Ischemia / reperfusion injury of the liver : pathophysiologic hypotheses and potential relevance to human hypoxic hepatitis

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Abstract

For the last decade, numerous experimental studies have demonstrated that the main part of liver injury caused by low or no flow states does not occur at the time of hypoxia, but during reperfusion. These experimental studies have a crucial clinical impact, because ischemia/reperfusion injury is involved in situations such as temporary vascular exclusion during liver surgery for trauma or tumors, preservation injury before liver transplantation, and liver cell necrosis observed in hypoxic (ischemic) hepatitis. The aim of the present review is to clarify the sequence of pathophysiological events responsible for ischemia/reperfusion injury of the liver, and to examine the potential contribution of liver ischemia/reperfusion injury to the syndrome of human hypoxic hepatitis. (Acta gastroenterol. belg., **2000**, 63, **336-347**).

Introduction

In 1948, in order to explain the tissue damage observed in conditions of shock state, Moon wrote : «*Anoxia is highly important in the vicious circle by which shock tends to progress to an irreversible stage. The anoxia may be anoxic as in asphyxia, or anemic as when the red corpuscules have been reduced below physiologic limits by hemorrhage or otherwise, or stagnant as by circulatory stasis, or toxic as from the effects of poisons upon the tissue cells. In any instance, anoxia is the factor which ultimately stops the machine and wrecks the machinery*» (1, p 238). By emphasizing the ultimate role of hypoxia, Moon was certainly a precursor. Nevertheless, he was only partially right. Indeed, for the last decade, numerous studies have demonstrated that hypoxia was not the sole actor in the pathogenesis of tissular injury secondary to low flow states, arterial hypoxemia or temporary vascular exclusion, but instead, that a large part of the injury may follow the restoration of oxygen delivery to the tissues. Such a situation, where a cell culture, a tissue, an organ or a whole organism is submitted to a hypoxic stress, then is reperfused and reoxygenated is experimentally called an ischemia/reperfusion (I/R) injury. From those experimental studies, there is mounting evidence that ischemic injury is a misnomer (2) and that most of the tissular injury occurs at the time of reperfusion. The clinical impact of these experimental studies on I/R is tremendous and explains why more than 4000 papers devoted to this topic were published from 1990 to 1994 according to a Medline Search (3). Indeed, in the field of liver injury, I/R encompasses several clinical settings such as the temporary interruption of the liver blood flow during liver surgery for trauma or tumors, the organ preservation before liver transplantation and all conditions of low flow state where the lack of oxygen is generally not absolute.

The aim of the present review is an attempt to clarify the nature and, if possible, the sequence of the different pathophysiological events leading to liver I/R injury. The last part of this review will be devoted to the potential impact of the I/R phenomenon on the pathogenesis of clinical hypoxic hepatitis. But before developing the pathophysiological mechanisms involved in I/R injury of the liver, it may be interesting to briefly comment different experimental tools that were useful in the study of the I/R injury.

Research tools in the field of liver ischemia / reperfusion injury

First, warm and cold ischemia have to be distinguished (4). Studies on cold ischemia are particularly interesting in the field of organ storage before transplantation. Since the clinical condition of low flow state corresponds more to warm I/R models, the following review on common experimental tools will address more specifically to this form. Several experimental models of warm I/R injury of the liver have been designed. The most widely used is the in-vivo partial I/R of the rat liver where the vascular pedicle of the median and left lobe are clamped while the right lobe remains perfused. This model has been largely promoted by the research team of Harmut Jaeschke (5-8). The advantage of a partial ischemia of the liver is to avoid intestinal venous congestion because the venous mesenteric blood can flow through the right lobe during the ischemic period. A disadvantage of this model is the preferential shunting of the blood flow through the right (non-ischemic) lobe during the reperfusion when the clamp is released. For

this reason, some investigators advocate to perform a resection of the right lobe at the start of the reperfusion and others have devised a model of total ischemia with an extracorporeal porto-systemic derivation (9). Another common model of liver I/R, is the isolated, perfused liver. By modulating the infusion rate, this model allows to study a condition of low-flow state/reperfusion (more similar to the clinical setting) instead of the condition of no-flow state/reperfusion (10-14). An interesting feature of this model is to avoid the contamination of the infused solution by components either naturally present in the blood such as platelets, leucocytes, coagulation factors and complement, or released in the blood from the intestine such as cytokines, platelet activating factor (PAF), endotoxins and other bacterial products. Less frequently, liver I/R has been studied in vitro on cell cultures submitted to hypoxia (15, 16) and in vivo on whole animals submitted to hemorrhagic shock followed by blood restoration (17-20). Finally, Neil Granger and his research team were the firsts to design a model of I/R of the gut and to study the impact on remote organs, particularly the liver and the lungs (21-23).

Actually, liver I/R injury corresponds partly to a hypoxic lesion and mainly to an inflammatory reaction. At least two inflammatory cells and a myriad of inflammatory mediators are implicated in a cascade of intricated events. The numerous experimental studies published in this field often share some similar methodological tools that will be briefly reviewed. The detrimental effect of inflammatory cells, mainly Kupffer cells and polymorphonuclear leucocytes (PMN) has been assessed by the use of stimulating agents and more often, of inhibiting agents such as specific monoclonal antibodies for PMNs (5, 24, 25) and gadolinium chloride for Kupffer cells (6, 8, 12). The microvascular (sinusoidal) injury known as the *no reflow* phenomenon has been assessed not only on fixed tissue by optic and electron microscopy but also in vivo by intravital fluorescence microscopy (22, 25-27), by scanning electron microscopy (28, 29), by laser doppler flowmetry (27, 30) and by reflectance spectrophotometry that allows assessment of tissue oxygenation (31, 32). The cell injury, either necrosis or apoptosis, has been studied by optic and electron microscopy, but also more dynamically by assessment of the time-course of cytosolic enzyme release (ALAT, ASAT, LDH). Moreover, in numerous studies, the viability of the cells (reversible or irreversible damage) has been studied by specific markers such as Trypan blue (10, 12, 13, 16, 33) or propidium iodide (11, 14, 34). Finally, countless proinflammatory mediators, mainly reactive oxygen species (ROS), cytokines, eicosanoids and PAF have been studied by measuring their blood and hepatic levels or for some of them, by measuring the expression of their mRNA during the I/R reaction, and by cutting their effect by antagonist agents or specific monoclonal antibodies.

Main pathophysiological mechanisms involved in liver ischemia / reperfusion injury

Despite numerous studies, the exact pathophysiological mechanisms involved in liver I/R and the precise sequence of events remains partly elusive and controversial. Liver I/R injury includes two phases, namely the ischemic injury and the reperfusion injury. In experimental conditions, both phases are clearly separated. However, in the clinical conditions of low flow state, these two phases are not clearly separated but overlap each other. Indeed, in clinical low flow states, the lack of oxygen is generally not absolute and hemodynamics can be often restored, at least temporarily, allowing hypoxic organs to be reoxygenated. These two phases, the ischemic and the reperfusion, will be successively commented.

The ischemic phase

Compared to other organs, the liver is resistant to ischemia. The classic statement that warm ischemia of the human liver by ligation of the vascular pedicle (Pringle manœuvre) cannot last over 15-20 min has been challenged by Huguet *et al.* two decades ago (35), then confirmed by other surgical teams. It is now admitted that total warm ischemia of the liver during surgery for hepatic trauma or tumors can be extended up to 90 min (36), particularly when venous congestion of the gut is avoided by venous bypass (37). In addition, experimental studies in the rat have shown that ischemia shorter than 30 min generally does not result in irreversible liver cell necrosis (10, 11, 26, 28, 33, 38, 39) and in some experiments does not provoke substantial injury even at the time of reperfusion (38). Conversely, ischemia lasting more than 90 min generally induces irreversible liver cell necrosis already at the time of ischemia (28, 33, 40) while ischemia between 30-90 min leads to some degree of liver cell necrosis during ischemia and more extensive lesions during reperfusion (10, 11, 26, 38, 39).

Of note, liver injury secondary to pure anoxia has been the purpose of few recent studies (38, 41-43) by comparison with the numerous studies devoted to reperfusion injury. Using optic microscopy, the first histological lesions observed during pure anoxia were swelling of the hepatocytes and sinusoidal cells resulting in narrowing of the sinusoids (42). These changes were predominantly observed in the midzonal regions (10, 11, 42, 43). In one study (33), areas of infarction were evident only after 180 min of ischemia. At electron microscopy, hepatocytes appeared deformed by blebs i.e. protrusions of the cell plasma membrane that tended to grow, coalesce and protrude into the sinusoidal lumen, while sinusoids were found to be stuffed with membrane-surrounded material corresponding to shed blebs (34, 38, 43). These changes were still reversible and thus, at this stage, shedding of blebs could result in

entry of cytosolic enzymes into the systemic circulation without cell death (41). After 90 min of ischemia, sinusoids were filled with amorphous cytoplasmic material, the sinusoidal wall was desintegrated and the membranes of surrounding hepatocytes were indiscernable (38, 41, 43). From a metabolic viewpoint, the first observed event was the rapid depletion of high-energy molecules such as adenosine triphosphate secondary to mitochondrial dysfunction (44). The loss of energy metabolism led to membrane cell function impairment with loss of intracellular ion homeostasis resulting in excessive intracellular accumulation of calcium and sodium (43-46). Another effect of energetic failure of the cell was activation of degradative enzymes such as proteases, phospholipases and endonucleases (43). As a consequence, xanthine dehydrogenase (XDH) was transformed in xanthine oxidase (XO) and concomitantly with the degradation of adenosine triphosphate in hypoxanthine, an ideal environment was set up for an oxidative stress as soon as flow and oxygen were reentered (40, 44). It may be worthy to point out that I/R experiments (particularly after cold ischemia) have showed that sinusoidal cells were particularly susceptible to ischemic insult (47-49), that human hepatocytes were more resistant to hypoxia than rodent hepatocytes (15) and that cell death caused by hypoxia included as well true necrosis than excessive apoptosis (50-53).

The reperfusion phase

When the clamp is removed from the vascular pedicle in experimental conditions, or when systemic hemodynamics are restored in clinical settings, blood and oxygen reenter the liver and, in relation with the duration of ischemia, may cause a severe reperfusion injury. The complex inflammatory reactions occurring at the time of reperfusion have been better delineated recently and at least four phenomena have gained some acceptance: 1/ the oxidative stress at the start of reoxygenation, 2/ the early activation of the Kupffer cells, 3/ the microvascular (sinusoidal) disturbance known under the term of "no-reflow" phenomenon and 4/ the late activation of PMNs leading to cellular injury (fig. 1).

1. The oxidative stress

At the onset of liver reperfusion, all conditions are theoritically met for an intracellular oxidative stress. As earlier reported, a substantial part of XDH has been converted to XO and the metabolites, hypoxanthine and xanthine are aboundant owing to the catabolism of ATP. If both XDH and XO degrade xanthine to uric acid, the reaction performed with XO results in production of superoxide anion, a potent ROS (3). Coupled together with rapid depletion of reduced glutathione, the generation of ROS could lead to severe cell injury by lipid peroxidation and covalent binding to proteins (3, 54, 55). After the initial description by Granger *et al.* in 1981 (21), the XO hypothesis gained widespread acceptance and was rapidly considered as the first step in the I/R chain reaction in all organs (3). The reality of an oxidative stress was demonstrated at first indirectly by the use of antioxidants agents such as allopurinol which inhibits XO and superoxide dismutase that neutralizes superoxide (10, 11, 56), then more directly by measuring the products of oxidative stress such as aldehyde adducts (12, 54), by measuring the increase in XO activity (57-59), or by assessing the consumption of reduced glutathione (GSH) and the concomitant increase in oxydized glutathione (GSSG) (60, 61). Recently however, it became increasingly obvious that the hypothesis was too simplistic and that there were organ specific differences (59, 60). Indeed, in the liver submitted to hypoxia, the conversion of XDH to XO proceeds very slowly and the rise in XO is very modest. Studies have shown that the conversion of XDH to XO began only after two hours of hepatic no-flow warm ischemia (62) and that the activity of XO increased modestly from 25 % of the total oxidoreductase activity in normal state to around 45 % in hypoxic state (58, 59). Moreover, no causal relationship could be established between the increase in XO and the liver cell injury (59). These observations have raised the question of the real relevance of the XO hypothesis in the pathogenesis of I/R injury of the liver (59-61). Nowadays, it is more widely agreed that the oxidative stress plays an important role in the reperfusion injury of the liver, but not through the pathway of the XDH conversion to XO, neither through an excessive mitochondrial production of the superoxide anion, another possible source of intracellular oxidative stress (63, 64). A growing body of evidence supports the recent hypothesis that the oxidative stress imposed to sinusoidal cells and hepatocytes during the I/R reaction has an extracellular (vascular) origin and comes from activated Kupffer cells in the early phase of the reaction (1-3 hours) and from activated PMNs in the late phase (6-24 hours) (6, 60, 65). The precise relevance of either an intracellular or an extracellular (vascular) oxidative stress remains, nevertheless, a highly controversial issue (66, 67).

2. The activation of the Kupffer cells

During the early phase of the liver I/R injury, the most detrimental event is probably the activation of Kupffer cell (6, 65). Kupffer cells are macrophages, i.e. adherent monocytes. Their first role is to clear by phagocytosis, exogenous products such as bacterial endotoxins (LPS) released into the mesenteric blood from the gut. They are also powerful reactive cells capable, more than any other cells, to release huge amounts of inflammatory mediators when they are activated. In normal state, these cells are quiescent but they can evolve into two levels of activation, the first called "priming" and the second corresponding to the "triggering" of their activation. When macrophages are primed, they do not release inflammatory mediators but they are more susceptible to triggering agents and release more mediators when stimulated.

Fig. 1. — Schematic view of the potential sequence of pathophysiologic events leading to liver reperfusion injury.

LIVER REPERFUSION

→ **EARLY EVENTS** (< 1 H)
- ***OXIDATIVE STRESS***
  - Intracellular : ROS originating from the transformation of XDH into XO and from the mitochondrial failure
  - Extracellular : Kupffer cell release of ROS
- ***KUPFFER CELL ACTIVATION***
  - Mediators : ROS, Sinusoidal factors, Complement activation, LPS
  - Results : Release of – inflammatory mediators (ROS, cytokines, eicosanoids, PAF), – chemokines

→ **INTERMEDIATE EVENTS** (1-6 H)
- ***NO-REFLOW PHENOMENON***
  - Mechanisms : Cellular blebs, Erythrocyte and platelet agregates, Neutrophil plugging, Perisinusoidal edema, Sinusoidal vasomotor tone

→ **LATE EVENTS** (6 – 24 H)
- ***NEUTROPHILIC HEPATITIS***
  - Mechanisms : – Neutrophil margination, rolling, adhesion to sinusoidal cells, diapedesis and transmigration
    – Neutrophil adhesion to parenchymatous liver cells
    – Neutrophil release of cytopathic agents (ROS and proteases)

Abbreviations : LPS : lipopolysaccharide (endotoxin) – PAF : platelet activating factor – ROS : reactive oxygen species – XDH : xanthine dehydrogenase – XO : xanthine oxidase.

Several substances have been shown to prime Kupffer cells and include, for instance, propionibacterium acnes and galactosamine in the common experimental model of LPS-induced liver injury (7, 68-76). Most interestingly in the setting of liver I/R injury, hypoxia has been shown to prime Kupffer cells. Indeed, Kupffer cells harvested from animals previously submitted to hypoxia release more inflammatory mediators when triggered by LPS (77), and human macrophages exposed to anoxia release TNF even without stimulation (78). As for the triggering agents of Kupffer cells, the best known and most studied is the lipopolysaccharidic moiety of endotoxin (LPS). When stimulated by LPS, primed Kupffer cells may release huge amounts of inflammatory mediators. These include ROS, eicosanoids, such as leukotrien B4, PAF and mainly proinflammatory cytokines such as tumor necrosis factor (TNF), Interleukine-1 (IL-1) and IL-8 (65, 79, 80). Macrophages have been compared to a "cytokine factory" (80) and are now considered as the major source of the key mediators of septic shock (74), the best example of clinical conditions linked to activation of the macrophages. Beside sepsis, the I/R reaction is another typical example of regional or systemic activation of the macrophages (65). Several lines of evidence have demonstrated that Kupffer cells are activated and play a major role in the early stage of liver reperfusion injury: 1/ optic and electron microscopy examinations have shown that Kupffer cells displayed typical features of activated macrophages (28, 47); 2/ inhibition of Kupffer cells by gadolinium chloride or methylpalmitate reduced the extension of liver injury (6, 8, 12, 28), while stimulation of Kupffer cells by latex particles or phorbol myristate extended the lesion (6, 28); 3/ Kupffer cells harvested from animals submitted to I/R release more inflammatory mediators such as ROS and TNF than non-activated cells (8, 77, 81).

Which substances trigger the release of proinflammatory mediators by Kupffer cells during the early phase of reperfusion remains elusive and controversial. Reactive oxygen species (ROS) generated at the onset of the reperfusion are potential candidates. Indeed, Kupffer cells not only produce ROS, but their production of inflammatory mediators such as TNF and PAF is also enhanced by ROS (50, 65, 82-85). Another possibility could be putative factors released by sinusoidal endothelial cells during the anoxic injury. Sinusoidal cells appear more susceptible than other liver cells to ischemic insult and morphologic features of severe

damage are present early during the ischemic period (47-49). Complement activation has also been proposed as a stimulatory agent for Kupffer cell. Complement is rapidly activated through the classical pathway in I/R injury (86) and Jaeschke *et al.* have shown that inhibition of complement activation decreased by half the oxidative stress and the extent of necrosis in a model of LPS-induced hepatitis while PMN recruitment was not modified (7). Finally, LPS is a potential triggering factor of Kupffer cells in experimental models of I/R and in clinical low flow states whereby the ischemic gut is a source of endotoxins and other inflammatory mediators released in the portal blood flow (87). Studies on the gut I/R injury have clearly shown that the release of LPS or PAF in the mesenteric blood flow can induce injury in remote organs rich in adherent macrophages such as the liver and the lung (the so called "*gut-liver-lung axis*") (22, 87, 88). However animal models of liver I/R injury free of bacterial products such as the isolated perfused liver, demonstrated that LPS was not absolutely required in the early phase of I/R.

Once triggered, Kupffer cells release powerful inflammatory mediators that have cytopathic effect, such as ROS, cytokines and PAF. Several studies have clearly established a relation between the generation of ROS in the early phase of I/R injury and the extent of liver cell necrosis (6, 12). Cytokines are soluble hormone-like proteins that have mainly a paracrine or autocrine effect. Their action remain generally confined to the environment of the producing cells (65, 78, 89). Two peculiarities of these cytokines are their ability to initiate cascade reactions ("cytokine network") and their amplification effect (65, 89, 90). For instance, TNF a powerful cytokine released by activated macrophages may not only trigger the release of other inflammatory mediators such as IL-1 or PAF, but can also stimulate its own production (65, 89, 90). Targets of cytokines are often also the source. This explains how a physiological reaction may become excessive and detrimental. Another important proinflammatory cytokine released by activated Kupffer cells is IL-1. When injected in animals, both TNF and IL-1 are able to trigger the main features of septic shock (89, 90). Another cytopathic inflammatory mediator also released by activated Kupffer cells and able to reproduce features of septic shock is PAF, a lipid mediator. Similarly to TNF, PAF is both a product and a stimulating agent of activated macrophages (91, 92). In the early phase of the I/R reaction, the combined effect of these cytopathic mediators released by activated Kupffer cells might be at the origin of two subsequent pathologic events: the sinusoidal damage leading to the "*no-reflow*" phenomenon and the "*neutrophilic hepatitis*" caused by transmigrating PMNs.

3. The sinusoidal injury: the "no-reflow" phenomenon

Some decades ago, investigators of shock states had already noted severe alteration of the liver microcirculation in low flow states. In 1965, Shoemaker *et al.* (93), using a method of in vivo photomicrography, showed the heterogeneity and slowing down of sinusoidal blood flow in animals submitted to hemorrhagic shock. Two years later, the term "*no-reflow*" was coined by Majno *et al.* (94) in a study of the brain microcirculation. The no-reflow phenomenon refers to the lack of blood flow in the microcirculation (sinusoids for the liver) despite restoration of blood perfusion after a period of ischemia. Once controversial, the no-reflow phenomenon has been since demonstrated and is currently considered as a detrimental event, potentially responsible for cellular hypoxia in the course of I/R injury. The reality of the no-reflow phenomenon has been directly established by in vivo methods using intravital fluorescence microscopy (22, 25, 26, 27, 95) or scanning electron microscopy (28, 29) for the anatomic and dynamic features of the sinusoids, laser Doppler flowmetry for the red blood cells velocity (27, 30) and reflectance spectrophotometry for variations in tissular oxygenation (31, 32, 96). If earlier studies had shown that the cessation of blood flow through the sinusoids was already present at the start of the reperfusion, interestingly, more recent studies have shown that, depending on the duration of the ischemic period, the no-reflow phenomenon could be absent or mild at the onset of the reperfusion, then increased progressively, thereby demonstrating that the sinusoidal perfusion failure was not only an ischemic injury but also a reperfusion injury. Hence, in an in-vivo model of total liver I/R in the rat, sinusoidal blood flow was assessed by laser Doppler flowmetry for 60 min during reperfusion after ischemic periods of 30 sec (control), 30, 45 and 60 min (95). After both 45 min- and 60 min-ischemic periods, sinusoidal blood flow was severely impaired (mean flow less than 50 % of the baseline level) from the start of the reperfusion and remained similarly decreased throughout the reperfusion. Conversely after the ischemia period of 30 min, the reperfusion recorded a biphasic curve with a mean flow reaching more than 80 % of the baseline level from 10 to 20 min after reperfusion, then decreasing progressively to a mean flow of 52 % after 1 hour of reperfusion (95). Very similar results were recorded by Koo *et al.* with a partial liver I/R experiment in the rat under the control of intravital microscopy (26). Depending on the duration of ischemia, reperfusion resulted in an initial return of blood flow, then cessation of blood flow developed progressively. In this study, an ischemic period of 15 min did not result in any reperfusion injury or no-reflow phenomenon, while an ischemic period of 55 min resulted in severe reperfusion injury and a no-reflow phenomenon already fully developed at the start of the reperfusion. Conversely, after an ischemic period of 25 min, initial reflow was observed in almost all sinusoids, then progressively decreased (26). Interestingly again, some studies could establish a significant correlation between the degree of sinusoidal perfusion failure

assessed by intravital microscopy (14, 27) or laser Doppler flowmetry (27) and the extent of the reperfusion injury assessed by the ALAT elevation (27) or the number of non-viable hepatocytes (14). Therefore, these studies demonstrated that, during the reperfusion injury, cell necrosis was, at least in part, caused by an hypoxic mechanism linked to the no-reflow phenomenon. It must be, however, outlined that, although the reality of the no-reflow phenomenon is nowadays agreed, its relevance in the pathogenesis of liver I/R injury remains matter of debate (88).

The pathophysiologic mechanisms involved in the no-reflow phenomenon are numerous, incompletely understood and include complement activation (7, 86), imbalance between the procoagulant and anticoagulant activities at the surface of the sinusoidal entothelium in favor of the former (97, 98) and the release by activated cells of a myriad of vasoactive substances, inflammatory mediators, chemokines and adhesion molecules. At present, it is impossible to establish a hierarchy of cellular or humoral components because of their intensive interactions and potential synergisms. The sinusoidal perfusion failure observed in the no-reflow phenomenon is otherwise not specific to the I/R injury and has been well described in the endotoxin-induced injury of the liver (95). The general term of "*basic microvascular inflammatory response*" has been proposed (95). Inferred from morphologic observations, two main features have been reported to explain the no-reflow phenomenon: 1/ the narrowing and 2/ the plugging of the sinusoidal lumens. The narrowing of the sinusoidal lumens is the first event. It results from the swelling of the sinusoidal cells and hepatocytes with blebs formation (38), from the perisinusoidal edema following the increase in capillary permeability (2) and mostly, from an imbalance in the vasomotor tone of the sinusoids (99). Indeed, the diameter of the sinusoids does not rely only on passive strenght but is actively regulated by potent vasoactive agents released by the sinusoidal wall, some of them such as endothelin-1 having powerful vasoconstrictive effect, other such as nitric oxid (NO, reported earlier as EDRF, i.e. endothelial derived relaxing factor) or prostacyclin having vasodilatator effect. The main target of these vasoactive agents could be the liver-specific pericytes, earlier known as perisinusoidal cells, fat-storing cells or Ito cells and currently called hepatic stellate cells. With their stellate expendages wrapping around sinusoids, these cells are able to control the vascular tone and the diameter of the sinusoids (99). In response to injury, hepatic stellate cells transform into myofibroblasts and become hyper-reactive to the stimulation of endothelin-1 (99). An accumulating body of evidence shows that endothelin-1 plays a major role in the sinusoidal perfusion failure during reperfusion injury. Recent studies have indicated that hypoxia induced endothelin gene expression and endothelin release by endothelial cells (100). In models of liver I/R, endothelin-1 concentrations started to increase immediately at the onset of reperfusion and the levels could be correlated with the timing and the severity of the sinusoidal perfusion failure (31). Inhibition of endothelin-1 by antiserum, monoclonal antibodies or antagonists such as bosentan decreased the sinusoid injury (14, 31, 101). So, it has been hypothesized that the no-reflow phenomenon in liver sinusoids during reperfusion was mediated by endothelin-1 (14, 31). On the other hand, numerous studies have recently demonstrated that the endothelin-induced vasoconstriction of sinusoids could be effectively counterbalanced by the vasodilating effect of NO released by endothelial cells and under some circumstances by Kupffer cells. Stimulation of endogenous NO production by L-arginine, the substrate for NO synthase, protected from the reperfusion injury (13, 102, 103) while inhibitors of NO synthase such as L-NAME accentuated the reperfusion injury (13, 14, 20, 102, 103).

The second main morphologic feature of the no-reflow phenomenon is the plugging of the sinusoidal lumens by aggregates of blood cellular elements. During low flow states, sinusoidal blood flow decreases early and becomes jerky. Red blood cells loose their usual deformability, change in shape and tend to form rouleaux that engorge sinusoids (93). Mixed aggregates of red blood cells and platelets adhere to the sinusoidal wall (93). But, the main event responsible for progressive plugging of the sinusoids during reperfusion might be the trapping of the PMNs. Several lines of evidence suggest that the progressive trapping of neutrophils inside the sinusoids during the reperfusion phase might be the pivotal mechanism responsible for the no-reflow phenomenon (104-107).

4. The role of neutrophil activation: a "neutrophilic" hepatitis

Recently, the role of leucocyte-endothelial cell interaction, followed by the transmigration and activation of PMNs inside the liver parenchyma has been increasingly acknowledged as a crucial determinant of liver I/R injury. This "neutrophilic" hepatitis includes three main steps: the first is the recruitment and adhesion of PMNs to the sinusoidal cells, the second is the diapedesis of the PMNs through the sinusoidal wall and then migration around parenchymatous liver cells (transmigration) and the third is the adhesion of PMNs to hepatocytes resulting in release of cytopathic agents and hepatocellular necrosis.

In the process of recruitment and adhesion, the first event is the margination and rolling of PMNs along the endothelial wall. Leucocytes that were flowing freely through the sinusoids are displaced from the axial stream and roll at much lower velocity along the sinusoidal wall (108-109). The rolling precedes firm adhesion to the sinusoidal wall then subsequent

transmigration. The process of recruitment-rolling is under the control of chemotactic agents (chemokines) and of adhesion molecules. Chemokines belong to a family of structurally related proteins sharing the ability to promote migration of leucocytes (110). They can be divided into a C-X-C subfamily and a C-C subfamily according to their aminoacid structure (110). For neutrophil chemoattraction, the most representative chemokines are members of the C-X-C subfamily such as IL-8 in humans and CINC (cytokine induced neutrophil chemoattractant factor) or KC the equivalent of IL-8 in the rats and the mouse, respectively (111-113). Chemokine upregulation is typically induced by proinflammatory cytokines such as TNF and IL-1 that are released mainly by activated Kupffer cells at the early phase of the reperfusion injury. A strong correlation could be established between the time course of C-X-C release and hepatic PMN sequestration (113, 114).

The next step is the firm adhesion of PMNs on endothelial cells. Adhesion molecules are glycoproteins involved first in the rolling of PMNs, then in their firm adhesion required for transmigration accross the sinusoidal wall. They belong to three main families: the selectins, the integrins and the immunoglobulin superfamily (111, 115). Selectins, either L-, E- or P-selectin according to their initial identification on lymphocytes, endothelial cells or platelets are expressed on endothelial cells and promote the rolling of PMNs (107, 116, 117). They are upregulated by inflammatory mediators such as cytokines, superoxide anion, complement products, and PAF (111, 118). Up to this stage, the role of PMNs remains passive. The firm adhesion of PMNs on endothelial cells requires the interaction between a leucocyte adhesion molecule, Integrin β2 (the complex CD11-CD18) and endothelial cell adhesion molecules of the immunoglobulin superfamily either ICAM-1 (intercellular adhesion molecule) or VCAM-1 (vascular cell adhesion molecule) (111, 112, 115, 118). Integrins on PMNs do not bind to endothelial cells unless PMNs are activated. Triggering mechanism required for promoting strong adherence are yet to be clearly defined but include chemokines, inflammatory cytokines, PAF, LPS, complement products (111, 115, 118). Once leucocytes attach, they can interact with flowing PMNs and platelets through adhesion molecules. Therefore, this massive recruitment of PMNs may result in tissular injury through impairment of regional blood flow (no reflow phenomenon). Several investigators have reported a significant correlation between the percentage of non-perfused sinusoids and the intensity of the PMN sequestration in liver models of I/R in the rat (22, 105).

After firm adherence to sinusoidal cells, PMNs can transmigrate through the sinusoidal wall. The key factors for PMN transmigration remained poorly understood up to very recently. In a model of endotoxin-induced hepatitis in rodents, the research team of Harmut Jaeschke has shown that endotoxin injected alone resulted in PMN sequestration in sinusoids but in minimal diapedesis and liver injury (119). Conversely, the injection of endotoxin together with galactosamine resulted in invasion of liver parenchyma by PMNs and liver cell necrosis (119). Very recently, the same team demonstrated that the critical signal for PMN transmigration and invasion was apoptosis (120, 121). Indeed, transmigration of PMNs and subsequent necrosis of liver cell were prevented if animals injected with galactosamine-endotoxin were pretreated by uridine with abrogrates apoptosis induced by galactosamine. Of note, was that uridine did not prevent PMN sequestration in sinusoids nor release of cytokines (TNF) or chemokines (120). Hence, in this model, apoptosis appeared to be a prerequisite event triggering widespread liver cell necrosis in the presence of neutrophil sequestration (120).

The mechanisms by which invading PMNs destroy parenchymatous liver cells have been reviewed in depth (111, 122). In brief, PMNs adhere to liver cells through integrin β2-ICAM1 interaction and are activated for production and release of ROS and proteases. Reactive oxygen species come mainly from a plasma membrane-enzyme termed the NADPH oxidase that generate ROS (122), while proteases come from intracytoplasmic granules that fuse with the membrane and release powerful cytopathic agents such as elastase, gelatinase, collagenase... (111, 122).

In conclusion, there is mounting evidence from these experimental studies that a complete reperfusion injury includes two phases, with an initial injury (1-2 h after reperfusion) mediated by activated Kupffer cells and a later injury (6-24 h after reperfusion) mediated by activated PMNs (neutrophilic hepatitis). The biphasic temporal pattern of reperfusion injury is well illustrated by the observation of two peaks, one early and mild, the other late and more important, of oxidative burst (6) and ALAT elevation (5, 82) separated by a plateau-phase. Neutrophils have a narrower repertoire of inflammatory responses than macrophages but are 10- to 20-fold more common in areas of inflammation explaining the more severe hepatocyte injury observed during the late phase of reperfusion injury (123). Besides these two phases of liver cell injury induced by mediators released from inflammatory cells, the progressive obstruction of the sinusoids (no-reflow phenomenon, also called "capillary perfusion failure") might also represent a relevant anoxic mechanism.

Is hypoxic (ischemic) hepatitis a liver ischemia / reperfusion injury ?

Recent advances in the field of liver I/R injury have considerable clinical implications. The consequences of temporary interruption of liver blood flow during surgery for trauma or tumors and the preservation injury of transplanted liver are clinical models of I/R injury.

Multiple organ failure (MOF), the leading cause of death in surgical intensive care units (124, 125) is another clinical model of I/R injury where hemodynamic instability might lead to monocyte/macrophage activation, neutrophilic inflammation and microvascular disturbance in the liver. The current hypothesis for the liver damage observed in the setting of MOF includes not only a I/R injury of the liver, but rather an ischemic injury of the gut, followed by a reperfusion injury of the liver. Accordingly, this hypothesis is acknowledged as the *gut-hypothesis* (124). In case of traumatic, septic or cardiogenic shock, the gut is rapidly affected by a fall of oxygen delivery. Decrease in splanchnic blood flow leads to ischemia of the intestinal mucosa and loss of its barrier function (126-128). Central to the gut hypothesis is the phenomenon know as "*translocation*" whereby bacteria, endotoxins and gut-derived products pass from the intestinal lumen to the regional lymph nodes and the blood circulation (125, 126, 129). After translocation, bacterial products enter the portal and systemic circulation and, if hemodynamics is restored, induces an inflammatory cascade typically observed in remote organs rich in resident macrophages such as the liver and the lung (126, 130). The increasing evidence of the role of gut in MOF has led to consider the gut as the "*motor*" of MOF (131) or as an "*undrained abscess*" (132). It must be noted that, although the gut hypothesis has gained large acceptance, it remains nevertheless controversial (133). Finally, the best clinical model of liver I/R injury could be hypoxic hepatitis, better known under the terms of "*ischemic hepatitis*" or "*shock liver*". Hypoxic hepatitis generally occurs in the setting of hemodynamic instability, particularly in severe congestive heart failure and is characterized by a dramatic increase in serum cytosolic enzyme activities (ASAT, ALAT, LDH) originating from a centrilobular liver cell necrosis (CLN) (134, 135).

Several clinical observations supported by experimental studies, strongly suggest that the course of hypoxic hepatitis follows a typical process of I/R injury. It is noteworthy that early autopsy studies had shown that the presence of CLN in patients dying from cardiac failure or shock was correlated with the duration of shock. In 1938, Wilson *et al.* (136) observed that CLN was absent or mild at autopsy of all 8 patients dying from thermal injury in less than 24 hours, but was severe or very severe in 9 of 12 patients dying in more than 24 hours. Similarly, when the data of the studies of Bywaters published in 1948 (137) and Ellenberg *et al.* published in 1951 (138) are cumulated, CLN in patients dying from shock was observed in 5 of 71 cases (7 %) when premortem period of shock lasted less than 10 hours but in 35 of 59 cases (59 %) when premortem shock lasted more than 10 hours. Yet, in 1950, Mallory *et al.* (139), from postmortem material of battle casualties, noted that the liver of patients dying after 4, 8 or 12 hours of prolonged shock showed little evidence of histologic damage but that a constant pattern of lesion was disclosed when material came from casualties who survived more than 18 hours after injury. For these early investigators of shock, the relation between the presence of CLN and the duration of the premortem hypotensive period was explained naturally by a longer period of liver hypoxia. Under the lights of recent advances, the hypothesis may be raised that the time-relationship between the duration of shock and the development of liver cell necrosis was explained not by a longer period of hypoxia but by the existence of one or several periods of reperfusion. Indeed, in these trauma patients who died in the course of shock state lasting more than 24 hours, it is difficult to believe that they could have survived so long without some transient periods of hemodynamic recovery and organ reperfusion.

Since, several indirect clinical arguments and some direct experimental observations have supported the hypothesis that CLN observed in hypoxic hepatitis occurs at the reperfusion phase instead of at the ischemic phase. For the clinical arguments, the first is that a protracted shock is not required for the development of hypoxic hepatitis (134, 135). On the contrary, although a period of acute hemodynamic instability is usual, this period may be short, poorly symptomatic and even unrecognized (140). Second, a shock state that cannot be temporarily reversed before death, generally does not progress to hypoxic hepatitis. For the preparation of two clinical studies on hypoxic hepatitis (134, 135), we collected two control groups of patients dying consecutively from irreversible cardiogenic shock in less than 24 hours after the onset of shock. The first group was collected from January 1988 to March 1989 and included 25 patients, the second was collected in 1991 and included 22 patients (personal results, partially published in 134, 135). Both groups were very similar and results may be cumulated. The median duration of shock before death was 3 hours (range 1-24). None fulfilled the clinical criteria that allow the diagnosis of hypoxic hepatitis without histologic confirmation (141). Serum aminotransferase activities equal or greater than 10 fold the upper limit of normal (ULN) were recorded in only 3 cases (6 %), reaching 10 ULN in two cases and 16 ULN in 1 case. Immediate postmortem puncture of the liver was performed in 25 cases and CLN, the typical histologic lesion of hypoxic hepatitis, was observed in only 2 cases, one with serum aminotransferases at 10 ULN and one with normal serum aminotransferases. In this latter case, CLN was just perivenular. Liver histology was also available for the patient with serum aminotransferase at 16 ULN and showed cirrhosis with mild centrilobular congestion. A third clinical argument comes from histologic studies of the liver in hypoxic hepatitis that have occasionally shown a midzonal (acinus zone II) pattern of liver necrosis. This particular pattern has been reported in early studies (137, 142) and was the purpose of a more recent study (143). In this

latter study, the authors observed a midzonal pattern of necrosis in 8 % of a series of 214 CLN from autopsic material. They stated that in some patients, the midzonal pattern of necrosis was strictly related to the selective survival of perivenular hepatocytes, but they found no explanation for this observation. It may be hypothesized that this midzonal necrosis resulted from reperfusion injury after partial reperfusion of the lobule progressing from the portal tract to the midzonal area, while the central part remained anoxic. Following this hypothesis, the periportal (acinus zone I) and the pericentral (acinus zone III) areas of the lobule remained intact, the first because sufficient oxygen delivery, the latter because anoxia without reperfusion, while the midzonal areas were destroyed at the ischemia/reperfusion front. Finally, a more direct clinical argument arose from an Italian group that demonstrated the existence of an oxidative stress in patients with hypoxic hepatitis (144). In a series of 26 patients with hypoxic hepatitis, they reported, 6 hours after hemodynamic recovery, the presence of an oxidative stress by measuring erythrocyte malondialdehyde (a product of lipid peroxidation) as well as plasma protein-aldehyde adducts and lipoperoxides. Protein-aldehyde adducts were detectable only in plasma of patients with HH and their levels were strongly correlated (r = 0.714) with the elevation of LDH 5 (144).

As for the experimental studies of liver I/R in animals, they also strongly support the role of reperfusion in the pathogenesis of hypoxic hepatitis. Indeed, some of these animal models, such as the low-flow I/R on isolated liver (10-14) or the hemorrhage-induced shock followed by recovery (17-20) reproduce fairly well the clinical condition of hypoxic hepatitis, and all of them resulted in CLN and massive release of cytosolic enzyme activities, occurring mainly at the time of reperfusion. Moreover some of these studies have shown that the central necrosis could be preceeded by a midzonal necrosis, supporting the harmful role of oxygen reentry (10, 11, 42, 144).

In conclusion, hypoxic hepatitis could be a clinical situation of I/R injury. This explains probably why Brunson *et al.* observed a surprising, unexplained increase of acute liver necrosis in the University Hospital of Minnesota during the early 1950s (146). Among 3,229 autopsies collected during a 10-year period from 1946 to 1955, they observed 62 cases of hepatic necrosis due to cardiac or circulatory failure. They noted that the incidence had strikingly increased during the last 3 years with 17 cases collected during the 7 first years among 2107 autopsies (0.8 %) versus 45 cases collected during the last 3 years among 1122 autopsies (4 %). The single risk factor that they could elicit was that the increase in liver necrosis coincided with the increasing use of vasopressor amines. They rose the hypothesis that the administration of these substances allowed hepatic lesion to develop by prolonging the agonal period. They were probably right and the reason is now termed "*reperfusion injury*".

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Abbreviations

ALAT : serum alamine aminotransferase activity
ASAT : serum aspartate aminotransferase activity
CLN : centrilobular liver cell necrosis
IL : interleukine
I/R : ischemia/reperfusion
LDH : serum lacticodehydrogenase activity
LPS : lipopolysaccharide
MOF : multiple organ failure
PAF : platelet activating factor
PMN : polymorphonuclear
ROS : reactive oxygen species
TNF : tumor necrosis factor
ULN : upper limit of normal