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Oxidative Stress Produced by Suprahepatic Occlusion and Reperfusion

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In this article the spontaneous chemiluminescence and the steady-state concentration of hydrogen peroxide were determined in rat liver as indicators of oxidative stress in the tissue. Hydroperoxide-initiated chemiluminescence and the activity of antioxidant enzymes (catalase, superoxide dismutase and glutathione peroxidase) were also measured to evaluate antioxidant defenses and serum activity of lactate dehydrogenase and aspartate aminotransferase. Mitochondrial morphology and mitochondrial respiratory control ratio were measured as indicators of cell and mitochondrial damage. Xanthine dehydrogenase and xanthine oxidase activities were determined as a possible source of oxyradicals. No significant changes were observed after 10 or 30 min of vena cava occlusion in any of the measured parameters. In contrast, 10 min of occlusion followed by 10 min of reperfusion increased chemiluminescence (from 18 ± 3 to 32 ± 5 cps/cm²), hydrogen peroxide (from 0.10 ± 0.01 to 0.17 ± 0.01 μ mol/L), lactate dehydrogenase (from 80 ± 2 to 330 ± 30 U/L), and aspartate aminotransferase (from 42 ± 2 to 100 ± 10 U/L). Liver reperfusion was also associated with mitochondrial swelling and decreased mitochondrial respiratory control (from 5.6 ± 0.3 to 2.6 ± 0.1). The activity of the antioxidant enzymes and xanthine oxidase was instead without change. After 30 min of vena cava occlusion and 10 min of reperfusion a more marked increase in chemiluminescence (37 ± 5 cps/cm²), hydrogen peroxide (0.30 ± 0.01 μ mol/L), lactate dehydrogenase (730 ± 10 U/L) and aspartate aminotransferase (140 ± 10 U/L) was observed. No further changes were found in either mitochondrial morphology or respiratory control (2.4 ± 0.1) in isolated mitochondria. A parallel decrease in the activity of cytosolic (36%) and mitochondrial (57%) superoxide dismutase, catalase (34%) and

glutathione peroxidase (34%) was also observed without any change in xanthine oxidase activity. Chain-breaker antioxidants were also diminished as indicated by an increase in the hydroperoxide-initiated chemiluminescence (40%). The results indicate the occurrence of an oxidative stress in association with a sequence of 10 or 30 min of occlusion followed by 10 min of reperfusion. The unchanged activity of xanthine oxidase, the modifications in mitochondrial morphology, the decrease in mitochondrial respiratory control and the relatively high inactivation of the mitochondrial superoxide dismutase indicate that the mitochondria are the main source of oxyradicals during reperfusion. An increased rate of superoxide radical and hydrogen peroxide generation by mitochondria, associated with a decreased activity of antioxidant enzymes, would be the major causes of oxidative stress and the related cell damage. (HEPATOLOGY 1993;18:881-889.)

Hepatic oxidative stress can be understood as a situation derived either from an enhanced rate of generation of oxygen radicals or from a diminished level of antioxidant (enzymatic or nonenzymatic) defenses (1, 2). Moreover, a biological situation associated with oxidative stress could be estimated by a physicochemical condition in which an increase in the steady-state concentrations of oxidative species (i.e., O₂⁻, H₂O₂, HO[•], R[•], ROO[•] and ¹O₂) occurs. This increased steady-state level of oxidants may lead, in sequence, to reversible or irreversible cell damage and eventually to cell death (2).

Oxygen free radicals are produced in a series of biochemical reactions that will normally occur within the cellular compartment. Mitochondria and the endoplasmic reticulum are the most important sources of oxygen free radicals in the liver (3).

The relatively high rate of generation of oxidative species in the liver under physiological conditions is balanced by the relatively high activity of various enzymes, including superoxide dismutase (SOD), catalase and glutathione peroxidase associated with a relatively high concentration of nonenzymatic antioxidants in the organ (3). As a result cytosolic steady-state concentration of superoxide anion and hydrogen peroxide are maintained in steady state at about 10⁻¹¹ mol/L (4) and 10⁻⁷ to 10⁻⁸ mol/L (5, 6), respectively. Furthermore, a continuous low rate of generation of

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excited species has been detected by a spontaneous chemiluminescence of 18 cps/cm² at the liver surface, which is consistent with a singlet oxygen steady-state level of about 10⁻¹⁴ mol/L (7).

The obstruction of the efferent liver venous flow is characteristic of acute congestive heart failure, tricuspid failure, the Budd-Chiari syndrome and other pathophysiological states that lead to severe liver damage (8, 9). Occlusion of the suprahepatic veins leads to blood reapeal in the splenoportal axis associated with derivation of the circulation to the collaterals, which leads to the development of portal hypertension. Venous occlusion is also associated with a redistribution of the intrahepatic blood flow and an increase in the circulation by arteriovenous shunts that elude the capillary system (8, 10). A direct consequence of these changes is the onset of ischemia in wide sectors of the hepatic parenchyma. Compensatory changes of the hepatic microcirculation, associated with alterations in the diffusional gradients of oxygen, will make the tissue normoxic once again; thus stasis-oxygenation sequences (i.e., ischemia-reperfusion cycles) will occur that are associated with the occlusion of the hepatic efferent pathways. Consistent with this idea, Marotto, Thurman and Lemasters (11) have reported that the rat liver perfused at a low flow rate displays higher susceptibility to irreversible injury in those cells at the border between anoxic and normoxic regions, as compared with the fully anoxic cells.

It thus seems likely that ischemia-reperfusion and stasis-oxygenation in the liver share molecular mechanisms of oxidative stress and cell damage. The hypothesis that postulates the conversion of xanthine dehydrogenase (XD) to the oxidase form (XO) during tissue ischemia, with a concomitant increase in the rate of superoxide anion production during reperfusion (12), appears applicable to intestine and other tissues with high activities of XD (12, 13). However, the same hypothesis fails to explain reperfusion injury observed in human heart and other tissues in which XD is practically undetectable (14-16). An alternative hypothesis for the generation of oxygen free radicals in mitochondria after reperfusion (17, 18) seems more applicable to tissues with a high metabolic rate including heart, liver, brain, muscle and kidney. The rat liver, which is an example of tissue with a high metabolic rate, also displays a high activity of XO. Therefore both hypotheses should be considered at first as equally contributing to the oxidative stress related to ischemia-reperfusion. However, Gonzalez-Flecha, Cutrin and Boveris (17) have recently provided experimental evidence showing that mitochondria prevail as oxyradical sources over both intracellular and endothelial XO, neutrophil infiltration and antioxidant enzyme inactivation in quantitative and kinetic terms.

In this article we describe the main features of oxidative stress and cell injury caused in rat liver by occlusion and release of the suprahepatic inferior vena cava.

MATERIALS AND METHODS

Experimental Model. Male Wistar rats weighing 180 to 200 gm were used throughout the studies. Animals were fed a

laboratory diet and water *ad libitum*. Rats were fasted for 24 hr before surgery, anesthetized with pentobarbital (50 mg/kg, intraperitoneally) and divided into five groups. The liver was exposed by a laparoscopic opening at the abdominal wall followed by clamping of the inferior vena cava at the suprahepatic level. In the experimental groups the clamp was removed after 10 or 30 min of occlusion, and the circulation was restored for 10 min (reperfusion period). Experimental groups of six rats each were submitted to a 10-min occlusion-10-min reperfusion (10/10) or a 30-min occlusion-10-min reperfusion (30/10) cycle. Three control groups not subjected to a reperfusion cycle were used corresponding to 0-min (0/0), 20-min (20/0) or 40-min (40/0) occlusion. After surgical excision of the liver, wet weight was determined. Liver wet weight was significantly increased during the occlusion period ($p < 0.01$, $n = 6$) and returned to control values after reperfusion (usually in 10 to 20 min). Liver wet weight was 2.5 ± 0.2 gm/100 gm body weight for controls, 2.9 ± 0.2 and 3.5 ± 0.3 gm/100 gm body weight after 10 and 30 min of occlusion, respectively. Systemic maximal, minimal and medium as well as portal pressure were measured. Values obtained from different rats at various times and sequentially for the same rat were compared. Systemic medium pressure under control conditions and immediately after occlusion were 93 ± 4 mm Hg (minimal = 81 mm Hg; maximal = 115 mm Hg) and 76 ± 6 mm Hg (minimal = 70 mm Hg; maximal = 100 mm Hg), respectively ($p < 0.01$, $n = 6$). No further changes were observed between 10 and 30 min of occlusion, but systemic pressure returned to control values (91 ± 3 mm Hg; minimal = 79 mm Hg; maximal = 114 mm Hg) immediately after the clamp was removed. Control portal pressure was 7.0 ± 0.4 mm Hg, which significantly increased after 10 or 30 min of occlusion (9.0 ± 0.4 and 10.0 ± 0.5 mm Hg, $p < 0.01$). For *in vitro* determinations livers were excised at the appropriate times and rapidly placed in 150 mmol/L NaCl at 0° to 4° C. All animals received care according to previously approved institutional guidelines as outlined in the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health (NIH publication no. 86-23, 1985).

Spontaneous Liver Chemiluminescence. Spontaneous *in situ* liver chemiluminescence was measured with a Johnson Foundation photon counter (19) (Johnson Research Foundation, University of Pennsylvania, Philadelphia, PA). Photon emission was expressed as counts per minute per square centimeter of liver surface (19). A high-pass cutoff filter (Wratten number 25; Eastman Kodak, Rochester, NY), allowing wavelengths greater than 600 nm, was placed in the optical path to avoid hemoglobin interference. Photon counting decreased only by 15% to 20%, thus indicating that 80% to 85% of the emitted light had a wavelength longer than 600 nm and could thus be regarded as singlet oxygen emission.

Steady-state Concentration of Hydrogen Peroxide. The hydrogen peroxide steady-state concentration was measured by use of a modification of a method originally described by Puntarulo, Sanchez and Boveris (20). In brief, tissue slice approximately 0.1 mm thick were incubated in a solution containing 150 mmol/L NaCl and 20 mmol/L phosphate buffer (pH 7.2) at 30° C and at a tissue/saline ratio of 1:3 (wt/vol). Samples of the supernatant were taken at various times and processed to determine the H₂O₂ concentration diffused out of the tissue. The H₂O₂ concentration was determined by measuring the chemiluminescence of the reaction with luminol, with CoCl₂ as catalyst. The assay conditions for measuring H₂O₂ were as follows: the supernatant was diluted 1:3 (vol/vol) with 0.5 mol/L ammonia buffer (NH₄OH/NH₄Cl (pH 10.3)) and added with 1 μmol/L luminol and 5 μmol/L CoCl₂. Chemiluminescence was measured with a liquid scintillation counter.

tillation counter (Packard Tri-Carb 3320; Packard Instruments, Chicago, IL) in the out-of-coincidence mode.

Marker Enzymes of Liver Damage. Serum samples taken at various times were processed to determine lactate dehydrogenase (LDH) and AST activity with conventional laboratory kits.

Electron Microscopy. Livers were removed and immediately sectioned into 1-mm slices. Samples were fixed in cold 3% (wt/vol) glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.4), postfixed in 2% (wt/vol) osmium tetroxide in 0.2 mol/L sodium cacodylate, dehydrated and embedded in Poly-Bed 812 (Poly-Doleysciences Inc., Warrington, PA). Ultrathin sections were stained with lead citrate and uranyl acetate (21) and examined with a transmission electron microscope (JEM-100C; Japan Electron Microscope, Tokyo, Japan). Electron micrographs were examined morphometrically under a grid to determine volume density and individual volume (22).

Determination of Mitochondrial Respiratory Control. Liver mitochondria were isolated in a medium containing 0.23 mol/L mannitol, 0.07 mol/L sucrose, 1 mmol/L EDTA and 5 mmol/L Tris-HCl (pH 7.2) (17). Mitochondrial respiratory rates were measured with a Clark oxygen electrode (YSI-Thomas Science, Swedesboro, NJ) in a medium containing 0.25 mol/L sucrose, 20 mol/L KCl, 5 mmol/L $MgCl_2$, 1 mmol/L EDTA, 10 mmol/L Tris-HCl and 7 mmol/L phosphate (pH 7.2) at 30° C. The respiratory control ratio was determined as described by Estabrook (23) with 8 mmol/L succinate and 0.5 mmol/L ADP.

XO and XD Determination. Liver samples were homogenized in 140 mmol/L KCl, 1 mmol/L PMSF, 1 mmol/L dithiothreitol and 20 mmol/L Tris-HCl (pH 7.6) and centrifuged at 15,000 *g* for 20 min at 0° to 4° C. XO activity was measured in the postperoxisomal supernatant including microsomes and cytosol in a Perkin-Elmer 356 spectrophotometer (Perkin-Elmer Cetus Corp., Norwalk, CT) after uric acid formation at 293 to 320 nm ($E = 12.4 \text{ (mmol/L)}^{-1} \cdot \text{cm}^{-1}$) in a reaction solution containing 0.1 mol/L Tris-HCl (pH 8.6) and 50 $\mu\text{mol/L}$ xanthine. XD activity was determined after NADH appearance at 340 to 375 nm ($E = 2.88 \text{ (mmol/L)}^{-1} \cdot \text{cm}^{-1}$) in the same reaction solution containing 100 $\mu\text{mol/L}$ NAD⁺ (17).

Tissue Homogenates. Liver samples of 0.5 to 1.0 gm wet weight were homogenized in 120 mmol/L KCl, 30 mmol/L phosphate buffer (pH 7.2) at 0° to 4° C. Whole-liver homogenates were centrifuged at 600 *g* for 10 min at 0° to 4° C. Pellets containing nuclei and cell debris were discarded, and supernatants were used as homogenates (17).

Tert-butyl Hydroperoxide-initiated Chemiluminescence. Tert-butyl hydroperoxide-initiated chemiluminescence from the homogenized tissue was measured with a liquid scintillation counter in the out-of-coincidence mode (17, 24). Homogenates were placed in low-potassium glass vials (diameter = 25 mm and height = 50 mm). The background level of emission of empty vials was 2,500 to 3,000 cpm. The solution used to assess the reaction consisted of 120 mmol/L KCl and 30 mmol/L phosphate buffer (pH 7.5). Chemiluminescence measurements were started by addition of 3 mmol/L tert-butyl hydroperoxide. Protein content was adjusted to 0.5 to 1.0 mg/ml of tissue homogenate. Determinations were carried out at 30° C with occasional stirring. Results are expressed in counts per minute per milligram protein.

Antioxidant Enzymes. SOD activity from liver homogenates was determined from the inhibition of the rate of autocatalytic adrenochrome formation in a reaction medium containing 1 mmol/L epinephrine and 50 mmol/L glycine-NaOH (pH 10.2) (17). The activity of the mitochondrial isoenzyme was assayed by adding 1 mmol/L KCN to the reaction medium (25). Catalase activity was determined by measuring the decrease in 240-nm absorption in a reaction medium consisting of 50

mmol/L phosphate buffer (pH 7.2) and 2 mmol/L H_2O_2 , thereby determining the pseudo-first-order reaction constant (k') of the decrease in H_2O_2 absorption. Results are expressed as catalase content in picomoles per milligram protein (17) ($k = 4.6 \times 10^7 \text{ mol/L}^{-1} \cdot \text{sec}^{-1}$) (3). Glutathione peroxidase activity was determined after NADPH oxidation at 340 nm in the presence of 0.17 mmol/L glutathione, 0.2 U/ml yeast glutathione reductase and 0.5 mmol/L tert-butyl hydroperoxide in 50 mmol/L phosphate buffer (pH 7.2) (26).

Protein Determination. Protein was measured by the method of Lowry et al. (27), with BSA as standard.

Chemicals. Tert-butyl hydroperoxide was from Aldrich Chemical Co. (Milwaukee, WI), luminol, glutathione, glutathione reductase, glycine, epinephrine, Tris, mannitol, sucrose, PMSF, dithiothreitol, xanthine, NAD⁺ and NADPH were from Sigma Chemical Co. (St. Louis, MO). Other reagents were of analytical grade. Kits for LDH and AST determinations were kindly provided by Boehringer Mannheim GmbH (Buenos Aires, Argentina).

Statistics. Results are indicated as the mean value of six independent experiments \pm S.E.M. Statistical significances were analyzed by ANOVA followed by Dunnett's test for other comparisons (28).

RESULTS

Spontaneous Rat Liver Chemiluminescence. Exposure of liver to a sensitive phototube allows the direct detection of spontaneous chemiluminescence of the *in situ* organ under physiological conditions. The constant emission obtained from a normal liver ($18 \pm 3 \text{ cps/cm}^2$; Fig. 1A) and the spectral definition (85% greater than 600 nm) is consistent with a physiological steady-state level of singlet oxygen of about 10^{-14} mol/L as reported previously (7, 19). After the vena cava was clamped, a slight decrease in chemiluminescence was observed (Fig. 1). Subsequent reperfusion of the liver produced a marked increase in the maximal values of chemiluminescence recorded between 2 and 5 min after the clamp was removed (Fig. 1B). A 10-min reperfusion period was chosen from the chemiluminescence reading (Fig. 1), at which time the overshoot in chemiluminescence started to decline. Increases in chemiluminescence after 10 min (and not longer reperfusion times) have been previously reported by us in rat intestine (13), consistent with the fact that the oxidative stress related to reperfusion is an early event. The maximal photoemission during reperfusion was significantly higher (180% to 205%) than the spontaneous liver photoemission (Fig. 2). Because hemoprotein absorption occurs mainly at wavelengths below 600 nm, the red emission of liver chemiluminescence will not be significantly affected by changes in hemoprotein absorption or hemoprotein conditions.

Steady-state Concentrations of Hydrogen Peroxide. Intracellular concentration of H_2O_2 assessed from the concentration of H_2O_2 in the incubation media of liver slices when the intracellular and extracellular concentrations reach diffusion equilibrium (20). No differences were found in the intracellular concentration of H_2O_2 in slices of liver removed either at 10 or 30 min of occlusion compared with the slices obtained from control (preocclusion) livers. In contrast, when the slices were obtained from reperfused livers, a twofold to

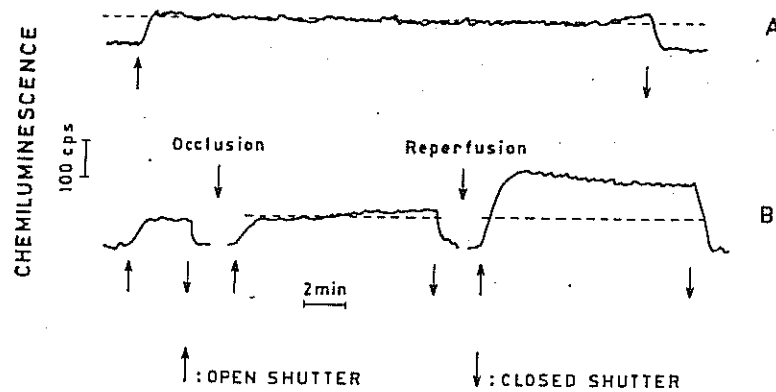


FIG. 1. Spontaneous chemiluminescence of rat liver *in situ*. The liver surface was exposed to the photon counter by laparotomy. Dark current corresponds to the light emission with the shutter closed. After the shutter was opened, photon emission of the liver surface was recorded (A) in physiological conditions and (B) during clamping of the vena cava for 10 min and reperfusion for 10 min. The chemiluminescence pattern reproduces the mean value of six independent experiments.

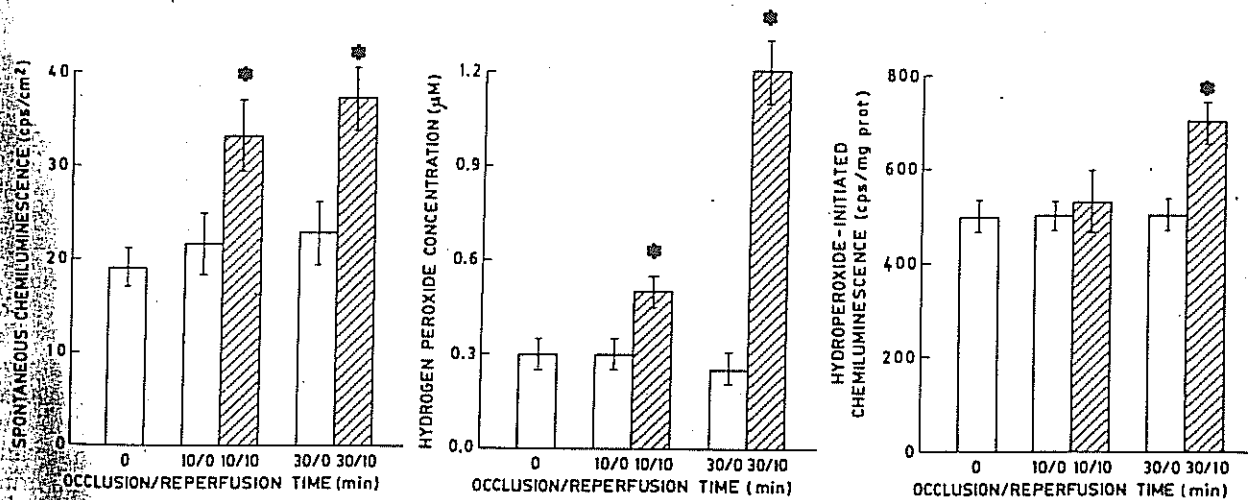


FIG. 2. Spontaneous chemiluminescence of the organ *in situ*. Steady-state concentration of hydrogen peroxide determined in liver slices and hydroperoxide-initiated chemiluminescence in homogenates during occlusion (open bars) or occlusion-reperfusion (filled bars). Time zero corresponds to control values. Bars indicate mean values of six independent experiments \pm S.E.M. * $p < 0.001$.

fold enhanced concentration of H_2O_2 was detected (Fig. 2) ($p < 0.001$).

Tert-butyl Hydroperoxide-initiated Chemiluminescence. The tert-butyl hydroperoxide-initiated chemiluminescence determines the integral level of endogenous chain-breaker antioxidants and, indirectly, the previous occurrence of oxidative stress in a tissue (2, 24). The hydroperoxide-initiated chemiluminescence of samples from reperfused tissue after 30 min of occlusion was higher than the chemiluminescence obtained from 10 min of occlusion and control samples (Fig. 2) ($p < 0.001$).

Hepatic Damage. Various markers of hepatic injury were also considered. Neither changes in the morphological appearance of the liver nor changes in the serum activity of AST were found during occlusion. A slight increase in LDH leakage was found after 30 min of occlusion, probably indicating an increase in the overall

membrane permeability that was not accompanied by AST leakage. An increase of threefold to 10-fold in LDH and AST activity was found in the serum samples obtained after occlusion-reperfusion periods as compared with the control (preocclusion) values (Table 1). The mitochondrial ultrastructure assessed by electron microscopy was not substantially modified after occlusion-reperfusion. Mitochondria had minimal to moderate matrix rarefaction and swelling depending on the length of occlusion. In some cases intracrystal swelling was also observed, a condition that seems to indicate a loss of the active and selective permeability of the inner mitochondrial membrane (Fig. 3). Mitochondrial volume experienced condensation (20/0) followed by expansion (40/0) on occlusion. Reflow produced a marked (approximately 50%) increase in mitochondrial volume (Table 1). Ultrastructural mitochondrial changes resembled the ones previously reported by

TABLE 1. Enzymatic serum markers of liver injury and mitochondrial ultrastructure in hepatic occlusion-reperfusion

Parameters	Occlusion/reperfusion time (min)				
	0/0	20/0	40/0	10/10	30/10
AST (U/L)	42 ± 4	42 ± 4	40 ± 4	100 ± 10 ^a	140 ± 10 ^a
LDH (U/L)	80 ± 8	100 ± 10	128 ± 10 ^a	330 ± 30 ^b	730 ± 30 ^b
Mitochondrial morphology					
Volume density	0.21 ± 0.02	0.16 ± 0.02	0.24 ± 0.02	0.33 ± 0.01 ^a	0.32 ± 0.02 ^a
Individual volume	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.5 ± 0.1 ^a	1.5 ± 0.1 ^a

Values represent the mean ± S.E.M. of six independent experiments.

^ap < 0.01.

^bp < 0.005.

TABLE 2. Respiratory activity of isolated mitochondria from rat livers subjected to occlusion and reperfusion

Parameters	Occlusion/reperfusion time (min)				
	0/0	20/0	40/0	10/10	30/10
Oxygen uptake at occlusion (ng-at O)					
State 4	36 ± 3	33 ± 3	32 ± 3	31 ± 2	31 ± 3
State 3	230 ± 10	200 ± 10	195 ± 10	76 ± 4 ^a	81 ± 9 ^a
Respiratory control ratio	6.5 ± 0.3	6.0 ± 0.2	6.1 ± 0.4	2.4 ± 0.1 ^a	2.6 ± 0.2 ^a

Values represent the mean ± S.E.M. of six independent experiments.

ng-at O = nanogram atoms of oxygen.

^ap < 0.005.

Ferreira et al. (15) for human heart subjected to the ischemia-reperfusion associated with revascularization surgery.

Mitochondrial Respiratory Activities. Mitochondrial function was also affected by occlusion-reperfusion cycles. No change was observed in the rate of state 4 respiration (resting or controlled respiration), but a marked decrease (60%) in state 3 respiration (active respiration; maximal physiological rate of O₂ uptake and ATP production) was observed for both occlusion times. The respiratory control ratio, a sensitive indicator of the coupling between oxidation and phosphorylation and of mitochondrial integrity, was drastically diminished for both occlusion times (Table 2) (p < 0.005).

XD and XO Activities. The conversion of XD to the oxidase form was tested as a possible source of oxyradicals during reperfusion. No significant changes (p > 0.1) in the XO activity were observed during occlusion periods of 10 or 30 min (Table 3).

Activity of Antioxidant Enzymes. SOD, catalase and glutathione peroxidase activities diminished significantly (35% to 60%) after occlusion times of 30 min. Mitochondrial SOD showed a more marked decrease in activity than cytosolic SOD (Table 4) (p < 0.01).

Components of Increases of H₂O₂ Steady-state Concentration. As previously described (17), the steady-state concentration of H₂O₂ is reached when the rate of H₂O₂ production equals the rate of H₂O₂ utilization. This assumption allows the calculation of the rate of H₂O₂ production (17). With this experimental approach increased rates of hydrogen peroxide production of 40% and 160% were calculated for occlusion times of 10 and 30 min followed by 10 min of reperfusion, respectively

(Table 5). Nevertheless, some catalase inactivation was also found for 30-min occlusion-10-min reperfusion that further amplifies the effect of increased H₂O₂ production yielding a higher H₂O₂ steady-state concentration.

DISCUSSION

A decrease or the complete interruption of oxygen supply to a tissue produces biochemical changes at the mitochondrial and cytoplasmic levels (14, 17, 29), thus making cells susceptible to oxidative injury on reoxygenation, even when oxygen is supplied at low concentrations (30). This phenomenon has been termed reperfusion injury. It is generally accepted that oxygen free radicals are involved in the generation of reperfusion injury, but not in the structural and functional changes that occur during either ischemia or hypoxia (2, 14, 15, 17, 29-31).

No changes were observed in the markers of cell damage (LDH and AST serum activities and electron microscopy). Significant changes were nonetheless found in systemic and portal blood pressure. Thus it appears unlikely that hemodynamic changes are responsible for the cell damage observed on reperfusion. The maintenance of the singlet oxygen steady-state concentration, as indicated by chemiluminescence, seems to indicate the maintenance of a low PO₂ in the tissue (probably by lateral circulation, blood reapeal or both) and a low PO₂ requirement for the free radical reactions leading to singlet oxygen production. After a short period (10 min) of blood stasis, reperfusion almost immediately (less than 2 min) produced a marked increase in the steady-state level of singlet oxygen, as

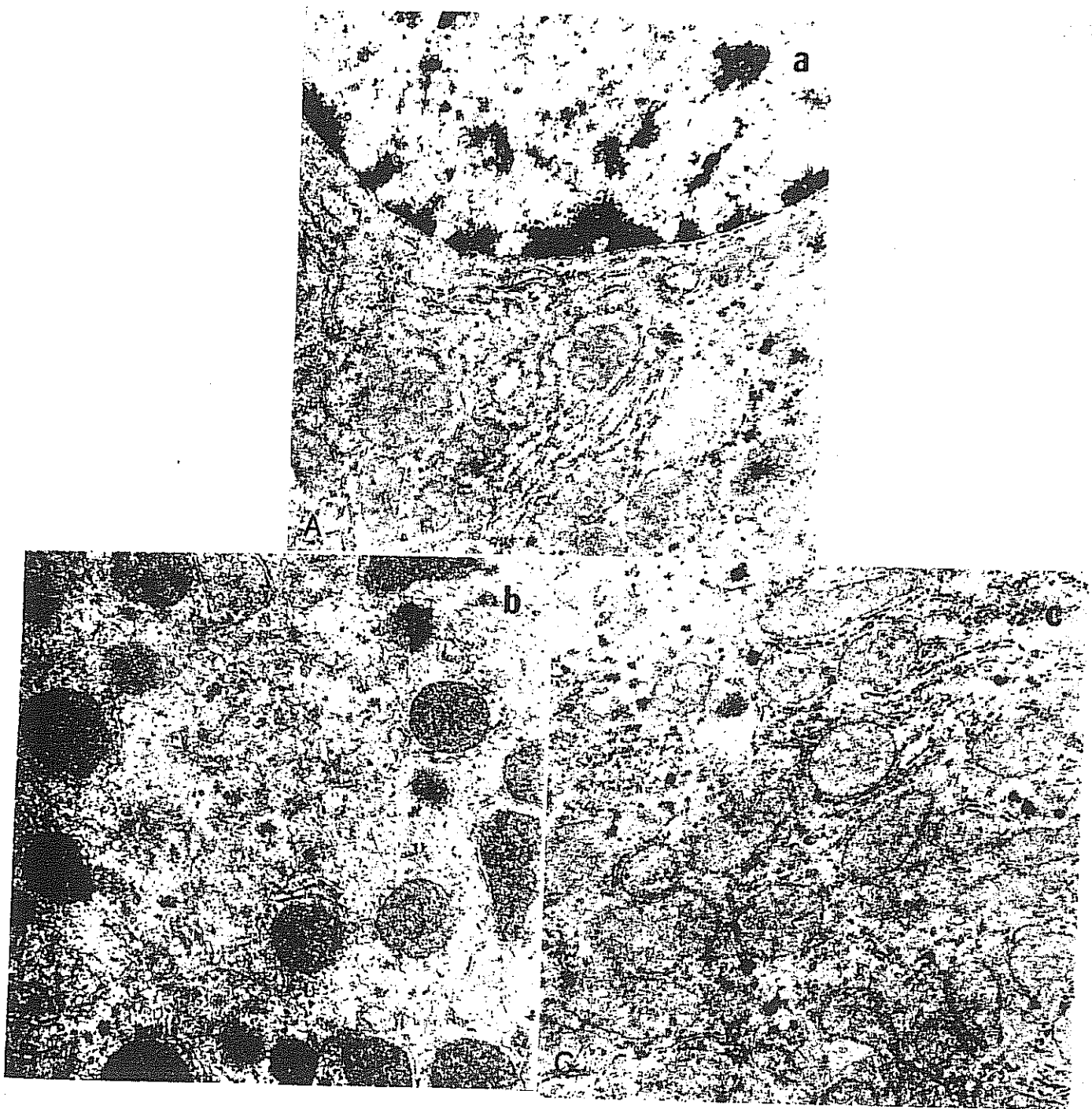


FIG. 3. Transmission electron micrographs of liver mitochondria before and after occlusion-reperfusion. (A) Control samples, (B) 40-min occlusion and (C) 30-min occlusion and 10-min reperfusion (original magnification $\times 20,000$).

detected by spontaneous liver chemiluminescence. Increased chemiluminescence has been reported both after 3 to 6 min of reperfusion of ischemic rat intestine (13) and after 3 min of anoxia and 2 min of reperfusion in suspensions of the amoeba *Acanthamoeba castellanii* (32). This indicates that this phenomenon occurs in most aerobic cells, including unicellular eukaryotes and mammalian tissues, and always appears at the onset of the reperfusion period. Hence, short-term reperfusion is

accompanied by oxidative stress even before other by-processes associated with long-term reperfusion, including neutrophil infiltration, occur.

An increased chemiluminescence of rat liver after occlusion and reperfusion ranged from moderate (1.5-fold) to marked (threefold) when occlusion time ranged from 10 to 30 min. Increases in spontaneous chemiluminescence have been previously reported in other oxidative stress situations, such as tumor bearing

TABLE 3. XD and XO activity after hepatic occlusion-reperfusion

Xanthine	Occlusion/reperfusion time (min)		
	0/0	10/10	30/10
XD (mU/gm liver)	220 ± 30	230 ± 30	230 ± 40
XO (mU/gm liver)	23 ± 2	23 ± 4	31 ± 6

Values represent the mean ± S.E.M. of six independent experiments. No significant differences were found among groups ($p > 0.1$).

TABLE 4. SOD, catalase and glutathione peroxidase activities in hepatic occlusion-reperfusion

Enzyme	Occlusion/reperfusion time (min)		
	0/0	10/10	30/10
SOD (U/gm liver)			
Mitochondrial	110 ± 5	107 ± 5	45 ± 2 ^a
Cytosolic	740 ± 40	680 ± 40	460 ± 10 ^a
Catalase (nmol/gm liver)	1.10 ± 0.04	0.96 ± 0.04	0.76 ± 0.04 ^a
Glutathione peroxidase (μmol/min/gm liver)	800 ± 30	530 ± 10 ^a	530 ± 10 ^a

Values represent the mean ± S.E.M. of six independent experiments.

^a $p < 0.01$.

mice (33), long-term ethanol treatment (34) and vitamin E and selenium deficiency in rats (35) in which an increase in oxyradical levels was sufficiently high to be above the threshold needed to cause hepatic injury.

Reflow after 10 min of inferior vena cava clamping produced a moderate increase in both the cell injury markers (LDH and AST) and in the indicators of mitochondrial damage (ultrastructure and respiratory activities), as well as in those of oxidative stress (chemiluminescence and H_2O_2). Mitochondrial respiratory control after reflow was also markedly decreased, indicating a significant impairment in mitochondrial function. With a 10-min occlusion period no decrease was found either in the activity of antioxidant enzymes or in the level of nonenzymatic antioxidant (as detected by the hydroperoxide-initiated chemiluminescence). Furthermore, no conversion of XD into XO was observed. Thus the moderate oxidative stress associated with 10-min occlusion appears to be caused by an increase in the rate of generation of oxyradicals without changes in the rate of free radical utilization. Such a situation appears to produce moderate damage, as indicated by the slight release of LDH and AST and the changes of mitochondrial morphology.

Reperfusion after an occlusion period of 30 min, in contrast, produced a more marked increase in H_2O_2 steady-state concentration and a leakage of LDH and AST without further changes in either spontaneous chemiluminescence, mitochondrial morphology, respiratory control or XO activity. Under these conditions, the developed oxidative stress appears to be aggravated by a diminution in the rates of oxyradical detoxification, as indicated by the marked decrease in both the activity of antioxidant enzymes and the level of nonenzymatic antioxidants.

Because LDH and AST are released into the general circulation on removal of the suprahepatic occlusion, it is unclear whether the rise in the enzymatic activity reflects injury occurring during occlusion measured

after the ligature has been removed or injury occurring after reperfusion. However, the absence of changes in mitochondrial function, in hydrogen peroxide steady-state concentrations and in spontaneous and hydroperoxide-initiated chemiluminescence, added to the previously reported correlation found between chemiluminescence and tissue damage (24, 35), seems to indicate that injury occurs during the reperfusion phase. The morphological changes that occurred during occlusion were much less marked than the one observed after reflow.

The measured XO activity and the ratio of univalent and bivalent electron fluxes at nearly neutral pH previously reported by Fridovich (36) allow the calculation of the rate of O_2^- and H_2O_2 production as by-products of the reaction of XO. The calculated rates are 0.08 to 0.1 $\mu\text{mol/L} \cdot \text{sec}^{-1} O_2^-$ and 0.30 to 0.42 $\mu\text{mol/L} \cdot \text{sec}^{-1} H_2O_2$ for control and 30-min occlusion-10-min reperfusion, respectively. The measured (17) or calculated (Table 5) rates of mitochondrial H_2O_2 production were 1.25 and 1.40 to 3.70 $\mu\text{mol/L} \cdot \text{sec}^{-1} H_2O_2$, respectively, 4 to 10 times higher than the expected H_2O_2 production by XO.

The marked increase in mitochondrial H_2O_2 generation (Table 5) is consistent with the observed inhibition of mitochondrial electron transfer and the decreased state 3 respiration. For production of the maximal rate of O_2^- and H_2O_2 as by-products of the activity of the mitochondrial respiratory chain, it is necessary to block the main pathway of electron transfer. This blocking is usually achieved *in vitro* by the use of mitochondrial inhibitors of electron transfer, such as rotenone and antimycin (37). The results reported herein suggest that an endogenous inhibitor blocks electron transfer (state 3 respiration) and increases mitochondrial H_2O_2 production occlusion-reperfusion cycles. This increased production and the consequent oxidative inactivation of antioxidant enzymes set an autoenhanced process.

Other putative liver sources of oxygen free radicals,

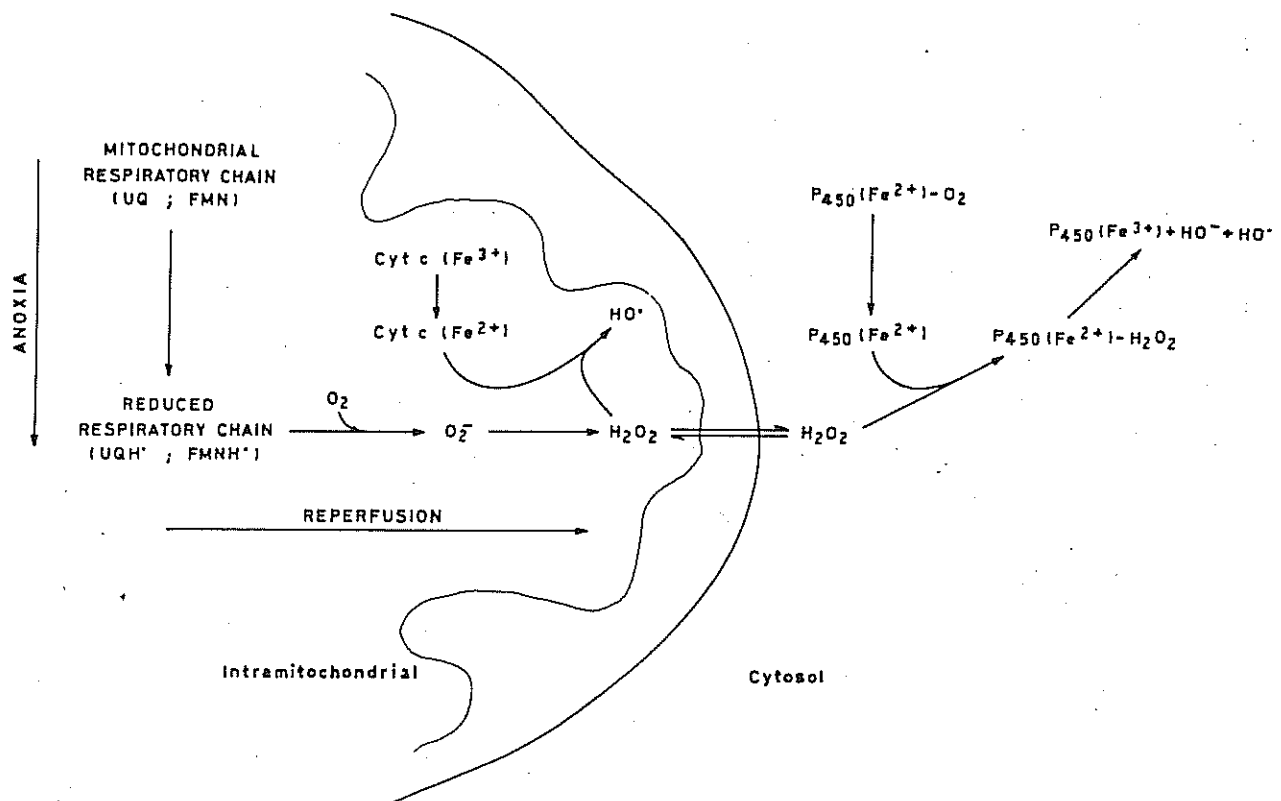


FIG. 4. Scheme of the biochemical events related to liver ischemia- or anoxia-reperfusion. *UQ* and *UQH•* = oxidized and partially reduced ubiquinone, respectively; *FMN* and *FMNH•* = oxidized and partially reduced NADH dehydrogenase; *cyt c* = cytochrome c; and *P₄₅₀* = cytochrome P-450.

TABLE 5. Intracellular concentration and production rate of hydrogen peroxide in hepatic occlusion-reperfusion

Parameters	Occlusion/reperfusion time (min)		
	0/0	10/10	30/10
H_2O_2 ($\mu\text{mol/L}$) ^a	0.10 ± 0.01	0.17 ± 0.01	0.30 ± 0.05
$d[H_2O_2]/dt$ ($\mu\text{mol/L} \cdot \text{sec}^{-1}$) ^b	4.6	6.2	12.2
Participation in the increase of H_2O_2 steady-state concentration (%) ^b			
Increase in H_2O_2 production	—	100	80
Decrease in catalase	—	0	20

$d[H_2O_2]/dt$ = the rate of H_2O_2 production, where $d[H_2O_2]$ is the change in H_2O_2 concentration and dt is the change in time.

^aValues represent the mean \pm S.E.M. of six independent experiments.

^bValues represent the mean of six independent experiments.

including monocytes and Kupffer cells, have been shown to need at least 60 min of ischemia and 60 min of reperfusion to produce significant oxidative stress in rat liver (38, 39). Neutrophils do not seem to play a major role in the early liver reperfusion injury as shown by Jaeschke and Farhood (39), who found no correlation between the number of neutrophils and the oxidized glutathione levels in the reperfused rat liver. The biochemical mechanism that seems to account for the generation of a highly reactive species able to initiate free radical chain processes is shown in Figure 4.

Because suprahepatic occlusion also affects lower

extremities and abdominal organs, it is not possible to discern whether the involved organs may also contribute to the hepatic changes herein reported. However, because of the short occlusion/reperfusion time used, it seems unlikely that oxidative stress enhancers—including those produced in the kidney, bowels or adrenals—could reach the liver and cause the observed changes (40, 41). The contribution of the local hemodynamic changes to the rate of free radical generation appears to be negligible because no clear correlation appears between increases in portal pressure and markers of oxidative stress and tissue damage was observed.

In summary, among the putative intracellular (XO, mitochondria and inactivation of antioxidant enzymes) and extracellular (endothelial XO, Kupffer cells, neutrophils and other organs) sources of oxygen free radicals, liver mitochondria seem to play a major role as the primary subcellular site in which oxidative damage on cellular components occurs in this model system.

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REFERENCES

- Sies H. Oxidative stress: introductory remarks. In: Sies H, ed. Oxidative stress. San Diego: Academic Press, 1985:1-7.
- Gonzalez-Flecha B. Oxidative stress in human disease: assays of clinical application. *Acta Biochim Clin Latinoam* 1990;24:67-74.
- Chance B, Sies H, Boveris A. Hydroperoxide metabolism in mammalian organs. *Physiol Rev* 1979;59:527-605.
- Tyler DD. Polarographic assay and intracellular distribution of superoxide dismutase in rat liver. *Biochem J* 1975;147:493-504.
- Oshino N, Chance B, Sies H, Bucher T. The role of hydrogen peroxide generation in perfused rat liver and the reaction of catalase compound I and hydrogen donors. *Arch Biochem Biophys* 1973;154:117-131.
- Giulivi C, Turrens JF, Boveris A. Chemiluminescence enhanced by trypanocidal drugs and by inhibitors of antioxidant enzymes in *Trypanosoma cruzi*. *Mol Biochem Parasitol* 1988;30:243-252.
- Cadenas E, Boveris A, Chance B. Low-level chemiluminescence of biological systems. In: Pryor WA, ed. Free radicals in biology. Vol 6. New York: Academic Press, 1984:211-238.
- Perez V. Liver diseases. Buenos Aires: El Ateneo, 1964:323-350.
- Bosch J, Rodes J. Portal hypertension. In: CSIC, ed. Hepatology. Madrid: Raycar Press, 1990:255-289.
- Terada T, Ishida F, Nakanuma Y. Vascular-plexus around intrahepatic bile ducts in normal livers and portal hypertension. *J Hepatol* 1989;8:139-149.
- Marotto ME, Thurman RG, Lemasters JJ. Early midzonal cell death during low-flow hypoxia in the isolated, perfused rat liver: protection by allopurinol. *HEPATOLOGY* 1989;8:585-590.
- Roy RS, McCord J. Superoxide and ischemia: conversion of xanthine dehydrogenase to xanthine oxidase. In: Greenwald R, Cohen G, eds. Oxyradicals and their scavenger systems. New York: Elsevier Science, 1983:145-153.
- Roldan EJA, Pinus CR, Turrens JF, Boveris A. Chemiluminescence of ischaemic and reperfused intestine *in vivo*. *Gut* 1989;30:184-187.
- McCord J. Free radicals and myocardial ischemia: an overview and outlook. *J Free Rad Biol Med* 1988;4:9-14.
- Ferreira R, Burgos M, Llesuy S, Molteni L, Milei J, Gonzalez-Flecha B, Boveris A. Reduction of reperfusion injury with mannitol cardioplegia. *Ann Thor Surg* 1989;48:77-84.
- Al-Khaidi OAS, Chaglasian TH. The species distribution of xanthine oxidase. *Biochem J* 1965;97:318-320.
- Gonzalez-Flecha B, Cutrin JC, Boveris A. Time course and mechanism of oxidative stress and tissue damage in rat liver subjected to *in vivo* ischemia-reperfusion. *J Clin Invest* 1993;91:456-464.
- Jaeschke H, Mitchell JR. Mitochondria and xanthine oxidase both generate reactive oxygen species in isolated perfused rat liver after hypoxic injury. *Biochem Biophys Res Commun* 1989;160:140-147.
- Boveris A, Cadenas E, Reiter R, Filipowsky M, Nakase Y, Chance B. Organ chemiluminescence: non-invasive assay for oxidative radical reactions. *Proc Natl Acad Sci USA* 1980;77:347-351.
- Puntarulo S, Sanchez R, Boveris A. Hydrogen peroxide metabolism in soybean embryonic axes at the onset of germination. *Plant Physiol* 1988;86:626-630.
- Stempak JG, Ward RT. An improved staining method for electron microscopy. *J Cell Biol* 1964;22:697-702.
- Blouin RP, Weibel ER. Stereological analysis in electron microscopy. *J Cell Biol* 1977;72:441-445.
- Estabrook RW. Mitochondrial respiratory control and the polarographic measurements of ADP:O ratios. *Methods Enzymol* 1967;10:41-47.
- Gonzalez-Flecha B, Llesuy S, Boveris A. Hydroperoxide-initiated chemiluminescence: an assay for oxidative stress in biopsies of heart, liver and muscle. *Free Radic Biol Med* 1991;10:93-100.
- Beauchamp CO, Fridovich I. Isoenzymes of superoxide dismutase from wheat germ. *Biochim Biophys Acta* 1973;317:50-64.
- Flohe L, Gunzler WA. Assays of glutathione peroxidase. *Methods Enzymol* 1984;105:114-121.
- Lowry OH, Rosebrough AL, Farr AL, Randall R. Protein measurement with the folin phenol reagent. *J Biol Chem* 1951;193:265-275.
- Winer BJ. Statistical principles in experimental design. New York: McGraw Hill, 1971:201-204.
- Jaeschke H. Glutathione disulfide as index of oxidant stress in rat liver during hypoxia. *Am J Physiol* 1990;21:G499-G505.
- Jones DP. The role of oxygen concentration in oxidative stress: hypoxic and hyperoxic models. In: Sies H, ed. Oxidative stress. San Diego: Academic Press, 1985:151-189.
- Marubayashi S, Kiyohiko D, Ochi K, Kawasaki T. Role of free radicals in ischemic rat liver cell injury: prevention of damage by tocopherol administration. *Surgery* 1986;90:184-192.
- Lloyd D, Boveris A, Reiter R, Filipowsky M, Chance B. Chemiluminescence of *Acanthamoeba castellanii*. *Biochem J* 1979;184:149-156.
- Boveris A, Llesuy S, Fraga C. Increased liver chemiluminescence in tumor-bearing mice. *Free Radic Biol Med* 1985;1:131-138.
- Boveris A, Fraga C, Varsavsky A, Koch O. Increased chemiluminescence and superoxide production in the liver of chronically ethanol-treated rats. *Arch Biochem Biophys* 1983;227:534-541.
- Fraga C, Arias RF, Llesuy S, Koch O, Boveris A. Effect of vitamin E- and selenium-deficiency on rat liver chemiluminescence. *Biochem J* 1987;242:383-386.
- Fridovich I. Quantitative aspects of the production of superoxide anion radical by milk xanthine oxidase. *J Biol Chem* 1970;245:4053-4057.
- Boveris A, Cadenas E. Production of superoxide radicals and hydrogen peroxide in mitochondria. In: Oberley LW, ed. Superoxide dismutase. Vol. II. Boca Raton, Florida: CRC Press, 1982:15-30.
- Caldwell-Kenkel JC, Currin RT, Tanaka Y, Thurman RG, Lemasters JJ. Kupffer cell activation and endothelial cell damage after storage of rat livers: effect of reperfusion. *HEPATOLOGY* 1991;13:83-95.
- Jaeschke H, Farhood A. Neutrophil and Kupffer cell-induced oxidant stress and ischemia-reperfusion injury in rat liver. *Am J Physiol* 1991;260:G355-362.
- Joannidis M, Bonn G, Pfaller W. Lipid peroxidation: an initial event in experimental acute renal failure. *Renal Physiol Biochem* 1989;12:47-55.
- Takenaka M, Tatsukawa Y, Dohi K, Ezaki H, Matsukawa K, Kawasaki T. Protective effects of tocopherol and coenzyme Q 10 on warm ischemic damages of the rat kidney. *Transplantation* 1981;32:137-141.