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# METHANE SUPPLEMENTATION IMPROVES GRAFT FUNCTION IN EXPERIMENTAL HEART TRANSPLANTATION

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**Abstract**

**Background:** Maintenance of cell viability during cold storage is a key issue in organ transplantation. Methane ($CH_4$) bioactivity has recently been recognised in ischaemia/reperfusion conditions; we therefore hypothesised that cold storage in $CH_4$-enriched preservation solution can provide an increased defence against organ dysfunction during experimental heart transplantation (HTX).

**Methods:** The hearts of donor Lewis rats were stored for 60 min in cold histidine-tryptophan-ketoglutarate (CS) or $CH_4$-saturated CS solution (CS-$CH_4$) (n=12 each). Standard heterotopic HTX was performed, and 60 min later the left ventricular (LV) pressure-volume relationship (LVSP), systolic and diastolic pressure changes (dP/dtmax and dP/dtmin) and coronary blood flow (CBF) were measured. Tissue samples were taken to detect pro-inflammatory parameters, structural damage (by light microscopy), endoplasmic reticulum (ER) stress and apoptosis markers (CHOP, GRP78, GSK3β, VLDR, Caspase 3 and 9, Bcl2 and Bax), while mitochondrial functional changes were analysed by high-resolution respirometry.

**Results:** LVSP and dP/dtmax increased significantly at the largest preload volumes in CS-$CH_4$ grafts as compared to the CS group (114.5±16.6 vs 82.8±4.6 mmHg and 3133±430 vs 1739±169 mmHg/s, respectively); the diastolic function and CBF (2.4±0.4 vs 1.3±0.3 ml/min/g) also improved. Mitochondrial oxidative phosphorylation capacity was more preserved (58.5±9.4 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 pro-apoptotic proteins were significantly lower in CS-$CH_4$ grafts.

**Conclusion:** The addition of $CH_4$ during 1hr 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.

**Introduction**

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 defence 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 recognised 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, and an influence on the pathways involved in pyroptosis, the proinflammatory form of programmed 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 immunological 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 ischaemia/reperfusion (I/R)-induced apoptosis by inhibiting the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt)/glycogen synthase kinase-3β (GSK-3β) pathway and nuclear factor-erythroid2 p45-related factor 2 (Nrf2) 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 haemodynamic 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, 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 (ATET) under licence 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 h 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, while the stability of the solution was checked by near-infrared laser-based photoacoustic spectroscopy (PAS). The solution containing 6.57±0.27 µmol/ml $CH_4$ was freshly prepared and stored at 4°C before use (Supporting Information File).

**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 min (cold ischaemia time), which was followed by heterotopical transplantation and a 60-min reperfusion period. During transplantations, the aorta and the pulmonary artery of the donor heart was 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* haemodynamic measurements were

performed in the recipient to evaluate early graft functions; thereafter, biopsies were taken from the left ventricle (LV) 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 (see Supplementary Appendix).

The animals were randomly allocated into three 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 ischaemia and storage and were not transplanted. In group 2 (n=12), the explanted grafts were stored in CS solution at 4ºC during the 60-min cold ischaemic period, while in group 3 (n=12) the grafts were stored in $CH_4$-enriched CS during the 60-min cold ischaemic period. In this group, the cold cardioplegic CS solution used to arrest the heart was also supplemented with $CH_4$ (Fig 1.).

**Haemodynamic measurements in the graft**

We have employed a heterotopic heart transplantation 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 LV. 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-min reperfusion, a 3F latex balloon catheter (Edwards Lifesciences Corporation, Irvine, CA, USA) was introduced in situ into the left ventricle (LV) via the apex.The maximal LV systolic pressure (LVSP), the dP/dtmax (maximal slope of systolic pressure increment) and dP/dtmin were determined with a Millar micromanometer (SPR-838, Millar Instruments) 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 flow meter (Transonic Systems Inc., Ithaca, NY, USA) 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-fuelled, in the presence of complex I inhibitor rotenone), oxidative phosphorylation capacity (OxPhos) and cytochrome c release (an indicator of inner mitochondrial membrane damage) were determined as described previously.[25]

**Quantitative real-time PCR (qPCR) analysis**

Myocardial mRNA expression was analysed by qPCR (Applied Biosystems, Foster City, CA, USA) 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), hypoxia-inducible factor 1-alpha (HIF1α), glycogen synthase kinase 3 beta (GSK3β) and very low-density lipoprotein receptor (Vldlr).

**Histology and immunohistochemistry**

The samples were fixed in buffered paraformaldehyde solution (4%) and embedded in paraffin. 5-μm thick sections were cut and then stained with haematoxylin and eosin (H&E). Structural damage assessment was performed according to a previously described histological scoring system (see Supplementary Appendix).

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 postischemic heart. Based on this background SERCA1 expression was detected with standard immunohistochemical staining technique (using #S1189 ab, Sigma Aldrich, Budapest, Hungary) in the LVs of transplanted rat heart grafts.

**Results**

**Haemodynamic parameters of the transplanted grafts**

After transplantation, increasing LV balloon volumes ('preload') resulted in elevated LVSP and dP/dtmax, which were both significantly increased at the largest preload values in the CS-$CH_4$ group as compared to CS alone (Fig. 2A–B). A similar change in diastolic function was noted at higher preload volumes, bringing about significantly elevated dP/dtmin values ($p < 0.05$) compared to CS, reflecting better myocardial relaxation (Fig. 2C). CBF was also significantly ($p < 0.05$) higher after 60

min of reperfusion in CS-$CH_4$ storage as compared to the CS group (Fig. 2D). There was no statistically significant difference in HR values within and between experimental groups (Fig. 2E).

**Cardiac mitochondrial function**

Complex II-linked basal respiration was significantly higher in the CS-$CH_4$ grafts than in the CS group 60 min after reperfusion (data not shown). After adding saturating amounts of ADP, the OxPhos capacity was significantly higher in the CS-$CH_4$ group (Fig. 3C). Mitochondrial respiration in response to cytochrome c (Fig. 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, GSK3β and Vldlr) was significantly lower in the CS-$CH_4$ group (Fig. 4). The expression of caspase-3 and caspase-9, and the pro-apoptotic Bax were not significantly decreased. However, the anti-apoptotic 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 (Fig. 4).

**Oxidative stress markers**

XOR is a key enzyme in reperfusion-induced ROS production; in addition, it can catalyze the reduction of nitrates and nitrites to nitric oxide (NO). XOR activity and tissue NOx levels were both significantly decreased when CS-$CH_4$ was applied during the cold ischaemia period relative to the data for the CS group (Fig. 5A-B). MPO is mostly produced by activated polymorphonuclear leukocytes. While tissue MPO was significantly increased as compared to that of the control group, MPO activity was significantly reduced when $CH_4$-CS was applied (Fig. 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 (Fig. 5D).

**Laboratory parameters of myocardium-specific enzyme changes**

$CH_4$ admixture in the CS-$CH_4$ group resulted in significantly lower plasma LDH, CK, CK-MB and troponin T levels as compared to CS storage alone (Table 1).

**Histology**

H&E staining showed only a mild disorganisation 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 to the controls (Fig. 6A-B). The architecture of cardiac myocytes was nearly normal in the CS-$CH_4$ storage group (Fig. 6C). These changes were not significantly different from those in the CS group, thus indicating nearly equal potential for tissue protection (Fig. 6D). The number of SERCA1 immunoreactive cardiac myocytes increased significantly in sections from CS-stored grafts as compared to the controls (Fig. 6E-F). In contrast, the number of immunoreactive cells was significantly reduced in the CS-$CH_4$ group (Fig. 6G-H).

**Discussion**

The aim of this study was to investigate whether adding $CH_4$ to a cold preservation solution modifies the graft function in experimental HTX. The haemodynamic efficacy of CS-$CH_4$ storage was evidenced by increased LV systolic pressure, cardiac contractility and coronary circulation, as compared to 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 pro-apoptotic signalling 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 ischaemia, 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 NO, carbon monoxide (CO) and hydrogen sulphide ($H_2S$) 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 nontoxic *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 which demonstrate that $CH_4$ can modulate NO-, CO- and $H_2S$-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 Nrf2/Keap1 or NF-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 pro-inflammatory activation (evidenced by reduced TNF-α, IL-1β, MPO activity and oxidative DNA damage) and maintained cardiac function four weeks post-infarction.[11]

The immediate hemodynamic circulatory consequences of $CH_4$-enriched graft storage included a significantly improved myocardial contractility and a parallel increase in coronary blood flow during the 60 min reperfusion. These data suggest that in this short time frame exogenous $CH_4$ can restrain or counteract those mechanisms, which 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 four weeks post-infarction, with improved left ventricular ejection fraction, diastolic volume and contractility, among other improvements, compared to non-$CH_4$-treated rats.[11]

In spite of the wide range of research to map the biological effects, the role of $CH_4$ in cold ischaemia or organ transplantation settings has not yet been investigated. Therefore, we manufactured a $CH_4$-saturated HTK solution according to reported protocols and in a concentration range, which 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 ischaemia 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 ADP utilisation, which contributed to the maintenance of OxPhos capacity. Furthermore, cytochrome-c release, a sign of mitochondrial inner membrane injury, was also reduced (Fig. 7).

The process of cold ischaemia-induced cellular damage with the dual contribution of ER and mitochondria is relatively well characterised. Hypoxic conditions trigger changes in cytoplasmic resting potential and, through the activation of ER-mediated $Ca^{2+}$ transport, increase the expression of HIF1α, 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 HIF1α expression, mRNA expression of VLDLr

and the pro-apoptotic transcription factor CHOP are also increased, and by the end of this process, CHOP upregulates Bim mRNA expression and activates Bax protein to translocate from the cytosol to the mitochondria.[31, 35] Our results demonstrate that cold ischaemia 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 HIF1α expression in cardiomyocytes. In addition, higher intracellular $Ca^{2+}$ can activate GSK3β, the pro-apoptotic factor in the intrinsic mitochondrial apoptotic pathway.[36] These mitochondrial changes raise the expression of Bax protein and its activation, while modified pro-apoptotic Bax and anti-apoptotic Bcl2 levels lead to further pro-apoptotic 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 signalling pathway was also reduced (Fig. 7).

Our study has several limitations. Firstly, 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 roles in the reduction of tissue damage in this setup. Secondly, a rat model of heterotopic abdominal heart transplantation 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 preclinical 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 ischaemia and isogenic HTX in rats. Oxidoreductive imbalance is an inevitable consequence of *ex vivo* periods and a basis for a cascade of pro-inflammatory 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.

**Conflict of Interest Statement and Funding Sources**

The authors declare no conflicts of interest. This study was supported by a National Research Development and Innovation Fund of Hungary (NKFI Grant K120232 and NVKP_16-1-2016-0017 ('National Heart Program')), an Economic Development and Innovation Operative Programme grant (GINOP-2.3.2-15-2016-00015), an FIKP programme grant (TUDFO/47138-1/2019-ITM) and a Human Resources Development Operational Programme grant (EFOP-3.6.2-16-2017-0006).

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Figure 1.

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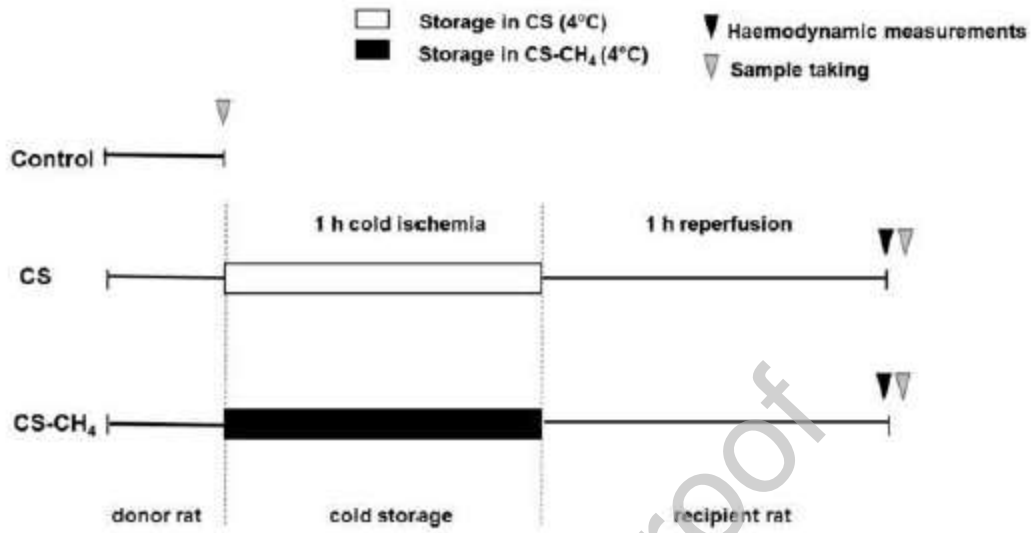


**Fig. 1. Experimental protocol.** The heart grafts were explanted from the donors and stored for 60 min in cold preservation solution before heterotopical heart transplantation (HTX). 60 min after the start of reperfusion, haemodynamic measurements were performed in the recipients to evaluate early 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 ischaemia period; in the CS-$CH_4$ group, the protocol was identical, except that $CH_4$-enriched CS solution was used.

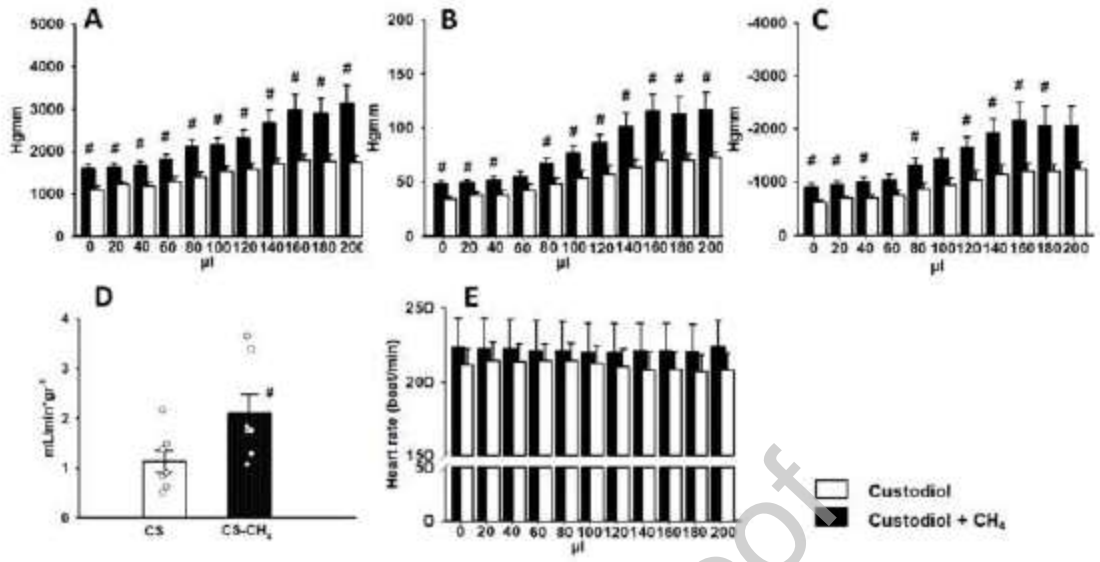


**Fig. 2. Left ventricular (LV) pressure-volume relations and coronary blood flow (CBF) changes.** (A) Maximal slope of the systolic pressure increment (dP/dtmax), (B) maximal left ventricular systolic pressure (LVSP), (C) diastolic pressure decrement (dP/dtmin) (D) coronary blood flow (CBF) and (E) 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).

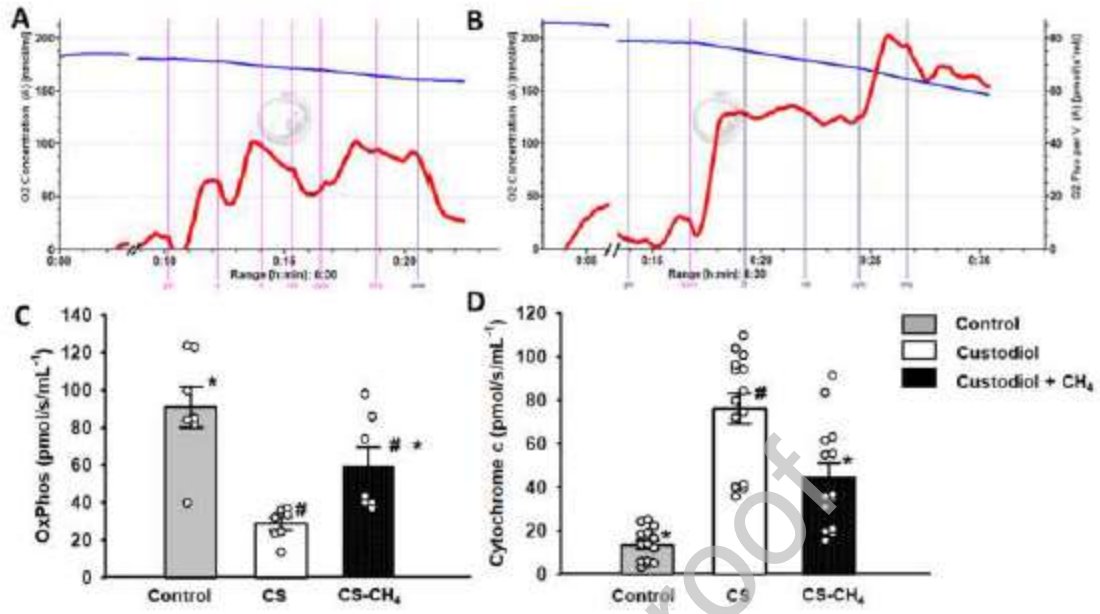


**Fig. 3. Oxygen consumption of cardiac mitochondria (pmol/s/mL$^{-1}$).** The upper charts (A; B) demonstrate representative records of mitochondrial oxygen consumption of (A) CS-stored or (B) CS-$CH_4$-stored samples measured by high-resolution respirometry. The blue line represents the instantaneous oxygen concentration in the respiration chamber, while the red line indicates the simultaneous oxygen consumption of the sample. The lower right-hand chart shows oxidative phosphorylation (OxPhos) capacity, and the lower left-hand chart demonstrates cytochrome c release data. Grey 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).

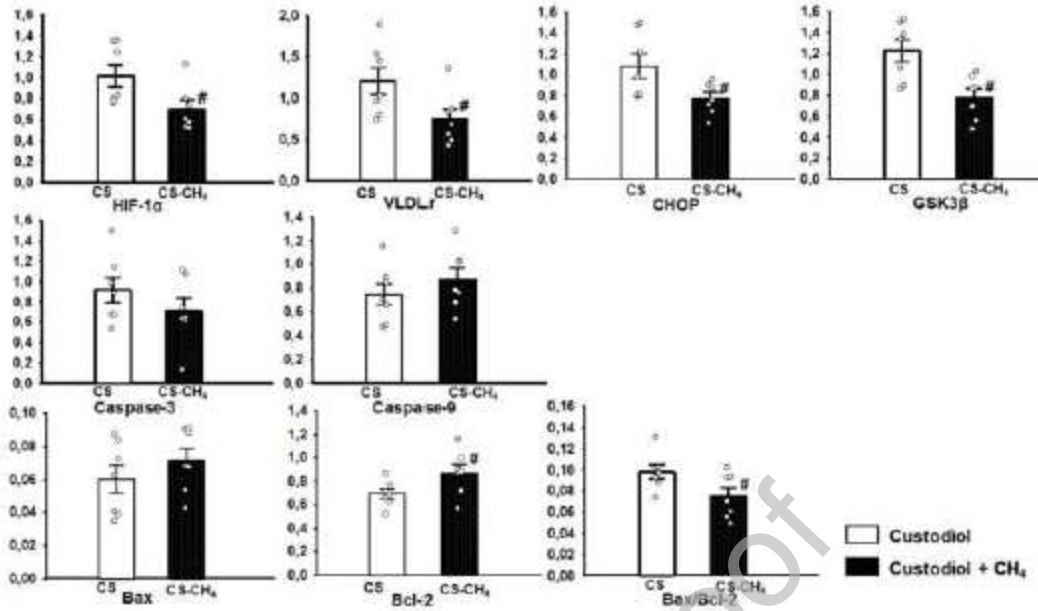


**Fig. 4. Gene expression changes.** White columns: CS group; black columns: CS-$CH_4$ group. Data are presented as means ± SEM, individual data points are shown (n=7-7). #P<0.05 vs. CS (one-way ANOVA, Tukey's test). HIF1α: hypoxia-inducible factor 1-alpha; VLDLr: very low-density lipoprotein receptor; CHOP: CCAAT/enhancer binding protein (C/EBP) homologous protein; GSK3β: glycogen synthase kinase-3 beta. Bcl-2: B-cell lymphoma 2; Bax: bcl-2-like protein 4.

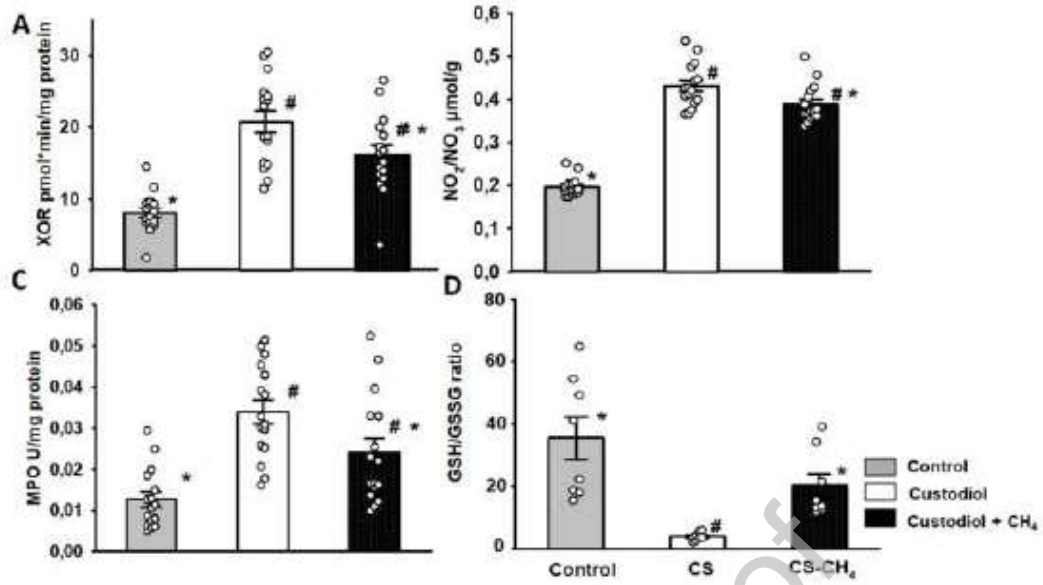


**Fig. 5. Biochemical assays for oxidoreductive stress parameters.** (A) Tissue xanthine oxidoreductase (XOR) activity, (B) nitrite/nitrate ($NO_2/NO_3$) levels, (C) myeloperoxidase (MPO) activity and (D) reduced glutathione and oxidized glutathione disulfide (GSH/GSSG) ratio. Grey columns: control group; 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 duplicates).*P<0.05 vs. CS; #P<0.05 vs. control (one-way ANOVA, Tukey's test).

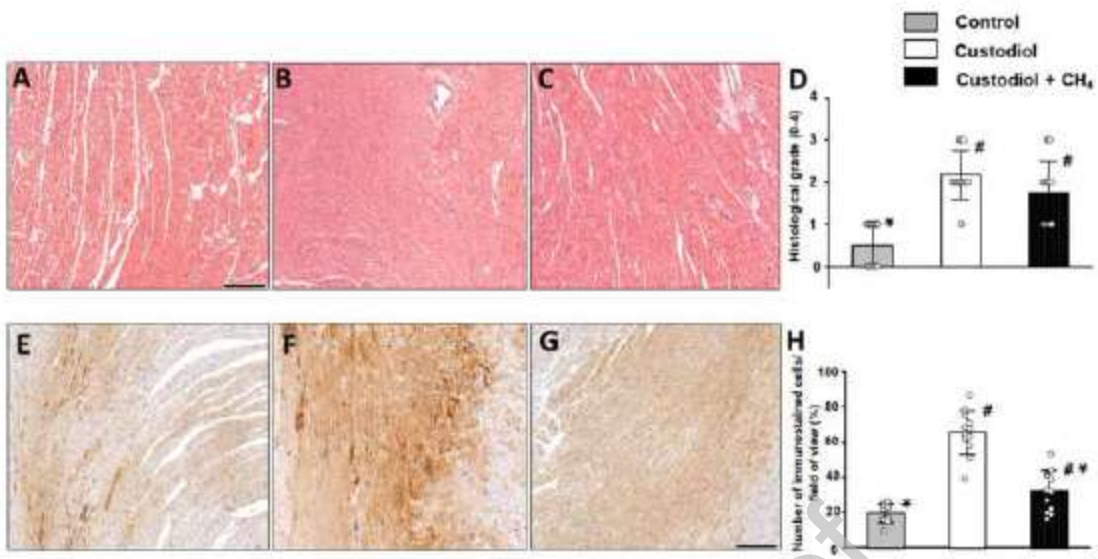


**Fig. 6. Histology and immunohistochemistry.** Haematoxylin and eosin (H&E) staining (A-C) of heart sections. (A) Control group, (B) CS group and (C) CS-$CH_4$ group. (D) Histological grading of groups represents a composite of number of damaged myocytes and number of foci of damage (n=12-12).

SERCA1 immunostaining (E–G) of heart sections. (E) Control group, (F) CS group, (G) CS-$CH_4$ group and (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: 200x. Marker: 200 µm.

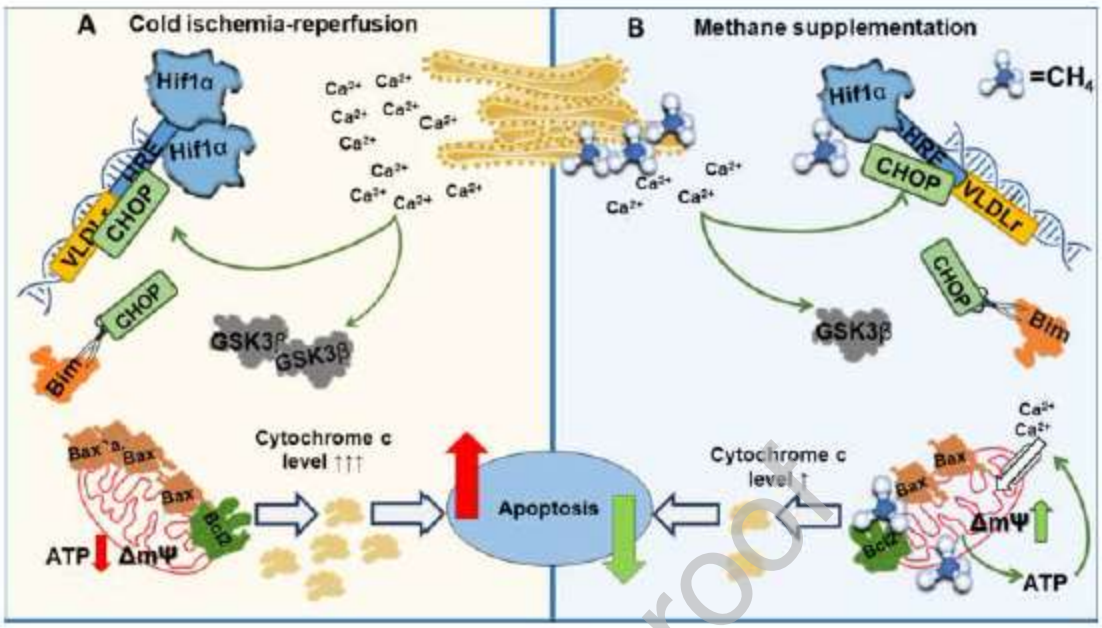


**Fig. 7. Proposed effects of methane supplementation on cold ischaemia-induced intracellular changes. Panel A:** Cold storage, ischemic and hypoxic states will lead to perturbations in normal endoplasmic reticulum (ER) functions. ER stress is accompanied by intracellular $Ca^{2+}$ overload, which modulates the activation of pro-apoptotic glycogen synthase kinase-3β (GSK3β) and leads to caspase activation. Hypoxia increases hypoxia-induced factor 1α (HIF1α) expression as well, which interacts with the promoter of the very low density lipoprotein receptor (VLDLr) and the pro-apoptotic transcription factor CCAAT/enhancer binding protein (C/EBP) homologous protein (CHOP) by direct binding and by the non-classical hypoxia responsive element (HRE). By the end of this process, CHOP upregulates the Bcl-2 family BH3 protein Bim mRNA expression, which directly activates Bax (also known as bcl-2-like protein 4) to translocate from the cytosol to the mitochondria. These events will trigger cardiomyocyte apoptosis.

**Panel 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 HIF1α decreases,

affecting the mitochondrial pathway of apoptosis by lowering the Bax/Bcl2 ratio, thus creating an anti-apoptotic milieu for cardiac muscle cells.

**Table 1. Myocardium-specific enzyme changes.** Data are presented as means ± SEM. *P<0.05 vs. CS; #P<0.05 vs. control (one-way ANOVA, Tukey's test).

| | **CK (U/L)** | **CK-MB (U/L)** | **LDH (U/L)** | **Troponin T (ng/L)** |
|---|---|---|---|---|
| Control (n=12) | 503±57* | 206±31* | 358±79* | 42±9 * |
| CS (n=12) | 2327±23$^{\#}$ | 527±43$^{\#}$ | 938±108$^{\#}$ | 172±36$^{\#}$ |
| **CS-$CH_4$ (n=12)** | **1507±49*$^{\#}$** | **328±52*$^{\#}$** | **732±96*$^{\#}$** | **110±21*$^{\#}$** |