clinical status was observed daily. Twenty-four hours post-laparotomy, three rats of group A and three rats of group B were intraperitoneally injected with 1 ml solution of the $O_2^{\bullet -}$ trap dihydroethidine (DHE) (described below in detail). The injection was given in the right lower quadrant of the abdomen after topical application of an antiseptic solution of Povidone Iodine 10% and under light ether anaesthesia. Seventy-five minutes later these animals were sacrificed by decapitation and immediately the intestine, liver, kidneys and brain were quickly removed for further analyses. Tissue was immediately stored at -70°C and homogenized no more than 2 days after storage (it can be stored frozen for at least 1 week without any effect on superoxide radical determination). The exact procedure of DHE injection and sacrifice was carried out for the remaining animals on the 5th and 10th day from surgery.

The experiments were carried out according to international standards on animal welfare (86/609/EEC) and to the guidelines of the Ethics Committee of Patras University Hospital, Patras, Greece. The study protocol was approved by the local ethics committee.

Reagents

Dihydroethidine (DHE), horseradish peroxidase, DNA type III (from salmon testes), Bovine serum albumin (BSA, fraction V), Coomassie Brilliant Blue G-250 (CBB-G250), Triton X-100 and Dowex 50X-8(mesh 400) were from Sigma (St. Louis, MO). Dimethyl sulphoxide, acetone, chloroform, acetonitrile, absolute methanol and ethanol, hydrogen peroxide, sodium cyanide, ammonium ferrous sulphate, sorbitol and trifluoroacetic acid were from Merck (Darmstadt, Germany). Hydrophobic Oasis HLB 1 cm^3 (30 mg) extraction cartridges were from Waters Corp (Milford, MA). All reagents and solvents used were of the highest purity.

Administration of DHE ($O_2^{\bullet -}$ trap)

The DHE solution, which was injected intraperitoneally to the rats, was prepared fresh (before its administration) as follows. Specifically, for a rat of 300 g weight 2.5 mg DHE was initially dissolved in 0.4 ml 100% DMSO. This solution was then brought to 40% DMSO by drop-wise addition of 0.6 ml distilled-deionized water, followed each time by vortexing in order to avoid cloudiness. DHE was in excess so as to trap efficiently the $O_2^{\bullet -}$ formed in the tested organs over the experimental incubation period of 75 min. For ensuring this, several doses of DHE were injected in the control/tested organisms over different incubation intervals and the rate of $O_2^{\bullet -}$ and unreacted DHE in the tested organs was determined. After running these preliminary experiments, the proper DHE dose was established based on following criteria: (i) 2-OH-E^+ formation rate is constant at several incubation periods up to 75 min

and (ii) that unreacted HE is detected in the rat tissues [12,13].

Tissue treatment

The removed intestine, liver, kidney and brain tissues were homogenized with a glass-glass Potter-Elvehjem homogenizer in 1:1 tissue wet weight:volume ice-cold phosphate buffer (50 mM, pH 7.8, containing 10 mM sodium cyanide as inhibitor of endogenous non-specific peroxidases, which are possible decomposers of 2-HO-ethidium in the presence of hydrogen peroxide) [12,13]. A non-toxic alternative to the highly toxic sodium cyanide would be the addition of 5 U catalase to the homogenization buffer in order to destroy any endogenous H_2O_2 substrate for non-specific peroxidases.

Superoxide radical assay

The assay is based on the 1:1 molar stoichiometric reaction of $O_2^{\cdot -}$ with DHE that results in the formation of the specific product 2-OH-ethidium, the formation rate of which is measured and converted to $O_2^{\cdot -}$ production rate [12,13]. 2-OH-Ethidium was estimated after being extracted from the tissue in alkaline acetone, isolated via cation and hydrophobic microcolumn chromatographies and quantified by the use of its fluorescence properties and its destruction by hydrogen peroxide (catalysed by horseradish peroxidase). Fluorescence measurements were performed in a quartz microcuvette (internal dimensions $4 \times 4 \times 45$ mm) fit into its appropriate holder, using a Shimadzu RF-1501 spectrofluorometer set at 10 nm excitation/emission slit width and at high sensitivity. The concentration of 2-OH-ethidium was quantified by its standard curve constructed from a stock solution made by the X/XO superoxide generating system in the presence of certain concentration of DHE [12,13]. The concentration of 2-OH-ethidium in this stock solution was equal to the concentration of consumed DHE since 2-OH-ethidium is the sole product of the reaction of $O_2^{\cdot -}$ with DHE. Thus, the standard curve of 2-OH-ethidium vs fluorescence units (FU) at ex/em 515/567 nm (in the presence of 0.12 mg/ml DNA) can be determined and the 2-OH-ethidium formed *in vivo* can be quantified by the $O_2^{\cdot -}$ assay. In order to calculate the production rate of $O_2^{\cdot -}$, the stoichiometric molar ratio 1:1 for the rate of $O_2^{\cdot -}$ (i.e. the rate of 2-OH-ethidium at 0–70 nM/min $O_2^{\cdot -}$ formation) was used [12,13].

Protein concentration assay

Protein in sample homogenates was determined by a modification of a Coomassie Brilliant Blue-based method [14]. Specifically, 0.063 ml of various dilutions of the homogenate was mixed with 0.02 ml 0.5% (v/v) Triton X-100 and 0.017 ml 6 N HCl. The mixture was incubated at 100°C for 10 min, brought to room temperature and mixed with 0.9 ml 0.033% (w/v) Coomassie Brilliant Blue G-250 stock reagent (made in 0.5 N HCl, stirred for 30 min and filtrated through Whatman #1 filter paper by water pump aspiration and stored in dark) and incubated for 5 min at room temperature. The absorbance at 620 nm (against appropriate sample and reagent blanks) of the mixture was converted to protein mg from a 0–0.05 mg BSA standard curve using a Shimadzu UV-VIS 1201 spectrophotometer.

Statistical analysis

Data were analysed using the SPSS statistical package (SPSS Inc, 2001, Release 11.0.0, USA). An independent-samples *t*-test was applied for comparison between sham-operated and BDL rats. Multiple-groups comparisons were performed by means of 1-way ANOVA, followed by Bonferroni's post-hoc test, since variances across groups were equal. A *p*-value of less than 0.05 was considered as significant.

Results

All animals survived the experiment and the samples were successfully analysed. During the experimental period of 10 days, operated animals did not develop signs of significant pain [15] and were clinically jaundiced within 3 days. At each studied post-operative time (1st, 5th and 10th day) there were no differences in body weight between sham and BDL rats or with pre-operative values for each group separately. In addition, at reoperation on 1st, 5th and 10th days, it was found that the ligation and division of the common bile duct in rats of group B had been successful in all cases and resulted in dilatation of the common bile duct remnant proximal to the ligature without signs of bile leakage.

Measurements of superoxide radical production showed a statistically significant elevation in the liver, intestine, kidney and brain tissues of BDL rats being sacrificed on the 1st, 5th and 10th day as opposed to the corresponding sham-operated rats (for comparison between BDL and sham animals in each organ and time lapse after operation p-value was < 0.05). In all four organs of sham and BDL rats, $O_2^{\cdot -}$ levels gradually increased from 1st day to 10th day ($p < 0.02$, ANOVA Bonferroni post-hoc test) except for the brain where the sham group showed no significant increase at the 10th vs the 1st day; in BDL rats the pair-wise multiple comparisons analysis showed that the $O_2^{\cdot -}$ difference between 1st and 5th day reach significant levels for intestine, liver and brain ($p < 0.05$). Since the $O_2^{\cdot -}$ formation rate in the four organs were differently affected by the sham operation, the net effect of BDL was assessed by

calculating the percentage of $O_2^{\cdot -}$ value increase in BDL rats over their corresponding sham-operated rats at each time point [(BDL $O_2^{\cdot -}$ − sham $O_2^{\cdot -}$) × 100/sham $O_2^{\cdot -}$]. In the BDL animals sacrificed on the 1st, 5th and 10th day the superoxide radical increased in the liver by 45%, 50% and 96%, in the intestine by 76%, 81% and 118%, in the kidneys by 64%, 71% and 110% and in the brain by 76%, 95% and 142%, respectively (Figure 1).

Discussion

ROS are highly unstable molecules that demonstrate a continuous turn-over being neutralized by specific cellular defense systems. The dramatic increase of ROS levels, a situation known as oxidative stress, has been recognized as part of many and diverse pathological conditions, including obstructive jaundice [4,16]. This study presents direct evidence of oxidative stress in all examined organs, confirming the results of a previous study [7], which showed 10 days after BDL an increase of $O_2^{\cdot -}$ levels by 136% in the intestine (as opposed to 118% in the current study), by 104% in the liver (96% in the current study) and by 95% (110% in the current study) in the kidney of jaundiced rats over sham groups. In addition, increase of $O_2^{\cdot -}$ production in the brain (142% on the 10th day after BDL) was also shown here. The main novelty of this study is the evaluation of oxidative stress over time. The findings of our experiments showed that as early as 24 h after initiation of BDL $O_2^{\cdot -}$ was raised in the brain and the kidneys as well as in the liver and the intestine. In each of four organs a comparable, gradual increase of $O_2^{\cdot -}$ production over time from the 1st to 5th and 10th day following BDL was demonstrated. One could hypothesize that oxidative stress may precede or be more severe in the gut-liver axis than in remote organs. However, this was not suggested by our findings. Instead, a systemic reaction triggered by BDL was postulated. It appears that circulatory factors contributed in various organs cause similar changes of oxidative state in diverse body tissues. It is notable that, 10 days after BDL the percentage $O_2^{\cdot -}$ increase was higher in the brain of BDL rats than in their other three organs. A methodological issue concerns the absolute values of $O_2^{\cdot -}$ concentration in the sham groups which were ~ 3-fold higher than those in the previous study [7]. This dissimilarity, however, which is possibly due to metabolic variations of the tested rats, did not affect the results since the two studies showed a comparable increase of $O_2^{\cdot -}$ in the all examined organs of BDL rats.

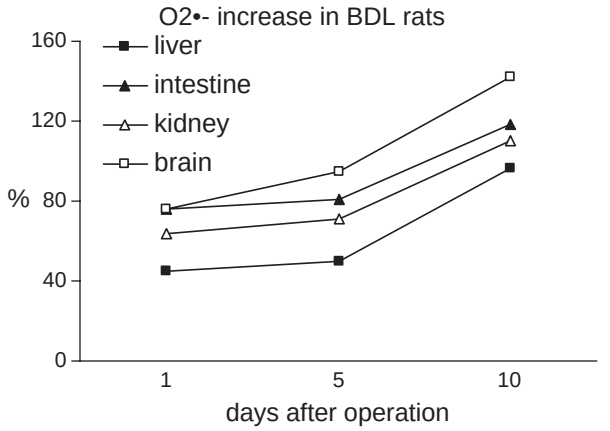

Figure 1. Percentages of superoxide radical ($O_2^{\cdot -}$) increase in bile duct ligated (BDL) rats over the corresponding sham rats for each organ and time lapse after operation.

The applied fluorescent assay is particularly advantageous in the BDL model that exerts multisystemic reactions, since it enables the detection of $O_2^{\cdot -}$ abnormalities in a specific tissue or organ *in vivo*. It is conceivable that the indirect markers of oxidative stress like malondialdehyde, oxidized glutathione and protein disulphide, which are accumulative, could be originated in the liver and thereafter transported by circulation to various other organs away from the site of production. Unlike the above oxidative products, $O_2^{\cdot -}$ has a very short lifetime (a few microseconds) and therefore the DHE-based assay measures the *in situ* formation of this radical, offering direct evidence of oxidative stress in a particular organ. In the present study, these direct oxidative stress measurements, made over time, provide a reliable estimation of the oxidative stress evolution in the liver, the intestine, the kidneys and the brain associated with obstructive cholestasis.

Biliary obstruction is a major injurious insult for the normal function of the gut–liver axis; absence of bile from the gut lumen contributes to intestinal barrier dysfunction, whilst bile retention into liver promotes cholestatic liver injury [8,9]. Accumulating evidence over the last several years have implicated oxidative stress as a key factor in the pathogenesis of cholestatic liver injury [17,18], while our group has recently shown the important role of oxidative stress in the obstructive jaundice-induced intestinal injury as well [8,9,19]. Using the 10 days' BDL model we have previously demonstrated that oxidative stress in the gut-liver axis in obstructive jaundice is not only associated with increased lipid peroxidation and glutathione oxidation, but also with a general imbalance between protein or non-protein thiols and protein or non-protein disulphides (symmetric or mixed) [8,10,20]. Increased levels of superoxide radical formation in both organs seem to play a pivotal role in these oxidative alterations [7]. The clear time-related response of superoxide formation rate in both the liver and the intestine indicates the close association of oxidative stress with the progression of cholestasis and emphasizes the importance of early bile flow restoration.

The underlying pathophysiology of renal impairment as a consequence of obstructive jaundice has not been completely understood, regardless of extensive clinical and experimental studies on this issue. Pre-renal events, such as peripheral vasodilation, impaired cardiac function and metabolic alterations together with jaundice itself are held responsible for renal complications [21]. In animal BDL models, a contributing role in nephrotoxicity has been suggested for bilirubin, bile acids and oxidative stress [21–24]. In the present study, the occurrence of oxidative stress appeared from 1 day after BDL. In accordance with our findings, early alterations of renal function were suggested in a previous study of cirrhotic rats, which demonstrated increased plasma rennin activity and serum creatinine day post-BDL, although histological changes of the kidney became apparent 2 days later [25].

Regarding the brain, accumulating evidence suggests a fundamental role of oxidative stress in the neuropathology associated with obstructive jaundice. Assessment of oxidative state by measuring the standard indirect markers have shown, 5–7 days after BDL, an increase of lipid peroxidation and decrease of reduced glutathione and protein thiols occurring to variable degree in different brain areas [6,26,27]. The direct assessment of oxidative stress by determination of $O_2^{\bullet -}$ levels confirmed the existence of oxidative stress 5 days after BDL in the cerebral cortex and midbrain, but not in the cerebellum [11]. The present study revealed an even earlier (24 h after BDL) initiation of increased $O_2^{\bullet -}$ in the brain, which further increased with progression of cholestasis up to 10 days. The pathophysiological patterns that lead to increased oxidative load in the brain in the setting of jaundice and liver failure are not entirely clear. In cases of liver failure, animal studies propose a double role of ammonia in the induction of oxidative stress in the brain; reduction of the antioxidant capacity, as well as enhancement of ROS formation [28,29]. The latter is believed to occur as a result of mitochondrial impairment or, alternatively, activation of N-methyl-D-aspartate (NMDA) receptor, which leads to excitotoxic neuronal death [30]. The end result of these metabolic derangements of the brain would be an excessive increase of superoxide radical (and other radicals derived from it) which, in turn, contribute to the neurotoxicity. Our animal model of obstructive jaundice was studied in its acute phase (up to 10 days) before development of severe and irreversible parenchymal liver injury (4–6 weeks of BDL). In this phase, cholemia predominates over parenchymal liver disease and thus the above mechanisms may contribute to brain injury to a lesser extend. Significant contributory factors in oxidative brain injury in acute obstructive jaundice could also be bilirubin neurotoxicity involving impaired glucose utilization, oxidative phosphorylation, disturbance of mitochondrial function and glutamate excitotoxicity and endotoxemia [31,32].

The findings of the present study indicate that obstructive jaundice is associated with a progressive evolution of the oxidative process in diverse organs and support the systemic extent of oxidative stress in obstructive cholestasis. The potential mechanisms of high systemic oxidative stress in obstructive jaundice have been extensively reviewed previously [7,10,19,20]. Briefly, increased levels of bile acids, systemic endotoxemia and the subsequent inflammatory response, up-regulation of inducible nitric oxide synthase expression, increased neutrophil chemotaxis and superoxide anion generation and decreased systemic levels of the antioxidant vitamin E contribute to the promotion of the oxidative process in obstructive jaundice.

In conclusion, the present study, employing a DHE-based assay for the direct measurement of $O_2^{\bullet -}$ *in vivo* in the BDL rat model, disclosed an early (1^{st} post-operative day) appearance of oxidative stress in the liver, intestine, kidneys and brain. The increase of $O_2^{\bullet -}$ was higher on the 5^{th} and even higher on the 10^{th} post-operative day in all investigated organs, further supporting the parallel evolution and causal relation with obstructive jaundice. Restoration of bile flow remains the only causal treatment for biliary obstruction. However, considering the systemic spread of oxidative stress and its close relation with progression of cholestasis, it would be reasonable to hypothesize that antioxidants might be an important supplementary treatment option. These expectations remain to be elucidated in future clinical studies.

***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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