THE PROTECTIVE ROLE OF METHANE IN MITOCHONDRIAL AND METABOLIC PATHWAYS OF ISCHEMIA/REPERFUSION-INDUCED CARDIAC DAMAGE

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Doctoral (Ph.D.) dissertation

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CONTENTS

List of abbreviations

$_3$H	Tritium
A/R	Anoxia re-oxygenation
AIF	Apoptosis inducing factor
Akt	Protein kinase B
Ama	Antimycin A
ATP	Adenosine triphosphate
Bak	Bcl-2 homologous antagonist killer
Bax	Bcl-2 associated protein X
Bok	Bcl-2 related ovarian killer
Bcl-2	B-cell lymphoma protein-2
Ca^{2+}	Calcium ion
CBF	Coronary Blood Flow
CCCP	Carbonyl cyanide m-chlorophenyl hydrazine
CH_4	Methane
CHOP	CCAAT/enhancer binding protein homologous protein, same as Ddit3
CK	Creatine kinase
CO_2	Carbon-dioxide
CS	Hystidine-tryptophane-ketoglutarate, Custodiol
Ddit3	DNA damage-inducible transcript 3, same as CHOP
DIABLO	Direct Inhibitor of Apoptosis-Binding protein with LOw pI
ER	Endoplasmic reticulum
ETC	Electron transport chain
GSH	Reduced glutathione
GSK-3β	Glycogen synthase kinase-3β
GSSG	Oxidized glutathione disulfide
Hif1α	Hypoxia-inducible factor 1-alpha
HRR	High-resolution respirometry
HTX	Heterotopic transplantation
Hsp	Heat shock protein
I/R	Ischemia-reperfusion
LDH	Lactate dehydrogenase
LV	Left ventricle

LVSP	Left ventricle systolic pressure
Mcl-1	Induced myeloid leukemia cell differentiation protein
MiRO5	Mitochondrial respiratory medium
MPO	Tissue myeloperoxidase
mPTP	Mitochondrial permeability transition pore
mRNA	Messenger RNA
Na^+	Sodium ion
NADH	Nicotinamide adenine dinucleotide hydride
N_2	Nitrogen
NO	Nitrogen monoxide
NOx	Tissue nitrite/nitrate level
Nrf2	Nuclear factor-erythroid2 p45-related factor 2
OxPhos	Oxidative phosphorylation capacity
PAS	Photoacoustic spectroscopy
PI3K	Phosphoinositide 3-kinase
qPCR	Quantitative real-time PCR
RET	Reverse electron transport
ROS	Reactive oxygen species
Rot	Rotenone
ROX	Residual oxygen comsumption
Smac	Second mitochondria-derived activator of caspase
SERCA1	Sarco(endo)plasmic reticulum Ca^{2+} ATPase
Succ	Succinate
URL	Upper reference limit
VLDLr	Very low-density lipoprotein receptor
XOR	Xanthine oxidoreductase

List of original papers relating to the subject of the thesis

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Horváth T, **Jász DK**, Baráth B, Poles MZ, Boros M, Hartmann P. (2020) Mitochondrial Consequences of Organ Preservation Techniques during Liver Transplantation. *INT J MOL SCI. 10;22(6):2816*. doi: 10.3390/ijms22062816. **IF: 4.556**

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Baráth B, **Jász DK**, Horváth T, Baráth Be, Maróti G, Strifler G, Varga G, Sándor L, Perényi D, Tallósy Sz, Donka T, Jávor P, Boros M, Hartmann P (2022). Mitochondrial Side Effects of Surgical Prophylactic Antibiotics Ceftriaxone and Rifaximin Lead to Bowel Mucosal Damage. *INT. J. MOL. SCI. 23(9):5064*, doi:10.3390/ijms23095064 **IF:5.924**

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1. INTRODUCTION

1.1. Ischemia-reperfusion (I/R)

Cardiac muscle cell contraction is essential for maintaining the life-long need of blood flow in our body. This mechanism, however, requires loads of oxygen to work in proper way. Most common cardiac conditions (e.g. myocardial infarction, hypertrophy, etc.) involve ischemia (decrease in blood flow and consequential loss of tissue oxygen level) in their pathomechanism. Warm ischemic changes are built around the hypoxic intracellular milieu of cardiac muscle cells. Low intracellular oxygen level leads to mitochondrial deceleration, which causes adenosine-triphosphate (ATP) depletion [1]. Disability of mitochondrial electron transport chain (ETC) also causes upstream accumulation of succinate (Succ) and nicotinamide adenine dinucleotide hydride (NADH), which activates several alternative metabolic pathways. ATP loss of the cell also realized in the diminished function of primarily or secondarily ATP dependent ion channels [2]. These changes can hinder intracellular homeostasis and can cause adaptive changes to improve survival of cardiac muscle cells. Although ischemic condition can modify cellular physiology, it is important to prevent any further damage, which will occur when cardiac muscle cells are re-flooded with oxygen in the reperfusion period. Subsequent overdosage of oxygen and previously decelerated mitochondria together lead to production and release of reactive oxygen species (ROS). Cold ischemia is based on the same concept of hypoxia as warm ischemia, however, cold (4°C) environment decelerates metabolic rate of organs and tissues, so it can be used to induce effective protection against harmful metabolic changes. This characteristic makes cold ischemia suitable for organ preservation in transplantation. However, recent studies explored some other issues regarding cold ischemic injury [3]. Cold ischemia also exhausts energy stores while ATP production is decreased and anaerobic metabolic pathways activate. These changes result in development of acidic environment. Increased proton levels stimulate the type 1 Na^+/H^+ antiporter, while the Na^+/K^+ ATPase is also disturbed, which causes intracellular accumulation of Na^+. Excessive load of Na^+ alters resting membrane potential of the cell, thus exchange of Na^+ and Ca^{2+} become more significant which is characterized by the release of large amount of Ca^{2+} in the cell. Intracellular Ca^{2+} overload is taken up by the mitochondria, but remained high intracellular Ca^{2+} concentration further activates pro-apoptotic enzymes, such as phospholipases, calmodulin regulated proteases and endonucleases. Ca^{2+} is also released from the endoplasmic reticulum (ER) as a consequence of cold environment. Ca^{2+} not only provokes pro-apoptotic enzyme activity, it promotes

changes in gene expression via the hypoxia-inducible factor 1-alpha (Hif-1α) pathway and even take part in the inner pathway of apoptosis by modulating mitochondrial death signals of Bax/Bcl2 system [4].

1.2. Mechanism of I/R-induced mitochondrial dysfunction

Avoiding mitochondria-connected damage of the graft should be considered as the main goal to improve organ preservation methods. Life of the cell is strongly attached to mitochondrial metabolism through mechanisms such as ATP synthesis, pro- and anti-apoptotic signaling pathways. I/R initiates organ dysfunction through intracellular hypoxic damage, in which the mitochondria are highly involved. This contributes to unsuccessful graft transplantation [5-7]. Absence of blood flow and consequential ischemia causes a relatively well-discovered metabolic shift into anaerobic glycolysis involving the reduction of electron transport through ETC. Hypoxia also inhibits the function of succinate dehydrogenase (respiratory complex II), thus leading to NADH and succinate accumulation; which is considered as a metabolic marker of ischemia [8-10].

Under hypoxic condition, oxidative phosphorylation of the mitochondria produces lower amount of ATP. Low intracellular ATP levels decrease the function of Na^+/K^+ ATPases, therefore resting membrane potential changes. This state can be characterized by elevated intracellular Ca^{2+} levels due to the altered function of Na^+/Ca^{2+} transporter and increased Ca^{2+} efflux from the ER and mitochondria. Accumulated intracellular Ca^{2+} is proved to influence mitochondrial permeability, potentiating ROS production, both of which are enhanced during reperfusion [9]. Reperfusion is characterized by subsequent oxygen overload. Therefore, the reaction of previously leaked electrons and highly accessible oxygen result in ROS accumulation through the impaired ETC. ROS-mediated damage to ETC complexes leads to more pronounced ATP depletion, completing the vicious circle of I/R, eventually initiating apoptosis [10]. Increased ROS disintegrates mitochondrial lipid membrane, which opens mitochondrial permeability transition pores (mPTPs) promoting Ca^{2+}-induced mitochondrial swelling. Mediators of the intrinsic apoptotic pathway are released into the cytoplasm due to increased mitochondrial membrane permeability.

1.3 Cardiac transplantation

In practice, transplantation involves both warm ischemic (during the surgical process) and cold ischemic (during cold storage) injury in its pathomechanism. Therefore, reduction of

reperfusion damage to improve graft function and minimize secondary cytokine storm or graft rejection is a highly important clinical goal [11] .

Transplantation is necessary in the treatment of end-stage organ failure according to several guidelines, however improving graft survival and providing patient safety is still in the focus of clinical sciences [12]. The necessity of allograft protection and procurement cannot be emphasized enough as these factors correlate to graft survival rate in the clinical practice. Nowadays, static cold storage is still the most applied method for organ preservation after surgical explantation [13]. Nevertheless, currently used techniques provide limited protection against transient anoxia or reperfusion-induced tissue damage, therefore, seeking for advanced methods to avoid cold storage-related organ dysfunction is highly researched [14].

1.4. Chemical properties and bioactivity of methane (CH_4)

CH_4 is a colorless, odorless, omnipresent gaseous molecule, while being inert at ambient temperature and pressure. In the upper layer of the atmosphere CH_4 plays a role in ozone formation by reacting with hydroxyl radicals, however, physico-chemical reactions of CH_4 in eukaryotic organisms are still not fully discovered [9]. Early studies described that intraarterially administered ^{14}C-labelled CH_4 transformed into [^{14}C]CO_2 in sheep [15]. Later [^{14}C]CO_2 transformation into bound ^{3}H and carbon was proved in mammalian liver tissue [16].

These results indicated further investigation to explore the biological role of endogenous CH_4 in several eukaryotic organisms (fungi, algae, plants and animals) and proofs are now available for the production of CH_4 as a result of aerobic metabolic processes [9, 17-19].

Exogenously administered CH_4 was also investigated by many research groups [20-22]. *In vivo* studies described the biological effects of CH_4 on intestinal peristalsis and motility parameters [23-25] and improved survival rate in hemorrhagic shock in rats [26]. CH_4-enriched fluids are proved to be protective against the consequences of I/R-induced conditions: inhalation of normoxic air containing 2.5% CH_4 has anti-inflammatory effect in intestinal and liver I/R injury [22]. Further experimental data demonstrated anti-oxidative, anti-apoptotic, and anti-inflammatory effects of CH_4 inhalation or CH_4-enriched fluid therapies in I/R and in sepsis. Most importantly, CH_4 administration reduced the sepsis-induced ER-stress in the kidney through the suppression of GRP78/ATF4/CHOP/caspase-12-mediated apoptotic pathway [27].

Potentially dangerous characteristics of (5-15% of CH_4 in gas mixture can be flammable and explosive) explain that dissolving CH_4 in fluids would insure more safety in terms of its

application. CH_4–enriched fluids and treatments were investigated in multiple experimental designs, where CH_4 gas was perfused in different composition of physiological saline solution [5, 23, 25, 28-31]. Nevertheless, the effects of CH_4 in the settings of organ transplantation and the circumstances and conditions that ensure the best efficiency in transplantation solutions have not been described yet.

1.4.1. The effect of CH_4 on I/R injury

Distribution of CH_4 is well-characterized *in vivo*. Due to its apolar property, CH_4 is capable of penetrating membranes and diffusing into mitochondria; therefore, the potential effect of CH_4 on mitochondrial respiration can also be highly emphasized. Mitochondria are intracellular organelles with bacterial origin, so it can play various physiological roles, such as energy supply, ROS formation, Ca^{2+} homeostasis, and programmed cell death. Several studies have demonstrated that mitochondria can be influenced by exogenously administered CH_4 in I/R injury via potential non-specific physico-chemical alterations of phospholipid bilayer of biological membranes [5, 28]. Mitochondrial respiration and ETC can also be modified by CH_4 administration as loads of *in vivo* and *in vitro* studies have been investigated [5, 31, 32].

I/R injury affects the ETC, which is embedded in the inner layer of mitochondrial membrane. Studies proved that CH_4 restored mitochondrial functions by nuzzling the rigidity of ETC to endure more significant oxidative stress during reperfusion [5, 22]. Similar to other simple molecules, CH_4 is likely to exert site-specific action on mitochondrial proteins or protein complexes. It seems that complex IV (cytochrome c oxidase), one of the protein complexes of the mitochondrial ETC, may be responsible for the action of CH_4 [5].

Several studies suggested that CH_4 modulates the intrinsic pathway (also known as mitochondrial pathway) of apoptosis [5, 25, 26, 30-33]. While CH_4 proved to be an anti-apoptotic agent, the mechanism behind its effect is not properly understood. Studies demonstrated that apoptosis reducing effect of CH_4 was associated with the release of cytochrome c and regulation of Bcl-2 family proteins, the latter causing interplay between pro-apoptotic and anti-apoptotic proteins [5, 6, 28].

1.5. The role of mitochondria in I/R damage

Mitochondria are intracellular organelles, which function mainly is to provide energy for cell metabolism in the form of ATP. Also, mitochondria take part in several biochemical pathways of cellular metabolism, such as β-oxidation of fatty acids, Krebs cycle and

alternative carbohydrate metabolic pathways. Although energy production through mitochondrial ETC is substantial in terms of the life of eukaryotic cells; however, oxidative phosphorylation of mitochondria can be responsible for only the metabolism of the cell but also its death. In response to noxious stimuli, mitochondrial proteins can emerge into the cytoplasm to play essential role in the apoptotic process [31, 34, 35]. These proteins include e.g. caspases and procaspases, adenylate kinase 2, apoptosis inducing factor (AIF) and even proteins of the respiratory chain, such as cytochrome c, Smac/Diablo (recently discovered caspase coactivator) and some heat shock proteins (Hsp10, Hsp60). Apoptotic activity of mitochondria and protein release into the cytoplasm as a pro-apoptotic signal may be regulated, triggered or blocked by mitochondrial proteins of the Bcl-2 family. They can be distributed into two classes: pro-apoptotic proteins (such as Bcl-2 associated X protein (Bax), Bak, and Bok) and anti-apoptotic Bcl-2 family proteins (such as Bcl-XL, Bcl-w, Mcl-1, A1, Bcl-Rambo, Bcl-L10, and Bcl-G). Primarily, Bcl-2 reduces cytochrome c release to develop its anti-apoptotic characteristics. In contrast, stress-induced activation of pro-apoptotic members of Bak or Bax leads to increased permeability of mitochondrial outer membrane and subsequent emission of intermembrane space proteins e.g. cytochrome c and procaspases. Cytochrome c is a small peptide loosely associated with the outer side of the inner mitochondrial membrane and physiologically carries electrons between complex III and IV to provide the sufficient function of ETC. Membrane damage provokes its release into the cytoplasm where it initiates caspase cascade-mediated apoptosome formation, thus leading to apoptosis [28].

Mitochondrial ETC can be the main character in developing reperfusion injury. Overcharging the ETC with oxygen in the reperfusion period leads excessive function of ATP synthase enzyme known as complex V. However, oxygen overload not only effects ATP synthesis, but also accelerates the electron transport, which is leading to electron leakage through different complexes. Leaked electrons can be redirected into the ETC, or can reduce NAD^+ leading to NADH accumulation. Another possibility for decreasing the locally elevated electron levels is using the already high amount of oxygen in the system thus creating ROS which is the main cause of oxido-reductive stress during reperfusion. High ROS levels can generate the apoptotic milieu of the cell, which later can kill the cell internally. Increased amount of intracellular ROS can lead to DNA damage, formation of inflammosomes and apoptosomes, and can influence intracellular transport system, such as through the Golgi apparatus and ER or through the nucleus and cytosol.

2. MAIN GOALS

We hypothesized that addition of CH_4 to a cold preservation solution modifies the I/R-damage of the graft (Study I).

- Therefore, we examined the effect of CH_4-enrichment on the oxido-reductive and nitrosative stress pathways in the myocardium in a rat model of isogenic heart transplantation.
- We also investigated the expression of ER stress and mitochondrial apoptosis markers as possible underlying mechanisms of the cytoprotective action of CH_4 enrichment.

As a next step, we hypothesized that CH_4 influences the extramitochondrial Ca^{2+} streams, which play a major role in the ER–mitochondria interactions (Study II).

- For this purpose, we investigated the effects of exogenous Ca^{2+} on the membrane potential changes of isolated cardiac mitochondria.
- Finally, we aimed to explore the potential target site on ETC responsible for the mitochondrial effects of CH_4 by using selective substrates and inhibitors of the respiratory complexes.

3. MATERIALS AND METHODS

3.1. Study I. Investigation of the effects of CH_4-enrichment of preservation solution on heart grafts in an experimental transplantation setup

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.

3.1.1. Production of CH_4-enriched Custodiol (CS)

Commercially-available 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 [36]. 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.

3.1.1.1. Gas chromatography

The CH_4-enriched products were analysed with an Agilent 4890 gas chromatograph using an Equity-1 column connected to a flame ionization detector. HP Chemstation software was used for data evaluation. CH_4 calibration was performed with the multi-point syringe technique, which included the low range area. 15 μl aliquots of CS solution were added to 2 ml gas-tight vials, which were previously degassed in an ultrasonic bath for 15 min. 50 μl gas samples (controls or graft-exposed) were injected into the gas chromatograph three times in a row. The average CH_4 concentration in the fluid phase was 6.57 ± 0.27 μmol/ml.

3.1.1.2. Photoacoustic spectroscopy (PAS) measurements

The stability of dissolved CH_4 concentration in the CS solution was determined by PAS, a special mode of spectroscopy which measures optical absorption indirectly via the conversion of absorbed light energy into acoustic waves [37]. The set-up allows for dynamic, on-line measurements of CH_4 concentrations with a minimum detectable concentration of 0.25 ppm. The CH_4 measurements were performed in duplicates over a period of 120 min at 4°C. After

30', 60', 90' and 120 min equilibrium, the CH_4 concentration of the solution was measured in a gas-tight cuvette (containing 10 cm^3 of airspace and 10 cm^3 of liquid, respectively). The CH_4 concentration in the airspace of the cuvette is proportional to the CH_4 content dissolved in the liquid, and the content of dissolved gas bubbled through the liquid phase can be calculated based on the measured concentrations and the volume flow. The average CH_4 concentration in the fluid phase remained stable (105 ± 17.5 ppm/ml) during the 120-min investigations.

3.1.2. Experimental protocol

Isogenic male Lewis to Lewis heterotopic transplantation (HTX) (n=36) was performed as described previously [6]. Briefly, after excision from the donors, the grafts were cold stored in a transplantation solution for 60 min (cold ischemia time), which was followed by HTX and a 60-min reperfusion period. At the end of the reperfusion, hemodynamic measurements were performed 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.

The animals were randomly allocated into three groups. In group 1 (control, n=12), donor rats underwent the same surgical procedure until the explantation, but the hearts were not subjected to cold storage and were not transplanted. In group 2 (n=12), the explanted grafts were stored in CS solution at 4°C during the 60-min cold ischaemic period, while the grafts were stored in CH_4-enriched CS during the cold ischaemic period in group 3 (n=12) (Fig 1).

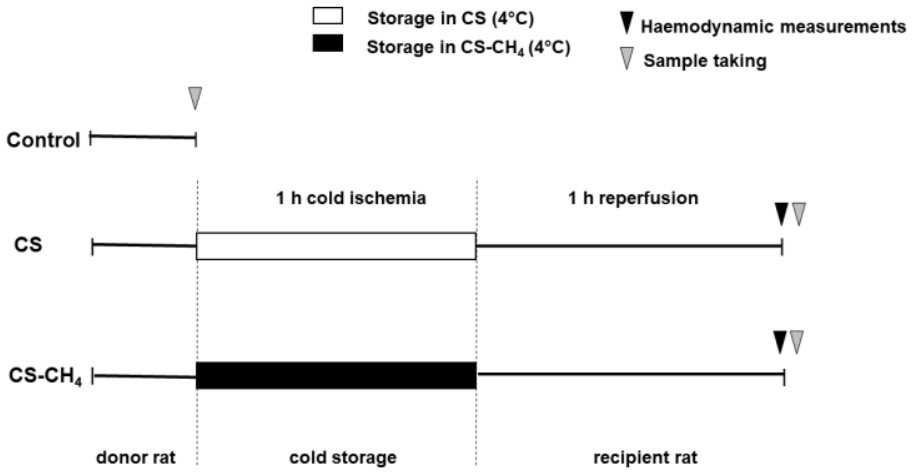

Fig 1. Experimental protocol. The heart grafts were explanted from the donors and stored for 60 min in cold preservation solution before HTX. 60 min after the start of reperfusion, hemodynamic measurements were performed in the recipients to evaluate early post-transplant graft function. Thereafter, 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.

3.1.3. Rat model of heterotopic heart transplantation (HTX)

HTX was performed in isogenic Lewis rats to avoid organ rejection. The technique has previously been described in detail [6]. Briefly, donor rats were anaesthetised with isoflurane, and heparin (25000 IU) was administered intravenously. A bilateral thoracotomy was performed to expose the heart and cold (4°C) cardioplegic CS solution (Dr. Franz Köhler Chemie GmbH, Bensheim, Germany, n=12) or CH_4-enriched CS (CS-CH_4, n=12) was infused into the coronaries via the aorta. The superior and inferior vena cava and the pulmonary veins were tied en masse with a suture, and the heart was excised with the aortic arch for the measurement of hemodynamics, including the coronary blood flow (CBF). After excision, the grafts were stored either in cold CS or CS-CH_4 for 60 min. Control hearts underwent the same surgical procedure but were not subjected to cold storage and transplantation (Control, n=12). The recipient rats were anaesthetised with isoflurane and

then heparinized (400 IU/kg iv), and body temperature was maintained at 37°C on a heating pad. Approximately 2-cm segments of the infrarenal aorta and the inferior vena cava were isolated and occluded with small-vessel forceps. The aorta and the pulmonary artery of the donor heart were anastomosed end to side to the abdominal aorta and the vena cava of the recipient, respectively. The duration of the implantation was standardized at 60 min (ischaemic period) to minimize time-related variability between experiments. After completion of the anastomoses, protamine (400 IU/kg iv) was administered to antagonize heparin effects. The occlusion was released, and the donor heart was reperfused with blood *in situ* for 60 min (see further as in Fig 1).

3.1.4. Hemodynamic measurements

60 min after transplantation, a 3F latex balloon catheter (Edwards Lifesciences Corporation, Irvine, CA, USA) was introduced into the left ventricle (LV) via the apex to determine the maximal LV systolic pressure (LVSP), dP/dtmax (maximal slope of systolic pressure increment) and dP/dtmin by a Millar micromanometer (SPR-838, Millar Instruments) at different LV volumes (20-180 μl). LV pressure-volume relationships were constructed from these data. The CBF of the graft was measured with an ultrasonic flow meter (Transonic Systems Inc., Ithaca, USA) mounted on the donor ascending aorta.

3.1.5. Examination of mitochondrial function

The efficacy of the mitochondrial respiration was assessed from tissue samples homogenized in 3 ml mitochondrial respiration medium (MiRO5) by high-resolution respirometry (HRR, 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 [5]. The respirometry data were normalized to the protein content determined by Lowry's method [5].

3.1.6. 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 (CHOP)), Hif1α, GSK-3β and very low-density lipoprotein receptor (VLDLr).

3.1.7. Biochemical assays

3.1.7.1. Tissue xanthine oxidoreductase (XOR) activity

Heart biopsies were homogenized in a phosphate buffer (pH 7.4) containing 50 mM Tris-HCl, 0.1 mM EDTA, 0.5 mM dithiotreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg ml^{-1} soybean trypsin inhibitor and 10 μg ml^{-1} leupeptin. The homogenate was centrifuged at 4°C for 20 min at 24 000 g and the supernatant was loaded into centrifugal concentrator tubes. The XOR activity was determined in the ultrafiltered supernatant by a fluorometric kinetic assay based on the conversion of pterine to isoxanthopterine in the presence (total XOR) or absence (XO activity) of the electron acceptor methylene blue [38].

3.1.7.2. Reduced glutathione and oxidized glutathione disulfide (GSH/GSSG) ratio in heart homogenates

The GSH/GSSG ratio was determined using a Fluorimetric Gluthatione Assay Kit (Sigma Aldrich, Budapest, Hungary). The GSH content of the sample can be determined by quantifying the thiol concentration in biological samples by reacting with the thiol groups they contain. The adduct can be detected by fluorimetry at 478 nm. The GSSG content of the sample was calculated following the manufacturer's recommendations.

3.1.7.3. Tissue myeloperoxidase (MPO) activity

The MPO activity was measured in biopsies using a method developed by Kuebler at al [39]. Briefly, the tissue was homogenized with Tris-HCl buffer (0.1 M, pH 7.4) containing 0.1 M polymethylsulfonyl fluoride to block tissue proteases, and then centrifuged at 4°C for 20 min at 24000 g. The MPO activity of the samples was measured at 450 nm (UV-1601 spectrophotometer; Shimadzu, Japan), and the data were referred to the protein content.

3.1.7.4. Heart nitrite/nitrate (NOx) levels

The levels of NOx, the stable end products of NO in the tissues were measured with the Griess reaction. This assay is based on the enzymatic reduction of nitrate to nitrite, which is then converted into a coloured azo compound, which is detected spectrophotometrically at 540 nm [40].

3.1.7.5. Laboratory serum diagnostics

Serum myocardial biomarkers were measured using a chemiluminescence assay (Cobas 6000 automatic chemical analyser (Roche, Hungary). The upper reference limit (URL) for the

creatine kinase (CK), CK-MB isoenzyme, lactate dehydrogenase (LDH) and Troponin T were 2000 U/L, 2000 U/L, 1000 U/L and 10000 ng/L, respectively.

3.1.8. Histology and immunohistochemistry

The hearts were immersed and fixed in buffered paraformaldehyde solution (4%) embedded in paraffin, and 5-μm thick sections were stained with haematoxylin and eosin (H&E). Immunohistochemical staining for the ER stress marker sarco(endo)plasmic reticulum Ca^{2+} ATPase (SERCA1) was performed with SERCA1-specific antibody (#S1189, Sigma Aldrich, Budapest, Hungary) as previously described [41]. Histological assessment was performed independently and blindly on coded slides by two investigators (K.D.J. and Z.S.) using a previously described 0–4-grade histological scoring system, representing a composite of number of damaged myocytes and number of foci of damage [42]. SERCA1 immunoreactivity was determined using open-resource imaging software (ImageJ version 1.8) and expressed as a percentage of immunopositive cells quantified per field of view.

3.1.9. Statistical analysis

The statistical analysis was performed with SigmaStat 13.0 statistical software (Jandel Corporation, San Rafael, CA, USA). Normal distribution was tested with the Kolmogorov–Smirnov test. In the case of a normal distribution, one-way ANOVA with Tukey's post-hoc test or the two-sample t-test was used. Where data showed no normal distribution, ANOVA on rank was run. Data are expressed as mean ± SEM. $P < 0.05$ was considered as statistically significant.

3.2. Study II. *In vitro* effects of CH_4 during anoxic stress and evaluation of mitochondrial membrane potential changes

We examined the effects of dissolved CH_4 on isolated mitochondria of rat cardiac muscle cells under anoxia re-oxygenation (A/R).

The experimental protocol was in accordance with EU Directive 2010/63 for the protection of animals used for scientific purposes, and it was approved by the National Scientific Ethics Committee on Animal Experimentation (National Competent Authority) under license number V./148/2013. This study also complied with the criteria of the US National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

3.2.1. Isolation of cardiac mitochondria

Adult Sprague Dawley rats were anesthetized to harvest the heart using sodium pentobarbital (45 mg/kg ip). The hearts were homogenized with a glass Potter homogenizer, and the mitochondria were isolated using Gnaiger's method [43]. Isolated mitochondria were suspended in 1 ml mitochondrial respiration medium (MiRO5) for respirometric analysis and were treated as follows: 2 h normoxia (95 % air and 5 % CO_2) or anoxia (100 % N_2) was followed by re-oxygenation (with or without CH_4) for 30 min. At the end of the experiments, mitochondrial functions were tested.

3.2.2. Experimental protocol

Isolated cardiac mitochondria were treated as follows: anoxia was induced using 100% N_2 persufflation for 2 h into a 2 ml volume cuvette containing 1 ml respiratory medium and 1 ml airspace. Anoxia was followed by a re-oxygenation period (95% air and 5% CO_2) with or without 2.2% CH_4 supplementation for 30 min (the A/R and A/R+CH_4 groups, respectively) (n=12–16). In the control groups, the mitochondria were kept in normoxic cuvettes (95% air and 5% CO_2) with or without 2.2% CH_4 supplementation (the normoxia and normoxia+CH_4 groups, respectively). Then the mitochondria were subjected to HRR (Fig 2).

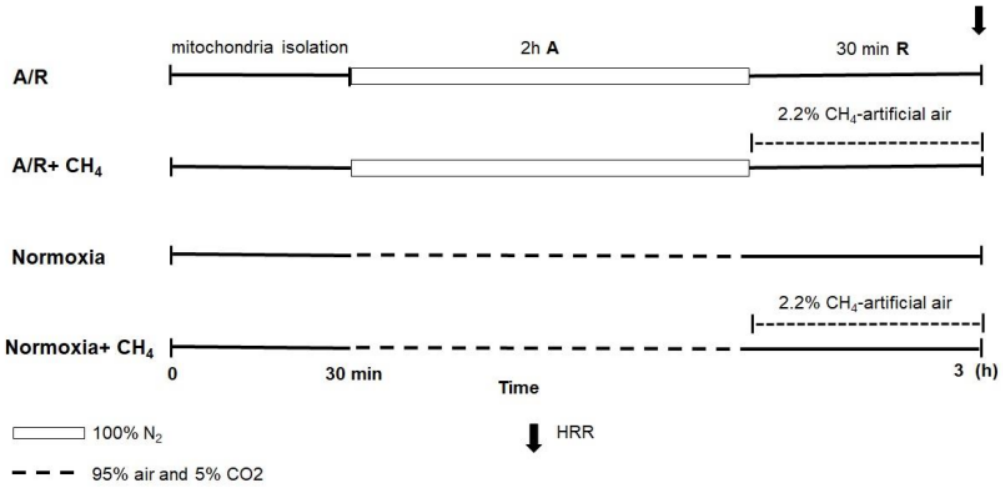

Fig 2. Protocol for isolated mitochondria. The mitochondria were isolated using Gnaiger's method and treated as follows: 2 h normoxia (95% air and 5% CO_2) or anoxia (A) (100% N_2) was followed by re-oxygenation (R) (with or without CH_4) for 30 min. At the end of the experiments, mitochondrial function was tested. CH_4: methane; CO_2: carbon dioxide; N_2:

nitrogen.

3.2.3. Examination of mitochondrial functions

HRR by Oxygraph-2k (Oroboros Instruments, Innsbruck, Austria) was used to examine the oxygen consumption of the isolated cardiac mitochondria under stimuli of different mitochondrial complexes, to determine mitochondrial hydrogen peroxide (H_2O_2) production and to detect changes in mitochondrial membrane potential.

3.2.4. Mitochondrial H_2O_2 production

In this series, mitochondrial H_2O_2 release as a marker of ROS (i.e. superoxide anion) production was monitored fluorimetrically with the Amplex Red/horseradish peroxidase system, whereby Amplex Red (non-fluorescent) is oxidised to Resorufin. H_2O_2 production was calibrated with known amounts of H_2O_2. In this setup, ROS release was investigated by adding oxidizing substrates (10 mM glutamate, 5 mM malate, 20 mM S, 5 mM ADP) to the mitochondria. On isolated mitochondria, the reverse electron transport (RET)-initiated H_2O_2 flux was measured when mitochondria were incubated with 20 mM Succ; it was then blocked by the addition of 1 μM rotenone (Rot). The residual oxygen consumption was estimated after addition of 1 µM antimycin A (Ama, an inhibitor of Complex III) to exclude the effects of oxidative side reactions. Then, free radical leak was also determined as the percentage of oxygen consumption diverted to the production of H_2O_2 in State 3.

3.2.5. Mitochondrial membrane potential

Mitochondrial membrane potential was measured fluorimetrically using the fluorophore agent safranin. First, we added 1μM Rot, 10mM Succ and 1μM CCCP. Finally, residual oxygen consumption (ROX) was determined by adding 1 µM Ama.

4. RESULTS

4.1. Results of Study 1.

4.1.1. Hemodynamic 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 3A–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 3C). 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 3D). There was no statistically significant difference in HR values within and between experimental groups (Fig 3E).

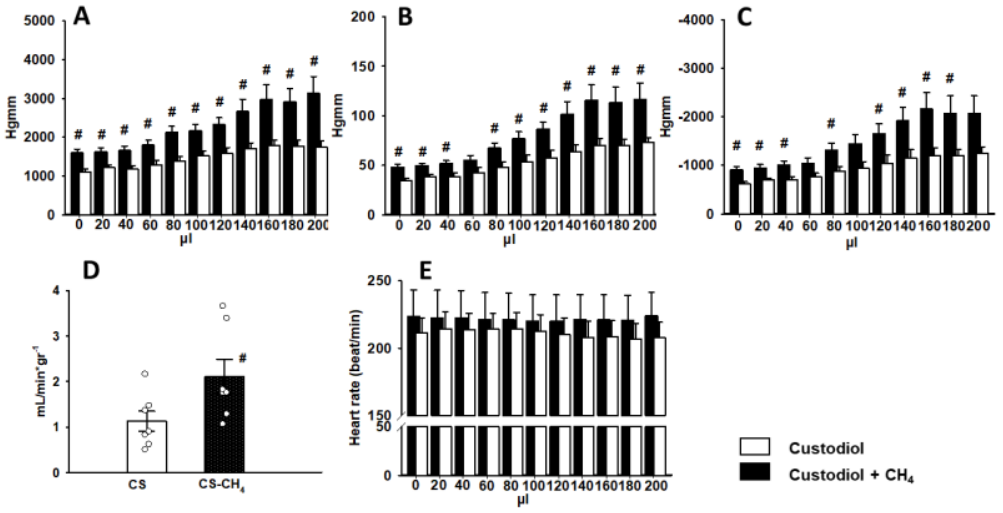

Fig 3. Left ventricular (LV) pressure-volume relations and coronary blood flow (CBF) changes. A. Maximal slope of the systolic pressure increment (dP/dtmax). **B.** Maximal left ventricular systolic pressure (LVSP). **C.** Diastolic pressure decrement (dP/dtmin). **D.** Coronary blood flow (CBF) and **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).

4.1.2. 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 was significantly higher in the CS-CH_4 group (Fig 4C). Mitochondrial respiration in response to cytochrome c (Fig 4D) 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.

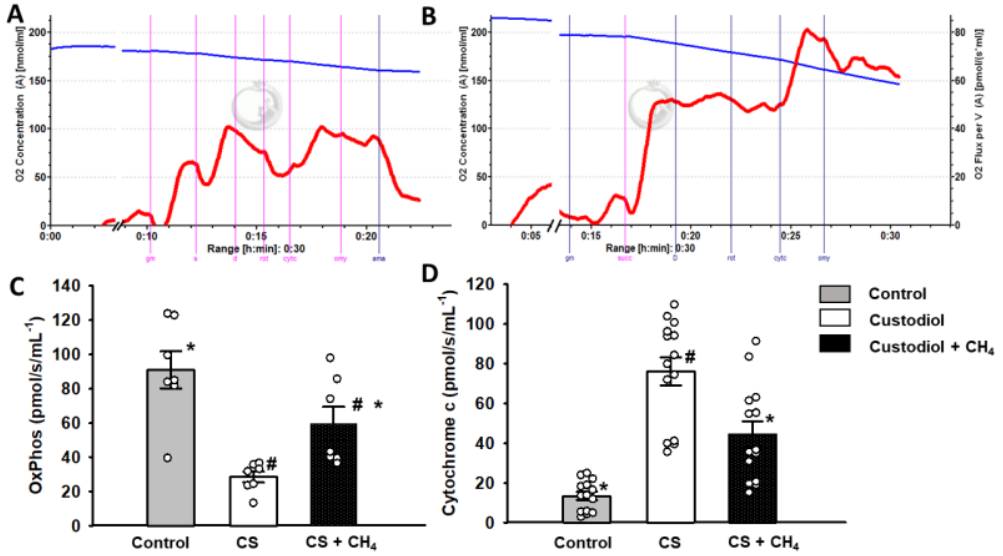

Fig 4. Oxygen consumption of cardiac mitochondria (pmol/s/mL^{-1}). The upper charts (**A. and B.**) demonstrate representative records of mitochondrial oxygen consumption of CS-stored **(A.)** or CS-CH_4-stored **(B.)** samples measured by high-resolution respirometry. The blue line represents the instantaneous oxygen concentration in the respiration chamber, while the red line indicates the simultaneous oxygen consumption of the sample. **C. and D.** The lower right-hand chart shows OxPhos, 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. Individual data points are shown for each of the columns. Data are presented as means ± SEM. *P<0.05 vs. CS; #P<0.05 vs. control (one-way ANOVA, Tukey's test).

4.1.3. 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 (Fig 5). 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 5).

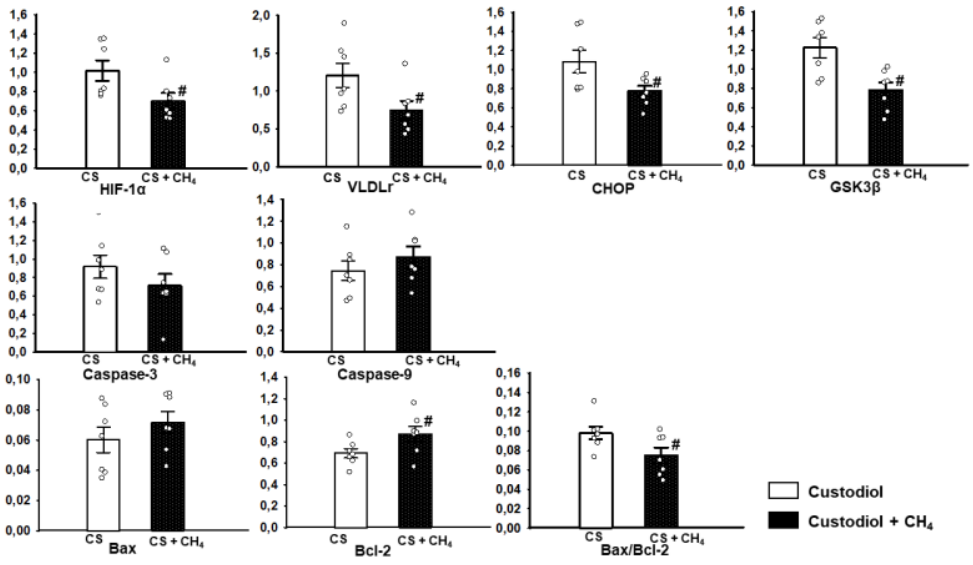

Fig 5. Gene expression changes. White columns: CS group; black columns: CS-CH_4 group. Individual data points are shown for each of the columns. Data are presented as means ± SEM. #P<0.05 vs. CS (one-way ANOVA, Tukey's test). Hif1α: hypoxia-inducible factor 1-alpha; VLDLr: very low-density lipoprotein receptor; CHOP: CCAAT/enhancer binding protein (C/EBP) homologous protein; GSK-3β: glycogen synthase kinase-3 beta. Bcl-2: B-cell lymphoma 2; Bax: bcl-2-like protein 4.

4.1.4. Oxidative stress markers described by biochemical assays

XOR is a key enzyme in reperfusion-induced ROS production; in addition, it can catalyse the reduction of nitrates and nitrites to NO. XOR activity and tissue NOx levels were both significantly decreased when CS-CH_4 was applied during the cold ischemia period relative to the data for the CS group (Fig 6A-B). MPO is mostly produced by activated PMNs. 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 6C). The GSH/GSSG ratio is one of the most important markers of oxido-reductive 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 6D).

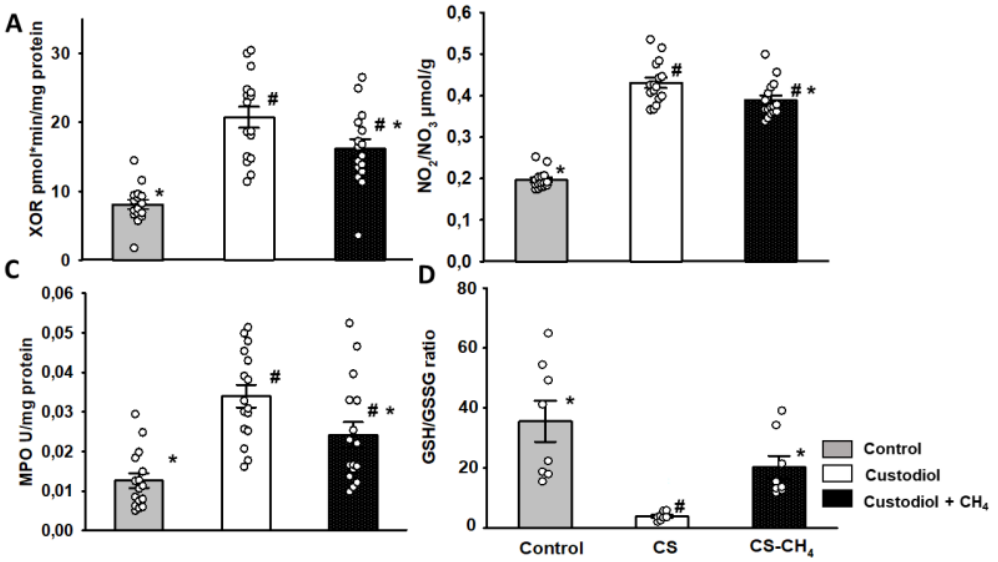

Fig 6. Biochemical assays for oxido-reductive stress parameters. A. Tissue xanthine oxidoreductase (XOR) activity. **B.** Nitrite/nitrate (NO_2/NO_3) levels. **C.** Myeloperoxidase (MPO) activity. **D.** Reduced glutathione and oxidized glutathione disulfide (GSH/GSSG) ratio. Grey columns: control group; white columns: CS group; black columns: CS-CH_4 group. Individual data points are shown for each of the columns. Data are presented as means ± SEM. *P<0.05 vs. CS; #P<0.05 vs. control (one-way ANOVA, Tukey's test).

4.1.5. 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).

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

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 post-hoc test).

4.1.6. Histology and immunohistochemistry

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 7A-B). The architecture of cardiac myocytes was nearly normal in the CS-CH_4 storage group (Fig 7C). These changes were not significantly different from those in the CS group, thus indicating nearly equal potential for tissue protection (Fig 7D). The number of SERCA1 immunoreactive cardiac myocytes increased significantly in sections from CS-stored grafts as compared to the controls (Fig 7E-F). In contrast, the number of immunoreactive cells was significantly reduced in the CS-CH_4 group (Fig 7G-H).

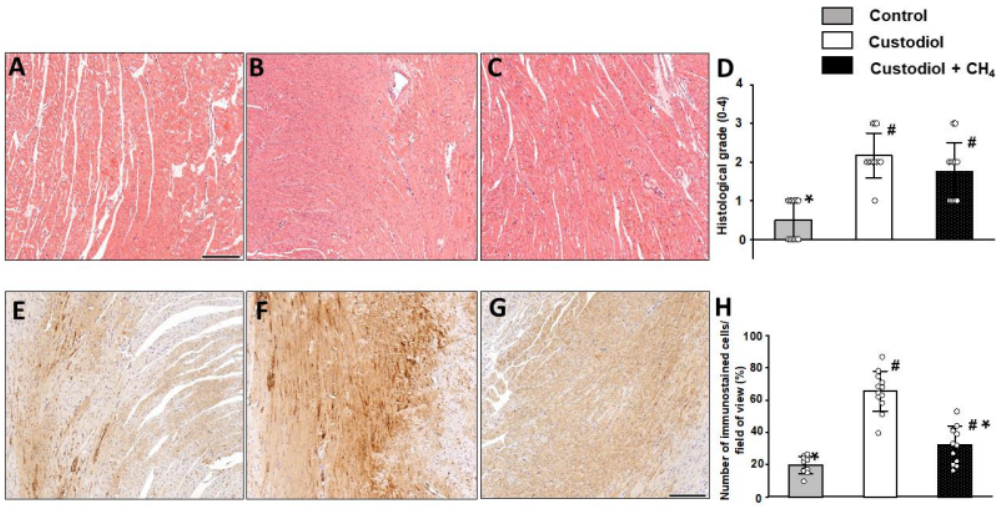

Fig 7. Histology and immunohistochemistry. Haematoxylin and eosin staining (**A-C**) of heart sections. **A.** Control group. **B.** CS group. **C.** CS-CH_4 group. **D.** Histological grading of groups represents a composite of number of damaged myocytes and number of foci of damage.

SERCA1 immunostaining (E–G) of heart sections. **E.** Control group. **F.** CS group. **G.** CS-CH_4 group. **H.** SERCA1 immunoreactivity is demonstrated as percentage of immunopositive cells quantified per field of view. Individual data points are shown for each of the columns. Data are presented as means ± SD. *P<0.05 vs. CS; #P<0.05 vs. control (ANOVA on ranks, Tukey–Kramer). Magnification: 200x. Marker: 200 μm.

4.2. Results of Study 2.

4.2.1. *In vitro* effects of CH_4 during anoxic stress and evaluation of mitochondrial membrane potential changes

Changes to mitochondrial membrane potential have been characterized by means of the potential-sensitive fluorophore safranin. Substrates of respiratory complexes induced a significant hyperpolarization in the mitochondrial membrane under normoxic conditions (Fig 8A). In contrast, hyperpolarization was eliminated in the A/R group. Substrate-induced changes in membrane potential were partially preserved by CH_4 supplementation (Fig 8A). CH_4 applied during the anoxic period lowered the amount of H_2O_2 production in leak states (Fig 8B). In terms of oxygen consumption, we investigated complex I and succinate-semialdehyde dehydrogenase (complex II)-linked respiration separately. CH_4 significantly decreased the oxygen consumption of complex I, while it had no effect on complex II-linked respiration under normoxic conditions. In contrast, CH_4 treatment in the A/R+CH_4 group significantly improved the oxygen consumption of complex II compared to complex I (Fig 8C).

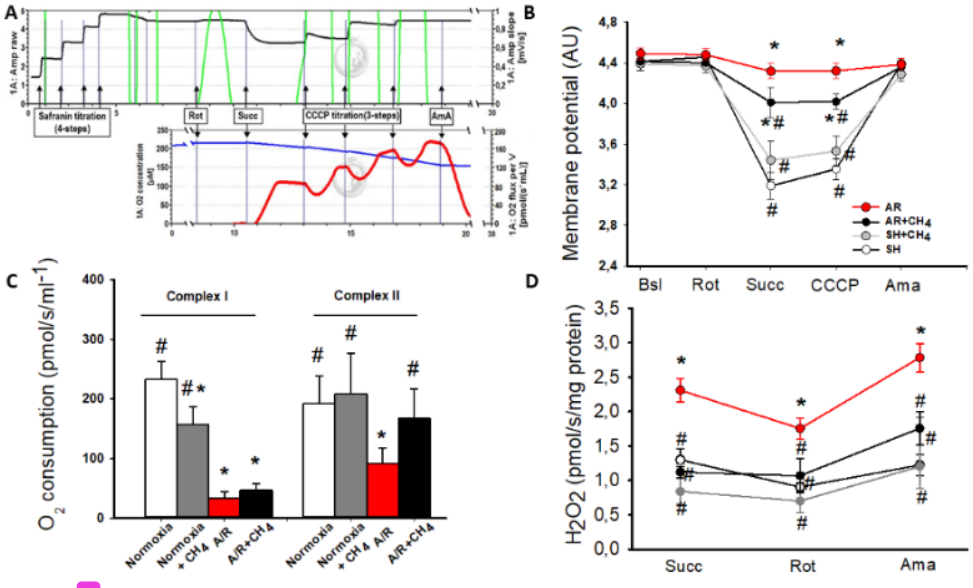

Fig 8. The effect of CH_4 on isolated cardiac mitochondria. The upper left-hand chart demonstrates representative records of mitochondrial membrane potential measured fluorimetrically by HRR. The continuous black line indicates changes in membrane potential; in parallel, the red line signifies the substrate-fuelled respiration. The upper right-hand chart

presents changes in membrane potential in the experimental groups. The A/R group is labelled with a red line, the A/R+CH_4 group with a black line, and the normoxia and normoxia+CH_4 groups with pale and dark grey lines, respectively. The lower left-hand chart shows complex I and II-driven mitochondrial oxygen consumption. The lower right-hand chart demonstrates H_2O_2 production in the case of RET.

4.2.2. Extramitochondrial Ca^{2+} -induced membrane potential changes

Membrane potential changes evoked by extramitochondrial Ca^{2+} in normoxic and anoxic environment was assessed in isolated cardiac mitochondria using a blue fluorescence sensor (excitation 465 nm; gain for sensor: 1000 and polarization voltage: 500 mV) on a high-resolution fluorespirometer. After the four-step titration of safranin, 100µl of samples were loaded into the chambers. Then, mitochondria were treated with 1µl of 0.5 µM Rot and 20µl of 10 mM Succ. The responsiveness of the fluorescent dye was monitored by exogenous 20 µl of 20mM $CaCl_2$ in three-step titration until reaching a plateau. The Ca^{2+} signal was eliminated with the addition of 1 µl of 1 µM Ama. Ca^{2+}-related changes were expressed as the rate of changes in fluorescent signal and average resting fluorescence.

In response to anoxia the extent of hyperpolarization was less prominent compared to normoxic group. This was most probably due to the non-selective Ca^{2+} efflux through mPTPs. However, CH_4 treatment resulted in significantly lower Ca^{2+} efflux. These results are in accordance with the membrane potential measurements where incubation of the mitochondria with 2.2% CH_4 maintained the hyperpolarization. Interestingly, CH_4 treatment in the normoxia group caused Ca^{2+} efflux to show no reactivity to substrates also seen in membrane potential measurements.

Of note, these results are preliminary because this method was originally developed to measure extramitochondrial Ca^{2+} movement on liver mitochondria. The magnitude of Ca^{2+} currents may be different in cardiac mitochondria; therefore, our method requires further validation.

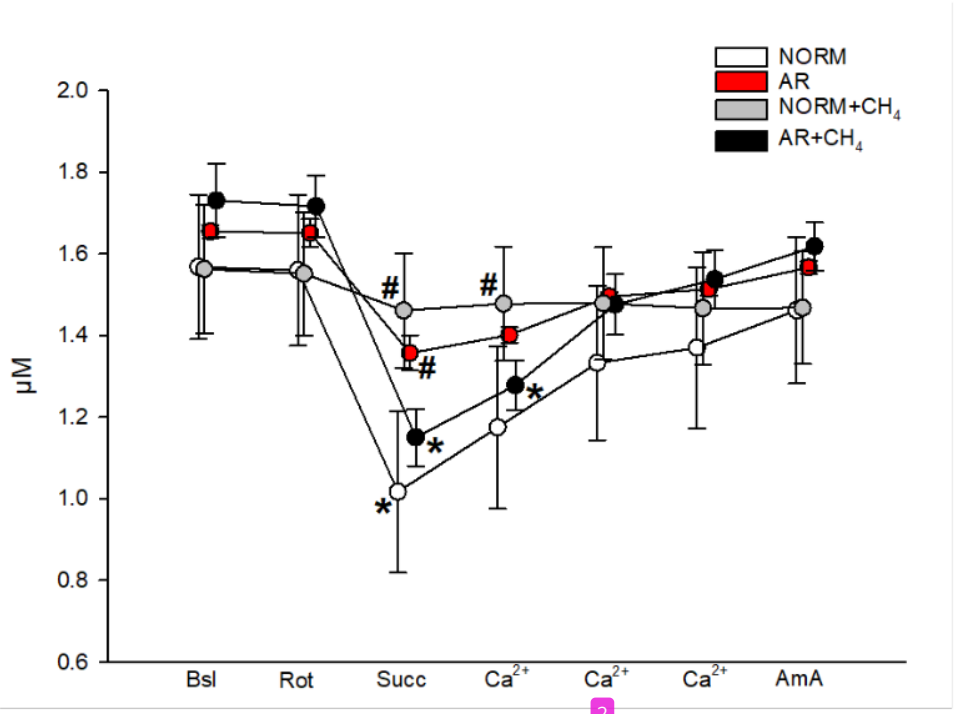

Fig 9. Ca^{2+} -induced membrane potential changes. The A/R group is labelled with a red line, the A/R+CH_4 group with a black line, and the normoxia and normoxia+CH_4 groups with pale and dark grey lines. Data are presented as means ± SEM. #P<0.05 vs. normoxia, *P<0.05 vs. AR; (one-way ANOVA, Tukey's test).

5. DISCUSSION

5.1. Effects of CH_4 during cold ischemia and reperfusion of graft.

The CH_4 supplementation of the transplantation solution improved structures and functions in experimental HTX. The hemodynamic efficacy of CS-CH_4 storage was evidenced by increased LVSP, cardiac contractility and coronary circulation, as compared to CS-treated grafts. The sum of biochemical data showed that the CH_4-containing Custodiol solution effectively reduced the degree of oxido-reductive stress in myocardial samples and significantly influenced several components of ER stress—mitochondria-related pro-apoptotic 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 heart transplantation model.

Myocardium has particularly poor tolerance to prolonged ischemia, and the issue of preservation is a major concern in transplantation. CS solution is generally used in clinical practice; therefore, it is an appropriate testbed for alternative options [44]. Several gas mediators, such as 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 [45, 46]. In contrast, CH_4 is non-toxic *in vivo*; considered to be 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 [24]. Nevertheless, there are pertinent data which demonstrate that CH_4 can modulate NO-, CO- and H_2S-linked reactions in living systems [47, 48]. 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 settings [5, 22, 29, 36]. 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 satisfactory cardiac function four weeks after infarction [29]. Despite of 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 CS solution according to reported protocols and in a concentration range, which demonstrated efficacy in I/R studies *in vivo* [36]. Many details of the mechanism are still unknown, but we have shown that this approach affected many

aspects of mitochondrial physiology during cold ischemia and after reperfusion, perhaps through an indirect influence on Ca^{2+} homeostasis [29, 36]. 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 utilization, which contributed to the maintenance of OxPhos. Furthermore, cytochrome c release, a sign of mitochondrial inner membrane injury, was also reduced (Fig 10).

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 Hif1α, one of the key initial factors in the cascade of events, which will finally lead to cell apoptosis or necrosis [49-52]. 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 [49, 53]. 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 Hif1α expression in cardiomyocytes. In addition, higher intracellular Ca^{2+} can activate GSK-3β, the pro-apoptotic factor in the intrinsic mitochondrial apoptotic pathway [54]. 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 from the mitochondrial membrane. In our study, 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 (Fig 10).

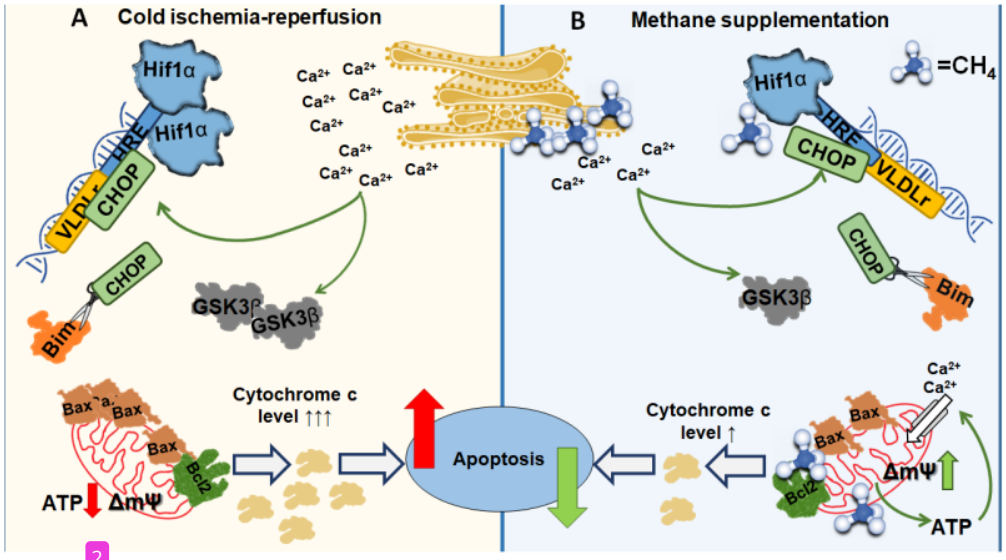

Fig 10. Proposed effects of CH_4 supplementation on cold ischemia-induced intracellular changes. Phospholipid membranes of the ER and the mitochondrion are possible targets. In the presence of a saturating amount of CH_4, the expression of genes downstream of HIF1α decreases, affecting the mitochondrial pathway of apoptosis by lowering the Bax/Bcl2 ratio, thus creating an anti-apoptotic milieu for cardiac muscle cells.

In conclusion, our study has demonstrated that CH_4 enrichment of CS solution results in increased graft protection during cold ischemia and isogenic HTX in rats. Oxido-reductive imbalance is an inevitable consequence of *ex vivo* periods and a basis for a cascade of pro-inflammatory events following re-oxygenation. Based on the totality of data, it seems that CH_4 supplementation conferred increased efficacy on CS 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.

5.2. The role of mitochondria in I/R damage

Expected mitochondrial effects of CH_4 have been characterized by HRR, and we have shown that the administration of CH_4 promotes mitochondrial protective effects on ETC and

apoptosis. Of importance, CH_4 preserved mitochondrial membrane potential (a marker of the integrity of the inner mitochondrial membrane) and several study shows decreased cytochrome c release (a sign of the integrity of the outer mitochondrial membrane) as well [4, 55].

Excessive oxidative stress is a major component of I/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 [56-59]. The concept of using reversible complex I inhibitors for ischemic preconditioning or as a pre-treatment to limit ROS generation and cardiac I/R injury has been demonstrated by several authors [60, 61]. Rotenone, an irreversible inhibitor of electron transport at the so-called ubiquinone (Q)-binding site, has been demonstrated to exert cardioprotection by decreasing RET in the early phase of reperfusion [58]. It should be added that two sites for superoxide production have recently been explored on respiratory complex I, the Q-binding and flavin sites, respectively [59]. The superoxide production at the flavin site is linked to the forward electron transport, and its rate depends on the reduction state of the matrix NAD pool. More importantly, the Q-binding site produces superoxide at much higher rates than the flavin site, driven by RET from complex II into complex I during the reperfusion [10].

In our model of cardiac I/R injury, we tracked mitochondrial ROS with HRR by using the fluorescent dye Amplex Red, while side by side analysis of mitochondrial membrane potential was occurred with potential-sensitive fluorescence dye safranin. Respiratory activity of complex I remained stunned in the re-oxygenation phase being interrelated with the overwhelming ROS production. CH_4 decreased the complex I-linked respiration in both control and ischemia-damaged mitochondria, but there were no changes in the presence of Rot, an irreversible complex I inhibitor, which suggests that CH_4 treatment reduced ROS generation via a partial blockade of electron transport in complex I.

In isolated cardiac mitochondria, the underlying mechanism of RET is the accumulation of Succ during ischemia and its subsequent rapid oxidation at reoxygenation in the presence of a high membrane potential [62]. However, recent discoveries have demonstrated that the difference between cold and warm ischemic injuries lies in the miscellaneous distribution of metabolic changes [4]. Our results suggest that the active site of CH_4 is similar to Rot, distal to the flavin site, since it enhances mitochondrial ROS generation when the electrons enter complex I from NADH while supporting the idea of NADH accumulation is a key issue in re-oxygenation injury. CH_4 also inhibits ROS generation by RET from complex II.

Large membrane potential is a prerequisite to drive the electrons against the gradient of redox potentials from complex II into complex I. It has been demonstrated in isolated mitochondria that only a 5% reduction in mitochondrial membrane potential will reduce peroxide production by 95% [63]. Any manipulation of the RET pathway could potentially influence the end outcome of ROS production, even by lowering the driving hyperpolarization of the mitochondrial membrane potential. Membrane potential also can be influenced by excessive Ca^{2+} stream occurring throughout the process of adapting to hypoxic conditions. Applied HRR protocols of our study can measure membrane potential changes in isolated mitochondria and simultaneously can describe Ca^{2+} flow through the outer mitochondrial membrane. Addition of normoxic CH_4 slightly reduced the substrate-induced hyperpolarization in control mitochondria, in contrast to the preservative effect seen in the case of the anoxia-damaged membrane, while it decreased the Ca^{2+} flow from mitochondria. The difference between cold and warm ischemia, is that warm ischemia involves metabolic changes such as Succ accumulation, which further decreases the RET and subsequent ROS production, while during cold ischemia, mitochondrial adaptation can lower cytoplasmic Ca^{2+} levels, thus protecting the cell against hypoxic danger and apoptotic signals.

The role of complex I and complex II in the post-ischemic cardiac mitochondrial respiration was addressed in more detail. As a result of CH_4 treatment, respiration was inhibited when glutamate + malate was used as a complex I substrate but not with Succ as a complex II substrate. This finding suggested that the addition of CH_4 resulted in a decreased electron flux through complex I but did not alter the Succ oxidation through complex II. Based on these findings, the drop in net ROS production from mitochondria with preserved Succ oxidation in the presence of CH_4 is most likely directly related to the inhibition of complex I. However, CH_4 appears to provide a blockade of electron transport in complex I in contrast to a complete blockade of oxidative metabolism during reoxygenation (Fig 11).

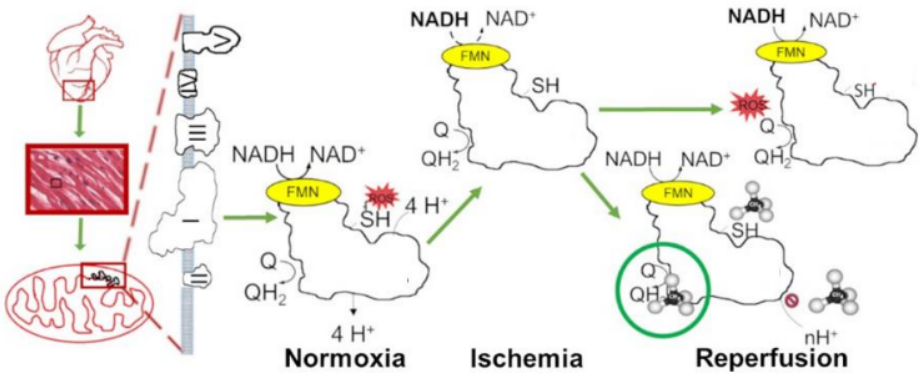

Fig 11. The effects of CH_4 on complex I. The mechanism of protection likely included a blockade of the electron transport in complex I and decreased ROS generation. Reversible deactivation of mitochondrial complex I is an intrinsic mechanism, which provides a fast response of the mitochondrial respiratory chain to oxygen deprivation. However, subsequent re-oxygenation leads to ROS generation due to the rapid burst of respiration. Under normoxic conditions, a high level of NADH can drive forward electron flow with superoxide generation at the flavin mononucleotide moiety located near the NADH binding subunit. During re-oxygenation, reverse electron flow driven by a reduced ubiquinone (ubiquinol) pool and high proton motive force can generate ROS when electrons flow back from ubiquinol to Complex I. CH_4 treatment restricts the forward electron transfer within complex I in control mitochondria while effectively inhibiting RET in post-ischemic mitochondria.

Mitochondrial complex I has two conformations with different catalytic activities: an active state (A) and a deactivated state (D), which are present in A/D equilibrium in a ratio of 9:7 under physiological conditions [64]. The A/D transition occurs during ischemia/anoxia, as an intrinsic mechanism which produces a rapid response of the mitochondrial ETC to oxygen deprivation [65]. Modulating factors of the A/D transition include the availability of oxygen, the reduced NAD pool in the matrix, the temperature and the pH. The physiological role of the accumulation of the D-form in anoxia is most probably to protect mitochondria from ROS generation due to the rapid burst of respiration following re-oxygenation [59, 66]. The transient preservation of complex I in the D-form was implemented as a successful strategy against reperfusion injury in the post-ischemic brain and heart [10, 62, 66]. Although the

entire NAD pool is reduced under ischemic/anoxic conditions, the nucleotide at the flavin site could be rapidly reduced by the RET directly from ubiquinol at the beginning of re-oxygenation (Fig 12).

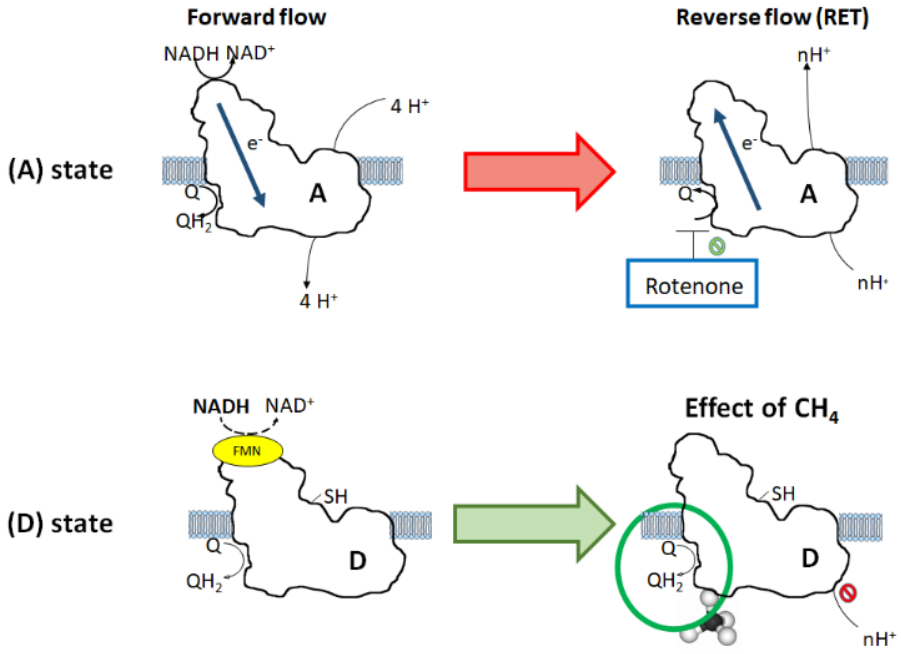

Fig 12. Transition of complex I between its activated (A) and deactivated (D) state. Forward flow of electrons occurs during the (A) state, which represents the physiological functioning of mitochondria. In the presence of complex I inhibitor rotenone, reverse electron flow can be triggered (upper right picture). During hypoxia complex I stays in its (D) state, while CH_4 can maintain this deactivated conformation during the re-oxygenation period, inhibiting the formation of RET, which is the main source of ROS.

However, the D-form of the enzyme is unable to catalyze the RET, and therefore de-activation may act as a protective valve by preventing reduction of the enzyme from downstream. Both the D-form of complex I and the rotenone-inhibited enzyme have restricted access to the quinone-binding pocket [12]. The functional outcome of this is, on the one hand, similar to the blockade of forward electron transfer within the A-form by a molecule of rotenone-like inhibitor bound at the quinone site. On the other hand, the RET occurring in the D-form of the enzyme can also be restricted, thereby abolishing the ROS formation [58, 64].

6. SUMMARY OF NEW FINDINGS

- Our study has demonstrated that CH_4 enrichment of preservation solution during cold ischemia results in increased graft protection in isogenic rat heart transplantation.

- The CH_4-containing CS effectively reduces the degree of oxido-reductive stress in myocardial samples as evidenced by maintained GSH/GSSG ratio and lower NOx level in the reperfused myocardium together with decreased tissue MPO and XOR activity.

- The underlying mechanism is related to the effect of CH_4 on ER stress and the structural and functional integrity of cardiac mitochondria.

- The interaction between the mitochondria and ER involves the changes in extramitochondrial Ca^{2+} fluxes.

- The Ca^{2+}-induced mitochondrial membrane potential changes are decreased by anoxia, however, CH_4 can restore the magnitude of hyperpolarisation.

- The anti-oxidative effects of CH_4 are related to the reduced mitochondrial ROS generation via a partial blockade of electron transport in complex I.

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