Examination of hepatic redox-homeostasis in different pathologic disorders (Experimental and human studies)

PhD thesis

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Introduction

Free radical reactions take part in every pathologic disorder, as well as in ischaemic-reperfusion damage and in tumorgenesis. Liver plays a central role int he body, so it is more affected by oxidative stress, and plays a definitive role in the function of defensive mechanisms of the body, as well. Therefore, the liver, as a shock-organ, became a perfect model of oxidative damage during the past few years. Numerous cellular mechanisms are already known, but there are lot of questions are to be answered. Therefore, I did studies on liver and other organs connected to it (blood, gut).

In Hungary the hepatic tumors, and hepatic metastases of gastrointestinal tumors can be found in high percentage among causes of mortality. Due to certain statistics benign liver tumors can be found in 10 % of the adults. Nowdays the study of the antioxidant status in tumor patients is an important aim worldwide. Detection of changes caused by benign and malign liver tumors int he redox-homeostasis may help in the diagnosis and therapy.

The most important therapeutic strategy of liver neoplasms is operation. Ischaemic-reperfusion damage doring operations is known as a risk factor of survival and cause of side effects. Low and relative high oxygen tension of the liver may damage the redox-homeostasis of the whole body through free radical reactions. Reactive oxygen species damage membranes of the cells (through lipide-peroxidation), as well as the DNA. Due to free radical reactions every known cellular pathways may be induced. Free radicals and phosphorilation cascades initialize the synthesis of antioxidant defense system through the induction/inhibition of transcription factors. During the damage metal ion homeostasis changes, as well. In case of serious damage apoptotic or necrotic celldeath occurs.

In case of operations damage of liver may be decreased by decreasing oxidative stress and changes in metal-homeostasis. This aim can be achieved by using natural antioxidant agents. The proper therapeutic protocoll still needs a long period of time, the exact amount of antioxidant and the duration of the therapy is still not known. In addition the antioxidative stress caused by overdose of antioxidant therapy may be harmful for patients.

In my work I wish to describe the changes of redox homeostasis during liver tumors and operations. The experimental and human studies will hopefully present useful results for clinicians.

Aims of the study

Changes in the redox-homeostasis of living organisms cause serious diseases and functional disorders, it is a central field of scientific research nowdays. During my studies my aim was to discover these changes with biochemical, molecular biological and analitical methods. Metal ion homeostasis and the study of lipid metabolism are needed in order to study redox-homeostasis becouse of their effect on molecular biological pathways.

In my experiments my aim was to describe a new, complex model for studying redox-homeostasis during liver operations. I used the model of 45 minutes of ischaemia with 15 minutes and 24 hours of reperfusion of the rat liver. Based on the literature changes of the redox-homeostasis during liver operations are assumed, I wanted to verificate these changes with biochemical examinations (total-scavenger capacity, H-donor ability, reducing power, free SH-group and dien-conjugate concentration), metal ion examination (ICP-OES method), examination of free fatty acids (gas chromatography) and with examination of enzyme activity (SOD, GSHPx). I used immunhistochemical (TUNEL method) and histological methods for the detectation of apoptotic and necrotic cell death. I examined these changes first in experimental model, after that I carried out a human study. I assumed that the redox-homeostasis changes in the intact liver lobes, as well.

The prevention of ischaemic-reperfusion may be possible with different natural antioxidant agents (betacyanins, poliphenols, flavonoids of beetroot), as well as drugs (metadoxine). The damage of the gut may be connected with the ischaemic-reperfusion damage of the liver, so I wanted to verify this damage.

In the human studies I wanted to examine the changes in the redox-homeostasis of the body due to benign and malign neoplasms, as well as to compare the behavior of primary and metastatic liver tumors. I also wanted to examine the total scavenger capacity, the H-donor ability, the reducing power and the free SH concentration of plasma, as well as the total scavenger capacity, antioxidant enzyme activity and content of elements and ATP of red blood cells.

With my work the pathomechanism of oxidative liver damage may be better understood. By the new in vivo results of the antioxidant system (enzymatic and non-enzymatic) more reliable data can be gained than results from in vitro and cell culture experiments. My results could be verified with histological and cellular biological methods.

Methods

General description of operations

I used 200-250 g male Wistar rats (Charles River Hungary). The rats were given water and standard rat chow ad libitum and housed in a temperature- and humidity-controlled room under a constant 12-hour light/dark cycle. . All studies were performed with the permission of the Animal Health and Food Control Station (770/004/04).

Surgery was performed under deep nembutal narcosis (35 mg/bwkg). After laparotomy, occlusion of the circulation of the left lateral and the medial liver lobes was induced. Reflow was initiated by the removal of the microclips, which selectively clamped the branches of the portal vein and hepatic artery.

Human study

11 patients with diagnosis of benign liver neoplasm (mean age: 48.73 ± 10.26 years; range: 32–62 years), 23 patients with diagnosis of HCC (mean age: 50.17 ± 8.04 years; range: 37–70 years) and 37 patients with diagnosis of CRLM of the liver (mean age: 53.08 ± 8.72 years; range: 39–72 years) who had not undergone any previous treatment for their liver tumors, were enrolled in this study. Patients were recruited from the I. Department of Surgery and from the Department of Transplantation and Surgery, Semmelweis University, Budapest, Hungary. 30 age and sex -matched volunteers (mean age: 54.42 ± 8.15 years; range: 36–62 years) served as controls. Specific exclusion criteria for the present study were the presence of hepatitis virus infection, diabetes mellitus, heart or renal failure and oral antioxidant supplementation at the moment of the enrollment. Some of the subjects were heavy drinkers, but none of them had consumed any alcohol, starting at least 48 hour prior to blood collection. Informed consent was obtained from all participants of the study before the blood collection.
The diagnosis of hepatic neoplasms was based on computer tomography and US-fine needle aspiration biopsy, this diagnosis was verified by the histopathological examination of their postoperative specimens. The study was approved by TUKEB Comittee of Semmelweis University, Budapest, Hungary (TUKEB No.: 15/2004).

Blood samples were drawn from the antecubital vein following an overnight fast, by venipuncture into tubes containing Na citrate. Plasma and erythrocyte samples were separated and stored at −20°C until assayed.

Laboratory diagnostic methods

Examinations were carried out by standard methods with spectrophotometry, with a Hitachi 717 machine.

Biochemical methods for examination of the redox-homeostasis

Total scavenger capacity

Reducing power

H-donor ability

Total antioxidant status

Free SH group concentration

Concentration of diene conjugates

Morphological examinations

Apoptosis with TUNEL assay

Histology

Metal ion- and element determination with ICP-OES machine

Molecular biological examination

Free fatty acid content determination with gas chromatography

ATP content determination with chemiluminescent method

Mathematical statistical analysis

The differences between two groups was examined by the Student's t probe using Statistica 6.0 software. Significance levels were determined at $p<0.05$.

Results and discussion

Connections between the changes of redox-homeostasis and element content and induction of apoptotic and necrotic cell death during hepatic ischaemia-reperfusion

First I verified the free radical reactions and changes in metal element and fatty acid content in hepatocytes due to ischaemia-reperfusion. Measuring only one parameter is not enough for describing redox-homeostasis. Therefore I planned to measure more parameters in my work. Apoptotic and necrotic pathways are induced by free radical generation. These experiment will serve as a proper model for testing antioxidant agents.

In the liver the significantly elevated chemiluminescent intensity is the marker of elevated free radical content, which is verified by the elevated concentration of diene conjugates, as well. The generated diene conjugates is the marker of membrane damage, as well. Parameters of non-enzymatic antioxidant defense system (H-donor ability and reducing power) strenghten that the rdox balance was damaged more seriously during reperfusion, than during ischaemia. The non-enzymatic defensive mechanisms induced in sham-operated and ischaemia groups are exhausted after 15 minutes of reperfusion. The value of TAS showed the same tendency.

Activity of GSHPx and SOD enzymes was significantly lower in reperfusion group and slightly lower in the ischaemia group than in the sham-operated group. The level of Mn and Zn decreased parallel with the activity of SOD enzymes in both of the experiment. The content of Se was significantly decreased after 15 minutes of reperfusion than in the controls, this could be the eplanation of decreased GSHPx enzyme function. Decreased GSHPx and SOD activity was compensated in the sham-operated animals, however due to the operations the enzymatic defense system was exhausted.

After 15 minutes of reperfusion the concentration of arachidonic acid was minimally altered, which may mean, that these molecules were used u pin the arachidonic acid cascade. The decrease in eicosapentaenoic acid and docosahexaenoic acid content also strenghtened this. After 24 hours of reperfusion the arachidonic acid content decreased significantly, which also showed the overfunction of arachidonic acid cascade. In this experiment docosahexaenoic acid content decreased significantly, whereas eicosapentaenoic acid content elevated significantly.

After 24 hours of reperfusion both apoptotic and necrotic processes were induced in the liver. Histology revealed massive necrotic areas. Numerous apoptotic hepatocytes were found with fluorescent technique.

Examination of redox-homeostasis may provide important informations about prevention of surgical liver damage. Our protocol seems to be proper for studying oxidative mechanisms. Measurement of induced free radical content tells us about the damaging agents generated in the tissues, whereas the global antioxidant parameters showes the defense system of the tissues. These examinations along with the metal ion content and the level of free fatty acids describe the homeostasis of the cells precisely.

Change in the redox-parameters in the intact lobes of the liver during ischaemia-reperfusion

In my experiment the fact of the damage was verified by the change of redox parameters. In the intact liver lobes diene conjugate concentration and chemiluminescent intensity were significantly higher than in the sham operated animals and in the control group. High level of lipid peroxidation is one of the markers of oxidative stress, which, along with chemiluminescent intensity, shows us the hepatocyte damage. Chemiluminescent intensity is considered as the free radical content of the tissue, this way the free radical generation was verified in these lobes, as well.

Based on my results the damage of the antioxidant system was detected in the intact lobes. The non-enzymatic defense system can be described with the measurement of H-donor ability, reducing power and free SH group concentration, which were decreased during the experiment. The members of the enzymatic defense system (SOD, GSHPx) decreased parallel with them. These changes describe well the damage of the hepatocytes.

Among the metal element especially the content of Ca, Fe, Mg and P elevated during hyperaemia, which may be explained with the damage of membranes. As the presence of Mn, Zn and Cu is essential for the function of SOD enzyme, the decrease of these elements may be an explanation for the decreased activity of this enzyme. Se is important for the activity of GSHPx.

Compared with the ischaemic-reperfusion damage of the liver the dysfunction caused by hyperemia is less serious, but it can be detected, so it may be interesting for liver operated patients.

Beneficial effects of the treatment of antioxidant drug during hepatic ischaemia-reperfusion

Most of the patients undergoing liver surgery takes some kind of drugs regularly, which may be important for the survival of the liver. Metadoxine is used for the treatment of different liver diseases, so it is possible that there are surgical patients who took this drug before operation.

The model built up in my previous experiments is useful for testing different agents during hepatic ischaemia-reperfusion. I assume that metadoxine enhances the survival of the liver.
By the measurement of antioxidant parameters it can be detected, that pretreatment with metadoxine has positive effect on the ischaemia-reperfusion tolerance of the liver. Redox-homeostasis became better both in the plasma and in the liver. Reducing power of the plasma was elevated, whereas the free radical content was lower. Significant changes were not found in erythrocytes. In the liver non-enzymatic parameters H-donor ability and reducing power were elevated due to the drug. Parallel with the non-enzymatic parameters the activity of antioxidant enzymes were higher. Until now there is no documentated side effect of metadoxine in the literature, this drug can be recommended for surgical patients against free radical damages.

Effects of natural antioxidant agents in different tissues during ischaemia-reperfusion

Besides drugs the proper food is very important for surgical patients in order to minimalize side effects. Beetroot (Beta vulgaris L. var rubra) contains numerous biological active agents which affect numerous cellular pathways.
Beetroot treatment had positive effects in the liver. Concentration of diene conjugates were lower due to the treatment, whereas H-donor ability and reducing power were elevated, which shows its positive effect on redox-homeostasis. Beetroot elevated the level of SOD and TAS in the liver, as well. The significantly decreased chemiluminescent intensity proves the decreased free radical content of the liver. Global antioxidant parameters (H-donor ability, reducing power) means that beetroot pretreatment was able to protect against damage of the redox balance. The level of TAS showed the same tendency. Activity of SOD enzymes was also higher in pretreated animals, which may be in connection with the extra intake of Zn and Cu elements.
In my experiment the concentration of arachidonic acid was significantly higher in the pretreated group, and the decrease in the content of eicosapentaenoic acid and docosahexaenoic acid may mean that the intensity of arachidonic acid cascade (and inflammatory reactions) decreased. The decreased level of short chain fatty acids may mean that these molecules were metabolised and were used up for the the synthesis of prostaglandines and eicosanoids.

In my experiment the pretreatment increased the content of Mg, which is essential for the synthesis of ATP and proteins. With the inhibition of free Fe content it has positive effect, as well. Changes in the content of Ca, Fe, K, Mg, Na and P in the liver can be explained with the damage of the membranes.

During hepatic ischaemia-reperfusion total scevenger capacity was increased in the bile, which may be the sign of the induced antioxidant defensive system. Due to the pretreatment chemiluminescent intensity of the bile came closer to the control values, which means lower level of oxidative stress.

Decrease in H-donor ability and reducing power shows the compensatoric effect, as well. There was no significant change in the concentration of free SH group during surgery.

Based on our results it may be stated that food containing betaine, betanin, quercetin and other flavonoids and polyphenols has beneficial effect during surgery against the oxidative stress of the whole body. My work proves the importance of proper food of surgical patients.

Oxidative stress with altered element content and decreased ATP level of erythrocytes in hepatocellular carcinoma and colorectal liver metastases

In my study I examined the possible connections between oxidant/antioxidant balance, element content and ATP content in the blood of patients with primary and metastatic liver tumor.

I assumed that in the blood of tumor patients alterations of the redox homeostasis and the element homeostasis and the dysfunction of antioxidant defense system can be detected. As the redox homeostasia needs energy, I also measured the ATP content of erythrocytes in tumor patients.

I have found elevated free radical content and increased level of oxidative stress of plasma and erythrocyte samples in hepatocellular carcinoma and metastatic groups versus controls and benign neoplasm groups. The worst antioxidant level was found in the metastatic group, this indicates, that the progress of neoplastic process may be a key factor of oxidative damage. Lipid peroxidation and oxidative stress is thought to be more prominent in erythrocytes than in plasma of cancer patients. In my study, the levels of SOD and GSHPx enzymes along with the content of Cu, Se and Zn elements in the erythrocytes of the HCC and CRLM groups were significantly lower than in the benign group and in healthy controls.

The content of certain elements in erythrocytes changed parallel with the lowered antioxidant parameters. We still don’t know, whether these changes are to be considered primary or secondary. Damage of membranes due to free radicals may be the explanation for the change

in the content of certain elements. Along with the generation of ROS decreased content of ATP was measured in the erythrocytes.

The results reveal an alteration in the antioxidant defense system with concomitant changes in the element content of red blood cells in cancer patients. Whether the alterations in the antioxidant status are the cause or consequence of the enhanced ROS generation remains unclear. It is hypothesized that an altered prooxidant–antioxidant balance may lead to oxidative damage and play an important role in hepatic carcinogenesis. It seems, that oxidative damage is closely connected with alterations of metal element content in erythrocytes. Depletion of elements, which are essential for antioxidant enzyme activities, such as Cu, Se and Zn, leads to a circulus vitiosus. Imbalance of redox state and element homeostasis may cause insufficient ATP generation and/or transfer. Low Mg content may play a key role in further ATP depletion.

Further research should be carried out in order to find out whether oxidative stress-related parameters could be used as differential diagnostic and prognostic tools in therapy of benign and malignant liver neoplasms. Presence of impaired antioxidant status may be a risk factor of both primary and metastatic tumorigenesis of the liver.

Based on our findings, it seems, that redox parameters of erythrocytes are sensitive factors for monitoring the clinical status of tumor patients. Damage of erythrocytes seem to be a key step in tumor developement and generation of metastases.

Conclusions

1. We have bulit up a model system proper for routine examination of redox-homeostasis during ischaemia-reperfusion.
2. We have verified the oxidative damage of intact liver lobes during hepatic ischaemia-reperfusion.
3. We have proved that there are significant alterations in the metal ion homeostasis in the liver during ischaemia-reperfusion.
4. We have showed, that the lipid metabolism changed in the liver during ischaemia-reperfusion.
5. We have verified, that natural antioxidant pretreatment has beneficial effects on the level of surgical damage.

6. By the examination of redox parameters we have found, that metadoxine pretreatment reduces the damage of hepatocytes significantly due to ischaemia-reperfusion.
7. We have verified, that free radical reactions play a key role in the generation and in the progression of both benign and malign liver tumors.
8. We have verified, that the level of parameters of redox-homeostasis is worse in case of metastatic liver tumors than in primary liver tumors. Erythrocytes are excellent markers of monitoring alterations due to malignant tumors.

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