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ORIGINAL PRE-CLINICAL SCIENCE

Methane supplementation improves graft function in experimental heart transplantation

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KEYWORDS:
methane;
cold ischemia-reperfusion;
preservation solution;
experimental heart transplantation;
endoplasmic reticulum stress;
mitochondria

BACKGROUND: Maintenance of cell viability during cold storage is a key issue in organ transplantation. Methane (CH_4) bioactivity has recently been recognized in ischemia/reperfusion conditions; we therefore hypothesized that cold storage in CH_4-enriched preservation solution can provide an increased defense against organ dysfunction during experimental heart transplantation (HTX).

METHODS: The hearts of donor Lewis rats were stored for 60 minutes in cold histidine-tryptophan-ketoglutarate (Custodiol [CS]) or CH_4-saturated CS solution (CS-CH_4) ($n = 12$ each). Standard heterotopic HTX was performed, and 60 minutes later, the left ventricular (LV) pressure-volume relationships LV systolic pressure (LVSP), systolic pressure increment (dP/dtmax), diastolic pressure decrement, and coronary blood flow (CBF) were measured. Tissue samples were taken to detect proinflammatory parameters, structural damage (by light microscopy), endoplasmic reticulum (ER) stress, and apoptosis markers (CCAAT/enhancer binding protein [C/EBP] homologous protein, GRP78, glycogen synthase kinase-3β, very low-density lipoprotein receptor, caspase 3 and 9, B-cell lymphoma 2, and bcl-2-like protein 4), whereas mitochondrial functional changes were analyzed by high-resolution respirometry.

RESULTS: LVSP and dP/dtmax increased significantly at the largest pre-load volumes in CS-CH_4 grafts as compared with the CS group (114.5 ± 16.6 mm Hg vs 82.8 ± 4.6 mm Hg and 3,133 ± 430 mm Hg/s vs 1,739 ± 169 mm Hg/s, respectively); the diastolic function and CBF (2.4 ± 0.4 ml/min/g vs 1.3 ± 0.3 ml/min/g) also improved. Mitochondrial oxidative phosphorylation capacity was more preserved (58.5 ± 9.4 pmol/s/ml vs 27.7 ± 6.6 pmol/s/ml), and cytochrome c release was reduced in CS-CH_4 storage. Signs of HTX-caused myocardial damage, level of ER stress, and the transcription of proapoptotic proteins were significantly lower in CS-CH_4 grafts.

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CONCLUSION: The addition of CH_4 during 1 hour of cold storage improved early in vitro graft function and reduced mitochondrial dysfunction and activation of inflammation. Evidence shows that CH_4 reduced ER stress−linked proapoptotic signaling.
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Transplantation is routine medical practice for treating end-stage organ failure, but research to improve outcomes and patient safety is still ongoing.[1] One of the decisive factors in clinical success is effective allograft protection after organ procurement. Several concepts have been attempted to date, but static storage in a cold solution is still the method of choice for organ preservation after surgical explantation.[2,3] Nevertheless, currently used techniques cannot provide a complete defense against transient anoxia or reperfusion-induced tissue damage, and therefore, the search for prevention or reduction of cold storage−related organ dysfunction and injury is a priority task.[4]

It is recognized that enrichment of preservation solutions with biologically active gases is a conceivable option to improve graft function because gas molecules in a fluid milieu are likely to have access to membranes, channels, and cell components involved in the maintenance of organ homeostasis.[5−7] Against this background, a link between methane (CH_4) supplementation and organ protection seems unconventional but reasonable. CH_4 is the most hydrogen-substituted form of carbon, and as a consequence of its physicochemical properties, it is distributed evenly across membrane barriers.[8] It is intrinsically non-toxic and widely regarded as physiologically inert.[8] However, various recent data have provided evidence for CH_4 bioactivity in various in vivo settings; most importantly, several studies have demonstrated modulator, anti-inflammatory potential for inhaled CH_4-based approaches in anoxia-reoxygenation experiments.[8−10] These results are supported by a series of studies where anti-apoptotic properties have been demonstrated for CH_4-enriched solutions, as well as an influence on the pathways involved in pyroptosis, the proinflammatory form of programed cell death.[11−14]

In this context, we set out to establish whether deteriorating graft functions might be modified by the CH_4 content of a preservation fluid. The effects of CH_4 in terms of organ storage or transplantation conditions have not yet been investigated, and therefore, the main purpose was to test the feasibility of CH_4 enrichment of a standard storage solution in a relevant experimental setting. With this aim, we have used the generally employed histidine-tryptophan-ketoglutarate (HTK) solution, with or without CH_4 admixture, in an isogenic rat model of heterotopic heart transplantation (HTX), devoid of immunologic effects.

Our next aim was to explore the cross-sectional details of the in vivo consequences and the underlying mechanisms of CH_4 action. Structural and functional mitochondrial damage and disturbed protein folding in the lumen of the rough endoplasmic reticulum (ER), defined as ER stress, are major upstream factors that govern the progression of graft dysfunction after organ procurement.[15,16] It has already been demonstrated that CH_4 can protect against ischemia/reperfusion (I/R)-induced apoptosis by inhibiting the phosphoinositide 3-kinase/protein kinase B/glycogen synthase kinase-3β (GSK-3β) pathway and nuclear factor erythroid 2-related factor 2 activation.[13,14,17] Other evidence suggests that CH_4 can possibly limit ER stress as well.[18,19]

Therefore, we put special emphasis on the detection of the most important hemodynamic variables, together with mitochondrial respiratory parameters and myocardial ER stress− and apoptosis-associated gene expression changes, to investigate the hypothesis that a cytoprotective action of CH_4 enrichment may target ER stress and its functional links to mitochondria in transplanted rat hearts.

Materials and methods

The experiments were carried out on male Lewis rats (250−350 g; Charles River, Sulzfeld, Germany) in accordance with EU Directive 2010/63 for the protection of animals used for scientific purposes and in compliance with criteria set down in the US National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. The study was approved by the national competent authority of Hungary National Scientific Ethical Committee on Animal Experimentation (ATET) under license number PEI/001/2374-4/2015.

Production of CH_4-enriched HTK

Commercially available Custodiol (CS) solution (Dr Franz Köhler Chemie GmbH, Bensheim, Germany) was saturated with pure CH_4 (>99.9%) under 0.4 MPa for 4 hours in a high-pressure vessel (Messer, Budapest, Hungary), as described previously.[20] The CH_4 concentration in the fluid phase was detected by gas chromatography, whereas the stability of the solution was checked by near-infrared laser-based photoacoustic spectroscopy. The solution containing 6.57 ± 0.27 μmol/ml CH_4 was freshly prepared and stored at 4°C before use (Supplementary Material, available online at www.jhltonline.org).

Experimental protocol

Isogenic male Lewis to Lewis HTX ($n = 36$) was performed as described previously.[21] Briefly, after excision from the donors, the grafts were cold-stored in a transplantation solution for 60 minutes (cold ischemia time), which was followed by heterotopical transplantation and a 60-minute reperfusion period. During transplantations, the aorta and the pulmonary artery of the donor heart were anastomosed end-to-side to the abdominal aorta and the inferior vena cava of the recipient rat, respectively, using microsurgical techniques. At the end of the reperfusion, in situ hemodynamic measurements were performed in the recipient to evaluate early

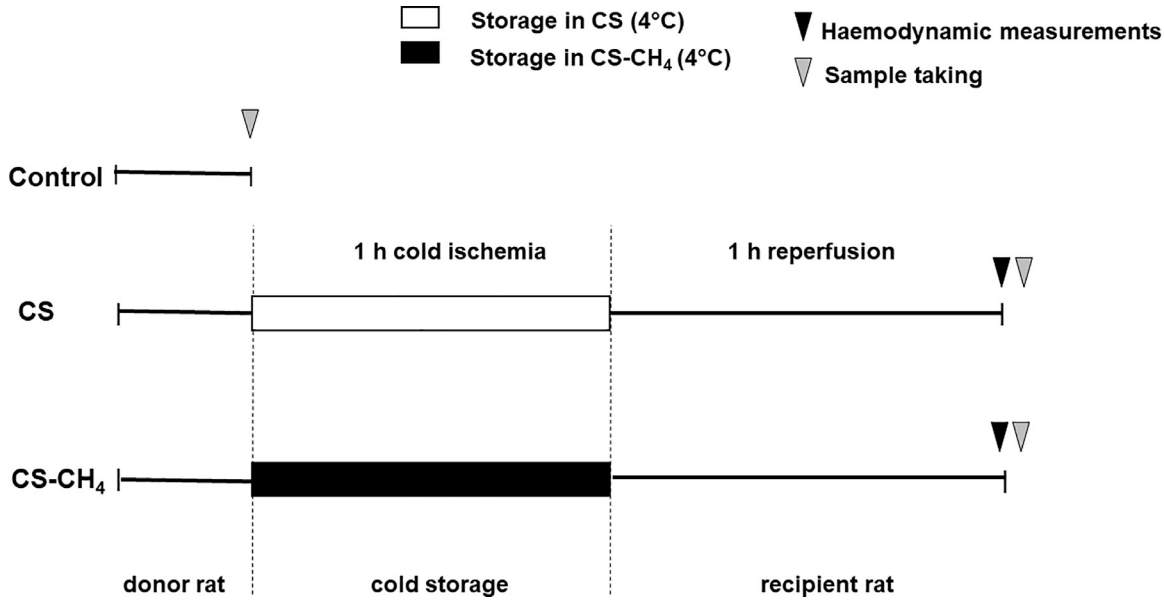

Figure 1 Experimental protocol. The heart grafts were explanted from the donors and stored for 60 minutes in cold preservation solution before heterotopical HTX. At 60 minutes after the start of reperfusion, hemodynamic measurements were performed in the recipients to evaluate early post-transplant graft function. Thereafter, samples were taken from the left ventricle for mitochondrial functional measurements, biochemical assays, qPCR analysis, and histology. Hearts in the control group underwent the same surgical procedure as those of the donors but were not subjected to cold storage and transplantation. Grafts in the CS group were stored in cold (4°C) CS solution during the cold ischemia period; in the CS-CH_4 group, the protocol was identical, except that CH_4-enriched CS solution was used. CH_4, methane; CS, Custodiol; HTX, heart transplantation; qPCR, quantitative real-time polymerase chain reaction.

graft functions; thereafter, biopsies were taken from the left ventricle of the grafts for mitochondrial functional measurements and biochemical assays. Tissue myeloperoxidase (MPO) and xanthine oxidoreductase (XOR) activity, reduced glutathione (GSH) and oxidized glutathione disulfide (GSSG) ratio, and tissue nitrite/nitrate (NOx) level were determined. Blood samples for serum biomarkers of myocardial injury were taken from the vena cava at the end of the reperfusion period (Supplementary Material online).

The animals were randomly allocated into 3 groups. In control Group 1 ($n = 12$), donor rats underwent the same surgical procedure until the explantation, but the hearts were not subjected to cold ischemia and storage and were not transplanted. In Group 2 ($n = 12$), the explanted grafts were stored in CS solution at 4°C during the 60-minute cold ischemic period, whereas in Group 3 ($n = 12$), the grafts were stored in CH_4-enriched CS during the 60-minute cold ischemic period. In this group, the cold cardioplegic CS solution used to arrest the heart was also supplemented with CH_4 (Figure 1).

Hemodynamic measurements in the graft

We have employed a heterotopic HTX model where the recipient aorta supplies the graft; that is, the blood flows from the recipient aorta to the aorta root of the graft. The aortic valve of the graft is competent, and therefore, the coronaries are perfused without entering the left ventricle. This virtually completely unloaded LV model is suitable for measurements of global hemodynamic parameters and the extent of functional damage of the experimentally transplanted hearts.[22,23] The LV pressure-volume relationships were determined as follows. After transplantation and 60-minute reperfusion, a 3F latex balloon catheter (Edwards Lifesciences Corporation, Irvine, CA) was introduced in situ into the left ventricle via the apex. The maximal LV systolic pressure (LVSP), the maximal slope of systolic pressure increment (dP/dtmax), and diastolic pressure decrement (dP/dtmin) were determined with a Millar micromanometer (SPR-838, Millar, Houston, TX) at different LV volumes (20−180 μl) with an injection of saline solution. The coronary blood flow (CBF) of the graft was measured indirectly with an ultrasonic flowmeter (Transonic Systems Inc, Ithaca, NY) mounted on the ascending aorta of the graft, which is the only outlet for circulating blood through the coronaries.[22,24]

Examination of cardiac mitochondrial functions

The efficacy of the mitochondrial respiration was assessed from heart homogenates by high-resolution respirometry (Oxygraph-2k, Oroboros Instruments, Innsbruck, Austria). Mitochondrial O_2 consumption (respiratory flux), complex II−linked baseline respiration (succinate-fueled, in the presence of complex I inhibitor rotenone), oxidative phosphorylation (OxPhos) capacity, and cytochrome c release (an indicator of inner mitochondrial membrane damage) were determined as described previously.[25]

Quantitative real-time polymerase chain reaction analysis

Myocardial mRNA expression was analyzed by quantitative real-time polymerase chain reaction (Applied Biosystems, Foster City, CA) for the following genes: *caspase-3*, *caspase-9*, *DNA damage-inducible transcript 3 (Ddit3;* also known as CCAAT/enhancer binding protein [C/EBP] homologous protein [CHOP]), *hypoxia-inducible factor 1-alpha (HIF1α)*, glycogen synthase kinase-3β (*GSK-3β*), and *very low-density lipoprotein receptor (VLDLr)*.

Histology and immunohistochemistry

The samples were fixed in buffered paraformaldehyde solution (4%) and embedded in paraffin. Next, 5-μm thick sections were cut and stained with hematoxylin and eosin. Structural damage assessment was performed according to a previously described histological scoring system (Supplementary Material).

Sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) is the major regulator of Ca^{2+} homeostasis and contractility in cardiac and skeletal muscle.[26] In addition, the ER stress response can induce an overexpression of SERCA isoforms, including SERCA1 in the post-ischemic heart. Based on this background, SERCA1 expression was detected with standard immunohistochemical staining technique (using #S1189 ab, Sigma Aldrich, St. Louis, MO) in the left ventricles of transplanted rat heart grafts.

Results

Hemodynamic parameters of the transplanted grafts

After transplantation, increasing LV balloon volumes (pre-load) resulted in elevated LVSP and dP/dtmax, which were both significantly increased at the largest pre-load values in the CS-CH_4 group compared with CS alone (Figure 2A and B). A similar change in diastolic function was noted at higher pre-load volumes, bringing about significantly elevated dP/dtmin values ($p < 0.05$) compared with CS, reflecting better myocardial relaxation (Figure 2C). CBF was also significantly ($p < 0.05$) higher after 60 minutes of reperfusion in CS-CH_4 storage than in the CS group (Figure 2D). There was no statistically significant difference in heart rate values within and between experimental groups (Figure 2E).

Cardiac mitochondrial function

Complex II−linked basal respiration was significantly higher in the CS-CH_4 grafts than in the CS group 60 minutes after reperfusion (data not shown). After adding saturating amounts of adenosine diphosphate, the OxPhos capacity was significantly higher in the CS-CH_4 group (Figure 3C). Mitochondrial respiration in response to cytochrome c (Figure 3D) was tested to determine the ability of exogenous cytochrome c to replace the enzyme in the mitochondrial membrane. In comparison with the CS group, the release of cytochrome c was significantly lower in the CS-CH_4 group.

Myocardial ER stress− and apoptosis-associated gene expression

The relative mRNA expression for hypoxia- and ER stress −associated genes (*HIF-1α*, *CHOP*, *GSK-3β*, and *Vldlr*) was significantly lower in the CS-CH_4 group (Figure 4). The expression of caspase-3 and caspase-9 and the proapoptotic bcl-2-like protein 4 (Bax) were not significantly decreased. However, the anti-apoptotic B-cell lymphoma 2 (Bcl2) and the ratio of Bax/Bcl2 expression were significantly different in the CS-CH_4 group, thus indicating the relative dominance of anti-apoptotic pathways (Figure 4).

Oxidative stress markers

XOR is a key enzyme in reperfusion-induced reactive oxygen species production; in addition, it can catalyze the reduction of nitrates and nitrites to nitric oxide. XOR activity and tissue NOx levels were both significantly decreased

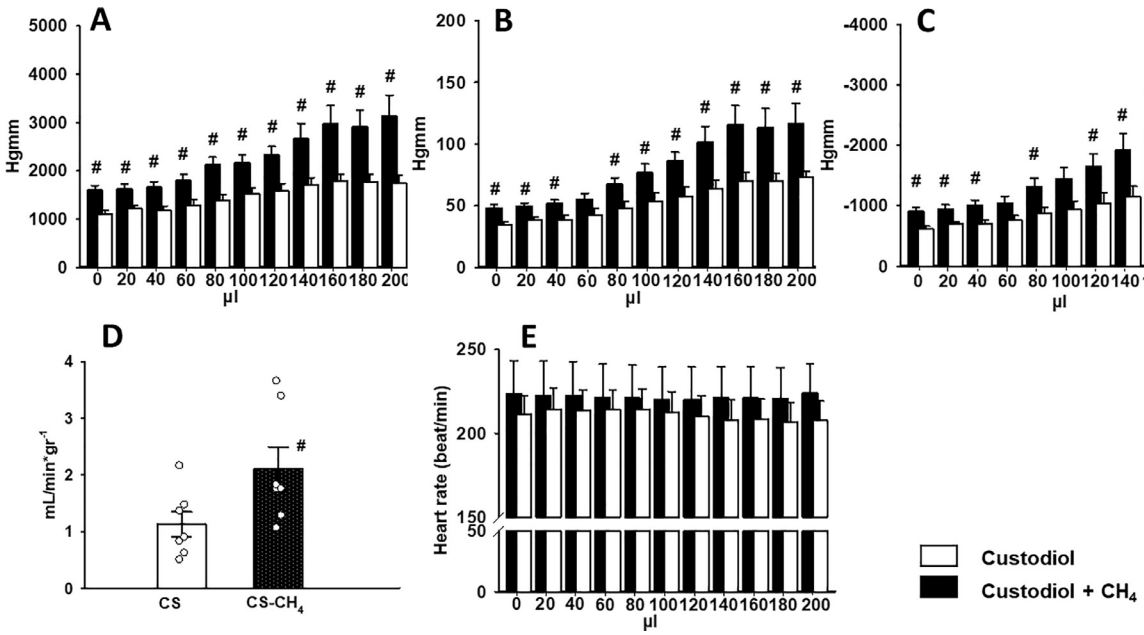

Figure 2 LV pressure-volume relations and CBF changes. (**A**) dP/dtmax, (**B**) maximal LVSP, (**C**) dP/dtmin, (**D**) CBF, and (**E**) heart rate. White columns: CS group; black columns: CS-CH_4 group. Data are presented as means ± SEM. #$p < 0.05$ vs CS (one-way ANOVA, Tukey's test). ANOVA, analysis of variance; CBF, coronary blood flow; CH_4, methane; CS, Custodiol; dP/dtmax, maximal slope of the systolic pressure increment; dP/dtmin, diastolic pressure decrement; LV, left ventricular; LV, left ventricular systolic pressure.

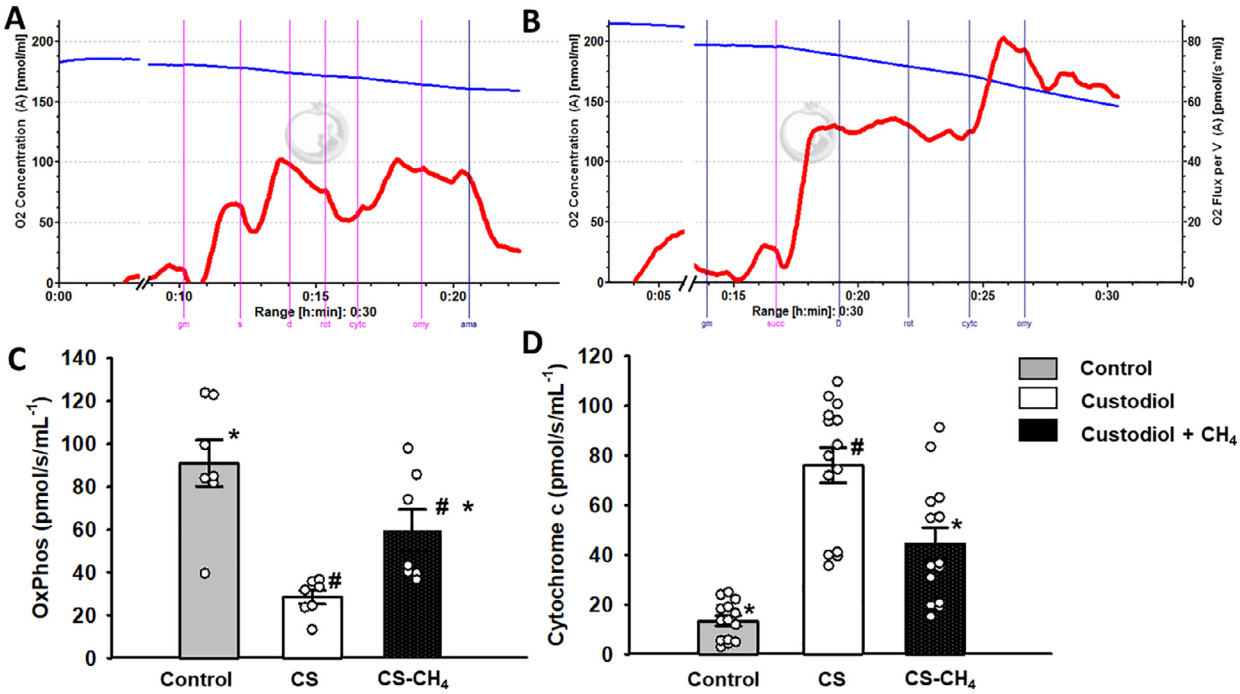

Figure 3 Oxygen consumption of cardiac mitochondria (pmol/s/ml^{-1}). (**A, B**) The upper charts demonstrate representative records of mitochondrial oxygen consumption of (**A**) CS-stored or (**B**) CS-CH_4−stored samples measured by high-resolution respirometry. The blue line represents the instantaneous oxygen concentration in the respiration chamber, whereas the red line indicates the simultaneous oxygen consumption of the sample. (**C**) The lower right-hand chart shows OxPhos capacity, and (**D**) the lower left-hand chart demonstrates cytochrome c release data. Gray columns: control group; white columns: CS group; black columns: CS-CH_4 group. Data are presented as means ± SEM, individual data points are shown (n = 7-7). Cytochrome c release measurements were made in duplicate. *$p < 0.05$ vs CS; #$p < 0.05$ vs. control (one-way ANOVA, Tukey's test). ANOVA, analysis of variance; CH_4, methane; CS, Custodiol; OxPhos, oxidative phosphorylation.

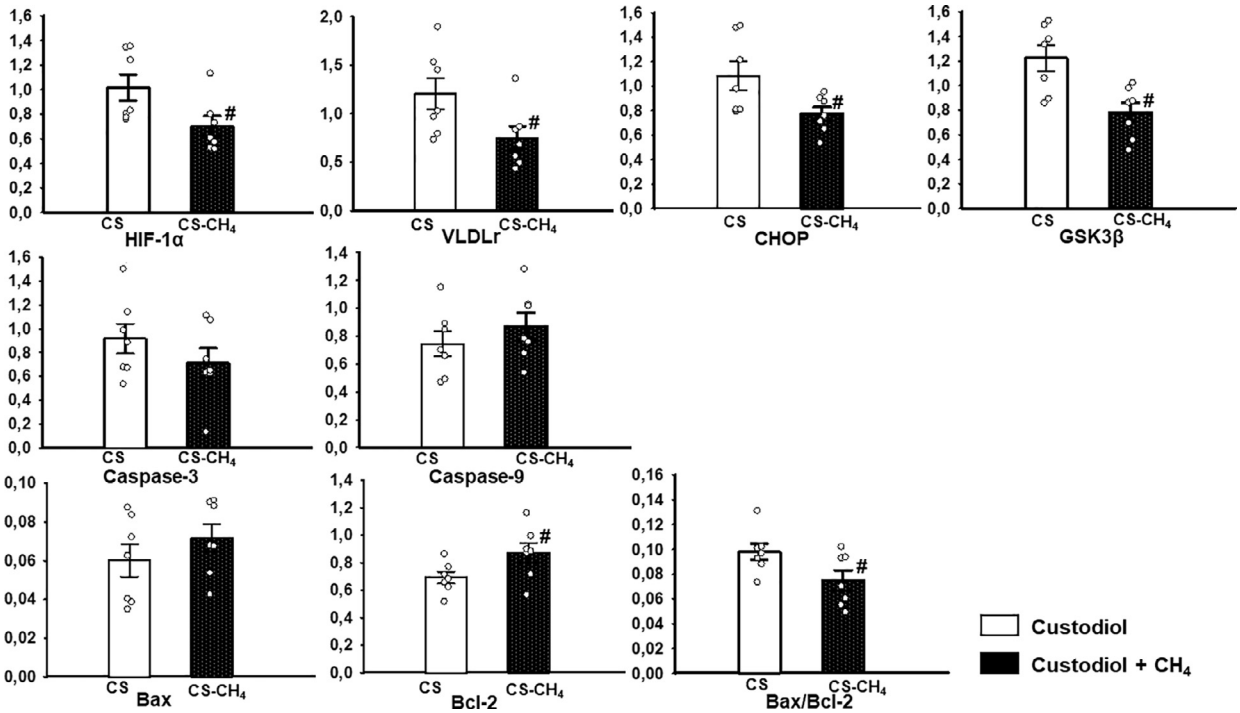

Figure 4 Gene expression changes. White columns: CS group; black columns: CS-CH_4 group. Data are presented as means ± SEM, individual data points are shown (n = 7-7). #$p < 0.05$ vs. CS (one-way ANOVA, Tukey's test). ANOVA, analysis of variance; Bax, bcl-2-like protein 4; Bcl-2, B-cell lymphoma 2; CH_4, methane; CHOP, CCAAT/enhancer binding protein (C/EBP) homologous protein; CS, Custodiol; GSK-3β, glycogen synthase kinase-3β; HIF-1α, hypoxia-inducible factor-1α; VLDLr, very low-density lipoprotein receptor. The y-axis shows fold-change in gene expression.

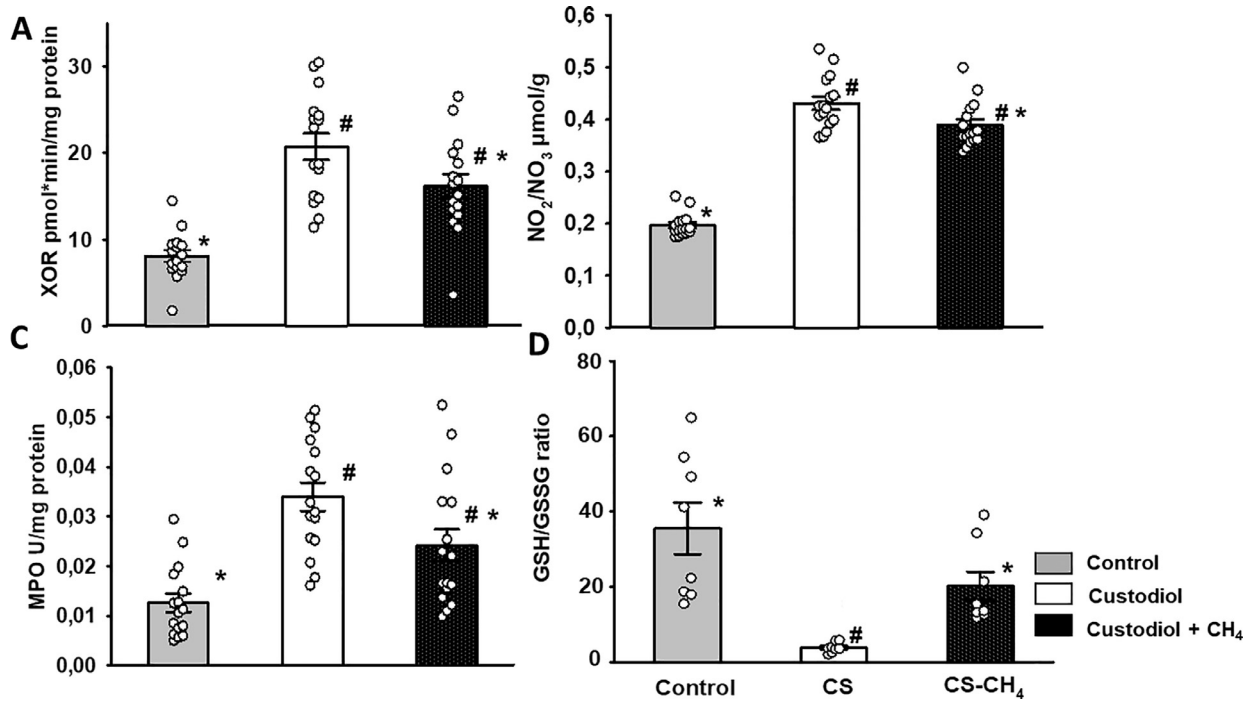

Figure 5 Biochemical assays for oxidoreductive stress parameters. (**A**) Tissue XOR activity, (**B**) NO_2/NO_3 levels, (**C**) MPO activity and (**D**) GSH/GSSG ratio. Gray columns: control group; white columns: CS group; black columns: CS-CH_4 group. Data are presented as means ± SEM, individual data points are shown for the columns (n = 8-8). XOR, MPO, and NO_2/NO_3 measurements were made in duplicate.*p < 0.05 vs CS; #p < 0.05 vs. control (one-way ANOVA, Tukey's test). ANOVA, analysis of variance; CH_4, methane; CS, Custodiol; GSH, reduced glutathione; GSSG, oxidized glutathione disulfide; MPO, myeloperoxidase; NO_2, nitrite; NO_3, nitrate; XOR, xanthine oxidoreductase.

when CS-CH_4 was applied during the cold ischemia period relative to the data for the CS group (Figure 5A and B). MPO is mostly produced by activated polymorphonuclear leukocytes. Although tissue MPO was significantly increased as compared with that of the control group, MPO activity was significantly reduced when CH_4-CS was applied (Figure 5C). The GSH/GSSG ratio is one of the most important markers of oxidoreductive stress. This ratio was significantly decreased in the CS group; however, preservation of grafts in CS-CH_4 resulted in a sustained GSH/GSSG ratio (Figure 5D).

Laboratory parameters of myocardium-specific enzyme changes

CH_4 admixture in the CS-CH_4 group resulted in significantly lower plasma lactate dehydrogenase, creatine kinase, creatine kinase myocardial band, and troponin T levels as compared with CS storage alone (Table 1).

Histology

Hematoxylin and eosin staining showed only a mild disorganization of the myofibrils with loss of striations and a combination of waviness, contraction bands, and disruption of plasma membranes of myocytes in the CS group as compared with the controls (Figure 6A and B). The architecture of cardiac myocytes was nearly normal in the CS-CH_4 storage group (Figure 6C). These changes were not significantly different from those in the CS group, thus indicating nearly equal potential for tissue protection (Figure 6D). The number of SERCA1 immunoreactive cardiac myocytes increased significantly in sections from CS-stored grafts as compared with the controls (Figure 6E and F). In contrast,

Table 1 Myocardium-Specific Enzyme Changes

Group	CK (U/L)	CK-MB (U/L)	LDH (U/L)	Troponin T (ng/L)
Control (n = 12)	503 ± 57[a]	206 ± 31[a]	358 ± 79[a]	42 ± 9[a]
CS (n = 12)	2327 ± 23[b]	527 ± 43[b]	938 ± 108[b]	172 ± 36[b]
CS-CH_4 (n = 12)	1507 ± 49[a,b]	328 ± 52[a,b]	732 ± 96[a,b]	110 ± 21[a,b]

Abbreviations: ANOVA, analysis of variance; CH_4, methane; CK, creatine kinase; CK-MB, creatine kinase myocardial band; CS, Custodiol.
Data are presented as means ± SEM.
[a]p < 0.05 vs CS
[b]p < 0.05 vs control (one-way ANOVA, Tukey's test).

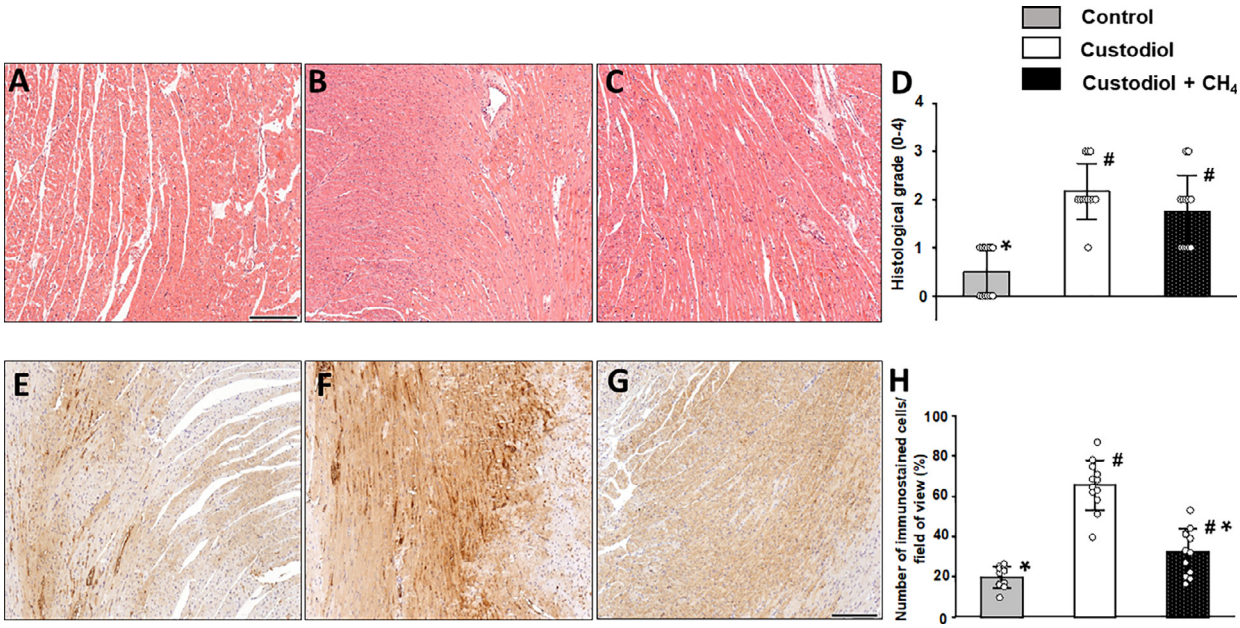

Figure 6 Histology and immunohistochemistry. (**A**−**C**) H&E staining of heart sections. (**A**) Control group, (**B**) CS group, and (**C**) CS-CH_4 group. (**D**) Histological grading of groups represents a composite of number of damaged myocytes and number of foci of damage (n = 12-12). (**E**−**G**) SERCA1 immunostaining of heart sections. (**E**) Control group, (**F**) CS group, and (**G**) CS-CH_4 group. (**H**) SERCA1 immunoreactivity is demonstrated as percentage of immunopositive cells quantified per field of view. Data are presented as means ± SD (individual data points are shown for the columns, n = 12-12). *$p < 0.05$ vs CS; #$p < 0.05$ vs control (ANOVA on rank, Tukey−Kramer). Magnification: × 200. Bar = 200 μm. ANOVA, analysis of variance; CH_4, methane; CS, Custodiol; H&E, hematoxylin and eosin; SERCA, sarco/endoplasmic reticulum Ca^{2+} ATPase.

the number of immunoreactive cells was significantly reduced in the CS-CH_4 group (Figure 6G and H).

Discussion

This study aimed to investigate whether adding CH_4 to a cold preservation solution modifies the graft function in experimental HTX. The hemodynamic efficacy of CS-CH_4 storage was evidenced by increased LVSP, cardiac contractility, and coronary circulation as compared with CS-treated grafts. The sum of biochemical data showed that the CH_4-containing HTK solution effectively reduced the degree of oxidoreductive stress in myocardial samples and significantly influenced several components of ER stress—mitochondria-related proapoptotic signaling pathways. In addition, high-resolution respirometry confirmed that CH_4 supplementation preserved the respiratory mechanism of cardiac mitochondria during cold storage. These pathways together may have contributed to improved structures and functions in this HTX model.

The myocardium has particularly poor tolerance to prolonged ischemia, and the issue of preservation is a major concern in transplantation.[27] The HTK solution is generally used in clinical practice; therefore, it is an appropriate testbed for alternative options.[28] The gas mediators nitric oxide, carbon monoxide, and hydrogen sulfide have already been tried as additives to solutions in transplantation models, assuming that a potential efficacy could be related to their tendency to react with biologically important molecules.[5,6,29] In contrast, CH_4 is intrinsically non-toxic in vivo; it is a simple asphyxiant, which means that hypoxia might occur when an increasing concentration of CH_4 displaces inhaled air in a restricted area and the concentration of oxygen is reduced.[30] Nevertheless, there are pertinent data that demonstrate that CH_4 can modulate nitric oxide−, carbon monoxide−, and hydrogen sulfide−linked reactions in living systems.[8,10] In addition, higher concentrations of exogenous CH_4 can lead to direct anti-cytokine effects via master switches, such as nuclear factor erythroid 2-related factor 2/Keap1 or nuclear factor-κB, and anti-inflammatory responses in experimental conditions.[9,11,20,25] In the case of myocardial I/R, treatment with CH_4-enriched saline significantly ameliorated the sequelae of proinflammatory activation (evidenced by reduced tumor necrosis factor-α, interleukin-1β, MPO activity, and oxidative DNA damage) and maintained cardiac function 4 weeks after infarction.[11]

The immediate hemodynamic circulatory consequences of CH_4-enriched graft storage included a significantly improved myocardial contractility and a parallel increase in CBF during the 60-minute reperfusion. These data suggest that, in this short time frame, exogenous CH_4 can restrain or counteract those mechanisms that would otherwise influence the cardiac contractility negatively. This conclusion is consistent with earlier results where CH_4 treatment maintained a satisfactory cardiac function measured at 4 weeks after infarction, with improved LV ejection fraction, diastolic volume, and contractility, among other improvements, compared with non−CH_4-treated rats.[11]

Despite the wide range of research to map the biological effects, the role of CH_4 in cold ischemia or organ transplantation settings has not yet been investigated. Therefore, we manufactured a CH_4-saturated HTK solution according to

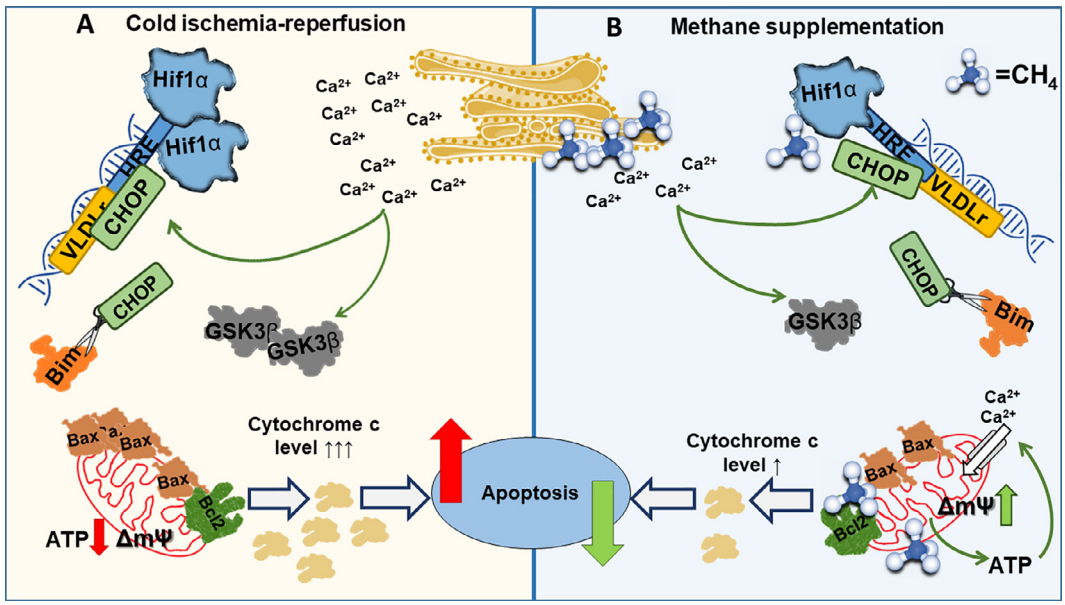

Figure 7 Proposed effects of methane supplementation on cold ischemia−induced intracellular changes. (**A**) Cold storage, ischemic, and hypoxic states will lead to perturbations in normal ER functions. ER stress is accompanied by intracellular Ca^{2+} overload, which modulates the activation of proapoptotic GSK-3β and leads to caspase activation. Hypoxia increases HIF-1α expression as well, which interacts with the promotor of VLDLr and the proapoptotic transcription factor CHOP by direct binding and by the non-classical HRE. By the end of this process, CHOP upregulates the Bcl-2 family BH3 protein Bim mRNA expression, which directly activates Bax to translocate from the cytosol to the mitochondria. These events will trigger cardiomyocyte apoptosis. (**B**) Phospholipid membranes of the ER and the mitochondrion are possible targets of CH_4. In the presence of a saturating amount of CH_4, the expression of genes downstream of HIF-1α decreases, affecting the mitochondrial pathway of apoptosis by lowering the Bax/Bcl2 ratio, thus creating an anti-apoptotic milieu for cardiac muscle cells. Bax, bcl-2-like protein 4; Bcl-2: B-cell lymphoma 2; Bim, bcl-2-like protein 11; CH_4, methane; CHOP: CCAAT/enhancer binding protein (C/EBP) homologous protein; ER, endoplasmic reticulum; GSK-3β: glycogen synthase kinase-3 β; HIF-1α, hypoxia-inducible factor-1α; HRE, hypoxia responsive element; VLDLr, very low-density lipoprotein receptor.

reported protocols and in a concentration range that demonstrated efficacy in I/R studies in vivo.[20] Many details of the mechanism are still unknown, but we have shown that this approach affected the mitochondrial physiology during cold ischemia and after reperfusion, perhaps through an indirect influence on Ca^{2+} homeostasis.[11,20] During circulatory arrest, depletion of mitochondrial substrates is a major contributor to Ca^{2+} influx−mediated membrane dysfunctions. As a result of CH_4 enrichment, the mitochondria were more responsive to adenosine diphosphate utilization, which contributed to the maintenance of OxPhos capacity. Furthermore, cytochrome c release, a sign of mitochondrial inner membrane injury, was also reduced (Figure 7).

The process of cold ischemia−induced cellular damage with the dual contribution of ER and mitochondria is relatively well characterized. Hypoxic conditions trigger changes in cytoplasmic resting potential and, through the activation of ER-mediated Ca^{2+} transport, increase the expression of HIF-1α, one of the key initial factors in the cascade of events, which will finally lead to cell apoptosis or necrosis.[31−34] More directly, as a consequence of HIF-1α expression, mRNA expression of VLDLr and the proapoptotic transcription factor CHOP are also increased, and by the end of this process, CHOP upregulates bcl-2-like protein 11 mRNA expression and activates Bax protein to translocate from the cytosol to the mitochondria.[31,35] Our results demonstrate that cold ischemia and graft storage activated all these participants, starting from higher SERCA1 protein levels reflecting an increased Ca^{2+} pump function in the ER and elevated HIF-1α expression in cardiomyocytes. In addition, higher intracellular Ca^{2+} can activate GSK-3β, the proapoptotic factor in the intrinsic mitochondrial apoptotic pathway.[36] These mitochondrial changes raise the expression of Bax protein and its activation, whereas modified proapoptotic Bax and anti-apoptotic Bcl2 levels lead to further proapoptotic events, such as cytochrome c release. CS-CH_4 storage did not influence the caspase enzyme system, but the Bax/Bcl2 ratio and the reduced cytochrome c release suggest that the intrinsic mitochondrial pathway of apoptosis was affected. More importantly, if the preservation solution was supplemented with CH_4, the expression of individual genes in the proposed signaling pathway was also reduced (Figure 7).

Our study has several limitations. First, animal models do not predict human responses precisely, and in prioritizing the interventions, we had to build on previously collected scientific information. Therefore, it is conceivable that other known or unknown mechanisms could also play a role in the reduction of tissue damage in this setup. Second, a rat model of heterotopic abdominal HTX provides important data on the dynamics of myocardial changes, but the unloaded reperfusion of the graft leads to relatively fast recovery. In other words, this method reduces experimental variability, but the relevant observation time is limited. Therefore, further pre-clinical transplantation studies should evaluate whether CH_4 supplementation can confer

in vivo tissue protection not only in rodents but also in larger animals with longer cold ischemia and prolonged reperfusion times.

In summary, our study has demonstrated that CH_4 enrichment of HTK solution results in increased graft protection during cold ischemia and isogenic HTX in rats. Oxidoreductive imbalance is an inevitable consequence of ex vivo periods and a basis for a cascade of proinflammatory events following reoxygenation. Based on the totality of data, it seems that CH_4 supplementation conferred increased efficacy on HTK to reduce signs of nitroxidative stress as shown by the maintained GSH/GSSG ratio, reduced MPO and XOR activity, and lower NOx level in the reperfused myocardium.

The underlying mechanism is attributed at least partly to an influence of CH_4 on myocardial ER stress and its link to mitochondrial structural and functional reactions. CH_4 enrichment is a simple and effective option for static organ preservation and also seems feasible for dynamic graft storage. Future research should particularly seek to answer the question of whether this approach confers long-term protection in immunologically challenged situations.

Disclosure statement

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Supplementary materials

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Supplementary data

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