Enrichment of preservation solution with methane – possible benefits for organ transplantation

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List of full papers relating to the subject of the thesis

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**

Jász DK, **Szilágyi ÁL,** Tuboly E, Baráth B, Márton AR, Varga P, Varga G, Érces D, Mohácsi Á, Szabó A, Bozó R, Gömöri K, Görbe A, Boros M (2021) Reduction of hypoxia-reoxygenation-induced myocardial mitochondrial damage with exogenous methane. J Cell Mol Med. Accepted for publication. **IF: 4.486**

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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 (Song et al., 2014). 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 (Brisson et al., 2017). 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. 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 (Hearse et al., 1991). Inflammatory cascades and oxidative stress can later induce a cytokine storm, which can result in cell death due to damage to cellular structures.

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 (Brisson et al., 2017, Jia et al., 2015), but 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.

According to their composition, there are two main types of preservative solutions: intracellular and extracellular. 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.

1.3. 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.

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 (Keppler et al., 2006, Althoff et al., 2014). The effects of exogenous CH_4 have also been demonstrated by many research groups, in I/R settings as well (Pimentel et al., 2006, Mathur et al., 2016). 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 (Boros et al., 2012). 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 (Boros et al., 2012). Nevertheless, the exact circumstances of administration of CH_4 dissolved in different transplantation solutions have not yet been explored.

1.4. 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 (Strifler et al., 2016). 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* (Chen et al. 2016, Wang et al., 2017)

1.5. The role of mitochondria in the process of apoptosis

Mitochondrial energy production is a substantial factor of the life of eukaryotic cells; However, mitochondria manage not only the metabolism of the cell but also its death. As a response to noxious stimuli, mitochondrial proteins can emerge into the cytoplasm to play essential role in the apoptotic process (Cui et al., 2017, Fan et al., 2016). These proteins include e.g. procaspases, apoptosis inducing factor (AIF), adenylate kinase 2, even proteins of the respiratory chain, such as cytochrome c, Smac/Diablo (recently discovered caspase coactivator) and some heat shock proteins (Hsp10, 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 mitochondrial outer membrane and subsequent release of intermembrane space proteins such as cytochrome c (Brenner et al., 2000). 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 (Mészáros et al., 2017)

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

3.1.1. Search strategy

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. No language limitation was applied. The end date for the literature search was 31 January 2017.

Inclusion criteria specified any randomized control trial 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, non-human and uncontrolled studies were excluded.

3.1.2. Outcomes

The primary outcome was primary non-function (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.

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.

3.1.3. Statistical analysis

All statistical calculations were performed using Stata 11 SE (Stata Corp) and Comprehensive Meta-analysis Software (Version 3, Biostat, Englewood). 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. 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. 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. We sought signs of a small study effect with the funnel plot.

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

3.2.1. Measurement of CH_4 concentration

The photoacoustic spectroscopy (PAS) technique for CH_4 detection was validated by our cooperative working group (Tuboly et al, 2013). In order to determine the solubility of CH_4, the measurements were made in a cuvette with a volume of 20 cm^3. The input aperture was

connected to the CH_4-containing gas bottle, and the output aperture was connected to the CH_4 detection system. Persufflation of the solution was made with a 2.2% CH_4–artificial air mixture at a flow rate of 200 ml/min.

3.2.2. 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 histidine-tryptophan-ketoglutarate (HTK) solution with different flow times (10 and 60 min). In the second step, the CH_4 solubility and stability were determined in NaCl and HTK solutions at 4 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 in two ways.

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

3.3.1. Isolation and culturing of neonatal rat cardiac myocytes (NRMCs)

NRMCs were isolated, as described previously (Görbe et al., 2010).

3.3.2. Experimental protocol

The experiments were performed using intact NRMCs. In this series, three-day-old cardiac myocytes were subjected to 4 h simulated ischemia. The cells were kept in a hypoxic chamber, and the culture medium was changed to a hypoxic solution. This was followed by a 2 h reoxygenation period in a culture medium when cells were kept under either normoxic conditions or in a chamber with normoxic air supplemented with CH_4. Control groups were kept in a normoxic incubator to maintain physiological conditions for 4 h, which was followed by a 2 h reoxygenation period in the normoxic incubator with or without CH_4 supplementation.

3.3.3. Investigation of cardiac mitochondrial function with high resolution respirometry (HRR)

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

Next, we applied a coupling control protocol to the permeabilized NRMCs.

3.3.4. 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 (Szarka et al., 2004).

3.3.5. Investigation of apoptosis and viability of NRMCs

Apoptosis of the NRMCs was detected with the TUNEL method. Cardiomyocyte viability was

determined with a calcein-based viability assay.

4. RESULTS

4.1. Results of Study 1

Demographic and clinical characteristics of donors and recipients were homogeneous in all trials. Our meta-analysis showed no significant difference in risk of PNF between the UW and CS solutions and between UW and HTK. 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. We found no evidence of a small study effect using the funnel plot analysis of the meta-analyses for the primary outcome.

Meta-analysis of the data showed no significant difference in the risk of OGS-1 between the UW and CS solutions or between the UW and HTK solutions. We also performed a subgroup analysis to compare all four solutions, including IGL-1. There was no significant difference between the solutions. The funnel plot analysis showed no evidence of a small study effect from either of the meta-analyses for the OGS-1.

4.2. Results of Study 2

We examined the solubility of CH_4 in three different solutions: H_2O, NaCl and HTK. The persufflation time did not influence the CH_4 solubility in different solutions. 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. 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.

4.3. Results of Study 3

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 was detected after the start of persufflation with a 2.2% CH_4–artificial air mixture ($1.5\times10^6\pm58.12$ ppm). This concentration was steadily maintained during the 2 h reoxygenation period. The dissolved CH_4 concentration was $1.46\times10^6\pm76381$ ppm in the cell culture medium and $1.41\times10^6\pm61314$ ppm in the MiRo5 respiration medium 5 min following CH_4 persufflation.

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. CH_4 treatment in the sI/R+CH_4 group significantly enhanced oxygen consumption. The leak respiration decreased during sI/R; however, it was

ameliorated as a result of CH_4 administration in the sI/R+CH_4 group. The sI/R significantly lowered the maximum respiratory capacity in comparison with the normoxia group. CH_4 treatment had no effect on the maximum respiratory capacity during sI/R. Flux values in different states were corrected for ROX.

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. Simulated I/R was accompanied by increased TUNEL positivity, which was diminished as a result of CH_4 incubation. 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. In contrast, sI/R was accompanied by increased cytochrome c oxidase activity, which was diminished as a result of CH_4 incubation.

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. Due to sI/R, the number of living cells decreased, a change shown by the significantly reduced calcein fluorescent intensity. Cell death due to sI/R was prevented with the CH_4 treatment in the sI/R+CH_4 group.

In the case of the LDH activity assay, there was no difference in this parameter between the two normoxic groups. The LDH concentration was significantly lower in the sI/R+CH_4 group than in the sI/R group.

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 (Rutger et al., 1993). It occurs in 2–6% of transplants and is unrelated to any direct surgical, immunological or other complications (Uemora et al., 2007). 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%) (Nardo et al., 2005). 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 (Kuznetsov et al., 2002, Meine et al., 2006). 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 (Adam et al., 2015). The contradictory conclusions can be explained with the selection bias of the database analysis (Nashan et al., 2015). 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 (Rutger et al., 1993, Uemora et al., 2007). A similar outcome was detected in a single-centre study with 140 patients that compared IGL-1 and UW solutions Dondéro et al., 2010). 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 (Adam et al., 2015). 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 (Stewart et al., 2009). 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 cold ischemia time (CIT) and less local and national export (Nashan et al., 2015, Stewart et al., 2009). 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.

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 (Mangus et al., 2007). 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 (Song et al., 2015, Fan et al., 2016, Brenner et al., 2000, Meng et al., 2018, Liu et al., 2012). 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 (F.S. Structures, F. Damp, PubChem CID: 297 Structure:, 1800.). Based on our result, it was not possible to increase the CH_4 concentration with a longer persufflation time. And the CH_4 concentration of the NaCl was less than the CH_4 concentration in H_2O and HTK solution. 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 (Clever et al., 2005). 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 (Chen et al., 2016). The anti-apoptotic and anti-oxidative effects of CH_4 have been demonstrated in other I/R settings as well (Boros et al., 2012, Strifler et al., 2016, Chen et al., 2016). 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 (Korge et al., 2017, Votyakova et al., 2001, Gorenkova et al., 2013, Babot et al., 2014). 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 (Ringe et al., 2005, Meng et al., 2018). 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.

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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