

# Protective Effects of Nitric Oxide and Phycocyanin Against Oxidative Stress Induced by Hepatic Ischemia/Reperfusion Injuries

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## Abstract

Liver ischemia-reperfusion induced hepatocellular damage that contributes to the morbidity and mortality<sup>1,2</sup> associated with shock, thermal injury, re-sectional surgery and liver transplantation. One of the earliest events associated with reperfusion of ischemic liver is the release of Reactive Oxygen Species (ROS) causing oxidative stress. The following review focuses on the antioxidant effects of nitric oxide (NO) and Phycocyanin (Pc) after cold ischemia/reperfusion injury (IRI). In this regard, this review investigates in the first part the effect of the addition of NO to the preservation solution at different concentrations (1000, 500 and 50 nM) and in the second part the effect of the addition of phycocyanin to the conservation solution at two doses (0.2 mg / ml / g of liver and 0.1 mg / ml / g of liver) on liver graft quality. In conclusion, phycocyanin and nitric oxide (at a low dose) are effective in preserving the hepatic graft and protecting it against IRI by acting as a potent antioxidant against the products of oxidative stress.

## Introduction

Organ transplantation was described in the early 20th century, in the development of the first successful vascular anastomosis by Carell<sup>3</sup>. But it was only in 1954 that the human kidney was successfully transplanted by Murray<sup>4</sup>. Since organ transplantation has been a success and a significant evolution through advances in the field of removal techniques and implants preservation which has allowed an increase in life expectancy and has a marked improvement in the quality of patient's life after transplantation<sup>5</sup>. Different metabolic changes take place during different steps of the liver transplantation procedure. After liver preservation and before transplantation, the hepatic graft undergoes diverse and complex molecular, cellular and biochemical alterations known as ischemia/reperfusion syndrome (IR)<sup>6,7</sup>. During transplantation, the hepatic graft is subject to two constraints: ischemia or lack of blood circulation and reperfusion. There are two types of ischemia, cold ischemia and warm ischemia followed by reperfusion<sup>8</sup>. During cold ischemia, the hepatic graft undergoes a serious damage associated with a reduction of cellular ATP levels, and an enhancement of cytosolic calcium levels<sup>9</sup>, leading to primary liver graft dysfunction. The restoration of blood flow during reperfusion has paradoxical consequences for the ischemic tissue. First, it will initiate a number of events responsible for the extension of tissue lesions. It is the paradox of oxygen<sup>10</sup>. The reperfusion phase is also characterized

by production and release of Reactive Oxygen Species (ROS) which are the main mediators of hepatic ischemia-reperfusion injury (IRI)<sup>11,12</sup>, and later by an inflammatory disorder mediated by the activation of Kupffer cells, endothelial and parenchymatous cells damage<sup>13</sup>. The graft cold preservation is one of the main principles of organ persistence that prolongs the preservation time and improves the quality of the graft. This review summarized recent findings about the improvement of the composition of the preservation solution by the addition of pharmacologically active substances to reduce IR lesions. The isolated perfused rat liver was used. The first part of this review focuses on the addition of sodium nitrite (NaNO<sub>2</sub>) to the conservation solution Institut Georges Lopez-1 (IGL-1) at different concentrations (1000, 500, and 50 nM). The second part focuses on the effect of added phycocyanin, the main pigment of *Spirulina platensis* to the Krebs Henseleit preservation solution (KH) at two different doses (0.1 mg/ml/g of liver, 0.2 mg/ml/g of liver) on the functional parameters of the liver.

### Conservation Solutions

The use of a preservation solution is an important part of organ preservation. Several preservation solutions have been developed to extend the viability of the graft. Thus in this work, two preservation solutions were used for liver graft preservation: IGL-1 solution and KH solution.

### Hypothermic Conservation

After hepatectomy, and in the first part of the present study, livers were immersed in a cylindrical plastic vial containing 40 ml of IGL and IGL+ NO solutions at 4° C and 40 ml of Krebs Henseleit bicarbonate buffer supplemented with mannitol and glutathione (reduced form), pH = 7.30 at 4 ° C (KH and KH + phycocyanin) for the second part of this study. Then, the plastic vial is placed in the refrigerator for 24 hours.

### Liver Perfusion Experiments

Livers were perfused with an isotonic Krebs Henseleit (KH) solution saturated with 95% O<sub>2</sub> / 5% CO<sub>2</sub>, according to the technique described previously<sup>14,15,16</sup>. Perfusions were performed in a closed circuit, in a humid thermoregulated chamber (37°C) during 120 minutes. The perfusion fluid circulation was provided by a peristaltic pump at constant pressure and calibrated for a basal hepatic flow rate about 3 ml/min/g liver<sup>17,18</sup>. Before starting the experimental protocol, livers were perfused in single-pass mode for 15 min to ensure the rinsing of liver and their stabilization. Once the peristaltic pump is running, the perfusion fluid returns to the livers through the portal vein and exits through the lower vena cava before returning to the pump. Livers have been perfused in a recirculation mode. Samples of liver effluents were collected every 10 min to determine liver toxicity parameters and tissue samples were taken

from the left lateral hepatic lobe at the end of reperfusion and subsequently frozen at -20 °C until malondialdehyde (MDA) and antioxidant enzymes assay.

## Discussion

### Effect of hypothermic conservation

Hypothermia is the essential element of the preservation. Hypothermic conservation has controversial effects upon the transplantation graft; it prolongs the preservation period and at the same time causes adverse effects to the cell by inhibiting metabolic enzyme activities<sup>19</sup>. In fact, cold ischemia is associated with lower cellular ATP levels, reduced oxidative phosphorylation, alteration of the calcium homeostasis and cellular edema<sup>19</sup>, while reperfusion leads to greater mitochondrial dysfunction and production of ROS responsible for oxidative stress<sup>20</sup>. All these events lead to the alteration of the microvascular system and lesions of the hepatic parenchyma. The effect of cold ischemia on the quality of hepatic graft, using the perfused isolated rat liver model, demonstrated an increase in hepatocellular lesions during reperfusion after 24 hours of cold ischemia. Livers stored for a period of 24h in the conservation solutions, release more transaminases; aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in the perfusion liquid after the end of the reperfusion (at t = 120 minutes). The presence of these enzymes in the perfusion fluid is a reflection of hepatocellular lysis caused by cold ischemia and reperfusion. During ischemia/reperfusion, several ROS are involved<sup>21</sup>, inducing a state of oxidative stress. In fact, the liver tissue of rats challenged with cold ischemia followed by reperfusion underwent a significant increase in lipid peroxidation (MDA). During reperfusion, the mitochondrial ROS generation increases notably<sup>22,23</sup> causing an alteration of the mitochondrial respiratory chain and an appearance of severe hepatocellular lesions<sup>24,25</sup>. Glutamate dehydrogenase (GLDH), a liver cell mitochondrial enzyme, is used as an indirect marker of mitochondrial lesions. We have noted a significant activity increase after 24h hypothermic storage. In conclusion, During cold ischemia, the electron transport chain of the mitochondria is disturbed, resulting in leakage of electrons and uncoupling of the respiratory chain during reperfusion, leading to high ROS production<sup>26,27</sup>. A significant increase in lipid peroxidation was accompanied by a real decrease in the protein thiol levels and a marked enhancement in the activities of the antioxidant enzymes glutathione peroxidase (GPx) and glutathione S-transferase (GST), after 24h of cold ischemia. These principal enzymes constitute the second line of enzymatic defense, which play an important role in the detoxification of the endogenous and the exogenous toxic compounds<sup>28</sup>. An acute inflammatory response, characterized by Kupffer cells activation takes place during reperfusion period<sup>29,30</sup>. Once activated, these cells generate ROS and release other inflammatory mediators, such as

cytokines and chemokines responsible of hepatocellular and endothelial lesions in the late period of reperfusion<sup>31</sup>.

### Effect of nitric oxide on hypothermic conservation

NO has a significant role in the prevention of I/R injury. Indeed, the bioavailability of NO is reduced at the beginning of hepatic graft reperfusion<sup>32</sup>. This decrease is associated with tissue damage and endothelial dysfunction that occurred during I/R<sup>33</sup>. Two NO synthesis pathways were developed: the endogenous pathway and the exogenous pathway. NO can be produced in the endogenous pathway, from a NO precursor, L-arginine, by endothelial nitric oxide synthase (e-NOS). L-arginine has shown to have protective potential during transplantation<sup>34</sup>. It promotes NO production by hepatocytes in order to limit hepatocellular lesions. The exogenous pathway requires the exogenous source of NO, either by direct inhalation or by NO donors such as sodium nitroprusside (SNP)<sup>35</sup> or S-nitroso-L-glutathione (GNSO)<sup>36</sup>. The beneficial effects of SNP have been demonstrated by Kuroki and associates<sup>37</sup>, who administered nitroprusside to the rat kidney during reperfusion and found an increase of hepatic microcirculation and reduced hepatocyte damage. Another exogenous source of NO would be nitrite anion ( $\text{NO}_2^-$ ) produced under hypoxia and acidosis conditions. In fact,  $\text{NO}_2^-$  can be reduced to NO by the action of xanthine oxidoreductase (XOR)<sup>38</sup>, deoxygenated hemoglobin<sup>39</sup> and the decrease in pH<sup>40</sup>. Several studies have evaluated the effect of nitrite on functional recovery of liver after warm ischemia<sup>41</sup>. Results showed that nitrite-dependent NO production significantly decreased I/R lesions in liver and heart. The beneficial effects of NO have been demonstrated in a recent study, in which ischemic livers were preserved at 4°C for 24 hours in the IGL-1 solution enriched with different concentrations of  $\text{NaNO}_2$  (1000, 500 and 50 nM). We concluded in this study that a low concentration of nitrite (50 nM) seems to be protective against I/R lesions, whereas a high concentration of nitrite (500, 1000 nM) can generate adverse effects. Indeed, a high concentration of  $\text{NaNO}_2$  induces the production of a large amount of NO, which can react with  $\text{O}_2^-$  to form the ONOO<sup>-</sup>, a very toxic compound for the cell<sup>42</sup>. Therefore, the addition of nitrite to the storage solution (IGL-1) at a low dose (50 nM) decreased oxidative stress (decrease in MDA concentration) and provided protection against mitochondrial damage (decrease in GLDH concentration), thereby maintaining the integrity of liver cells and protecting the graft against cytolysis (decreased transaminases)<sup>43</sup>.

### Effect of phycocyanin on hypothermic conservation

Phycocyanin (Pc) is the principal pigment of *Spirulina*, a microalgae which have been widely used in many countries as a dietary supplement<sup>44</sup>. Previously, several studies have demonstrated the hepatoprotective<sup>45</sup>, anti-inflammatory<sup>45,46</sup> and antioxidant<sup>47,48</sup> properties of Pc. The

presence of Pc in the preservation solution can reduce I/R damages and optimize the quality of the preserved graft. Its therapeutic power consists in eliminating the excessive formation of ROS. Its huge scavenger activity is mediated by its major component, phycocyanobilin (PCB) which is a chromophore-tetrapyrrole chain, similar to bilirubin<sup>31,49</sup>. The latter plays an antioxidant role for the reactive species<sup>50,51</sup>. Previous reports have shown that in experimental models *in vivo* and *in vitro*, Pc protects cells from lipid peroxidation damage induced by oxidative stress through scavenging ROS<sup>29,52</sup>. Recently, our research team demonstrated the beneficial effects of phycocyanin on the ischemically damaged rat liver. We have concluded that treatment with phycocyanin at the concentration of 0.2 mg/ml/g of liver normalizes levels of transaminases (AST and ALT), lipid peroxidation (MDA), and thiol groups as well as the activity of the antioxidant enzymes studied, whereas phycocyanin pretreatment at the concentration of 0.1 mg/ml/g of liver seems to reduce only GST and alkaline phosphatase (ALP) levels after 24 hours of hypothermic storage<sup>53</sup>.

### Conclusion

Taking into account these findings, it's clear that the improvement of the composition of the preservation solution is necessary in order to optimize the quality of the preserved graft and to limit ischemia/reperfusion injury. The additions of a dose of 50 nM of nitrite or 0.2 mg/ml/g of liver of phycocyanin to cold preservation solutions may protect livers from cold I/R injury through their strong antioxidant potentials.

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