# Controlled Rewarming after Hypothermia: Adding a New Principle to Renal Preservation

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**Abstract**

Early graft dysfunction due to preservation/reperfusion injury still represents a notable issue after kidney transplantation, affecting long term prognosis of graft viability. One trigger of postischemic cell dysfunction could be recognized in the abrupt temperature shift from hypo- to normothermia, leading to mitochondrial dysfunction and proapoptotic signal transduction. Here we propose a technique to cope with this "rewarming injury" by interposing a period of gentle warming up by hypo- to subnormothermic machine perfusion of the isolated graft prior to warm reperfusion. Porcine kidneys were subjected either to 18 hours of hypothermic machine preservation (HMP) or 18 hours static cold storage + 3 hours of gentle, machine controlled oxygenated rewarming (COR). Functional integrity was evaluated in both groups by subsequent normothermic reperfusion *in vitro*.

The functional benefit of COR was documented by an approximately twofold increase in renal clearances of creatinine as well as urea upon warm reperfusion, compared to controls. This was accompanied with a notable mitigation of postischemic mitochondrial dyshomeostasis. COR significantly improved renal oxygen consumption and maintained total NAD tissue content upon reperfusion. Mitochondrial initiation of cellular apoptosis, as evidenced by activation of caspase 9 was also largely prevented after COR but not in controls. The concept of gentle regenerative graft rewarming could become a valuable adjunct in renal transplantation. Clin Trans Sci 2015; Volume 8: 475–478

**Keywords:** rewarming, preservation, reperfusion injury, kidney, machine perfusion

## Introduction

Reperfusion injury after cold preservation still represents a significant problem in renal transplantation, favoring delayed graft function and consecutive alterations in long-term outcome.[1,2] Reduction of preservation injury by hypothermic machine perfusion (HMP) represented a notable advance in preservation technology, allowing for continuous supply of oxygen during preservation along with the removal of metabolic waste products and preservation of mechanical homeostasis to vascular endothelium by pulsatile flow conditions.[3,4] Thus, HMP improved 1 and 3 years survival after kidney transplantation.[5,6]

However, a yet merely addressed aspect of preservation induced graft injury lies in the cellular susceptibility to abrupt rewarming after prolonged periods in the cold.

There is experimental evidence that the imminent temperature shift from hypo- to normothermia, incurring upon reperfusion of organ grafts, triggers mitochondrial respiratory dysfunction[7] as well as induction of the mitochondrial apoptotic pathway[8] after cold preservation and may constitute a genuine factor contributing to reperfusion injury and graft dysfunction. This "rewarming injury" is even operative after complete redox homeostasis in the cold.[9]

In this paper we describe a technical approach to alleviate renal reperfusion injury after hypothermic preservation by transient extracorporeal cold to mid-thermic perfusion that allows for a gentle recovery of mitochondrial function at limited workload.

## Materials and Methods

Porcine kidneys were procured from German landrace pigs weighing between 25 and 30 kg. After nephrectomy the procured organ was flushed on the back-table with approximately 100-mL Custodiol-N solution at 4°C using gravity perfusion (100 cm H20).

All grafts were then randomly assigned to one of the following preservation methods.

Hypothermic machine perfusion (HMP): Grafts were preserved overnight by pulsatile machine perfusion using Custodiol-N solution including 5 g/dL of dextran 40.

Controlled oxygenated rewarming (COR): Kidneys were conventionally preserved by cold storage for 18 hours at 4°C. Immediately prior to reperfusion, grafts were put on a machine perfusion circuit for gentle rewarming from the cold by gradual increase of perfusate temperature up to 20°C. The solution was pumped through a hollow fibre-oxygenator fed with 100% oxygen. During the first period of COR, temperature was kept hypothermic at 8°C and was gradually increased (cf., *Figure 1*) by means of a programmable, external circulating cryothermostat, connected to the integrated heat exchanger of the oxygenator.

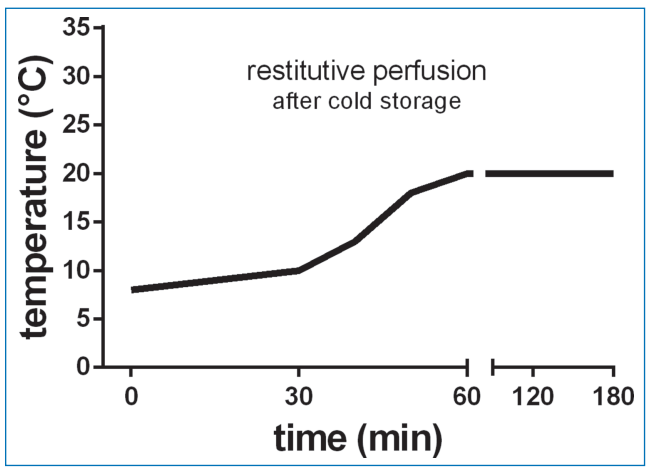


**Figure 1.** Controlled oxygenated rewarming: gentle elevation of graft temperature upon oxygenated extracorporeal machine perfusion prior to warm reperfusion.

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Short-term hypothermic machine perfusion after CS (CS+HMP): As additional control group, kidneys were cold stored for 18 h and then put on a machine for additional 3 h of oxygenated perfusion at constant temperature of 8°C.

Custodiol-N solution, including 5 g/dL of dextran 40 was used as machine perfusate in all groups.

### Reperfusion model

Graft integrity was tested thereafter by isolated reperfusion in vitro using an established model according to earlier studies.[10]

The perfusion medium consisted of 1,000 mL freshly prepared Krebs–Henseleit buffer containing 2.2% bovine serum albumin and 20 mL of concentrated amino acid solution (RPMI 1640–50×). Creatinine (0.1 g/L) and urea (1 g/L) were added to the perfusate to allow for calculation of respective renal clearances. The ureter was cannulated with PE-tubing. Urine volume was replaced every 30 minutes by adding equal amounts of saline solution to the perfusate.

Immediately before reperfusion, all organs were exposed to no flow conditions at room temperature for 20 minutes in order to imitate warm ischemia time in the clinical setting.

Concentrations of creatinine and urea were determined in perfusate and corresponding urine samples in a routine fashion at the Laboratory centre of the University Hospital. Clearances were calculated for the respective intervals as urinary creatinine (urea) × urine flow (mL/min)/perfusate creatinine (urea).

Renal tubular injury was approximated by the release of L-type fatty acid binding protein (LFABP), an intracellular carrier protein predominantly expressed in proximal tubular cells. Protein levels were measured on a micro plate reader (Tecan, Grailsheim, Germany) using analytic kits from USCN life science (Wuhan, China) according to the instructions of the manufacturer. Urinary FABP excretion was expressed in microgram FABP normalized per gram creatinine (μg/g Cr).

### Mitochondrial function and permeability transition pore

Oxygen consumption was calculated from the $pO_2$ differences between arterial and venous sites, measured in a pH-blood gas analyser (ABL 500 acid-base laboratory, Radiometer, Copenhagen) and expressed as mL/min/g according to transrenal flow and kidney mass.

Total content of NAD was determined from frozen cortical tissue samples by means of the enzyme cycling method using a commercial kit (Abcam, Cambridge, United Kingdom) and calculated from fluorescence at *Ex*/*Em* 540/590 nm, compared to baseline values from nonischemic tissue samples that were run in parallel.

Functional activity of caspase 9 was used to approximate the mitochondrial induction of the apoptotic pathway. Enzyme activity of caspase 9 was analysed using a commercial colorimetric assay kit (R&D Systems, Minneapolis, MN, USA) and activities in the experimental groups are presented as the percentage increase with respect to the baseline values obtained from nonischemic control tissue.

### Statistics

All values were expressed as means ± SEM of $n = 6$ animals per group. Differences between groups were tested by parametric comparison of the means using Instat 3.01 (Graph Pad software Inc, San Diego, CA, USA). Statistical significance was set at $p < 0.05$.

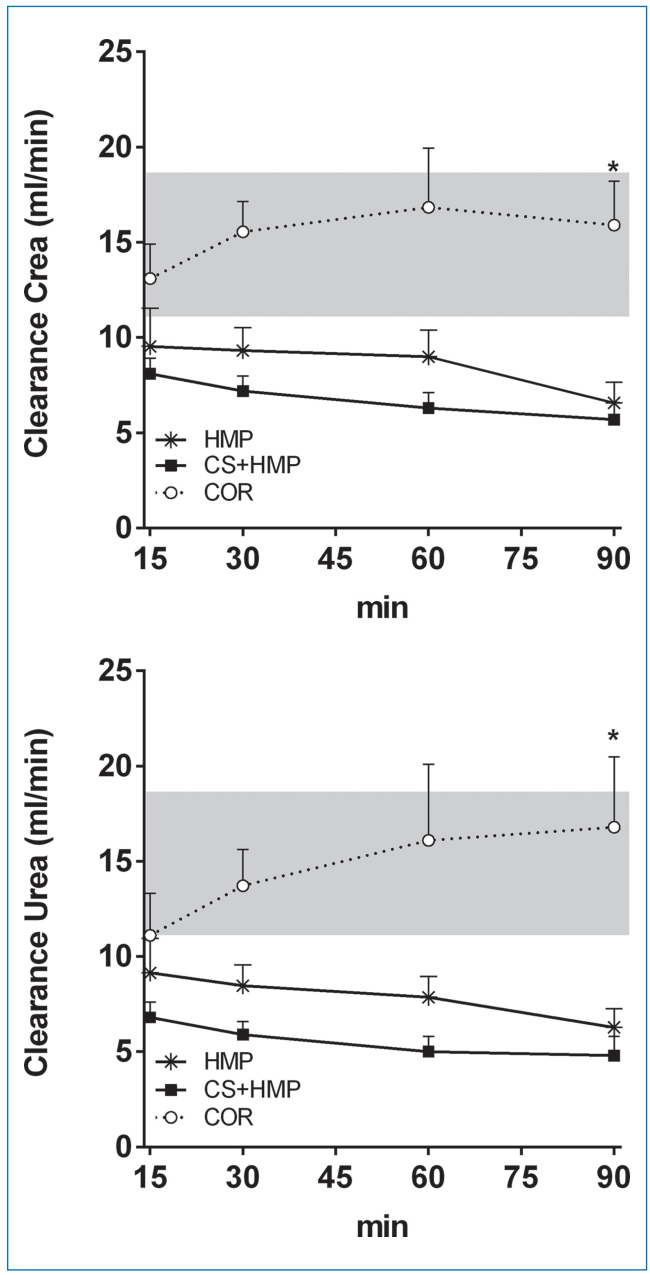


**Figure 2.** Renal clearances of creatinine and urea upon reperfusion after 18 hours of HMP (HMP), 18 hours cold storage + 3 hours of HMP (CS + HMP) or 18 hours of cold storage + 3 hours of controlled oxygenated rewarming (COR). $N = 7$ per group (*$p < 0.05$ [area under the curve, AUC] vs. HMP). Model specific normal range, obtained from nonischemic organs, is represented by the gray area, for comparison.

## Results

Vascular resistance after COR was 1.13 ± 0.22 mmHg/mL/min/g, but differences to the values observed after HMP (1.45 ± 0.36 mmHg/mL/min/g) or CS + HMP (1.62 ± 0.23 mmHg/mL/min/g) did not reach statistical significance.

Upon Reperfusion, however, COR resulted in a significant enhancement of renal functional recovery with clearances of creatinine, as well as urea being nearly twice as high than observed after HMP (cf., *Figure 2*).

Mitochondrial permeability transition pore opening of inactive or dysfunctional mitochondria is reflected by reduced concentrations

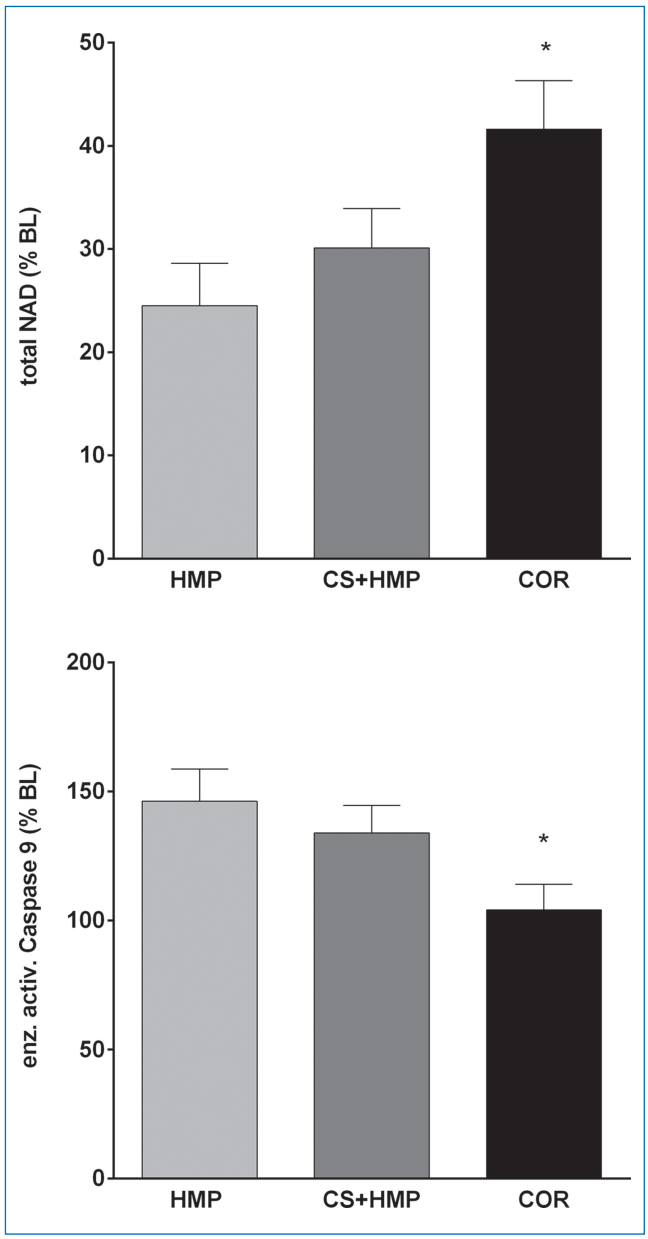


**Figure 3.** Effect of controlled oxygenated rewarming after cold storage (COR) compared to hypothermic machine perfusion (HMP) or 18 hours cold storage + 3 hours of HMP (CS + HMP) on total NAD content and caspase 9 activity upon warm reperfusion of isolated porcine kidneys (*$p < 0.05$ vs. HMP).

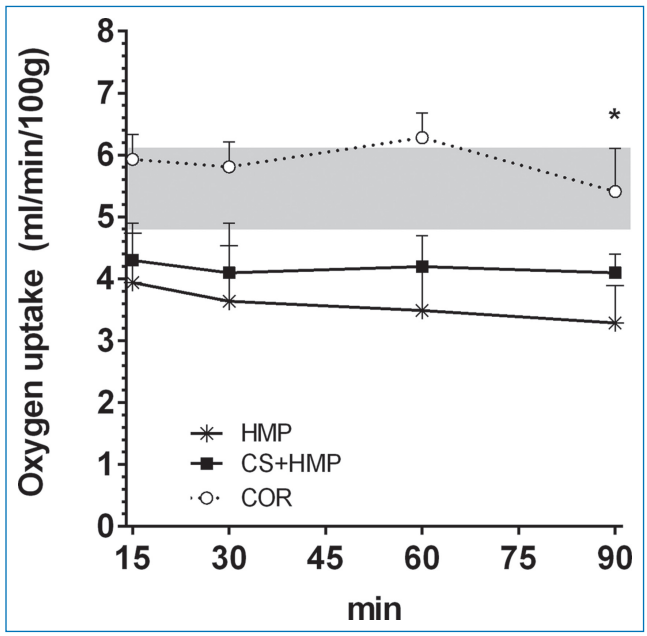


**Figure 4.** Renal oxygen consumption during reperfusion after 18 hours of HMP (HMP), 18 hours cold storage + 3 hours of HMP (CS + HMP) or 18 hours of cold storage + 3 hours of controlled oxygenated rewarming (COR). $N = 7$ per group (*$p < 0.05$ [area under the curve, AUC] vs. HMP). Model specific normal range, obtained from nonischemic organs, is represented by the gray area, for comparison.

of NAD in postischemic tissue[11] as NAD leaks out upon pore opening and is washed out during reperfusion. Total NAD was notably depleted after HMP, but significantly better preserved upon controlled rewarming prior to reperfusion (*Figure 3*). Mitochondrial induction of the apoptotic pathway by activation of caspase 9 was similarly mitigated by the gentle rewarming protocol that nearly kept caspase 9 activity at baseline values.

The positive effect of COR on functional mitochondrial recovery was further supported by the course of renal oxygen consumption during reperfusion, which was significantly improved after controlled rewarming (*Figure 4*).

Structural tubular cell injury was less affected by the preservation protocol. Urinary release of LFABP was rather low in all groups (3.8 ± 0.5; 3.9 ± 0.9, and 2.4 ± 0.7 µg/g Cr—HMP vs. CS + HMP vs. COR, respectively, $p > 0.05$), and least pronounced after COR.

## Discussion

The protective effect of controlled tissue rewarming after cold storage has already been shown in porcine livers after extended preservation times, where end-ischemic machine perfusion with gradual warming up resulted in superior functional recovery than perfusion at either hypo- or subnormothermia prior to warm reperfusion.[12]

Here we addressed the question, if controlled rewarming would also be of use when compared to optimally preserved grafts, like healthy kidneys, maintained by pulsatile hypothermic machine perfusion.

In line with previous reports,[13] short-term cold perfusion after preceding CS was able to supplant, but did not improve over the benefits of continuous HMP.

By contrast, an approximately twofold enhancement of postischemic renal function could be obtained, by controlled graft rewarming in a gentle process of extracorporeal perfusion along with a restitutive period ad subnormothermic temperatures.

It has already been shown in rabbit kidneys that mitochondrial function is impaired by cold storage but respiratory chain dysfunction severely aggravated upon warm reperfusion.[7] Reperfusion injury after cold storage also implicates the onset of mitochondrial membrane permeability transition (MPT),[14] initiating cell apoptosis by activation of caspase 9[15] and marked, for example, by the release and wash out of NAD.[11] Studies form Leducq et al. have shown a dominant association of MPT and defective oxidative phosphorylation with the abrupt shift in temperature.[9] In line with this, renal oxygen consumption

was depressed upon abrupt reperfusion in our model but nearly normal after controlled rewarming.

Moreover, controlled rewarming mitigated functional signs of mitochondrial permeability transition pore opening (Caspase 9 activation) and enabled a better preservation of total NAD.

It is conjectured, that the controlled transition from hypo- to subnormothermia protects cold preserved mitochondria from initial dysfunction and that the brief subnormothermic perfusion period helps to reverse mitochondrial dys-homeostasis prior to the challenge of normothermia. Warming up to higher temperatures was not attempted, as preservation solutions would not seem an appropriate media for normothermic physiology. Moreover, rewarming injury is not seen at temperatures above approximately 16°C.[16]

In conclusion, controlled rewarming seems to be a promising new aspect in renal preservation that could help to minimize graft dysfunction even in optimally preserved kidneys.

## Acknowledgments

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## References

**1.** Perico N, Cattaneo D, Sayegh MH, Remuzzi G. Delayed graft function in kidney transplantation. *Lancet*. 2004; 364(9447): 1814–1827.

**2.** Siedlecki A, Irish W, Brennan DC. Delayed graft function in the kidney transplant. *Am J Transpl.* 2011; 11(11): 2279–2296.

**3.** Mangino JE, Lindell S. Hypothermic machine perfusion of kidneys. In: Uygun K, Lee CY, editors. Organ preservation and reengeneering. Norwood, MA: Artech House. 2011; p. 35–57.

**4.** Hoffmann T, Minor T. New strategies and concepts in organ preservation. *Eur Surg Res*. 2015; 54(3–4): 114–126.

**5.** Moers C, Smits JM, Maathuis MH, Treckmann J, van Gelder F, Napieralski BP, van Kasterop-Kutz M, van der Heide JJ, Squifflet JP, van Heurn E, et al. Machine perfusion or cold storage in deceased-donor kidney transplantation. *N Engl J Med*. 2009; 360(1): 7–19.

**6.** Moers C, Pirenne J, Paul A, Ploeg RJ. Machine perfusion or cold storage in deceased-donor kidney transplantation. *N Engl J Med*. 2012; 366(8): 770–771.

**7.** Sammut IA, Burton K, Balogun E, Sarathchandra P, Brooks KJ, Bates TE, Green CJ. Time-dependent impairment of mitochondrial function after storage and transplantation of rabbit kidneys. *Transplantation.* 2000; 69(7): 1265–1275.

**8.** Salahudeen AK. Cold ischemic injury of transplanted kidneys: new insights from experimental studies. *Am J Physiol.* 2004; 287(2): F181–F187.

**9.** Leducq N, Delmas-Beauvieux MC, Bourdel-Marchasson I, Dufour S, Gallis JL, Canioni P, Diolez P. Mitochondrial permeability transition during hypothermic to normothermic reperfusion in rat liver demonstrated by the protective effect of cyclosporin A. *Biochem J.* 1998; 336(2): 501–506.

**10.** Minor T, Efferz P, Luer B. Hypothermic reconditioning by gaseous oxygen persufflation after cold storage of porcine kidneys. *Cryobiology.* 2012; 65: 41–44.

**11.** Di LF, Menabo R, Canton M, Barile M, Bernardi P. Opening of the mitochondrial permeability transition pore causes depletion of mitochondrial and cytosolic NAD+ and is a causative event in the death of myocytes in postischemic reperfusion of the heart. *J Biol Chem.* 2001; 276(4): 2571–2575.

**12.** Minor T, Efferz P, Fox M, Wohlschlaeger J, Lüer B. Controlled oxygenated rewarming of cold stored liver grafts by thermally graduated machine perfusion prior to reperfusion. *Am J Transpl.* 2013; 13: 1450–1460.

**13.** Gallinat A, Paul A, Efferz P, Luer B, Kaiser G, Wohlschlaeger J, Treckmann J, Minor T. Hypothermic reconditioning of porcine kidney grafts by short-term preimplantation machine perfusion. *Transplantation.* 2012; 93: 787–793.

**14.** Lemasters JJ. V. Necrapoptosis and the mitochondrial permeability transition: shared pathways to necrosis and apoptosis. *Am J Physiol.* 1999; 276(1 Pt 1): G1–G6.

**15.** Morariu AM, Maathuis MH, Asgeirsdottir SA, Leuvenink HG, Boonstra PW, van Oeveren W, Ploeg RJ, Molema I, Rakhorst G. Acute isovolemic hemodilution triggers proinflammatory and pro-coagulatory endothelial activation in vital organs: role of erythrocyte aggregation. *Microcirculation.* 2006; 13(5): 397–409.

**16.** Rauen U, Kerkweg U, de Groot H. Iron-dependent vs. iron-independent cold-induced injury to cultured rat hepatocytes: a comparative study in physiological media and organ preservation solutions. *Cryobiology.* 2007; 54(1): 77–86.