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**metabolic shift in rat liver: correlation between perfusion temperature and hypoxia inducible factor−1α**

Ferrigno A *et al*. Temperature changes cause aerobiosis/anaerobiosis shift

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## Abstract

**AIM**: to study at what temperature the oxygen carried by the perfusate meets liver requirements, in a model of organ perfusion.

**METHODS**: in this study, we correlated the hypoxia inducible factor (HIF)-1α to the perfusion temperature and the hepatic oxygen uptake in a model of isolated perfused rat liver. Livers from Wistar rats were perfused with an oxygenated medium at 10 °C, 20 °C, 30 °C, and 37 °C (6-h). Oxygen uptake was measured by an oxygen probe; lactate-dehydrogenase activity, lactate release and glycogen were measured spectrophotometrically; bile flow was gravitationally determined; pH of the perfusate was also evaluated; HIF-1α mRNA and protein expression were analyzed, respectively, by real time-polymerase chain reaction and ELISA.

**RESULTS**: livers perfused at 10 °C and 20 °C showed no difference in LDH release, after 6 h of perfusion (0.96 ± 0.23 and 0.93 ± 0.09 mU/min per g respectively, *P* = 0.47) and had lower hepatic damage as compared to 30 °C and 37 °C (5.63 ± 0.76 and 527.69 ± 45.27 mU/min per g respectively, 10 °C and 20 °C *vs* 30 °C: *P* < 0.004; 10 °C and 20 °C *vs* 37 °C: *P* < 0.0004). After 6 h, tissue ATP was significantly higher in livers perfused at 10 °C and 20 °C than in livers perfused at 30 °C and 37 °C (0.89 ± 0.06, 1.16 ± 0.05, 0.57 ± 0.09 and 0.33 ± 0.08 nmol/mg, respectively, 10 °C and 20 °C *vs* 30 °C: *P* < 0.001, 10 °C and 20 °C *vs* 37 °C: *P* < 0.0001). No sign of hypoxia was observed at 10 °C and 20 °C, as highlighted by low lactate released respect to livers perfused at 30 °C and 37 °C (121.4 ± 12.6, 146.3 ± 7.3, 281.8 ± 45.3, 1094.5 ± 71.7 respectively, 10 °C and 20 °C *vs* 30 °C: *P* < 0.02; 10 °C and 20 °C *vs* 37 °C: *P <* 0.0001), low HIF-1α mRNA expression (0.40 ± 0.08, 0.20 ± 0.03, 0.60 ± 0.2, 1.47 ± 0.3 respectively; 10 °C and 20 °C *vs* 30 °C: *P* < 0.05; 10 °C and 20 °C *vs* 37 °C: *P* < 0.01) and low HIF-1α protein expression (3.72 ± 0.16, 3.65 ± 0.06, 4.43 ± 0.41, 6.44 ± 0.82 respectively; 10 °C and 20 °C *vs* 30 °C: *P* < 0.05, 10 °C and 20 °C *vs* 37 °C: *P <* 0.02).

**CONCLUSION**: livers perfused at 10 °C and 20 °C show no sign of liver injury and anaerobiosis, differently from livers perfused at 30 °C and 37 °C.

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**Key words:** hypoxia inducible factor-1α; Anaerobiosis; Ischemia; Liver transplantation; Machine perfusion

**Core tip:**Among the techniques developed to improve the preservation of marginal organs for transplantation, hypothermic perfusion is the preferred choice. We show that it is possible to perfuse a rat liver at 20 °C, without incurring in ischemia. We evaluated liver injury, energetic status, lactate release and hypoxia inducible factor-1α. Twenty °C is the way-in-between: at higher temperatures symptoms of ischemia do appear, and at lower temperature no real advantage is detectable. These findings have interesting implications in liver preservation: maintaining the liver in a mild metabolism state could be useful for pharmacologic treatment and regeneration of the energetic status in ATP depleted organs.

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## INTRODUCTION

Orthotopic liver transplantation is the treatment of choice for end-stage liver disease. The employment of this technique is limited by the shortage of viable donor organs. Recently the donor acceptance criteria for organ retrieval have been expanded, including livers with low degrees of steatosis[1] and grafts from non-heart-beating donors[2].

The use of marginal livers for organ transplantation emphasized a fundamental flaw of conventional cold storage: despite all improvements, marginal organs are at greater risk of preservation-associated primary non function, because of increased sensitivity to preservation−induced ischemia/reperfusion injury[3]. Increasing grades of donor liver steatosis were associated with worse initial poor function[4,5] due to impaired metabolism of the steatotic hepatocytes[6,7], to the crystallization of lipids during cold ischemia[8], and to an increased sensitivity to oxygen radicals during reperfusion[9]. Livers from non−heart−beating donors exhibited postoperative biliary complications[10,11], due to the superimposing effects of cold ischemia and warm ischemia[12].

In order to overcome the limits of cold storage, animal studies are flourishing about preservation by machine perfusion, this technique reducing the ischemic injury usually associated with organ preservation. Different settings have been tested: Peter and colleagues showed that oxygenated, normothermic (sanguineous) machine perfusion (NMP) recovers ischemic livers to a viable level[13]; in a clinical trial, Guarrera *et al*[14] demonstrated improved clinical parameters and shorter duration of hospital stay in patients who received grafts stored by hypothermic machine perfusion (HMP), in comparison to patients who received grafts preserved by cold storage; Tolboom *et al*[15] showed how, in a rat liver transplantation model, the survival rate after 4 wk was 100% for animals receiving livers preserved by subnormothermic machine perfusion; on the contrary no cold stored graft survived after transplantation.

The mechanism by which machine perfusion better preserves marginal livers is not yet fully understood, nor a rationale was given for applying particular perfusion conditions. Nonetheless, cold storage compromises the ability to re−oxidise NAD(P)H through mitochondrial respiration[16] while machine perfusion is always associated with ATP and glycogen recovery[17,18], suggesting a decisive role of the oxygenation in the control of ischemic damage during preservation. For these reasons, it is of primary importance to ensure an adequate oxygenation during perfusion. The issue of oxygenation is strictly related to perfusion temperature: both the oxygen carried by the perfusate and liver oxygen requirement are strongly related to temperature, with the first decreasing and the second exponentially increasing at increasing temperatures[19].

In our previous works, we evaluated the machine perfusion at subnormothermic temperature for the preservation of ischemic[20] and steatotic[6] rat livers, in a model of *ex vivo* reperfusion. In this work we studied how the liver responds to different perfusion conditions, with the goal of find out at what temperature the oxygen carried by the perfusate and the liver oxygen requirement meet or, from a different point of view, at what temperature liver switches from aerobiosis to anaerobiosis, taking the road to ischemia.

We perfused rat livers at 10 °C, 20 °C, 30 °C and 37 °C, saturating the perfusion solution with O2-CO2 (95%:5%). The considered temperature range allows to maintain homogeneous perfusion conditions, while long term liver perfusion at 4 °C is usually performed at lower flow rate, and may require additives to prevent cell swelling. For these reasons, we did not include in the experimental design livers perfused at 4 °C.

Liver injury, function, and energetic status were evaluated. The switch to anaerobic metabolism was evaluated using lactate release and mRNA/protein expression of hypoxia inducible factor (HIF)-1α, a transcription factor that precociously responds to decreases in available oxygen in the cellular environment[21].

## MATERIALS AND METHODS

#### Animals and surgery and liver perfusion

Male Wistar rats (Harlan-Nossan, Italy) weighing 250-300 g, were allowed free access to water and food until the beginning of all experiments. The use and care of animals in this experimental study were approved by the Italian Ministry of Health and by the University Commission for Animal Care. All surgery was performed under anesthesia, and all efforts were made to minimize suffering. Rats were anesthetized with sodium pentobarbital (40 mg/kg intraperitoneally) and livers were isolated as already described[20,22]. Briefly: After median laparotomy followed by bilateral subcostal incisions, the animals received 200 units of heparin per 100 g of body weight via the inferior vena cava (5000 IU/mL, Marvecs Services, Agrate Brianza – MI). The bile duct was cannulated with a 50 G polyethylene tubing (Intramed, Becton-Dickinson, Loveton Circle, MD – United States), and the portal vein was cannulated with a 16 G catheter (Johnson and Johnson, Arlington, United Kingdom). The liver was washed out with 50 mL of modified Krebs-Henseleit buffer (KHB) *via* the portal vein cannula, then was freed from ligaments, removed and placed in a jacketed chamber for perfusion at different temperatures. At the end of liver perfusion, liver samples were immediately snap frozen in liquid nitrogen and stored at -80 °C.

Livers were divided into four experimental groups: group I, livers perfused at 10 °C (*n =* 6); Group II, livers perfused at 20 °C (*n =* 6); Group III, livers perfused at 30 °C (*n =* 6); Group IV: livers perfused at 37 °C (*n =* 6). Livers were placed in an organ chamber, connected to a recirculating perfusion system, and perfused for 6 h. The perfusion medium was a modified Krebs-Henseleit buffer (KHB)[18] continuously gassed with O2:CO2 (95%:5%). Perfusion flow was kept constant at 2.6 mL/min per g[20].

#### Assays

Liver parenchima viability was assessed through release of lactate dehydrogenase (LDH) into the effluent perfusate, as described by Bergmeyer *et al*[23]. The perfusion temperature was continuously monitored with a probe placed inside the isolated organ chamber. The portal venous pressure was continuously measured throughout the perfusion by means of a water column connected to the portal vein inflow catheter; pre-calibration was performed each time just before connecting the liver to the circuit. The basal perfusion pressure was about 12-14 mmHg. Dissolved oxygen in the perfusion solution was measured with a probe (OXY 340i, WTW GmbH, Germany) at intervals of one hour, both in the inlet and outlet perfusion solution; oxygen delivery rate (ODR) and liver oxygen uptake rate (OUR) were calculated. The pH was continuously evaluated both in the perfusion solution reservoir and in a reference solution, consisting of the perfusion buffer kept at the same temperature and pO2 conditions, but not circulated through the liver. Tissue ATP was measured with the luciferin-luciferase method using the ATPlite luminescence assay kit (Perkin Elmer Inc., United States) according to manufacturer’s instructions with minor changes. Briefly, frozen tissue was homogenized in ice cold 100 mmol/L phosphate buffer with 3 mmol/L EDTA; the homogenate was immediately precipitated in TCA 30% and centrifuged at 3000 x g x 15 min at 4 °C. The supernatant was diluted 50 x in 100 mmol/L phosphate buffer and assayed[20]. The glycogen assay was performed as described by Bennett *et al*[24]. Frozen samples were homogenized in a solution of 10% HClO4 and centrifuged at 280 g for 15 min. The pellets were resuspended with 2 ml of deionized H2O. Samples (0.1 ml) were mixed with 0.2 ml of 5% phenol and 1 ml of H2SO4. After 30 min, absorbance at 490 nm was measured[24]. HIF-1α mRNA was analyzed using real-time polymerase chain reaction (RT-PCR): total RNA was isolated from frozen liver samples with Tri reagent (Sigma-Aldrich)[25]. The cDNA was generated using iScript Supermix (Biorad). The RNA was assayed by measuring the absorbance at 260/280 nm. HIF-1α, UBC and GAPDH gene amplification efficiencies were established by means of calibration curves (respectively 108.8%, 98.6% and 97.4%). The expression of the house keeping gene remained constant in the considered experimental group. Primers had the following sequence: HIF-1α: 5’-ACA AGA AAC CGC CTA TGA CG-3’ (forward) 3’-TAA ATT GAA CGG CCC AAA AG-5’ (reverse); UBC: 5’-CAC CAA GAA CGT CAA ACA GGA A-3’(forward), 3’-AAG ACA CCT CCC CAT CAA ACC-5’ (reverse); GAPDH: 5’-AAC CTG CCA AGT ATG ATG AC-3' (forward), 5’-GGA GTT GCT GTT GAA GTC GTC A-3’ (reverse). Gene expression was analyzed using Platinum Sybr Green qPCR mix UDG. Ubiquitin c and GAPDH were used as reference gene. The amplification was performed through two-step cycling (95–60 °C) for 45 cycles, in an ABI prism 7000 sequence detection system (Applied Biosystems Deutschland Inc., Darmstadt, Germany), following the instructions of the supplier. All samples were assayed in duplicate. The results were normalized to the endogenous controls, and fold change of the gene expression was calculated using threshold cycle (Ct) values. At the end of the preservation, the nuclear fraction was immediately isolated from fresh tissue with the Nuclear Extraction Kit (Cayman, United States). The HIF-1α protein expression was analyzed on the nuclear fraction with an ELISA kit (HIF-1α Transcription Factor Assay Kit, Cayman, United States). The lactate was assayed using the Lactate Colorimetric Assay Kit (BioVision, United States).

#### Statistical analysis

Data are presented as the mean ± SE. Statistical analysis for multiple comparisons was performed through one-way ANOVA test with Bonferroni’s corrections.

## RESULTS

#### Liver oxygen uptake rate and oxygen delivery rate

In our experiment, OUR was constant during 6 hours of perfusion at 10 °C, 20 °C, and 30 °C. At 37 °C, after 3 h of perfusion OUR dropped down, probably due to massive necrosis of the liver (Figure 1A). We observed a strong linear correlation between the basal OUR and the perfusion temperature (*R*2 = 0.9979). A dependence of ODR on temperature was also observed: the available oxygen in the perfusion solution decreased with perfusion temperature (data not shown).

#### Release of LDH, portal pressure and bile production

Livers perfused at 10 °C and 20 °C showed a very low LDH release; livers perfused at 30 °C and 37 °C released, at the end of perfusion, significantly more LDH in comparison to livers perfused at 20 °C. Furthermore, LDH release rate was near to zero in 10 °C and 20 °C perfusion groups, suggesting a stationary condition, whereas it increased exponentially in livers perfused at 30 °C and 37 °C (Figure 1B). At the starting time point, portal pressure showed a correlation with perfusion temperature. Basal pressure was higher in livers perfused at 10 °C (5.8 ± 0.2 mmHg), intermediate in livers perfused at 20 °C and 30 °C (respectively 4.9 ± 0.1 mmHg and 4.9 ± 0.2 mmHg) and lower at 37 °C (4.2 ± 0.1 mmHg). Livers perfused at 20 °C and 30 °C had identical portal pressure and differed significantly from the other groups (*p <* 0.001). During perfusion, pressure did not significantly change within each group, with the exception of the 37 °C group; in this group portal pressure significantly increased from the 3rd hour of perfusion, rising rapidly to out-of-scale values after the 4th hour of perfusion (data not shown).

The increase in basal bile flow was logarithmically proportional to perfusion temperature. Basal bile flow in livers perfused at 10 °C and 20 °C was very similar, and was significantly different in comparison to livers perfused at both 30 °C and 37 °C (Figure 2A). Bile flow remained constant in livers perfused at 10 °C and 20 °C during the whole perfusion; on the contrary, in livers perfused at 30 °C and especially at 37 °C, bile flow fell rapidly after 2 h of perfusion (figure 2B).

#### ATP and glycogen in tissue

ATP was measured in tissue samples frozen at the end of 6th hour of perfusion. ATP in livers perfused at 20 °C was significantly higher in comparison to both 30 and 37 °C (*p <* 0.01 and 0.001). Interestingly, the ATP content in the 20 °C perfusion group was also higher when compared to livers perfused at 10 °C (*p <* 0.008, Figure 2C). In order to assess the state of the energy stores at the end of the 6-hour perfusion, glycogen was assayed in frozen tissue samples. The 37 °C group had significantly lower glycogen content in comparison to the other three groups. No difference was observed between the 10 °C, 20 °C and 30 °C groups (Figure 2D).

#### pH and lactic acid release

We observed that there was no significant acidification during perfusion at 10 °C and 20 °C. On the contrary, at 30 °C the pH of the perfusion solution was significantly lower in comparison to the basal values; furthermore, at 37 °C the pH significantly dropped starting at the 3rd hour of perfusion, in comparison to the pH values at 10 °C and 20 °C for the same time points (Figure 3A). To justify the pH fall at higher perfusion temperatures, we evaluated lactic acid release in perfusion buffer as index of anaerobiotic metabolism. Livers perfused at 10 °C and 20 °C did not release lactic acid during perfusion. In livers perfused at 30 °C, lactic acid concentrations values at the 2nd hour of perfusion were identical to the respective time points at 10 °C and 20 °C, but increased significantly in the subsequent time points. In livers perfused at 37 °C, lactic acid was significantly higher at the 2nd hour of perfusion, and increased dramatically during perfusion (Figure 3B).

#### HIF-1α mRNA and protein expression

HIF-1α mRNA and protein expression were assayed to confirm which livers were perfused in hypoxic conditions. We observed a slight increase of HIF-1α mRNA expression in livers perfused at 30 °C (*p <* 0.05 when compared to livers perfused at 10 °C and 20 °C) and a more accentuated rise of HIF-1α mRNA expression in livers perfused at 37 °C (*p <* 0.001 in comparison to livers perfused at 10 °C and 20 °C). Livers perfused at 10 °C and 20 °C did not show an increase in HIF-1α mRNA expression in comparison to control livers (Figure 4A). We used an ELISA kit to evaluate HIF-1α protein expression in liver tissues at the end of perfusion, obtaining results similar to those observed for HIF-1α mRNA expression (Figure 4B).

## DISCUSSION

The difficulty in perfusing the liver with acellular solutions at normothermic temperatures results from two different causes: (1) as stated by Henry’s Law, oxygen solubility decreases at higher temperatures; and (2) at increasing temperature, liver metabolism increases proportionally. These two aspects act synergistically so that oxygen carried by the perfusate and liver oxygen requirement inevitably diverge at increasing temperatures. Because of this, one of the major drawbacks of acellular perfusion at normothermic temperature is the inadequate oxygenation of the liver parenchyma, which leads to anaerobic glycolysis and acidosis[26]. In the isolated perfused rat liver model (IPRL) this problem is partially solved by raising the perfusion flow at higher-than-physiological levels: by speeding up the flow, the oxygen carried to the parenchyma will increase; on the other hand, according to Poiseuille’s Law, a higher flow is not associated with an abnormal increase in physiological portal pressure, due to the lower viscosity of acellular solutions compared to blood[26,27]. In our model, we increased the flow through the portal vein from the physiological value of 1.7 ml/min per g to 2.6 ml/min per g, obtaining a basal pressure similar to the physiological portal pressure. Unfortunately, this procedure is not sufficient to fulfill liver oxygen requirement at 37 °C. In our previous works, we showed that machine perfusion at subnormothermic temperature better preserve ischemic[20] and steatotic[6] rat livers, in a model of *ex vivo* reperfusion, respect to conventional preservation. Differently, the aim of this work was to determine at what temperature the oxygen carried by the perfusate and the liver oxygen requirement meet, allowing long term perfusion or, conversely, at what temperature liver metabolism shifts from aerobiosis to anaerobiosis. In isolated mitochondria, respiration rate increases exponentially with temperature[28]. Taking a look at the whole organ, Fujita *et al*[29] studied the oxygen requirement at different temperatures in perfused livers, and worked out an equation showing that liver oxygen requirement increases exponentially as a function of temperature. This observation has a key implication: below a certain temperature threshold, liver oxygen requirement slightly increases with temperature, whereas above such threshold it increases dramatically. The data obtained herein suggest that this threshold temperature may lie between 20 °C and 30 °C. In our perfusion model, the oxygen uptake rate (OUR) of livers perfused at 10 °C and 20 °C was identical to the theoretical oxygen requirement according to Fujita *et al*, but uptake rate of livers perfused at 30 °C and 37 °C was lower than theoretical oxygen requirement, suggesting that oxygen requirements are not completely fulfilled at higher temperatures (Figure 1B).

The data obtained on liver injury and function support this hypothesis: LDH release rate was near zero in livers perfused at 10 °C and 20 °C, while LDH increased significantly in livers perfused at 30 °C and 37 °C.

The literature clearly shows that bile formation depends on the activity of various ATP-driven pumps[30,31], and consequently is strictly related to mitochondrial respiration rate. Due to dependence on temperature of both respiration rate[28,32] and enzyme activity[33], a similar bile flow dependence is expected. In our model, the basal bile flow followed this trend (Figure 2A). Significantly, bile flow remained constant during perfusion at 10 °C and 20 °C, but dramatically dropped after 2 and 3 h of perfusion in both 30 °C and 37 °C perfused livers, suggesting that liver is unable to maintain baseline bile production rate, due to oxygen deficiency (Figure 2B). Accordingly, livers perfused at 30 °C and 37 °C contained significantly less ATP compared to livers perfused at 20 °C (*p <* 0.03 and 0.003, respectively) (Figure 2C). ATP content of 10°C livers was significantly higher when compared to 30 °C and 37 °C groups as well but, interestingly, was significantly lower than ATP measured in the 20 °C perfusion group (*p <* 0.008). This difference may be explained by a lower coupling efficiency of oxidative phosphorylation, usually occurring at low temperatures[28]. Furthermore, while glycogen stores are not affected at 30 °C, we observed a significant reduction of liver glycogen at 37 °C (Figure 2D).

These data suggest that livers perfused at 30 °C and 37 °C are in anaerobic conditions. Livers perfused at 20 °C and 10 °C show aerobic conditions during the whole perfusion. Accordingly, we observed that, contrary to livers perfused at 30°C and 37°C, livers perfused at 10 °C and 20 °C did not acidify the perfusion medium (Figure 3A). Lactic acid release is a suitable and sensitive marker of occurring anaerobic metabolism[34]. We assayed the lactic acid release at the 2nd, 4th, and 6th hour of perfusion for all the experimental groups. Ten °C and 20 °C livers showed identically low lactate release, and lactate release rate near to zero, demonstrating that, at these temperatures, the liver can support aerobic metabolism through the perfusion interval used in our experiments. Livers perfused at 30 °C showed a significantly higher lactate release rate, compared to 10 °C and 20 °C perfused livers. Livers perfused at 37 °C had dramatically higher lactate release rates, suggesting a huge difference between oxygen uptake and oxygen requirement at this temperature. Indeed, at 37 °C, in our model of acellular perfusion, the liver cannot support aerobic metabolism and his metabolism is mainly anaerobiotic, wasting ATP and glycogen stores, and releasing great amounts of unprocessed lactic acid in the perfusate (Figure 3B).

HIF-1α is a transcriptional activator of genes whose products, including glycolytic enzymes, are involved in systemic, local, and cellular responses to hypoxia such as, for example, inducing alternative metabolic pathways that do not require O2[35]. In hypoxic conditions, HIF-1α mRNA expression can increase[36,37]. To the best of our knowledge, HIF-1α mRNA and protein expression have not been assayed in a model of rat liver perfusion. We observed, for both mRNA and protein HIF-1α expression, an increase in livers perfused at 30 °C, and a more significant increase in livers perfused at 37 °C. Interestingly, both mRNA and protein expression did not differ in livers perfused at 10 °C and 20 °C, and were similar to controls.

These data clearly demonstrate that long term liver perfusion with simple acellular solutions is not possible above 30 °C. Livers perfused at 37 °C are evidently in anaerobic conditions; livers perfused at 30 °C seems to be in an intermediate state, showing the first signs of distress, but not as much as livers perfused at 37 °C, suggesting that the optimal temperature should certainly lie below 30 °C.

Should this optimal temperature be necessarily lower than 20 °C? Our data, both those registered as a time course along perfusion period, and those assayed in the tissue, demonstrate that livers perfused at 10 °C and 20 °C exhibit quite similar and stable conditions, suggesting that the temperature where oxygen liver requirement and oxygen delivery meet is certainly below 30°C, but not necessarily below 20 °C.

One of the most important applications of long term liver perfusion is machine perfusion for organ transplantation. Recently, liver machine perfusion has been evaluated as a suitable alternative to simple cold storage, particularly for marginal organ preservation[38,39]. Furthermore, liver hypothermic machine perfusion (4 °C to 8 °C) has recently been tested in a clinical trial with encouraging results[14,40,41]. In this trial, a starch-added solution was used to perfuse livers at 0.667 mL/min per liver g, without oxygenation. Subnormothermic perfusion has not yet been used in clinical trial, although some authors referred to the subnormothermic machine perfusion as “the way in between” that could potentially bypass the flaws of both hypothermic and normothermic machine perfusion[42,43]. The subnormothermic temperature should consent the use of low- viscosity solutions and consequently higher flow rates, sustaining a mild liver metabolism useful to restore energy stocks before transplantation, particularly in livers from donation after cardiac death. Moreover, the avoidance of hypothermia could be useful in fatty livers, and maintaining the liver in a mild metabolism state could be useful for genetic and immunologic manipulations before transplantation[41,42].

These data clearly show that livers perfused at 20 °C have no sign of anaerobiosis, therefore reducing the perfusion temperature below 20 °C is unlikely to further improve this technique. On the contrary, livers perfused at 30 °C start to show the symptoms of lack of oxygenation.

The adequate oxygenation of livers preserved by perfusion at 20 °C highlights this technique’s concrete potential to avoid ischemic insult, the real culprit of the preservation injury observed in cold storage transplanted organs. The subnormothermic temperature, allowing a less complicated setting, might also favor the successful translation of this technique from the experimental studies into clinical practice.

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## COMMENTS

### *Background*

Cold (0-4 °C) storage is considered the gold standard in organ preservation for transplantation. Currently, as new perfusion techniques are developed to improve the preservation of marginal organs, hypothermic machine perfusion is the most preferred choice, though it presents drawbacks as cold injury, a slowed down metabolism and a more complex setting.

### *Research frontiers*

Clinical trial showed improved clinical parameters and shorter duration of hospital stay in patients who received grafts stored by hypothermic machine perfusion (HMP), in comparison to patients who received grafts preserved by cold storage.

### *Innovations and breakthroughs*

Currently, new perfusion techniques have being developed for the preservation of marginal livers. The most preferred technique is hypothermic machine perfusion (0˗4°C). We demonstrated that livers perfused at 20°C show no sign of ischemia, therefore reducing the perfusion temperature below 20°C is unlikely to further improve this technique. On the contrary, livers perfused at 30°C start to show the symptoms of lack of oxygenation.

### *Applications*

The adequate oxygenation of livers perfused at 20°C highlights this technique’s concrete potential to avoid cold injury and ischemic insult during liver preservation for transplantation. Furthermore, the subnormothermic temperature, allowing a less complicated setting, might also favor the successful translation of this technique from the experimental studies into clinical practice. Finally, maintaining the liver in a mild metabolism state during preservation could be useful for pharmacologic, genetic and immunologic manipulations, and for regeneration of the energy status in ATP depleted organs, avoiding both ischemic and cold-induced insult. This possibility could be extremely suitable for the preservation of livers to date rejected, as non˗heart˗beating˗donor organs and steatotic organs.

### *Terminology*

Oxygenated Machine Perfusion is a new technique, currently under development, for the preservation of organs for transplantation. This technique is aimed to reduce the ischemic injury and better preserve marginal organs, unable suffer cold and ischemic injury, both present in static cold storage, the gold standard for organ preservation.

### *Peer review*

This manuscript showed that liver perfused at higher temperature (> 30 degrees) led to hypoxia and increased hypoxia inducible factor-1α expression.

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**Abbreviations:** HIF-1α: hypoxia inducible factor-1α; LDH: lactate-dehydrogenase; ODR: oxygen delivery rate; OUR: oxygen uptake rate; RT-PCR: real time-polymerase chain reaction; IPLR: isolated perfused rat liver.



**Figure 1 Liver oxygen uptake and lactate-dehydrogenase release in rat livers perfused for 6 h at 10 °C, 20 °C, 30 °C and 37 °C.** A: oxygen uptake rate (OUR) was constant during the 6 h of perfusion at 10 °C, 20 °C and 30 °C, and dropped in livers perfused at 37 °C; B: lactate-dehydrogenase (LDH) release slightly increased during perfusion at 10 °C and 20 °C. No significant difference was observed between livers perfused at these temperatures, and the LDH release rate was near zero. LDH release dramatically increased in livers perfused at 30 °C and 37 °C, and similarly, LDH release rate increased in an exponential manner when compared to 10 °C and 20 °C ( *n =*  6; b*P* < 0.01 *vs* 30 °C and 37 °C).

   

**Figure 2** **Bile flow, ATP and glycogen in rat livers perfused for 6 h at 10 °C, 20 °C, 30 °C and 37 °C.** A: Basal bile flow showed a strong correlation with perfusion temperature (*R*2 = 0.98); B: bile flow remained constant in livers perfused at 10 °C and 20 °C during the whole perfusion; on the contrary, in livers perfused at 30 °C and 37 °C, bile flow fell rapidly after 3 and 2 h of perfusion; C: ATP content in 10 °C and 20 °C *vs* 30 °C and 37 °C groups was significantly higher. ATP was significantly lower in 10 °C in comparison to 20 °C group; D: glycogen content in the 37 °C group was significantly lower in comparison to the other groups. No difference was observed between the 10 °C, 20 °C and 30 °C groups (*n* = 6; b*P* < 0.01 *vs* 30 °C and 37 °C; d*P* < 0.01 *vs* 20 °C; f*P* < 0.01 *vs* 37°C).



**Figure 3 pH levels and lactate release during a 6˗h perfusion in rat livers at 10 °C, 20 °C, 30 °C and 37 °C.** A: normalized pH of the perfusion solution in livers perfused at 10 °C and 20 °C remained constantly above 7.35, pH at 30 °C show a tendency to acidification (not significant), at 37°C the pH of the perfusate significantly decreased, reaching at the 6th hour the value of 7.14 ± 0.05; B: lactic acid release in the perfusate significantly increased in the 6th hour of 30 °C perfused livers, and at all the time points in 37 °C perfused liver (*n* = 6; b*P* < 0.01 *vs* 10 °C and 20 °C; c*P* < 0.05 *vs* 10 °C and 20 °C).

 

**Figure 4 Hepatic hypoxia inducible factor-1α mRNA and protein expression at the end of 6˗h oxygenated perfusion in rat livers at 10 °C, 20 °C, 30 °C and 37 °C.** A: hypoxia inducible factor (HIF)-1α mRNA expression increased in livers perfused at 30 °C and, more significantly, at 37 °C. No significant difference was observed in livers perfused at 10 °C and 20 °C when compared to control livers; B: HIF-1α protein expression increased slightly in livers perfused at 30 °C, and more significantly in the 37 °C group. No significant difference was observed between the 10°C and 20°C groups respect to control livers (*n* = 6; a*P* < 0.05 *vs* 10 °C and 20 °C; b*P* < 0.01 *vs* 10°C and 20°C; c*P* < 0.05 *vs* 10 °C and 20 °C).