Am J Transl Res 2023;15(8):5197-5205
www.ajtr.org /ISSN:1943-8141/AJTR0151136

Original Article

Successful temporary machine perfusion of marginal liver grafts early after retrieval or shortly before implantation

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Received May 8, 2023; Accepted June 29, 2023; Epub August 15, 2023; Published August 30, 2023

Abstract: Objectives: The benefit of machine perfusion during storage of liver grafts retrieved after cardiac death should be investigated as applied either at the beginning or near the end of the preservation period. Methods: Rat livers were explanted 20 min after cardiac arrest of the donor and cold-stored (CS) for 18 h. Other grafts were additionally subjected to 2 h of normothermic machine perfusion (MP) either 3 h after retrieval (early MP) or 3 h before reperfusion (late MP), thus extending total ischemic time to 20 h. The 3 h period should represent a short transport period between a resident regional pumping center and the explant or implant hospital, respectively. Viability of all livers was assessed thereafter by warm reperfusion in vitro. Results: In comparison to the controls, both regimens significantly improved hepatic recovery upon post-preservation reperfusion as evaluated by enzyme release, bile production, and energetic recovery. Molecular upregulation of pro-inflammatory signals was also significantly mitigated. No functional differences between early and late machine perfusion could be disclosed. Conclusion: Our data suggest that it might not be necessary to hurry with the attempt to connect the graft to a machine early after retrieval.

Keywords: Machine perfusion, reconditioning, liver

Introduction

Extracorporeal machine perfusion of marginal donor organs is about to translate from promising research projects into clinical routine [1, 2]. An international randomized controlled multicenter trial has shown that continuous normothermic perfusion of liver grafts from retrieval to implantation significantly reduces ischemic liver injury after transplantation [3].

The maintenance of an aerobic, quasi-physiologic metabolism during the extracorporeal period, including the transport to the implantation clinic, minimizes biochemical alterations, and at the same time allows for some degree of functional monitoring and evaluation [4, 5].

Normothermic ex situ machine perfusion of the isolated liver graft may hence become a useful means to minimize ischemic tissue injury during organ preservation but also represent a promising platform for a variety of therapeutic interventions aiming to enhance post-transplant outcome of the livers [6].

Thus, genetic engineering such as by delivery of silencing RNA may modulate the expression of specific genes and improve tissue resilience towards reperfusion injury [7].

Perfusion with defatting drugs has been proposed to reduce liver steatosis as a risk factor for later reperfusion injury [8], and infusion of mesenchymal stem cells during machine perfusion may ameliorate hepatic microcirculation and alleviate ischemic injury [9].

Ischemia is also prone to enhance cellular senescence that can effectively be counteracted by the use of specific senolytic drugs that suppress overabundant senescent cells which otherwise might foster increased inflammatory reactions upon reperfusion [10].

Last but not least, the perfusion itself can be used as a physical trigger, to induce molecular

chaperones by controlled heat shock treatment in order to increase graft resilience [11].

However, the continuous use of machine perfusion, including the transport from graft retrieval to the place of implantation, bears some logistic impediment in comparison to the traditional transport by simple cold storage in an icebox.

Therefore, some investigators propose the use of machine perfusion only after preceding conventional transport of the organ to the transplantation clinic [12, 13] for reconditioning or repair of the injured organ prior to reperfusion.

However, machine perfusion of marginal organ grafts remains a specialized procedure that requires trained personnel and dedicated technical infrastructure, even if operated under stationary conditions.

An attractive perspective may hence be seen in the creation of dedicated perfusion centers [14, 15].

The concentration of infrastructural requirements, technical expertise, and personnel on call would economize the expenses necessary to maintain a functioning graft perfusion service for everybody.

Thus, a certain number of transplantation clinics might be associated to one dedicated perfusion center, situated in proximity that may serve as a hub for machine perfusion and reconditioning of marginal donor grafts. Actual transplantation of the graft could be done after reconditioning in the perfusion center and a very short additional transport on ice to the implantation clinic that might not be associated with notable adverse effects [15].

On the other hand, one might also consider the alternative to place the respective hubs for machine perfusion not in proximity to the implant clinics, but rather centered in reach of the retrieval hospitals. That way, any liver damage encountered prior to or during graft retrieval, as for instance warm ischemia occurring upon donation after cardiac death, might be counteracted prior to a putative potentiation by subsequent cold ischemic transport.

Therefore, the two strategies of placing the machine perfusion period either at the beginning of a longer cold ischemic transport or as a post-hoc measure only shortly prior to transplantation, should be compared in an experimental pilot study.

Material and methods

The study is reported in accordance to the recommendations in the ARRIVE guidelines (PLoS Bio 8(6), e1000412,2010). Isolated livers retrieved from male Wistar rats with a weight between 250 g and 300 g were used for the experiments. The procedure of euthanasia for organ retrieval according to §4 Abs. 3 TSG (German Legislation on animal protection) has been discussed with and approved by the animal welfare officers of the University Hospital Essen. The federal law regulating the protection of animals and the principles of laboratory animal care (NIH publication vol 25, No. 28, revised 1996) were followed. Animals were killed in deep isoflurane-anesthesia by intracardiac injection of KCl.

The abdomen of the dead animal was exposed by midline incision and exsanguination induced by incision of the infra-renal caval vein. Twenty minutes after cardiac arrest the portal vein was cannulated and the liver rinsed via the portal vein with 60 mL of histidine-tryptophan-ketoglutarate (HTK) solution (Köhler Chemie, Germany).

After hepatectomy the liver was randomly assigned to one of the following groups (cf. **Figure 1**): 1) Controls: Livers were preserved overnight in HTK solution for 18 h at a temperature of 4°C regulated by a cryo-thermostat. 2) Early machine perfusion - EMP: Livers were cold-stored for 3 h in HTK solution (simulating the transport period to a perfusion hub near the retrieval hospital), then subjected to 2 h of normothermic machine perfusion (for details see below) and put back in static cold storage in HTK for another 18 h (simulating the transport to the recipient). 3) Late machine perfusion - LMP: Livers were cold stored in HTK solution for 18 h at a temperature of 4°C (simulating the transport to a perfusion hub near the implant hospital), then subjected to 2 h of normothermic machine perfusion (for details see below) and put back to static cold storage in HTK for another 3 h (simulating the transport to the recipient).

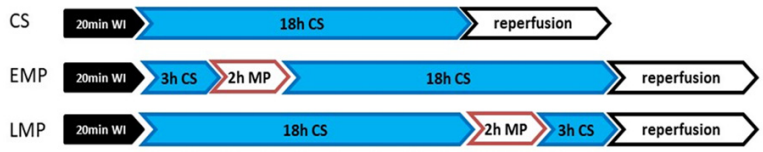

Figure 1. Schematic representation of experimental groups: cold storage (CS), early machine perfusion (EMP), and late machine perfusion (LMP). For details see text.

Normothermic machine perfusion

Livers were connected to a home-made machine perfusion circuit and 200 ml of oxygenated Aqix RS-I solution were recirculated through the portal vein at a constant pressure mode [16].

To mitigate putative tissue damage by abrupt temperature increase (rewarming injury) [17, 18], the temperature of the perfusate was slowly elevated from initially 8°C up to normothermia within the first 60 min of perfusion while adjusting the perfusion pressure from 3 mmHg to 6 mmHg.

Viability testing after preservation

An established recirculating perfusion system was used for the evaluation of organ recovery after preservation in the different groups. Details of this in vitro system had been described previously [16, 19]. In brief, the livers were perfused at 3 ml/g/min with 300 ml of oxygenated Williams E solution, that was supplemented with 3 g/100 ml of bovine serum albumin. The system was kept normothermic at 37°C by use of an external thermostat.

The activities of alanine aminotransferase (ALT) as well as glutamate dehydrogenase (GLDH) were determined in the circulating perfusate at regular intervals by photometric routine assays at the laboratory center of the University Hospital and taken as a general readout of hepatic cellular damage upon reperfusion.

Hepatic bile production was followed by cumulative collection of bile juice via a 27-gauge polyethylene tubing that was inserted into the common bile duct of the liver grafts.

Energetic status

Freeze drying of tissue specimen and subsequent perchloric acid extraction was performed as established previously [20].

Tissue content of adenosine triphosphate (ATP) was determined in the neutralized supernatant by means of a commercial test kit (Abcam, Cambridge, UK) according to the manufacturer's instructions and the results were normalized to g tissue dry weight.

Gene expression analyses

Total RNA was isolated from snap-frozen samples and analysed as described previously [21]. The amount of specific mRNA in the tissue was normalized for the respective individual quantities of transcripts of GAPDH, which was analysed as house-keeping gene. Results are expressed as relative deviation from baseline levels that were analysed from native rat liver samples that were processed in parallel. All reagents and primers for GAPDH (n° PPR06-557B), TNFα (n° PPR06411F), MHC2 (n° PPR-699851). TLR4 (n° PPR45931B) and ICAM1 (n° PPR42255A) were purchased from Qiagen GmbH (Hilden, Germany).

Histology

Tissue samples were cut into 3 mm blocks and fixed by immersion in 4% buffered formalin. The blocks were embedded in paraffin and 2-4 mm tissue slides were prepared using a microtome (SM 2000R, Leica Instruments, Nußloch, Germany).

Hematoxylin-eosin (H&E) staining was conducted adherent to in-house standards and used to assess morphologic integrity of the parenchyma.

Necrotic injury was examined at 200-fold magnification and graded semi-quantitatively as described elsewhere [22], ranging from 0 (no necrosis) to 3 (severe necrosis with disintegration of hepatic cords) by two independent examiners.

Statistics

All values are expressed as means ± standard deviation (SD) of n=6 animals per group. Differences among the groups were tested by analysis of variances followed by the Student-Newman-Keuls test. Statistical significance was set at p less than 0.05.

Table 1. Portal vascular flow and concentrations of lactate and liver enzyme activities (alanine aminotransferase - ALT and Glutamate dehydrogenase - GLDH) in the perfusate upon early or late reconditioning machine perfusion during liver preservation

	Early MP	Late MP	
Flow (ml/g/min)	4.3±1.3	6.9±0.6	*
Lactate (mmol/L)	2.2±0.59	1.6±0.7	ns
ALT (U/L)	16.2±8.1	20.1±22.4	ns
GLDH (U/L)	9.5±4.2	8.3±3.0	ns

Values given as mean ± standard deviation of n=6 experiments per group; *: P < 0.05, Student T-test.

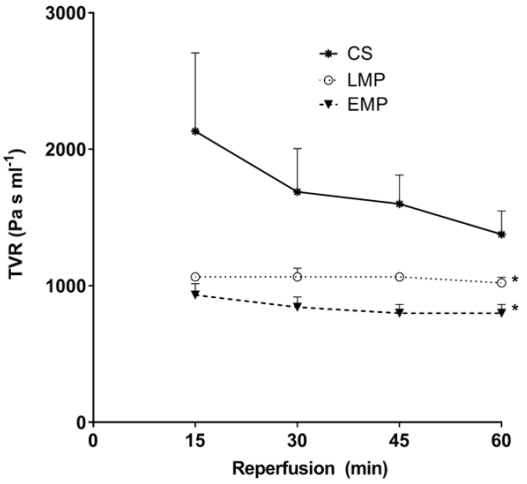

Figure 2. Total vascular resistance (TVR) upon reperfusion after cold storage (CS) or after cold storage with early (EMP) or late (LMP) intercalation of 2 h of normothermic machine perfusion (*: P < 0.05 vs. CS by ANOVA and SNK-test).

Results

Portal flow during machine perfusion was found to be affected by the temporal proximity to graft retrieval (cf. **Table 1**). However, apart from the fact that early reconditioning was associated with significantly higher flow values than late reconditioning, no differences could be substantiated between the two groups with regard to lactic acid accumulation or enzyme leakage of ALT or GLDH (**Table 1**).

Portal vascular resistance upon reperfusion is depicted in **Figure 2**. Untreated livers exhibited elevated initial values that slowly declined during ongoing reperfusion.

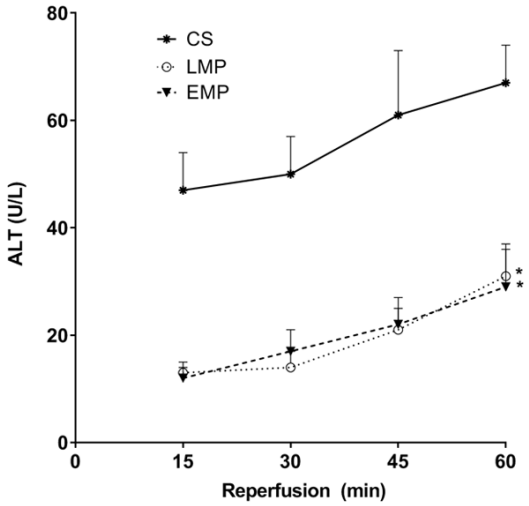

Figure 3. Perfusate activities of alanine aminotransferase (ALT) upon reperfusion after cold storage (CS) or after cold storage with early (EMP) or late (LMP) intercalation of 2 h of normothermic machine perfusion (*: P < 0.05 vs. CS by ANOVA and SNK-test).

By contrast initially low values were observed in the EMP as well as the LMP group that both remained constant and significantly lower than in the CS group throughout the observation period.

Enzyme leakage of the cytosolic alanine aminotransferase (ALT) and the intramitochondrial glutamate dehydrogenase (GLDH) was followed during reperfusion and taken as parameter of hepatocellular injury.

As depicted in **Figure 3**, perfusate levels of alanine aminotransferase (ALT) were significantly lower in the treated groups than after simple cold storage (CS), but no difference could be delineated between early or late machine perfusion.

Slightly different results were found with regard to the hepatic leakage of glutamate dehydrogenase (GLDH, **Figure 4**). In the LMP group, GLDH release was significantly lower than in the cold storage group, but although the values also tended to be attenuated in the EMP group, the difference with the CS group did not reach statistical significance.

Functional recovery of the livers is summarized in **Table 2**. Hepatic bile production upon reperfusion could be significantly and similarly

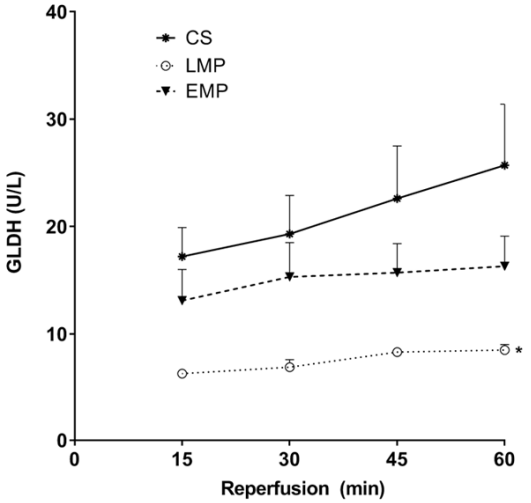

Figure 4. Perfusate activities of glutamate dehydrogenase (GLDH) upon reperfusion after cold storage (CS) or after cold storage with early (EMP) or late (LMP) intercalation of 2 h of normothermic machine perfusion (*: $P < 0.05$ vs. CS by ANOVA and SNK-test).

enhanced by EMP as well as LMP, when compared to cold storage alone.

In line with this, the clearance of lactic acid from the perfusate was only impaired in the cold storage group but kept in a normal range in both treatment groups.

Energetic recovery was judged by the hepatocellular content of ATP at the end of reperfusion. It was seen that both reconditioning protocols led to a significant increase in hepatic tissue ATP levels in comparison to the controls, but no relevant difference could be disclosed between EMP or LMP (cf. **Table 2**).

Pro-inflammatory reactions in the grafts were approximated by the expression of selected genes associated with immunogenicity and inflammation (cf. **Table 3**).

EMP as well as LMP significantly abrogated the upregulation innate and acquired immunity as reflected by the expression of toll like receptor 4 and major histocompatibility complex 2. At the same time, the expression of TNFα, a major proinflammatory cytokine was also significantly reduced in both treatment groups while the expression of ICAM1 did not disclose any influence of either type of machine perfusion in comparison to simple cold storage.

Histological analysis of tissue sections obtained at the conclusion of the experiments did neither reveal relevant morphological alterations nor apparent differences between the three groups. Structural alterations were mostly restricted to some cytoplasmic vacuolization. The quantitative injury score in the cold storage group was 1.22±0.34 versus 0.85±0.34 after EMP and 0.90±0.53 after LMP.

Besides its potential to recondition donor organs injured by warm or cold ischemic challenges, normothermic machine perfusion provides the opportunity to evaluate the isolated perfused organ and to aid in the decision, whether or not to use it for actual transplantation.

The correlation of biomarkers, obtained during ex vivo machine perfusion, with graft integrity upon posterior reperfusion was slightly different, dependent on whether the machine perfusion had been performed at the beginning or the near the end of the preservation period. Thus, perfusate concentrations of ALT on the machine fairly well correlated with those during reperfusion while a significant correlation was only seen in the LMP group (cf. **Figure 5**). By contrast perfusate activities of GLDH were barely predictive in either group showing r^2 values of 0.133 after LMP and 0.084 after EMP.

Discussion

At present, it is generally accepted that normothermic perfusion techniques may serve as effective means to improve graft recovery upon transplantation after preservation [23]. For logistic reasons, many investigators advise to make use of normothermic machine perfusions only after transport of the organ by simple static cold storage [12, 15, 23].

The establishment of centralized perfusion locations with specialized infrastructure will allow for cost-effective high-volume usage at potentially unified protocols, when organs undergo ex vivo perfusion prior to being transported to the individual implantation clinic [23].

In the present study, the respective benefits of normothermic machine perfusion (NMP) as performed early or late during liver preservation should be compared with each other and

Table 2. Functional recovery of livers upon reperfusion after 18 h of cold storage (CS), and early (EMP) or late (LMP) reconditioning machine perfusion during liver preservation

	CS	EMP	LMP
Bile production (µl/g/h)	7.3±5.1	16.1±6.8*	15.7±3.1*
Lactic acid (µmol/l)	3.2±0.7	1.3±0.5*	1.7±1.2*
ATP (µmol/g dw)	0.98±0.62	2.59±0.82*	2.97±1.44*

Values given as mean ± standard deviation of n=6 experiments per group; *: $P < 0.05$ vs. CS, ANOVA and SNK-test.

Table 3. Molecular expression of selected proinflammatory genes: tumor necrosis factor alpha (TNFα), major histocompatibility complex 2 (MHC2), toll-like receptor 4 (TLR4) and intercellular adhesion molecule 1 (ICAM1) in the respective groups

	CS	Early MP	Late MP
TNFα	103.4±93.8	33.7±14.0*	24.2±5.2*
MHC2	1.5±0.9	0.5±0.3*	0.7±0.2*
TLR4	0.3±0.1	0.1±0.04*	0.1±0.03*
ICAM1	29.5±29.4	23.9±11.6	21.5±15.3

CS: simple static cold storage; EMP: early machine perfusion; LMP: late machine perfusion. Values given as percent of baseline expression in naïve liver tissue and given as mean ± standard deviation of n=6 experiments per group; *: $P < 0.05$ vs. CS by ANOVA and SNK Test.

with respect to controls, that were preserved by cold storage only.

Within the conditions of the present model, the benefits obtained by both machine perfusion regimen were substantial and uniformly comprised a more than 50% improvement of structural, metabolic and functional parameters.

Especially the recovery of bile production and the restoration of tissue energetics have been shown to be most decisive parameters of graft function in rat liver transplantation and established readouts of graft liver viability in ischemia/reperfusion models [24-26].

An additional repercussion of hepatic ischemia reperfusion injury lies in the molecular upregulation of inflammatory responses [27], which are likely to negatively affect tissue integrity upon reperfusion in vivo. In line with previous observations where NMP reduced pro-inflammatory responses in transplanted human livers [28], the expression of selected pro-inflammatory genes could be significantly mitigated by NMP in our study. Moreover, it was seen that this effect was completely independent of whether the NMP had been executed early or late during the preservation period.

The results suggest that it might not be necessary to hurry with the attempt to connect the graft to a machine as early as possible after retrieval. An injurious effect or potentiation of tissue injury by sequential warm and cold ischemia [29] could not be substantiated in our set up. Either such effect was not operative, or it could be fully compensated for by late normothermic machine perfusion.

Nonetheless it must be conceded that although LMP has been effective in the face of 20 min of warm ischemia prior to cold storage, we cannot exclude more decisive effects in case of liver retrieval after more intensive warm ischemic injury in the donor.

However, a practical advantage of LMP over EMP lies in the fact that LMP is much closer to the actual transplantation and evaluation of graft viability should therefore be more precise and reliable. In clinical practice EMP will be followed by a longer and more variable time of static preservation and transport of the graft to the implantation clinic. Depending on the individual resilience of the livers against cold ischemia actual graft viability upon transplantation will more or less deviate from the prognostic results obtained during EMP.

By contrast, LMP will only be followed by a short and foreseeable period of cold storage and evaluative parameters may hence be more accurate.

In the present study, this aspect could not be represented in detail, since experimental conditions required a most uniform degree of initial liver injury as well as a constant preservation period after EMP. Taken together, putative variations of these parameters as described above and how they occur in real life could not be taken into account.

Further limitations of our study relate to the isolated *ex vivo* perfusion model, which only partly reflects the actual situation *in vivo*. Thus, secondary inflammatory reactions related to blood-

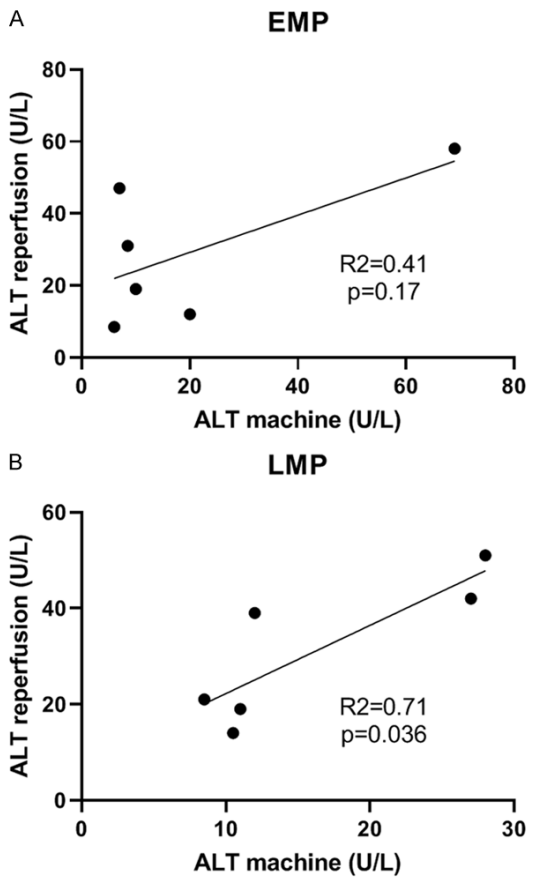

Figure 5. A: Correlation of perfusate activities of alanine aminotransferase (ALT) after 2 h of normothermic machine perfusion as performed at an early (EMP) time during preservation and later post-preservation reperfusion. B: Correlation of perfusate activities of alanine aminotransferase (ALT) after 2 h of normothermic machine perfusion as performed at a late (LMP) time during preservation and later post-preservation reperfusion.

vessel interactions and long-term results are not available.

The set up, however, has been shown to adequately monitor basic ischemia-reperfusion related tissue alterations and metabolic repercussions [30, 31] and serves as an established screening model. Based on the present results, further long-term experiments *in vivo* seem indicated to confirm the conclusion that machine perfusion shortly before implantation might be an adequate option for graft reconditioning and evaluation.

Disclosure of conflict of interest

None.

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