ENRICHMENT OF PRESERVATION SOLUTION WITH METHANE – POSSIBLE BENEFITS FOR ORGAN TRANSPLANTATION

PhD Thesis

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List of original papers

List of full papers relating to the subject of the thesis

I. **SZILÁGYI ÁL**, MÁTRAI P, HEGYI P, TUBOLY E, PÉCZ D, GARAMI A, SOLYMÁR M, PÉTERVÁRI E, BALASKÓ M, VERES G, CZOPF L, WOBBE B, SZABÓ D, WAGNER J, HARTMANN P (2018) Compared efficacy of preservation solutions on the outcome of liver transplantation: Meta-analysis. World J Gastroenterol. 24(16):1812-1824. doi: 10.3748/wjg.v24.i16.1812. Review. **IF: 3.411**

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VIDA N, MÁRTON A, JÁSZ DK, **SZILÁGYI ÁL**, DIÓSZEGI P, SZABÓ M, CSONKA CS, SZABÓ A, MOHÁCSI Á, BOROS M, HARTMANN P, TUBOLY E (2018) Metánnal dúsított perfúziós folyadék protektív hatásainak feltérképezése ex vivo patkány szív iszkémia-reperfúziós modellben.
http://erbetegsegek.com/image/archivum/2018_3_absztrakt.pdf

SZILÁGYI ÁL, TUBOLY E, MÁRTON A, JÁSZ DK, DIÓSZEGI P, SZABÓ M, CSONKA CS, SZABÓ A, MOHÁCSI Á, BOROS M, HARTMANN P (2018) Metánnal dúsított perfúziós folyadék hatása szív iszkémia-reperfúzió során kialakuló mitokondriális diszfunkcióra ex vivo patkány modellben.
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List of full papers not relating to the subject of the thesis

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CONTENTS

List of abbreviations

Ama	antimycin A
ATP	adenosine triphosphate
CCCP	carbonyl cyanide m-chlorophenyl hydrazine
CH_4	methane
CI	confidence intervals
CIT	cold ischemia time
COR	controlled oxygenated rewarming
CS	Celsior solution
DAPI	4’,6’-diamidino-2-phenylindole staining
DDLs	deceased donor livers
ECD	extended criteria donor
ETC	electron transport chain
ETS	electron transport system
G	glutamate
H_2O	distilled water
H_2O_2	hydrogen peroxide
HES	hydroxyethyl starch
HMP	hypothermic machine perfusion
HTK	histidine-tryptophane-ketoglutarate solution
HRR	high-resolution respirometry
I/R	ischemia-reperfusion
IGL-1	Institut Georges Lopez solution
M	malate
MiRo5	mitochondrial respiratory medium
MELD	model of end-stage liver disease
MP	machine perfusion
NMP	normothermic machine perfusion
O_2	oxygen
O2k	oxygraph-2k
Omy	oligomycin
OGS-1	one-year post-transplant graft survival
OxPhos	oxidative phosphorylation

PDF	primary dysfunction
PEG-35	polyethylene glycol
PNF	primary non-function
POD	post-transplant death within 30 days
RCT	randomized control trial
ROS	reactive oxygen species
Rot	rotenone
RR	risk ratio
RT	retransplantation rate
S	succinate
SCS	static cold storage
SNMP	subnormothermic machine perfusion
SUIT	substrate-uncoupler-inhibitor titration
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labelling
UW	University of Wisconsin solution

1. INTRODUCTION

1.1. Ischemia-reperfusion (I/R) injury of transplanted grafts

Organ transplantation is the only available treatment for end-stage organ failure. In recent decades, it has become a routine intervention performed in steadily increasing numbers worldwide. Nevertheless, the technique is still accompanied by many hazards [1]. The transplanted organ (graft) undergoes multiple warm and cold ischemic periods upon removal from the donor and storage, which is further aggravated by reperfusion injury as circulation recovers [2]. During cold ischemia, the input of oxygen and nutrients is ceased, and the anaerobic metabolism leads to the exhaustion of energy stores, all of which together result in the development of an acidic environment. The increased proton concentration stimulates the type 1 Na^+/H^+ antiporter; additionally, due to the decreased ATP level, the function of the Na^+/K^+ pump is also disrupted. These two processes result in intracellular Na^+ accumulation with a decelerated Na^+/Ca^{2+} exchange system. After a while, the Na^+/Ca^{2+} exchange turns around, thereby leading to the release of a large amount of Ca^{2+} in the cell [3]. Part of the Ca^{2+} is taken up by the mitochondria, and high intracellular Ca^{2+} concentration activates phospholipases, calmodulin-regulated proteases and endonucleases, which play a role in apoptotic processes in the cell. During the reperfusion phase, the implanted graft suffers from biochemical and metabolic alterations, including generation of reactive oxygen species (ROS), endothelial dysfunction, DNA damage and local inflammatory responses [4]. Inflammatory cascades and oxidative stress can later induce a cytokine storm, which can result in cell death due to damage to cellular structures [5].

1.2. Preservation techniques

A number of methods have been developed to reduce ischemia-related morbidity of grafts during storage and transportation and to maintain the viability of tissues [2, 6].

1.2.1. Static and dynamic perfusions

There are currently two approaches to preserving transplantable organs: static or dynamic. Simple static cold storage (SCS) is the main method for static storage, while hypothermic machine perfusion (HMP) and other perfusion-based methods, such as normothermic machine perfusion (NMP) and oxygen persufflation, comprise the methods for dynamic

preservation.

Since the 1960s, SCS has gradually become the gold standard method for organ preservation. SCS involves flushing the procured organ with preservation solution at 0–4°C, then immersing it into preservation solution at the same temperature until transplantation. The hypothermic environment is responsible for decreasing cellular metabolism, and the preservation solution reduces cellular metabolism and provides cytoprotection. I/R-induced injury increases the risk of early graft dysfunction and reduces long-term survival after transplantation. Meanwhile, the shortage of donor organs has led to the use of extended criteria donor (ECD) organs. Proper donor organ functional assessment and *ex vivo* repair/reconditioning of organs prior to transplantation have become necessary.

In addition to static techniques, attempts have been made to perform dynamic techniques in parallel. The most common dynamic technique is machine perfusion (MP). MP is a method involving organ perfusion with a controlled flow of perfusate. It facilitates the maintenance of organ microvasculature tone, provision of oxygen and nutrients in support of tissue metabolism, and removal of toxic metabolic waste.

1.2.2. Perfusions at different temperatures

Originally, organs were perfused at room temperature. In 1876, Bunge and Schmiedeberg added a water bath to the circuit to maintain perfusion of blood at physiological temperatures. Later, scientists began to speculate that the use of lower temperatures might attenuate organ damage during perfusion by abating cellular metabolism. For example, SCS at 0–4°C reduces the metabolic rate of the organ by approximately 5% of its physiological level. Different temperatures have been investigated for *ex vivo* machine perfusion, including NMP at 35–38°C, subnormothermic machine perfusion (SNMP) at 20–34°C, controlled oxygenated rewarming (COR) at 8–20°C and HMP at 0–8°C [7–9].

1.3. Preservation solutions

According to their composition, there are two main types of preservative solutions: intracellular and extracellular [8]. The University of Wisconsin (UW) and Institut Georges Lopez (IGL-1) solutions are among the intracellular preservation solutions with high K^+ and low Na^+ concentration. The histidine-tryptophan-ketoglutarate (HTK) and Celsior (CS) solutions are extracellular preservation solutions (Table 1).

Among intracellular solutions, UW has high viscosity and high K^+ content, and it is generally considered as the gold standard for flushing and cold storage of liver allografts

[10]. Despite its clinical success, the UW solution has potential drawbacks: high viscosity only allows a slow flush rate and the role of high Na^+ content has been suspected in endothelial and bile duct damage [11, 12]. Moreover, it is complicated to store at low temperature due to leakage of some particles from the containing bag and possible formation of adenosine crystals [13]. IGL-1 falls within the category of intracellular solutions; it combines a cationic inversion (lower concentration of Na^+) and replacement of hydroxyethyl starch with PEG. These properties of the IGL-1 could improve hepatic microcirculatory changes, thereby decreasing I/R injury [14].

Recently, HTK has been the most important transplantation solution in Europe, which is used for every type of organ transplantation. This extracellular-type solution was originally developed as a cardioplegic solution [15]. It has very low viscosity based on a buffer system (histidine) with two additional substrates (tryptophan and ketoglutarate) [15]. A lower viscosity index allows faster cooling and theoretically improves washout of blood cells from the graft.

Another extracellular solution, CS was initially used in heart transplantation and has since been proposed for kidney and liver transplantation for preservation of all organs with one solution. To prevent oxidative injury from ROS, CS contains reduced glutathione with mannitol and histidine. Like HTK, CS lacks colloids, resulting in a dramatic decrease in their viscosity and related improvement of their perfusability, thus not requiring liver flushing prior to reperfusion. Given its low viscosity, high Na^+ and low K^+ levels, and antioxidant properties, CS might be particularly suitable for preserving the liver. However, the preservation solutions listed here are not perfect; they too have some limitations.

The use of UW was associated with potential endothelial and bile duct damage and its storage is complicated at low temperatures. HTK was accompanied with an increased risk of graft loss and early graft loss. The most important limitation of CS is the absence of colloid components [16]. In the special condition of using a partially deceased donor liver graft, IGL-1 offered the best graft outcome. In another study, it was suggested that IGL-1 was superior to other solutions at preserving fatty livers by protecting against primary non-function (PNF) and early allograft dysfunction [17]. However, a prospective randomized study did not show any significant improvement in the subgroup of patients receiving IGL-1 preserved grafts [18].

	UW	HTK	CS	IGL-1
HES	0.25	-	-	-
PEG-35	-	-	-	0.03
Na^+	27	15	100	120
K^+	125	10	15	25

Table 1. Ingredients in preservation solutions. Concentrations are expressed in mmol/l. HES: hydroxyethyl starch; PEG-35: polyethylene glycol.

1.4. Chemical properties and bioactivity of methane (CH_4)

CH_4 is the smallest hydrocarbon, a colourless, odourless, omnipresent gaseous molecule and an inert gas at ambient temperature and pressure. CH_4 is well-known for its reaction with hydroxyl radicals in the mechanism of ozone formation; however, the physico-chemical reactions of CH_4 in the animal or human organism are not fully mapped. The first discovery regarding the *in vivo* mechanism of action of CH_4 was the transformation of intra arterially administered, $_{14}C$-labelled CH_4 into $[_{14}C]CO_2$ in sheep [19]. Further studies proved that $[_{14}C]CO_2$ transforms into bound $_3H$ and carbon in mammalian liver tissue [20].

Several studies have explored the biological role of endogenous CH_4 in eukaryotic organisms. In this respect, several studies have provided proof that fungi, algae, plants and even animals can produce CH_4 as a result of aerobic metabolic processes [21-23]. The effects of exogenous CH_4 have also been demonstrated by many research groups [24, 25], in I/R settings as well [26]. The findings have been reinforced by *in vivo* studies describing the biological effects of CH_4 on intestinal peristalsis and motility parameters [27–29] and improved survival rate in haemorrhagic shock in rats [30]. Several data have demonstrated that exogenous CH_4-enriched fluids protect against the consequences of I/R-induced pathologies. Our group was the first to describe that the inhalation of normoxic air containing 2.5% CH_4 has an anti-inflammatory effect in intestinal and liver I/R injury models in large and small animals [26]. These findings were supported by numerous experimental data demonstrating the anti-oxidative, anti-apoptotic and anti-inflammatory effects of CH_4 inhalation or CH_4-enriched fluid therapies in I/R, endotoxemia and sepsis [26, 29].

Due to the well-known characteristics of CH_4 (5–15% of CH_4 in gas mixture can be flammable and potentially explosive), dissolving it in fluids would increase the safety of its application. Indeed, several forms of CH_4 treatment have been investigated, in which CH_4

gas was perfused in physiological saline solutions [27, 29, 31-35]. Nevertheless, the exact circumstances of administration of CH_4 dissolved in different transplantation solutions have not yet been explored.

1.5. The effect of CH_4 on mitochondria in I/R injury

CH_4 has favourable distribution properties *in vivo* thanks to its characteristics of penetrating membranes and diffusing into mitochondria; therefore, the potential effect of CH_4 on mitochondrial respiration was also raised. Mitochondria are special intracellular structures that play various physiological roles, such as energy production, formation of ROS, Ca^{2+} homeostasis and apoptosis. Several studies have demonstrated that these physiological functions may be targets of exogenously administered CH_4 via non-specific physico-chemical alterations of phospholipid membranes in I/R injury [31, 32]. The modulatory effects of CH_4 on mitochondrial respiration have been demonstrated in animal models of I/R injury both *in vivo* and in hypoxic assays *in vitro* [31, 35, 36].

Under the impact of I/R injury, the addition of CH_4 restored the electron transport system (ETS) of the inner mitochondrial membrane, by changing its oxidative stress-related rigidity when oxygen concentration increased [26, 31]. In parallel, CH_4 seemingly exerts site-specific action on protein complexes. It seems that, among the protein complexes of the mitochondrial ETS, complex IV (cytochrome c oxidase) is a target of the CH_4 action [31].

Several studies have demonstrated that CH_4 modulates the intrinsic pathway of apoptosis also called the mitochondrial pathway [29–31, 34-36]. This effect of CH_4 was associated with the release of cytochrome c, and other inner mitochondrial membrane proteins are regulated by Bcl-2 family proteins through interplay between pro-apoptotic and anti-apoptotic proteins [31, 32, 37].

1.6. The role of mitochondria in the process of apoptosis

Mitochondrial energy production is a substantial factor in the life of eukaryotic cells. However, mitochondria not only manage the metabolism of the cell, but also cause its death. As a response to noxious stimuli, mitochondrial proteins can emerge in the cytoplasm to play an essential role in the apoptotic process [38–40]. These proteins include e.g. procaspases, apoptosis-inducing factor (AIF), adenylate kinase 2 and even proteins of the respiratory chain, such as cytochrome c, Smac/Diablo (a recently discovered caspase coactivator) and some heat shock proteins (Hsp10 and Hsp60). Mitochondrial protein release into the cytoplasm may be regulated, stimulated or inhibited by mitochondrial

proteins of the Bcl-2 family. They can be distributed into two classes: anti-apoptotic Bcl-2 family proteins (such as Bcl-XL, Bcl-w, Mcl-1, A1, Bcl-Rambo, Bcl-L10 and Bcl-G) and pro-apoptotic proteins (such as Bcl-2-associated X protein (Bax), Bak and Bok). Primarily, Bcl-2 blocks the release of cytochrome c to develop its anti-apoptotic characteristics. In contrast, stress-induced activation of pro-apoptotic members of the Bcl-2 family (Bak or Bax) leads to permeabilization of the mitochondrial outer membrane and subsequent release of intermembrane space proteins, such as cytochrome c. Cytochrome c is loosely associated with the outer side of the inner mitochondrial membrane and carries electrons between complex III and IV. Membrane damage provokes its release into the cytosol, where it initiates caspase cascade-mediated apoptosome formation, thus leading to apoptosis [32].

2. MAIN GOALS

The main goal of our studies was to develop a method that increases the efficacy of the currently available transplant solutions.

As a first step, we aimed to compare the efficacies of the solutions commonly used in solid organ transplantation. For this purpose, we performed a meta-analysis comparing the efficacy of the four most widely used transplant solutions (HTK, UW, Celsior and IGL-1) (Study 1).

Next, we aimed to design a transplantation solution with improved biological effects during cold storage of grafts over a longer period of time. Therefore, in Study 2 we constructed a system to optimize the efficacy of HTK storage with CH_4 enrichments and to investigate the temperature and pressure dependency of the solubility and stability of CH_4-containing solutions.

Finally, we intended to test the effect of dissolved CH_4 in a relevant *in vitro* model of transplantation-induced I/R damage. Thus, in Study 3 myocardial cell cultures were subjected to simulated I/R insults. In this set-up, we examined the effects of dissolved CH_4 on transient anoxia-induced mitochondrial dysfunction and cardiomyocyte apoptosis.

3. MATERIALS AND METHODS

3.1. Study 1. Compared efficacy of preservation solutions on the outcome of liver transplantation. Meta-analysis

We performed a meta-analysis in accordance with the PRISMA (Preferred Reporting Items in Systematic Reviews and Meta-Analysis) statement [41]. The review protocol was registered with the National Institute for Health Research PROSPERO system on 12 January 2017 and can be found online (Registration No. CRD42017054908) [42].

3.1.1. Literature search

A systematic literature search was performed using EMBASE/MEDLINE, PubMed, Scopus and Cochrane Library. Database searches were conducted with MeSH keywords, combined with various terms for organ transplantation and organ preservation solutions (Figure 1). No language limitation was applied. The end date for the literature search was 31 January 2017.

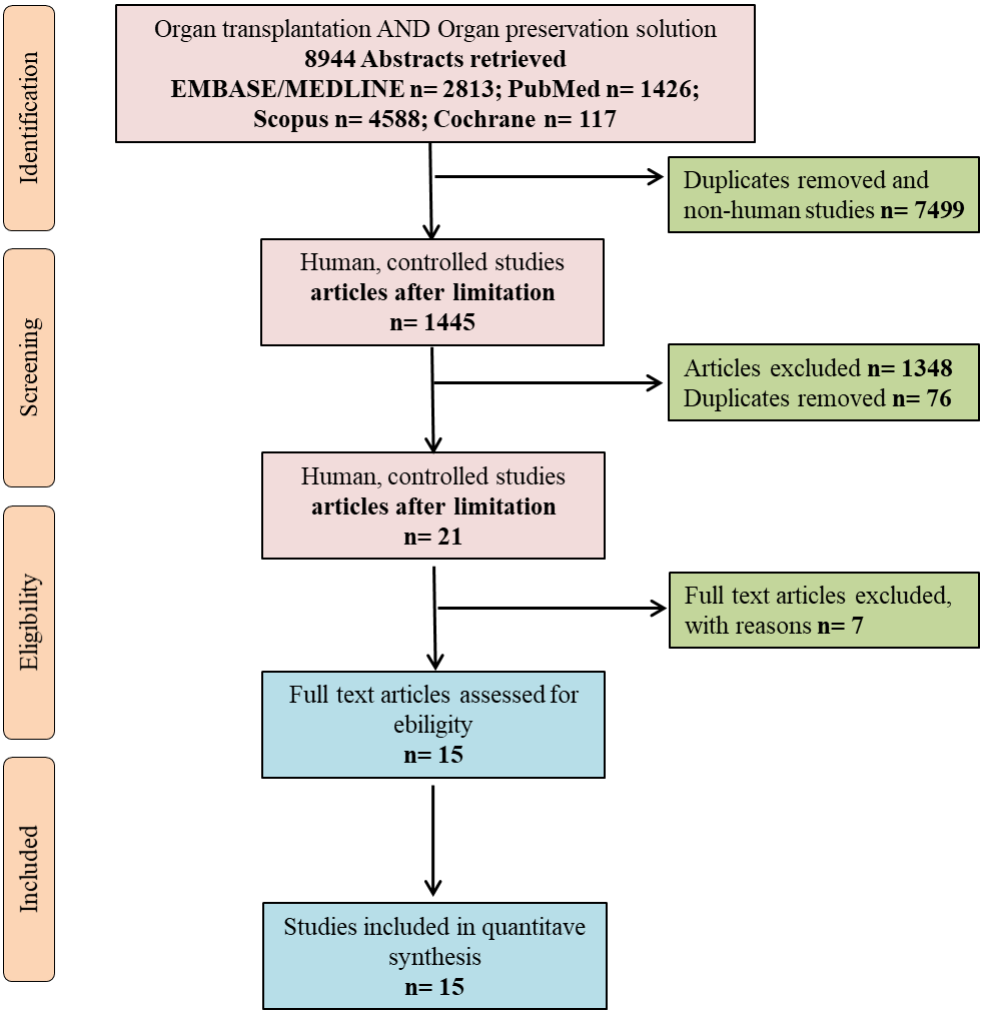

Figure 1. PRISMA flowchart

3.1.2. Inclusion criteria

Inclusion criteria specified any randomized control trial (RCT) comparing two or more preservation solutions for the SCS of deceased donor livers (DDLs), from both adult and paediatric donors. Living donor transplantation, multiple organ transplantation, retransplantation (RT), non-human and uncontrolled studies were excluded. Abstracts for inclusion were independently reviewed by two authors (Ágnes Lilla Szilágyi (ÁLSz) and Daniella Pécz (DP)), and disagreements were resolved by discussion with a third author (Petra Hartman (PH)) (Figure 1).

3.1.3. Outcome

The primary outcome was PNF of the liver grafts. PNF is a life-threatening condition after transplantation that leads to death or to the need for retransplantation within seven days of transplantation. It is characterized by hepatic cytolysis, elevated fasting transaminase levels, diminishing or absent bile production, coagulation deficit related to severely impaired liver function, high lactate levels, hypoglycaemia, respiratory failure requiring ventilation, circulatory failure requiring catecholamines, and the onset of renal and multi-organ failure [43].

The secondary outcome was the one-year post-transplant graft survival (OGS-1), since the one-year post-transplant time point was considered by an expert consensus opinion as the most suitable to evaluate the effect of the preservation solutions [44].

Other outcomes, such as model of end-stage liver disease (MELD) score, primary dysfunction (PDF), early RT rate, post-transplant death within 30 days (POD), cold ischemia time (CIT) and one-year post-transplant patient survival (OGS-1), were also evaluated. The MELD score incorporates parameters of recipients (such as abnormal coagulation, creatinine and serum bilirubin levels and the aetiology of cirrhosis) and serves as a predictive factor of mortality after liver transplantation [45]. PDF is a syndrome of acute graft injury that occurs within the first 72 hours after transplantation. Early RT takes place within 30 days after primary transplantation, and it has traditionally been associated with poor outcome in terms of morbidity and survival. CIT was defined as the time between clamping the donor aorta and connecting the anastomosis of the organ to the recipient's vascular system or organ disposal.

3.1.4. Data extraction

Demographic, quality and outcome data were extracted independently into Microsoft Excel

2016 by two authors (ÁLSz and DP) Data were collected from all articles describing the studies; in the case of discrepancies, the article with the largest number of patients was used. Any questions regarding data extraction were settled by discussion with a third author (PH).

3.1.5. Statistical analysis

The statistical analysis for this study was conducted by Péter Mátrai, Institute of Bioanalysis, University of Pécs, Pécs, Hungary. Risk ratios (RR) from individual studies were pooled statistically with the random effect model using the DerSimonian–Laird estimator and were displayed on forest plots. As RR allows for a comparison of two samples, the CS and HTK solutions were compared to UW. Summary RRs were calculated with 95% confidence intervals (CI) and *p* values to test if summary RR=1 can be rejected. $P<0.05$ was defined as a significant difference between solutions. In the analysis of outcome of PNF and PDF, we used a computational correction recommended in the Cochrane Handbook and proposed by Sweeting et al. to overcome the difficulty of dividing by 0 [46]. Statistical heterogeneity was tested using the I^2 statistic and the chi-square test to obtain probability values; $p<0.05$ was defined to indicate significant heterogeneity. All statistical calculations were performed using Stata 11 SE (Stata Corp) and Comprehensive Meta-analysis Software (Version 3, Biostat, Englewood). We sought signs of a small study effect with the funnel plot. To identify potential sources of heterogeneity, we defined *a priori* subgroup analysis with the MELD score and CIT. All other outcome related to the solutions were investigated by subgroup analysis.

3.2. Study 2. Investigation of the solubility of CH_4 gas in transplantation solution

After comparing the efficacy of different transplantation solutions, we examined the solubility and half-life of CH_4 in the HTK, the most commonly used transplantation solution in Europe.

3.2.1. Photoacoustic spectroscopy (PAS) measurement of CH_4 concentration

The PAS technique for CH_4 detection was validated by our cooperative working group [44]. PAS is a special mode of spectroscopy, which measures optical absorption indirectly via the conversion of absorbed light energy into acoustic waves. The amplitude of the generated sound is directly proportional to the concentration of the absorbing gas component. A standard photoacoustic gas detector consists of the following main parts: an excitation

source, a photoacoustic cell, a gas-handling system, and controlling and data-processing units. The gas sample is passed through the photoacoustic cell, in which signal generation takes place, and the photoacoustic signal produced is detected by a microphone. The signal of the microphone is amplified and the photoacoustic signal is measured using a digital lock-in technique. The complete photoacoustic measuring system (excluding the sampling chamber) is built into an easily portable, 19”4U instrument box (Figure 2). The set-up allows for online measurements of CH_4 concentration with a minimum detectable concentration of 0.25 ppm.

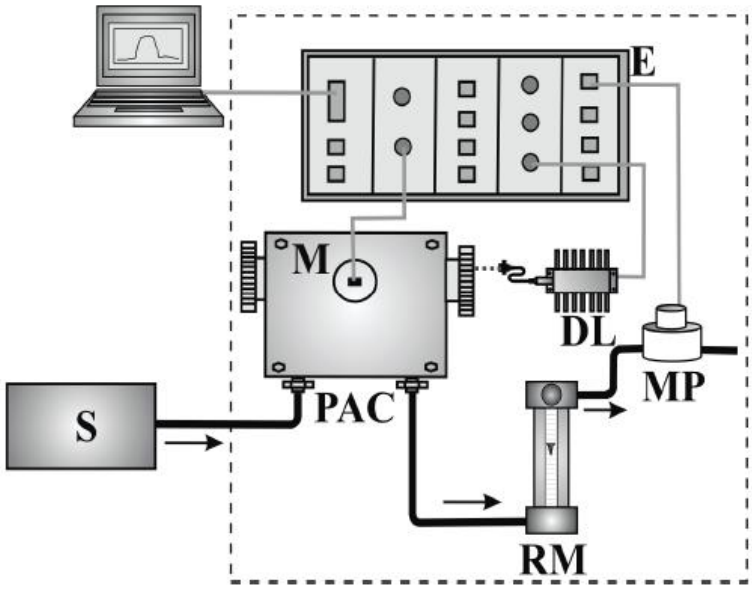

Figure 2. Schematic figure of the device and the photoacoustic detection set-up. Gas samples are drawn by a membrane pump into the photoacoustic cell, where the signal is generated by a diode laser and detected by a microphone. The gas flow rate can be adjusted with a rotameter. The electric unit provides system control and data processing, and a computer presents data graphically. **S:** gas sample; **MP:** membrane pump; **PAC:** photoacoustic spectroscopy; **DL:** diode laser; **M:** microphone; **RM:** rotameter; **E:** electric unit. Arrows indicate the direction of the gas flow. The dashed line encloses the components built into a 19”4U instrument box [44].

3.2.2. Experimental set-up

In order to determine the solubility of CH_4, the measurements were made in a cuvette with a volume of 20 cm^3 (10 cm^3 gas space, 10 cm^3 solution). The input aperture was connected to the CH_4-containing gas bottle, and the output aperture was connected to the CH_4 detection system (Figure 3). Persufflation of the solution was made with a 2.2% CH_4–artificial air

mixture at a flow rate of 200 ml/min.

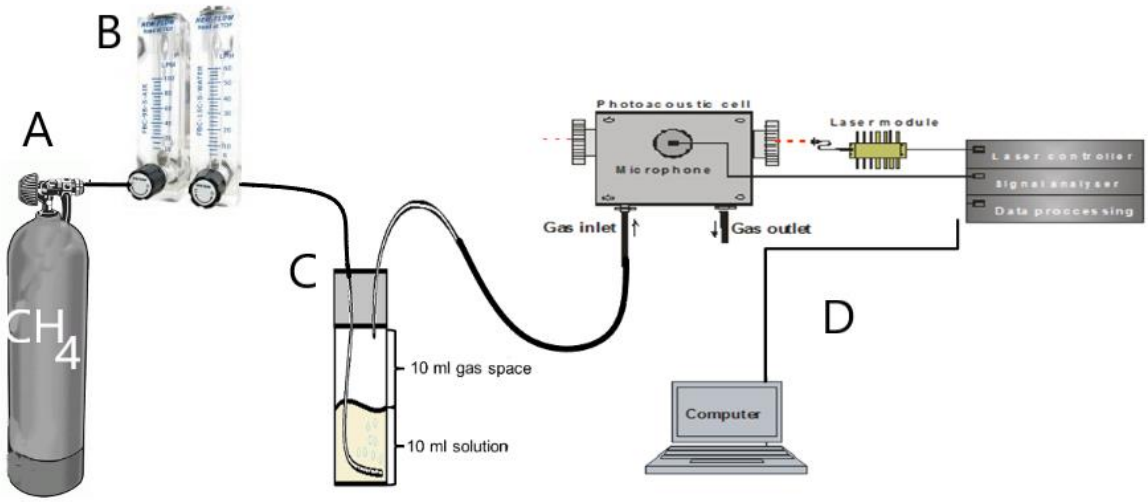

Figure 3. The experimental set-up. The CH_4 concentration measurements of different solutions were made in a cuvette with a volume of 20 ml (10 ml gas space, 10 ml solution). The input aperture was connected to the CH_4 gas bottle through a rotameter and the output aperture connected to the PAS device. The solution was perfused with a 2.2% CH_4–artificial air mixture at a flow rate of 200 ml/min. **A.** Gas bottle with 2.2% CH_4–artificial air mixture, **B.** Rotameter, **C.** Cuvette with a volume of 20 cm^3, **D.** PAS

3.2.3. Experimental protocols

We have employed two experimental protocols to determine the solubility of CH_4 and its stability in a fluid phase. We investigated the CH_4 solubility in distilled water (H_2O), in physiological saline (NaCl) and in HTK solution with different flow times (10 and 60 min) (Figure 4A). In the second step, the CH_4 solubility and stability were determined in NaCl and HTK solutions at 4 and 21°C (Figure 4B). The CH_4 content of the samples was determined after 10 min, 1 h, 3 h, 6 h and 24 h incubation in two ways. First, the CH_4 concentration measured in the gas space above the liquid is proportional to the CH_4 content dissolved in the liquid. Second, the total dissolved CH_4 content of a gas bubbled through a liquid can be calculated with the measured concentration and the volume flow. The dynamics of CH_4 concentration changes were detected by PAS, as described previously.

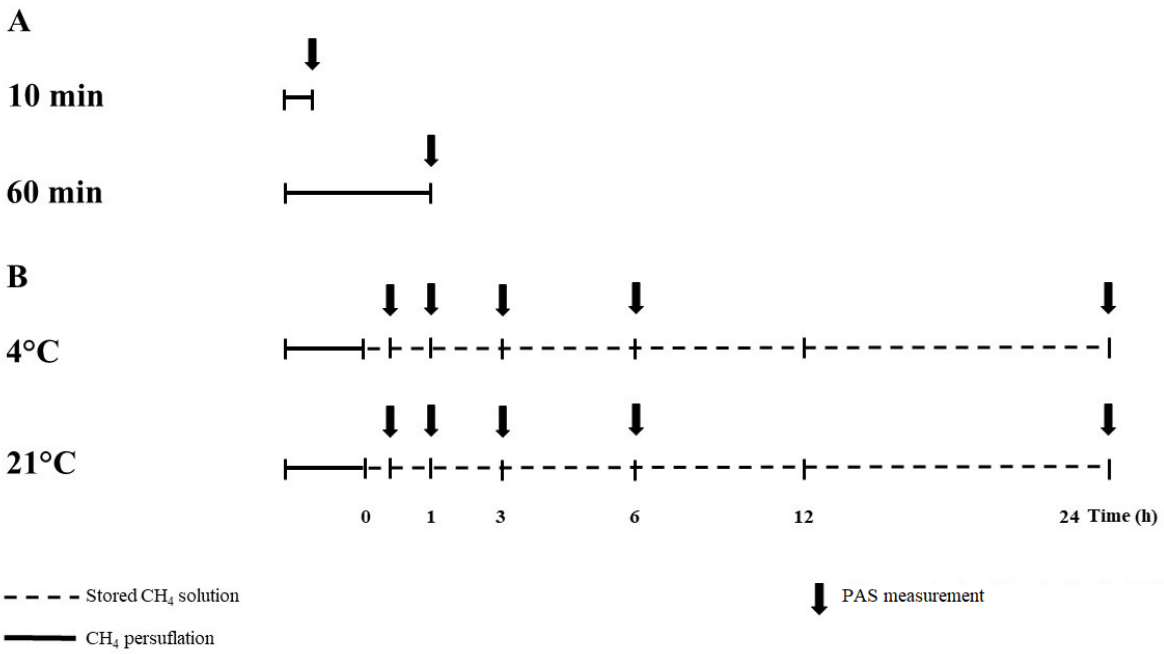

Figure 4. Protocols for CH_4 concentration measurements. A. CH_4 concentration measurements in three different solutions after 10 or 60 min persufflation. **B.** CH_4 concentration measurements in two different solutions at 4°C and 21°C. The CH_4 content of the samples was determined after 10 min, 1 h, 3 h, 6 h and 24 h incubation.

3.3. Study 3. Myocardial IR-induced mitochondrial damage, the *in vitro* effects of CH_4

After determining the solubility of CH_4 in different solutions, we examined the effects of dissolved CH_4 on neonatal rat myocardial cell under simulated I/R (sI/R). The experimental protocol was in accordance with EU Directive 2010/63 for the protection of animals used for scientific purposes, and it was approved by the National Scientific Ethics Committee on Animal Experimentation (National Competent Authority) under licence number V./148/2013. This study also complied with the criteria of the US National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

3.3.1. Cardiomyocyte cell culture

Neonatal rat cardiac myocytes (NRMCs) were isolated, as described previously [45]. Briefly, 1–3-day-old Wistar rats were sacrificed by cervical dislocation, and the hearts were excised and collected in ice-cold phosphate-buffered saline. After the atria were removed, the ventricles were minced with scissors and digested with 0.25% trypsin for 25 min. The cell suspension was centrifuged at 2000 rpm for 15 min at 4ºC. Pelleted cells were pre-

plated for 90 min at 37ºC to separate the cardiac myocyte-enriched fraction. Cardiac myocytes were collected and counted in a Burker chamber and plated into 24-well plates (7×104 cells/well) and into 75 cm^2 flasks (4×106 cells/flask). Cells were harvested in Dulbecco's Modified Eagle's growth medium (DMEM) supplemented with a 10% foetal bovine serum (FBS), 1% glutamine and 1% antibiotic/antimycotic solution for 24 h, and then the medium was changed to 1% FBS-containing growth medium to promote the differentiation of the cardiomyocytes. At the end of the three-day isolation protocol, the phenotype of NRMCs corresponds to that of cardiomyocytes isolated from adult rats. The cardiac myocytes were kept in a normoxic incubator to maintain physiological conditions (37ºC, 5% CO_2 and 95% air).

3.3.2. Experimental protocols

The experiments were performed using intact NRMCs. In this series, three-day-old cardiac myocytes were subjected to 4 h simulated ischemia (sI). The cells were kept in a hypoxic chamber (37ºC, 95% N_2 and 5% CO_2), and the culture medium was changed to a hypoxic solution (in mM: NaCl 119, KCl 5.4, $MgSO_4$ 1.3, NaH_2PO_4 1.2, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 5, $MgCl_2$ 0.5, $CaCl_2$ 0.9, Na lactate 20; bovine serum albumin (BSA) 0.1%, 310 mOsm/l, pH=6.4). This was followed by a 2 h reoxygenation period (R) in a culture medium when cells were kept under either normoxic conditions (37ºC, artificial air) or in a chamber with normoxic air supplemented with CH_4 (37ºC, 2.2% CH_4+artificial air) (the sI/R and sI/R+CH_4 groups, respectively) (n=8–16). Control groups were kept in a normoxic incubator to maintain physiological conditions for 4 h (normoxic solution containing in mM: 125 NaCl, 5.4 KCl, 1.3 $MgSO_4$, 1.2 NaH_2PO_4, 20 HEPES, 0.5 $MgCl_2$, 1 $CaCl_2$, 15 glucose, 5 taurine, 2.5 creatine-monohydrate and 0.1% BSA at pH 7.4), which was followed by a 2 h reoxygenation period in the normoxic incubator with or without CH_4 supplementation (the normoxia and normoxia+CH_4 groups). 4–5 isolation rounds were performed for each experimental series resulting in 2–4 flasks of NRMCs (4×10^6 cells/flask) for each isolation. Data for all individual wells were analysed. At the end of the isolation protocol, the NRMCs were subjected to HRR and cell viability assays (Figure 5). The CH_4 concentration was measured by PAS technique (described in 3.2.1. Photoacoustic spectroscopy (PAS) measurement of CH_4 concentration). The CH_4 concentration in the medium was measured over a period of 120 min, and samples were taken every 2 min.

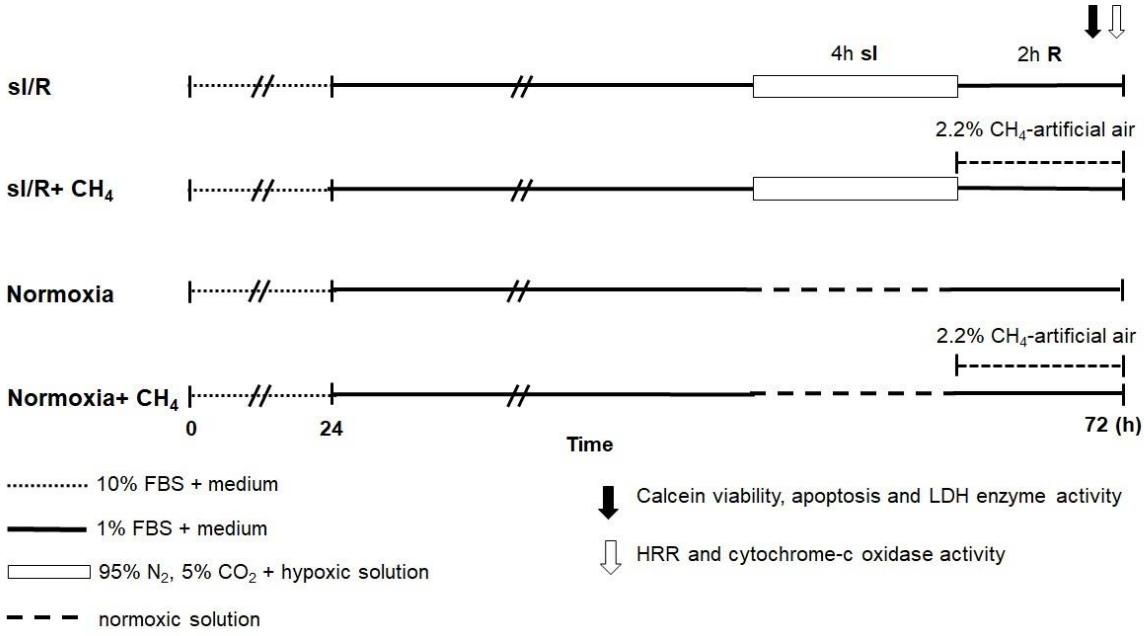

Figure 5. Protocol for the NRMCs. The neonatal rat cardiomyocytes (NRMCs) were incubated in growth medium supplemented with 10% FBS for 24 h, and then the medium was changed to a 1% FBS-containing medium, which promoted cell differentiation. The NRMCs were kept under physiological conditions in a normoxic incubator (37ºC, 5% CO_2 and 95% air). The three-day-old NRMCs were treated as follows: 4 h normoxia (normoxic solution and normoxic incubator), followed by reoxygenation (medium with or without CH_4) for 2 h. Ischemia (hypoxic solution + hypoxic chamber) was simulated for 4 h, followed by reoxygenation (medium with or without CH_4) for 2 h. At the end of the experiments, cell viability and mitochondrial function were tested. NRMCs: neonatal rat cardiomyocytes; CH_4: methane; FBS: foetal bovine serum; CO_2: carbon dioxide; N_2: nitrogen.

3.3.3. Examination of mitochondrial functions

HRR by Oxygraph-2k (Oroboros Instruments, Innsbruck, Austria) was used to examine the oxygen consumption of the NRMCs and isolated cardiac mitochondria in various mitochondrial metabolic states.

3.3.4. Coupling control protocol

Before the mitochondrial metabolic states were examined, a cell permeabilization protocol of the NRMCs was applied in the respirometer chamber (Figure 6).

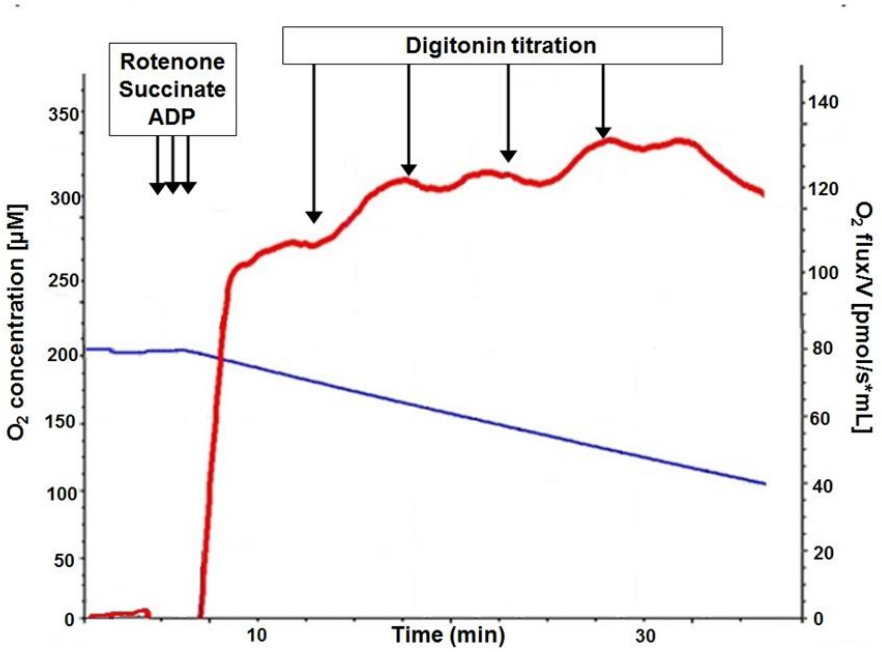

Figure 6. Cell permeabilization titration protocol. Cells were suspended in Miro5 respiration medium at a final cell density such that routine respiration yielded a cell number-specific oxygen flux of about 40 pmol/s*ml-1. After complex I-linked routine respiration was inhibited with rotenone, intact cells (viability >0.95) were stimulated with the addition of 10 mM succinate and 1 mM ADP substrates. Subsequent stepwise digitonin titration yielded gradual permeabilization of plasma membranes, as shown by the increase in respiration up to full permeabilization. Permeabilization at a digitonin concentration of 10 µg per 106 cells was optimal for ADP-stimulated respiration. The blue line shows the O_2 concentration in the chamber, while the red line shows changes in the O_2 flux of the sample.

Next, we applied a coupling control protocol to the permeabilized NRMCs. Routine respiration was defined without substrates. Then, the cells were permeabilized with digitonin, and the oxidative phosphorylation capacity (OxPhos, State 3) of the NRMCs was measured by adding 10 mM succinate (S) and 5 mM ADP substrates. Subsequently, ATP-independent respiration was measured using 0.5 µM oligomycin (Omy). Maximal mitochondrial respiratory capacity was then measured by titration of 1µM carbonyl cyanide p-trifluoromethoxy-phenyl-hydrazine (FCCP). Finally, residual oxygen consumption (ROX) was determined by adding 1 µM rotenone (Rot) and 1 µM antimycin-A (Ama).

3.3.5. Detection of cytochrome c oxidase activity

Cytochrome c oxidase activity was calculated via the time-dependent oxidation of cytochrome c at 550 nm, as described previously [49]. Briefly, a cytochrome c stock solution was freshly prepared by dissolving 10.6 mg cytochrome c (Sigma-Aldrich, Budapest, Hungary) in 20 ml H_2O. The cytochrome c was then reduced by adding 50 µl 0.1 M sodium dithionite, with the absorbance of the solution determined at 550 nm; the photometer was calibrated to this level. Heart samples were homogenized with a Potter grinder in 10× ice-cold Miro5 medium and then centrifuged at 800 g for 5 min at 4°C. 50 µl supernatant was added to 2.5 ml cytochrome c stock solution, and the increase in optical density at 550 nm was measured spectrophotometrically during 1 min intervals at 0, 30 and 60 min.

3.3.6. TUNEL and DAPI staining

Apoptosis of the NRMCs was detected with the TUNEL method. First, the cell number was detected, and then a cytocentrifuge (6 min, 600 rpm, 50.000 cells/slide) was used to create cytospin samples. Samples (n=6 each) were analysed for apoptotic cell staining with In Situ Cell Death Detection Kit TMR red (Roche, Cat. No. 12 156 792 910). The cytospin samples were fixed in 4% paraformaldehyde for 60 min and then permeabilized on ice for 2 min in 0.1% Triton X-100 in 0.1% sodium citrate. We used one-part enzyme solution and nine parts label solution for the TUNEL reaction mixture according to the manufacturer's instructions. The cytospin samples were incubated in the dark for 60 min at 37°C in a humidified atmosphere, followed by DAPI staining (Sigma-Aldrich, Cat. No. 10236276001). For each experimental series, one negative control (incubated only with the label solution), one positive control (digested with DNase I (Quiagen, Cat. No. 79254) together with the TUNEL reaction mixture) and three normal (only with the TUNEL mixture) samples were used. Three pictures were taken of each sample (negative control, positive control, three normal samples each) in each experimental series with a Zeiss AxioImager.Z1 microscope at 20x magnification. The number of apoptotic cells per field of view (524.19 µm×524.19 µm) was determined with Image J 1.47 software.

3.3.7. Cell viability assay and lactate dehydrogenase (LDH) release

The NRMCs were incubated with 1 µM calcein acetoxymethyl ester (Sigma-Aldrich, Cat. No. 56496 calcein-AM, Sigma, St. Louis, MO) dissolved in dimethyl sulfoxide at room temperature for 30 min to assess cell viability. Fluorescence intensity was measured with a

fluorescence plate reader (Fluostar Optima, BMG Labtech, Ortenberg, Germany). Cell viability was compared to that of vehicle control. Cytotoxicity was also measured with the level of LDH released from the damaged cells into the medium culture with the available commercial LDH activity assay kit (Sigma-Aldrich, Cat. No. MAK066 Sigma-Aldrich, Budapest, Hungary) according to the manufacturer's instructions.

4. RESULTS

4.1. Results of Study 1

Demographic and clinical characteristics of donors and recipients were homogeneous in all trials.

4.1.1. Model of end-stage liver disease score

MELD scores were reported in five studies. Subgroup analysis showed no significant difference in MELD score between the four solutions (RR=18.6, 95% CI=15.7–21.5, p=0.379).

4.1.2. Cold ischemia time

CIT was reported in five studies. Subgroup analysis showed no significant difference in risk of CIT between the four solutions (RR=484.7, 95% CI=445.4–524.0, p=0.1).

4.1.3. Primary non-function

PNF rates were reported in 15 studies (Table 2). In four studies, PNF was defined as patient death or retransplantation in the first week. In eleven studies, PNF was undefined. Overall rates of PNF were very low (range 0–13.7%). Our meta-analysis showed no significant difference in risk of PNF between the UW and CS solutions (z=0.41, p=0.680) and between UW and HTK (z=1.07, p=0.284) (Figure 7A). We found only one RCT that dealt with IGL-1, which was not sufficient for a meta-analysis to compare IGL-1 with the UW solution. We performed a subgroup analysis to compare the four solutions in the context of PNF. There was no significant difference between solutions (RR=0.02, 95% CI=0.01–0.03, p=0.356) (Figure 7B). We found no evidence of a small study effect using the funnel plot analysis of the meta-analyses for the primary outcome (p=0.846) (Figure 7C).

	Solution 1				Solution 2					
Study		**N**	**n**	**%**		**N**	**n**	**%**	**RR**	**P**
Cavallari et al., 2003	UW	90	1	1.100	CS	83	0	0.000	2.77	0.53
Lopez-Andujar et al., 2009	UW	104	2	1.900	CS	92	2	2.200	0.88	0.90
García-Gil et al., 2006	UW	40	0	0.000	CS	40	0	0.000	1.00	1.00
Nardo et al., 2001	UW	60	2	3.333	CS	53	0	0.000	4.43	0.33
Duca et al., 2010	UW	51	0	0.000	CS	51	0	0.000	1.00	1.00
García-Gil et al., 2011	UW	51	4	11.100	CS	51	4	11.100	1.00	1.00
Lama et al., 2002	UW	10	0	0.000	CS	10	0	0.000	1.00	1.00
Rayya et al., 2008	UW	68	1	1.471	HTK	69	1	1.449	1.01	0.99
Meine et al., 2006	UW	65	2	3.070	HTK	37	1	3.030	1.14	0.91
Erhard et al., 1994	UW	30	2	6.660	HTK	30	0	0.000	5.00	0.29
Mangus et al., 2008	UW	98	5	5.102	HTK	111	3	2.703	1.89	0.38
Dondéro et al., 2010	UW	92	4	4.350	IGL-1	48	1	2.080	2.09	0.51
Meine et al., 2015	HTK	65	2	3.100	IGL-1	113	3	2.700	1.16	0.87
Wiederkehr et al., 2014	HTK	125	1	0.700	IGL-1	53	0	0.000	1.29	0.88
Nardo et al., 2005	HTK	20	1	5.000	CS	20	0	0.000	3.00	0.49

Table 2. PNF rate in included studies. Studies are grouped by preservation solutions. PNF: primary non-function; N: number in group; n: number of PNF; RR: relative risk; UW: University of Wisconsin solution; HTK: histidine-tryptophan-ketoglutarate solution; CS: Celsior solution; IGL-1: Institut Georges Lopez solution.

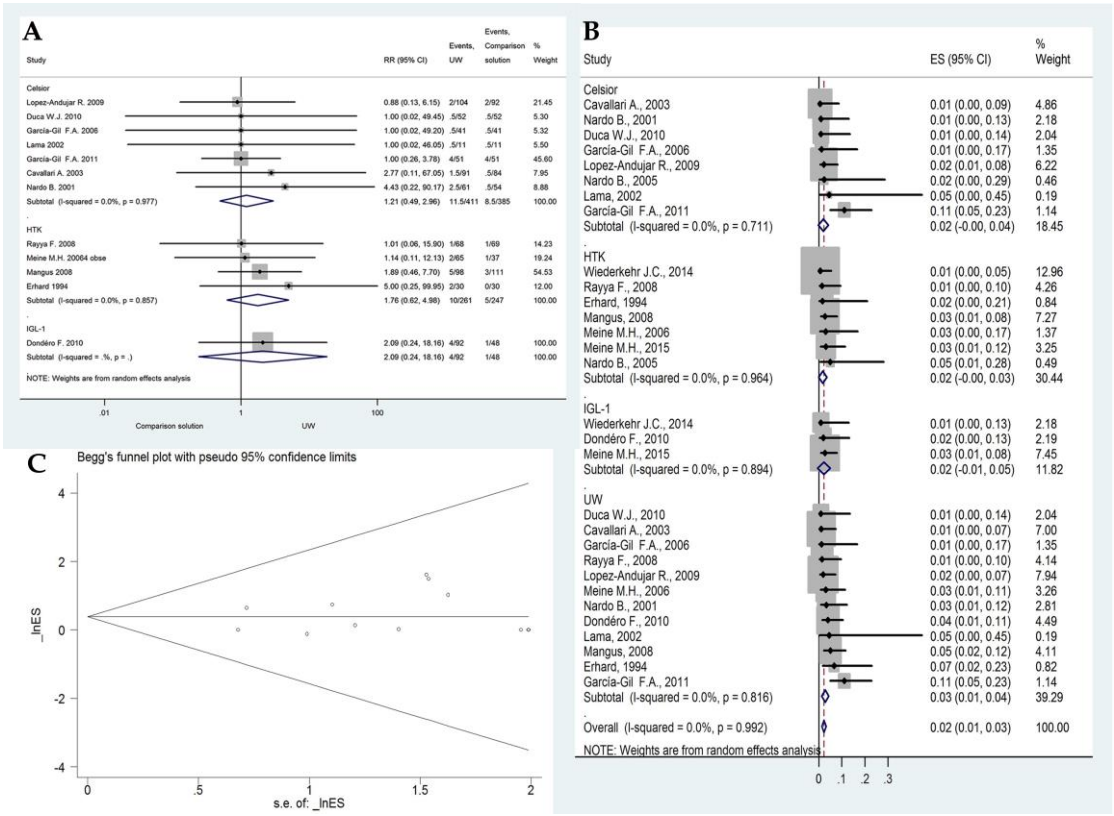

Figure 7. Effects of preservation solutions on PNF. A. Meta-analysis of the relative risk (RR) of PNF comparing studies using different preservation solutions: UW *vs.* Celsior and UW *vs.* HTK; **B.** Forest plot showing subgroup analysis of PNF; **C.** Funnel plot of PNF in studies. PNF: primary non-function; RCTs: randomized controlled trials; ES: effect size; CI: confidence interval; UW: University of Wisconsin solution; HTK: histidine-tryptophan-ketoglutarate solution; CS: Celsior solution; IGL-1: Institut Georges Lopez solution. Squares represent individual study effects, with the size of the box relating to the weight of the study in the meta-analysis. Diamond represents summary effect from meta-analysis. Horizontal bars represent 95% CIs. There is no evidence of a small study effect in the test or the formal plot.

4.1.4. One-year post-transplant graft survival

OGS-1 was reported in eleven studies (Table 3). No study was individually powered for small differences in graft survival, and no study reported a difference related to the preservation fluid used. Meta-analysis of the data showed no significant difference in the risk of OGS-1 between the UW and CS solutions (z=0.30, *p*=0.763) (Figure 8A) or between the UW and HTK solutions (z=0.01, *p*=0.991) (Figure 8A). We also performed a subgroup analysis to compare all four solutions, including IGL-1. There was no significant difference between the solutions (RR=0.80, 95% CI=0.80–0.80, *p*=0.369) (Figure 8B). The funnel plot

analysis showed no evidence of a small study effect from either of the meta-analyses for the OGS-1 (p=0.397) (Figure 8C).

	Solution 1				Solution 2					
Study		**N**	**n**	**%**		**N**	**n**	**%**	**RR**	**P**
Cavallari et al., 2003	UW	90	75	83.0	CS	83	71	85.0	0.97	0.69
Lopez-Andujar et al., 2009	UW	104	83	80.0	CS	92	75	81.0	0.98	0.76
García-Gil et al., 2006	UW	40	26	66.1	CS	40	31	78.0	0.84	0.22
Nardo et al., 2001	UW	60	54	90.0	CS	53	48	90.6	0.99	0.92
Duca et al., 2010	UW	51	31	60.6	CS	51	37	73.5	0.84	0.21
Rayya et al., 2008	UW	68	53	78.0	HTK	69	49	71.0	1.01	0.35
Meine et al., 2006	UW	65	61	94.0	HTK	37	35	94.0	0.99	0.88
Mangus et al., 2008	UW	98	82	84.0	HTK	111	95	86	0.98	0.70
Dondéro et al., 2010	UW	92	73	79.1	IGL-1	48	19	39.8	2.00	0.00
Meine et al., 2015	HTK	65	54	83.0	IGL-1	113	96	85	0.98	0.74
Nardo et al., 2005	HTK	20	15	75.0	CS	20	18	90.0	0.83	0.22

Table 3. OGS-1 rate in included studies. Studies are grouped by preservation solutions. OGS-1: 1-year post-transplant survival of the graft; N: number in group; n: number of OGS-1; RR: relative risk; UW: University of Wisconsin solution; HTK: histidine-tryptophan-ketoglutarate solution; CS: Celsior solution; IGL-1: Institut Georges Lopez solution.

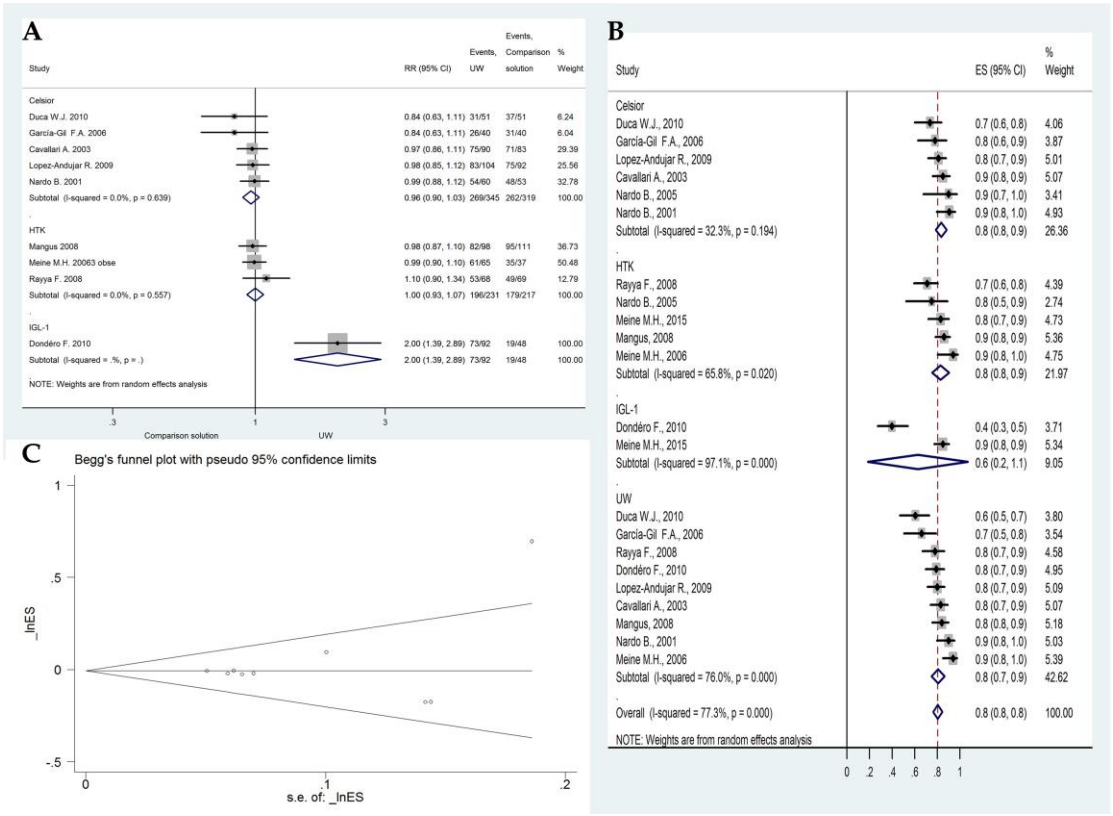

Figure 8. Effects of preservation solutions on OGS-1. A. Meta-analysis of the relative risk (RR) of OGS-1 comparing studies using different preservation solutions: UW *vs.* Celsior and UW *vs.* HTK; **B.** Forest plot showing subgroup analysis of OGS-1; **C.** Funnel plot of OGS-1 in studies. OGS-1: 1-year post-transplant survival of the graft; RR: relative risk; RCTs: randomized controlled trials; ES: effect size; CI: confidence interval; UW: University of Wisconsin solution; HTK: histidine-tryptophan-ketoglutarate solution; CS: Celsior solution; IGL-1: Institut Georges Lopez solution. Squares represent individual study effects, with the size of the box relating to the weight of the study in the meta-analysis. Diamond represents summary effect from meta-analysis. Horizontal bars represent 95% CIs. There is no evidence of a small study effect in the test or the formal plot.

4.1.5. Primary dysfunction

PDF rates were reported in six studies: five of them compared UW with CS, and one compared UW with HTK. Overall rates of PDF were very low (range 0–15.5%). The difference in PDF rate was found higher with the use of UW solutions in one study [50]. However, the subgroup analysis showed no increased risk of PDF in the UW group (= 0.1, 95% CI=0.0–0.1, *p*=0.582).

4.1.6. Early retransplantation rate

Early RT was reported in seven studies and ranged from 0.9% to 20%. None of the studies found a significant difference in early RT between groups; however, they were underpowered to detect such a low incidence outcome. Similarly, subgroup analysis showed no increased risk of early RT in the UW group (RR=0.0, 95% CI 0.0–0.1, p=0.698).

4.1.7. Post-transplant death within 30 days

POD rates were reported in seven studies. Overall rates of POD were very low (range 1.7–14.4%). The difference in POD rate was higher with the use of the CS solution compared with the UW solution in two studies [45, 51]; however, subgroup analysis showed no increased risk. In contrast, there was a significant difference when UW was compared to HTK or IGL-1 (RR=0.07, 95% CI=0.04–0.09, p<0.01).

4.1.8. One-year post-transplant patient survival

OGS-1 rates were reported in ten studies. No study was individually powered for small differences in graft survival, and no study reported a difference related to the preservation fluid used. Subgroup analysis showed no significant difference in risk of OGS-1 between the four solutions (RR=0.9, 95% CI=0.8–0.9, p=0.786).

4.2. Results of Study 2

4.2.1. CH_4 solubility in different solutions

We examined the solubility of CH_4 in three different solutions: distilled water (H_2O), physiological saline (NaCl) and HTK. After 10 min of persufflation, a significantly higher CH_4 concentration was measured in H_2O in comparison with NaCl (0.0206±0.0017 mg/100 ml *vs*. 0.0174±0.0008 mg/100 ml; p<0.005). A significantly higher CH_4 concentration was measured in HTK than in NaCl (0.0194±0.0005 mg/100 ml *vs*. 0.0174±0.0008 mg/100 ml; p<0.005) (Figure 9A). After 60 min, the CH_4 concentration measured in HTK was significantly higher than the CH_4 content in H_2O (0.0253±0.0032 mg/100 ml *vs*. 0.0166±0.0064 mg/100 ml; p<0.005) (Figure 9B). The persufflation time did not influence the CH_4 solubility in different solutions (H_2O: 0.0206±0.0017 mg/100ml *vs*. 0.0166±0.0064 mg/100 ml; p>0.005; NaCl: 0.0174±0.0008 mg/100 ml *vs*. 0.0237±0.0058 mg/100 ml; p>0.005; HTK: 0.0194±0.0005 mg/100ml *vs*. 0.0253±0.0032 mg/100ml; p>0.005).

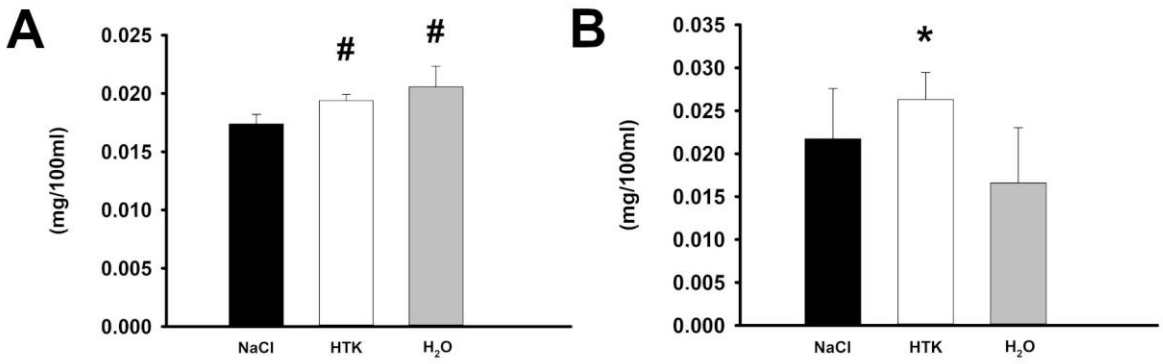

Figure 9. CH_4 solubility in different solutions. A. The CH_4 solubility in different solutions after 10 min incubation. **B.** The CH_4 solubility in different solutions after 60 min incubation. Black columns: physiological saline (NaCl); white columns: HTK solution; grey columns: distilled water (H_2O); $^{\#}p<0.05$ *vs.* NaCl; $*p<0.05$ *vs*. H_2O (one-way ANOVA, Bonferroni test)

4.2.2. Temperature dependence of CH_4 solubility and the half-life of the CH_4-enriched HTK solution

In this experimental series, we stored the CH_4-enriched HTK at 4°C and 21°C for one day. The CH_4 concentration of solutions at each temperature was measured at 10 min, 1 h, 3 h, 6 h and 24 h. As a result, the CH_4 concentration was significantly higher at 4°C at all examined time points in comparison with the storage at 21°C (10 min: 586.10±11.08 ppm *vs*. 442.30±15.10 ppm; $p<0.001$; 1 h: 476.56±15.33 ppm *vs*. 358.80±14.02 ppm; $p<0.001$; 3 h: 280.24±14.28 ppm *vs*. 113.34±13.91 ppm; $p<0.001$) (Figure 10). The CH_4 concentrations decreased steadily over the study period.

After 24 h of storage at 4°C, CH_4 was still present in a therapeutic concentration in the transplantation solution, while no detectable amount was present after 3 h when stored at 21°C (Figure 10).

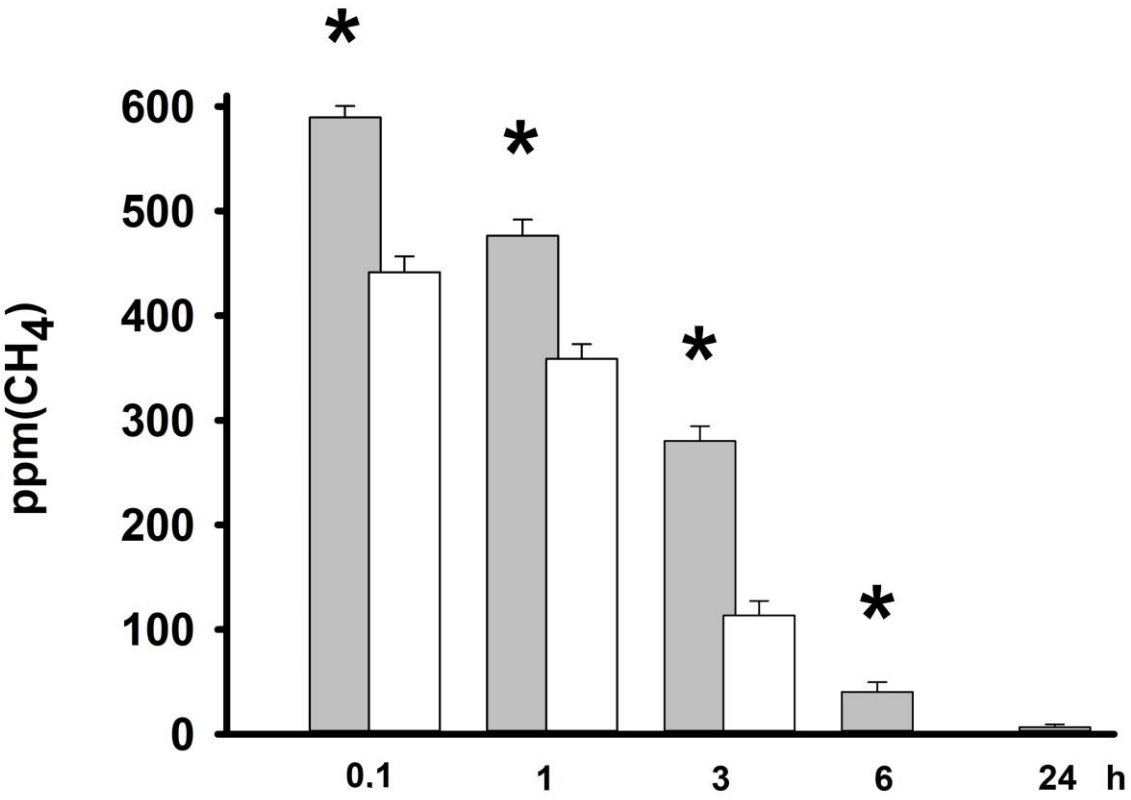

Figure 10. Temperature dependence of CH_4 solubility and the half-life of the CH_4-enriched HTK solution. The grey column is the CH_4-enriched HTK stored at 4°C, and the white column is the CH_4-enriched HTK stored at 21°C. *p<0.001 *vs*. 21°C HTK (one-way ANOVA, Bonferroni test).

4.3. Results of Study 3

4.3.1. CH_4 concentrations

The background CH_4 concentration in the airspace of the incubation chambers was $1.46\times10^4\pm94.95$ ppm, and a rapid two orders of increase (to $1.5\times10^6\pm58.12$ ppm) was detected after the start of persufflation with a 2.2% CH_4–artificial air mixture. This concentration was steadily maintained during the 2 h reoxygenation period (Figure 11A and B). The dissolved CH_4 concentration was $1.46\times10^6\pm76381$ ppm in the cell culture medium 5 min following CH_4 persufflation (Figure 11C and D).

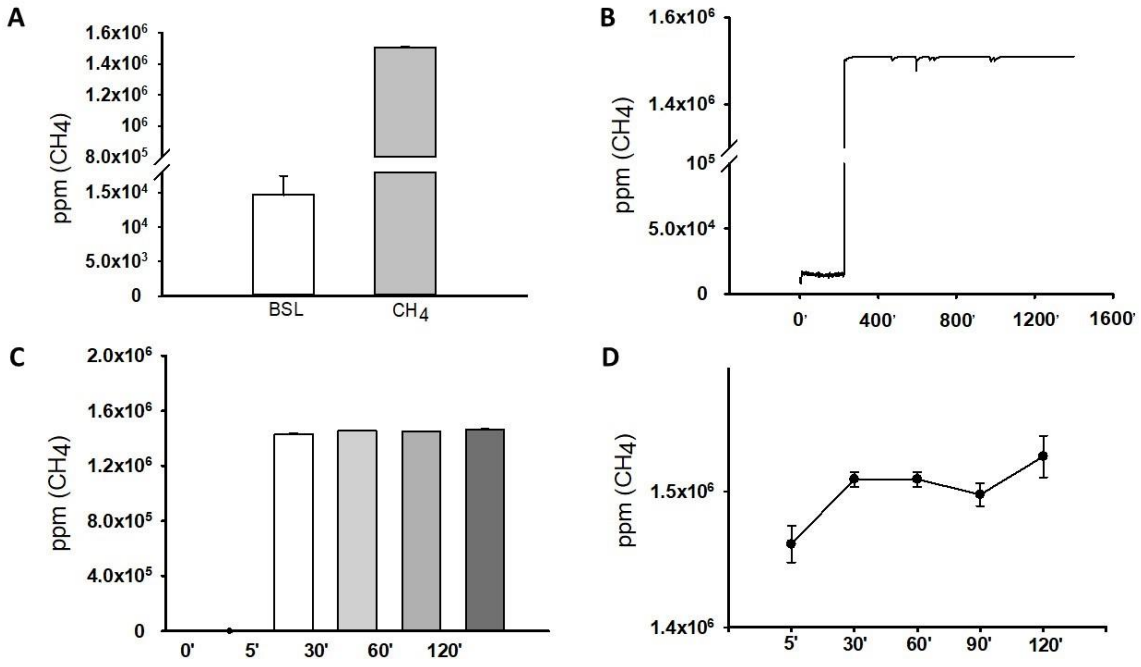

Figure 11. CH_4 concentrations measured by photoacoustic spectroscopy (PAS). A. The CH_4 concentration in the airspace of the incubator (white column: baseline/background concentration; grey column: concentration during persufflation with a 2.2% CH_4–artificial air mixture) **B.** Representative record of CH_4 measurement in the airspace of the incubator. **C.** The change in the dissolved CH_4 concentration of the cell culture medium during persufflation with the 2.2% CH_4–artificial air mixture. **D.** The CH_4 concentration of the medium shown in a narrower range. Data are presented as means ± SEM. #p<0.05 *vs*. baseline/background CH_4 concentration (one-way ANOVA, Tukey's test).

4.3.2. Effect of CH_4 on the mitochondrial functions of the NRMCs

The coupling control protocol provides an opportunity to analyse the leak respiration of the mitochondria. As a result, significantly lower OxPhos was measured in the sI/R group in comparison with the normoxia group (19.17±9.37 pmol*s^{-1}*ml^{-1} *vs*. 50.51±12.87 pmol*s^{-1}*ml^{-1}; p<0.001) (Figure 12). CH_4 treatment in the sI/R+CH_4 group significantly enhanced oxygen consumption (to 40.88±15.08 pmol*s^{-1}*ml^{-1}; p=0.004) (Figure 12). The leak respiration decreased during sI/R (13.54±2.66 pmol*s^{-1}*ml^{-1}; p<0.001); however, it was ameliorated as a result of CH_4 administration in the sI/R+CH_4 group (19.94±3.15 pmol*s^{-1}*ml^{-1}; p<0.001) (Figure 12). The sI/R significantly lowered the maximum respiratory capacity in comparison with the normoxia group (17.35±4.46 pmol*s^{-1}*ml^{-1} *vs*. 35.72±6.55

pmol*s^{-1}*ml^{-1}; p<0.001) (Figure 12). CH_4 treatment had no effect on the maximum respiratory capacity during sI/R (18.41±2.99 pmol*s^{-1}*ml^{-1}; p=0.986) (Figure 12). Flux values in different states were corrected for ROX (data not shown).

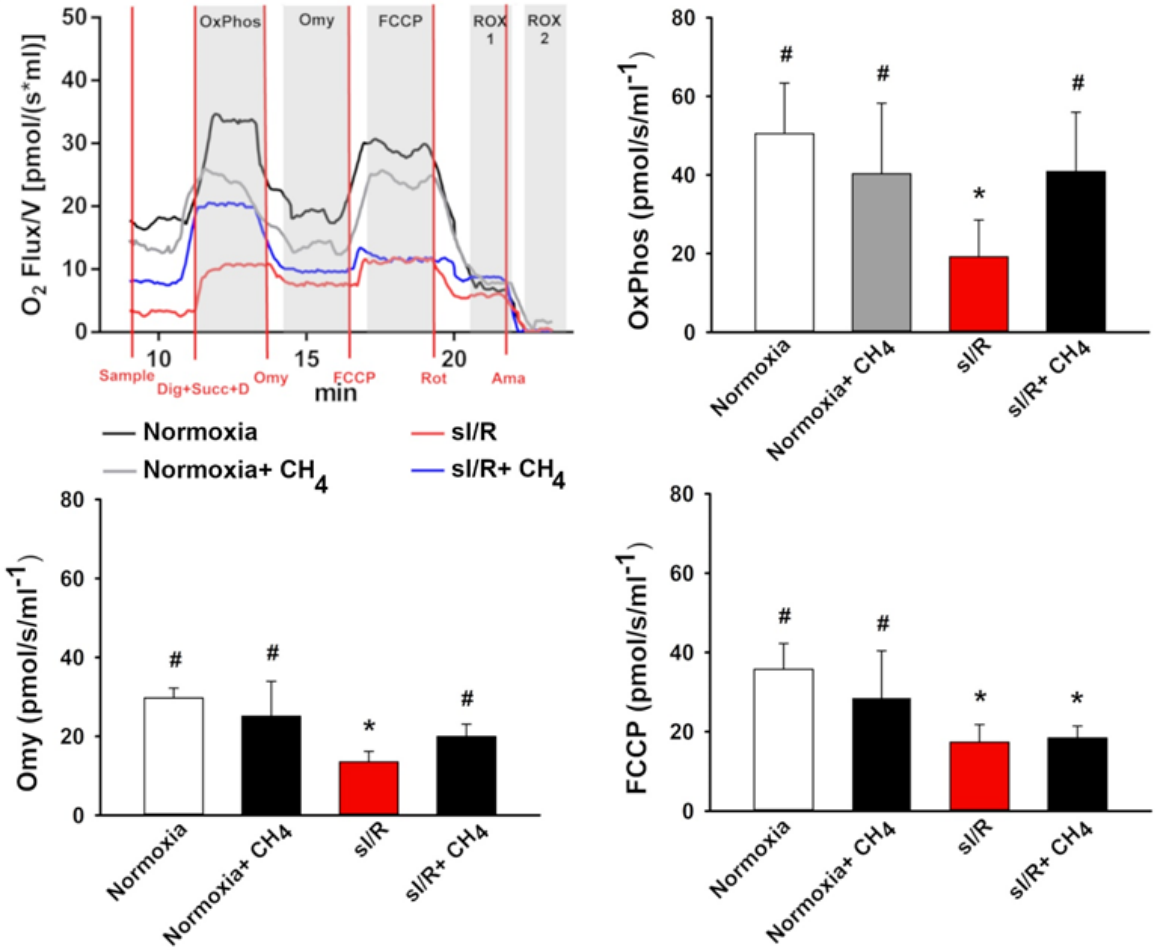

Figure 12. The effect of CH_4 incubation on neonatal rat cardiomyocytes (NRMCs). A. The oxygen consumption of NRMCs (pmol/s^{-1}*ml^{-1}). The upper left-hand chart demonstrates representative records of mitochondrial oxygen consumption measured by HRR. The upper right-hand chart shows the oxidative phosphorylation (OxPhos), the lower left-hand chart presents the oligomycin leak (Omy), and the lower right-hand chart displays the maximum respiratory capacity (FCCP). sI/R: simulated ischemia/reperfusion; CH_4: methane; Dig+Succ+D: 1µl digitonin+20 mM succinate+5 mM ADP; Omy: 0.5 µM oligomycin; FCCP: 1µM carbonyl cyanide p-trifluoro-methoxyphenyl hydrazine; Rot: 1 µM rotenone; Ama: 1 µM antimycin-A; OxPhos: oxidative phosphorylation; ROX: residual oxygen consumption; white columns: normoxia group; grey columns: normoxia+CH_4 group; red columns: sI/R group; black columns: sI/R+ CH_4 group. Data are presented as means ± SEM. *p<0.05 *vs.* normoxia; #p<0.05 *vs*. sI/R (one-way ANOVA, Tukey's test).

4.3.3. Effect of CH_4 on apoptosis, cytochrome c oxidase activity and viability of NRMCs

NRMCs were marked with TUNEL/DAPI staining to examine the presence of apoptosis. As expected, few TUNEL-positive cells were observed in the normoxia and normoxia+CH_4 groups (26±9% and 26.3±12% of cells, respectively; p=1.00) (Figure 13A–B and E). Simulated I/R was accompanied by increased TUNEL positivity (sI/R: 52.4±12 % of cells) (Figure 13C and E), which was diminished as a result of CH_4 incubation (sI/R+CH_4: 20.1±16.4 of cells; p=0.01) (Figure 13D and E). The mitochondrial cytochrome c oxidase activity was determined with a spectrophotometric analysis. Remodelling the mitochondrial membrane during I/R results in cytochrome c release to the cytosol; therefore, this event can be considered as an indicator of mitochondrial membrane damage. In the normoxia+CH_4 group, the enzyme activity did not change in response to CH_4 incubation as compared to the normoxia group (0.39±0.17 *vs*. 0.37±0.24; p=0.992) (Figure 13F). In contrast, sI/R was accompanied by increased cytochrome c oxidase activity (1.43±0.13; p<0.001) (Figure 13F), which was diminished as a result of CH_4 incubation (0.48±0.15; p<0.001) (Figure 13F).

Cardiomyocyte viability was determined with a calcein-based viability assay. During the measurements, calcein passed through the cell membrane and hydrolysed to green fluorescent calcein due to the endogenous esterases in the living cells. Compared to the normoxia group, the CH_4 treatment led to a small drop in viability in the normoxia+CH_4 group (93.48±14.32 *vs*. 83.89±12.91; p=0.891) (Figure 14A). Due to sI/R, the number of living cells decreased, a change shown by the significantly reduced calcein fluorescent intensity (61.74±9.76; p=0.041). Cell death due to sI/R was prevented with the CH_4 treatment in the sI/R+CH_4 group (86.63±12.03; p=0.003) (Figure 14A).

In the case of the LDH activity assay, there was no difference in this parameter between the two normoxic groups (0.22±0.12 *vs.* 0.35±0.11; p=0.15). The LDH concentration was significantly lower in the sI/R+CH_4 group than in the sI/R group (0.42±0.10 *vs.* 0.68±0.12; p=0.041) (Figure 14B).

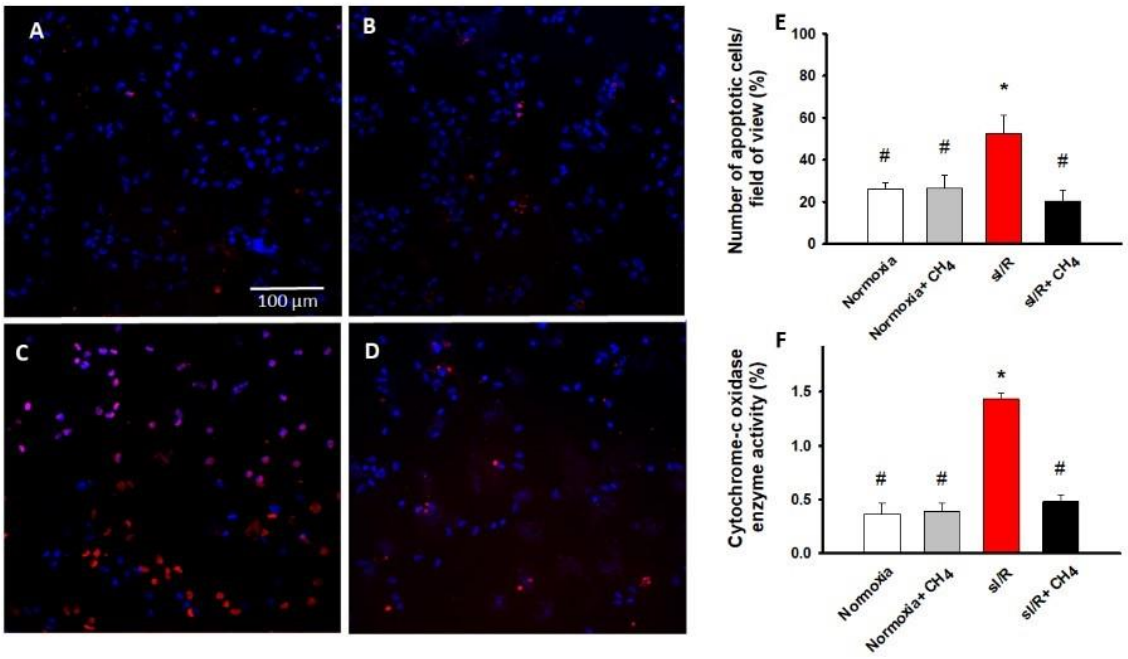

Figure 13. Cell apoptosis and cytochrome c oxidase activity. A. Normoxia group. **B.** Normoxia+CH_4 group. **C.** sI/R group. **D.** sI/R+CH_4 group. **E. The number of apoptotic cells (%). F. Cytochrome c oxidase activity (%).** White column: normoxia group; grey column: normoxia+CH_4 group; red column: sI/R group; black column: sI/R+CH_4 group. Data are presented as means ± SEM. **p*<0.05 *vs.* normoxia; #*p*<0.05 *vs*. sI/R (one-way ANOVA, Tukey's test).

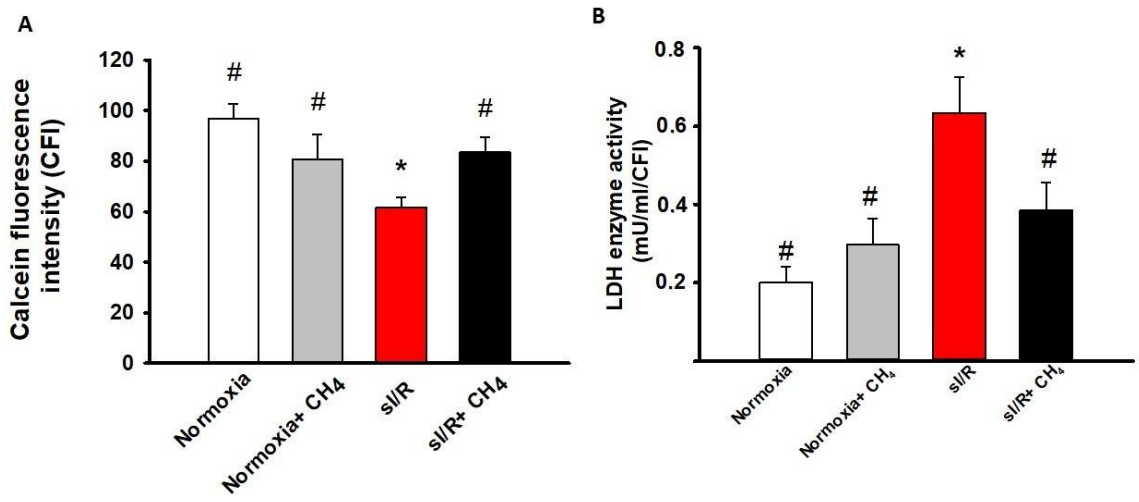

Figure 14. Cell viability of NRMCs. A. The number of living cells. B. Lactate dehydrogenase (LDH) enzyme activity. White column: normoxia group; grey column: normoxia+CH_4 group; red column: sI/R group; black column: sI/R+CH_4 group. Data are presented as means ± SEM. **p*<0.05 *vs*. normoxia; #*p*<0.05 *vs*. sI/R (one-way ANOVA, Tukey's test).

5. DISCUSSION

5.1. Efficacy of preservation solutions on the outcome of liver transplantation

We have summarized the current evidence and updated knowledge on four frequently used preservation solutions for static cold storage of DDLs for transplantation. The treatment groups were homogeneous in terms of donor and recipient characteristics; the prediction of primary and secondary outcomes (i.e. PNF and OGS-1) was thus likely independent of individual risk variables, patient selection or the overall severity of the disease during liver transplantation. More importantly, the analysis of outcome parameters (i.e. PNF and OGS-1) provided good evidence that UW was not outperformed by the CS, HTK and IGL-1 solutions in maintaining organ function and viability of liver grafts in cold storage.

PNF mainly depends on the organ preservation method [49]. It occurs in 2–6% of transplants and is unrelated to any direct surgical, immunological or other complications [50]. Our meta-analysis included 15 trials that evaluated the effectiveness of the UW solution as compared to either the CS or HTK solution. In accordance with the literature, the overall rates of PNF were very low, except in one trial (13%) [54]. When analysing the single studies, we found two trials with a higher incidence of PNF in the UW group than in the HTK group, but the difference did not reach statistical significance upon meta-analysis [48, 50]. It should be added that a recent analysis of the ELTR data demonstrated that use of HTK represented an individual risk factor for the development of PNF when compared to the UW solution [55]. The contradictory conclusions can be explained with the selection bias of the database analysis [56]. In either case, we found no difference between UW and the other solutions with regard to the risk of PNF. As regards IGL-1 and HTK, two prospective randomized clinical studies with 356 patients reported identical results [52, 53]. A similar outcome was detected in a single-centre study with 140 patients that compared IGL-1 and UW solutions [57]. This was confirmed in the current study, since IGL-1 showed a similar PNF risk to that of UW and HTK in our subgroup analyses.

In our study, OGS-1 was the secondary endpoint. Graft survival rates were evaluated one, three and five years after liver transplantation in single studies. The one-year term was chosen as an appropriate period to evaluate the effect of the preservation solutions because other factors could have a greater impact on this outcome parameter after this time. A retrospective analysis of the ELTR database demonstrated that HTK preservation was independently associated with higher mortality than UW, CS and IGL-1 in a multivariate

analysis [55]. Another analysis of a large national registry database (United Network for Organ Sharing, UNOS) has also demonstrated differences in graft survival rate between the HTK and UW solutions [58]. However, important risk factors among donors were not considered in the ELTR analysis, and selected groups of transplant patients were not homogeneous in the other analysis: HTK was utilized in allografts with more favourable recipient traits, as well as shorter CIT and less local and national export [56, 58]. In accordance with findings from numerous clinical trials, the meta-analyses and subgroup analyses in this study did not show a significant difference in risk of OGS-1 between UW and any of the solutions under examination. Similarly, there was no evidence for a difference between IGL-1 and UW solutions and between IGL-1 and HTK in the subgroup analyses.

Apart from preservation methods used to protect the organ from I/R injury, the final outcome of transplantation can also be linked to factors such as donor age, general condition and CIT [59]. A recent UNOS study showed a more pronounced risk for graft loss with longer CIT and donors over 70 years [60]. In our study, subgroup analysis showed that the included trials did not vary significantly and that the mean CITs were beyond the critical 12 h [61]. Several experimental studies demonstrated that the use of the UW solution allows for longer CITs with better graft preservation; however, it remains to be determined whether any of the alternative solutions is better than UW when CIT is prolonged over 12 h.

Recipient morbidity and MELD scores are also important contributing factors to the outcome of liver transplantation. Recipient parameters are incorporated into the MELD score, which indicates the state of health of the recipient; the MELD score-based organ allocation algorithm could thus significantly influence the graft survival rate [60]. In the present study, there was no significant difference between the preservation solutions in the context of the MELD score and other recipient characteristics.

In recent times, the crisis in organ supply has made it necessary to expand the scope of potential donors by using extended criteria donors (ECD). Although there is no precise definition of ECD, frequently cited characteristics are donor age, steatosis, donation after cardiac death (DCD), donors with increased risk of disease transmission and transplantation after prolonged CIT, and use of partial grafts (split grafts and living donor liver transplantation) [62]. Unfortunately, higher rates of graft failure were documented in this class of extended allograft; in addition, very little data is available on the influence of

preservation solutions on their post-transplant outcomes [63]. A single-centre study by Mangus et al. failed to find statistically significant differences in overall graft survival when they compared UW to HTK in ECD transplantations [64]. However, they suggested that HTK may be protective against biliary complications. In contrast, in 2009, the UNOS database analysis reported that HTK was associated with an increased risk of graft loss and early graft loss [55]. More recently, Adam et al. compared the four most frequently used preservation solutions and concluded that HTK is an independent risk factor for graft loss after ECD liver transplantations [55]. The remaining three solutions, UW, CS and IGL-1, provided similar results in post-transplant outcomes after ECD transplantations. In the special condition of using a partially deceased donor liver graft, IGL-1 offered the best graft outcome [55]. In another study, it was suggested that IGL-1 was superior to other solutions for preserving fatty livers by protecting against PNF and early allograft dysfunction [65]. However, a prospective randomized study failed to show any significant improvement in the subgroup of patients receiving IGL-1-preserved grafts [66]. In living donor liver transplantations, risk-adjusted analyses of single- and double-centre studies consistently reported that UW and HTK were equally effective and safe for cold preservation [67–70]. There is currently no evidence-based recommendation on the optimal preservation solution in ECD liver transplantations because the number and quality of RCTs are not sufficient. However, based on the above data, differences in the indications of various preservation solutions are expected.

This study has some limitations. There are so far only three small RCTs that compare IGL-1 with UW or IGL-1 with HTK. We were therefore not able to run a meta-analysis to compare IGL-1 with any of the solutions. In order to compare the risk of the four solutions for PNF, we had to perform a subgroup analysis. In addition, surgery time and haemoderivative transfusions due to recipient coagulation problems are often not cited as predictors of poor outcome in the literature [66]. This factor was not considered in the selected trials. Moreover, different trials presented some differences as regards the operative procedure. Furthermore, the included RCTs were homogeneous with regard to donor and recipient parameters. On the one hand, this provided the opportunity to rule out selection bias, but, on the other hand, the effects of preservation solutions in the case of longer CIT and involvement of expanded criteria donors could not be evaluated.

In conclusion, elucidating the role of preservation solutions in the outcome of liver transplantation is complicated by the intrinsic complexity of the clinical procedure, which is

made up of many different, but interactive phases. This review evaluated the best available evidence from comparisons of the four most frequently used preservation fluids in DDL transplantation. A direct meta-analysis comparison was made, and the sample size of the included trials was large enough to correctly estimate the risk of low-incidence outcomes, such as PNF or OGS-1. Based on our results, there is good evidence that the UW, CS, HTK and IGL-1 solutions are associated with nearly equivalent outcomes. Additional studies on larger patient populations, including marginal donors, longer cold ischemia time, multi-organ transplantations and economic aspects, are needed to evaluate the superiority of any alternative solution over UW.

5.2. The solubility of CH_4 gas in transplant solution

To date, several studies have demonstrated the biological effects of CH_4. Some of these studies examined the effects of CH_4-enriched saline on I/R damage. Specifically, supersaturated CH_4-physiological saline was used [34,39,40,71,72]. However, no study has yet been performed to investigate its effect in transplantation solutions. Therefore, in our experiments, the solubility of CH_4 gas and the stability of CH_4-enriched solutions were determined using PAS technology. We used a safe 2.2% CH_4–artificial air gas mixture with the appropriate O_2 concentration, which can be used for both inhalation and graft preservation. First, we determined the solubility of the CH_4 gas mixture in different solutions. According to literature data, after enrichment with 99.9% CH_4 under high pressure, 3.5 mg of CH_4 gas dissolves in 100 ml of water at 21°C [73]. 0.0206±0.0017 mg/100 ml of CH_4 concentration was measured after persufflation in H_2O for 10 minutes using a 2.2% CH_4–artificial air gas mixture. It was not possible to increase it with a longer persufflation time because slightly less, 0.0166±0.0064 mg/100 ml, was detected after 60 minutes of persufflation. After both persufflation times, the CH_4 concentration of the NaCl solution (0.0174±0.0008 mg/100 ml, 0.0237±0.0058 mg/100 ml) was less than the CH_4 concentration in the HTK solution (0.0194±0.0005 mg/100 ml, 0.0253±0.0032 mg/100 ml). This phenomenon is due to the fact that higher salt content reduces the solubility of gases, while higher protein content and lower heat increase it [74]. In HTK, the highest CH_4 concentration value was measured after 60 minutes of persufflation, but this value was not significantly different from that measured after 10 minutes of persufflation. Based on our data, if pure (99.9%) CH_4 gas was used to enrich the solutions instead of a gas mixture, it was possible to achieve 4.5 mg/100 ml concentration in NaCl and 4.9 mg/100 ml concentration in HTK.

As the next step, we investigated the temperature dependence of the solubility and stability of CH_4. The enriched HTK solutions were stored at various temperatures for one day, and the CH_4 content was examined at specified intervals. The results show that not only the solubility of the HTK solution, but also its stability are significantly better at lower temperatures than at 21°C. The maximum CH_4 concentration was 586 ppm in the 4°C HTK solution, which proportionally decreased with the incubation time. However, CH_4 was still present in detectable amounts (3 ppm) in the HTK solution after 24 h. Based on the results, the CH_4-enriched HTK solution may have a sufficiently protective biological effect to influence I/R damage even after 24 hours of incubation.

5.3. Reduction of myocardial IR-induced mitochondrial damage with exogenous CH_4

In this study, we outlined a possible mechanism linked to the *in vivo* biological efficacy of CH_4. The expected mitochondrial effects of CH_4 have been characterized by HRR, and we have shown that administering CH_4 reduces the sI/R-related mitochondrial ETC disruption and mitigates subsequent apoptotic consequences. Importantly, CH_4 decreased cytochrome c release (a sign of the integrity of the outer mitochondrial membrane) as well.

According to current knowledge, CH_4 is not involved in catabolic or metabolic biochemical processes in the eukaryotic cell. Interestingly, in a pre-clinical model of myocardial infarction (MI), CH_4 treatment significantly improved cardiac function and reduced the apoptosis of cardiomyocytes [33]. The anti-apoptotic and anti-oxidative effects of CH_4 have been demonstrated in other I/R settings as well [26, 31, 33]. These data all suggest that the underlying mechanism of action is intimately connected with mitochondrial functions.

Excessive oxidative stress is a major component of sI/R, and the mitochondrial ETC is a dominant source of ROS generation. Likewise, the majority of superoxide production is linked to complex I early in reperfusion [75–78]. This notion has been supported by studies showing that ischemic preconditioning or pre-treatment with reversible complex I inhibitors can limit ROS generation and cardiac I/R injury [73, 74]. In this study, interactions with complex I certainly occupy a key position in the protective mechanism of CH_4 treatment against I/R injury. It is likely that this action includes conformational changes of respiratory complex I rather than direct interaction with a membrane-associated binding site. Earlier notions about the molecular mechanism by which CH_4 exerts a non-specific action were linked to the physical properties of the molecule. Hydrocarbon gases may modulate the structure and function of biological membranes, which has been demonstrated in lipid bilayer models *in vitro* and in animal models *in vivo* [79–82]. As the smallest hydrocarbon

molecule, CH_4 may interact with the cell membrane, leading to haemolysis in erythrocytes in a concentration-dependent manner [79]. Conformational changes ranging from localized motions of side-chains to global structural changes are required for small molecules, even gases, to gain access to their target binding site [83]. Research on this subject has therefore shifted significantly toward the interaction of gases and proteins with membrane-mediated conformational change. Typical examples are anaesthetic gases, which have previously been known to exert their effect through the disruption of the membrane, yet accumulate and bind to multiple modulation sites of cellular membrane-embedded ion channels. Isoflurane and barbiturates have been identified as partitioning first in the lipid membrane and then binding to the transmembrane domain of the nicotinic acetylcholine receptor [79, 84]. In line with this, halothane has shown demonstrable effects on acetylcholine-activated ion channel kinetics through its conformational changes [85].

6. SUMMARY OF NEW FINDINGS

- We have provided strong evidence that the currently used preservative solutions – HTK, UW, Celsior and IGL-1 – can prevent graft damage with the same efficacy and that none of the preservative solutions offer complete protection against I/R injury in transplanted grafts.
- We have demonstrated that HTK, the most frequently used preservation solution in Europe, can be effectively enriched with 2.2% CH_4 gas. The CH_4-enriched HTK solution stored at 4°C may have a sufficiently high concentration even after 24 hours, which can exercise a protective effect against I/R damage.
- We have demonstrated the protective effect of CH_4 treatment against I/R injury on myocardial cell culture. Cardioprotection was evidenced by increased cell viability and a reduced number of apoptotic cells. The mechanism of CH_4 action is based on improved integrity of the inner mitochondrial membrane.

In summary, we have developed a method that can provide an opportunity to increase the efficacy of currently used transplant solutions.

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9. ANNEX