

Original Research

# $\Delta^9$ -Tetrahydrocannabinol ( $\Delta^9$ -THC) Improves Ischemia/Reperfusion Heart Dysfunction and Might Serve as a Cardioprotective Agent in the Future Treatment

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## Abstract

**Background:** Ischemia/reperfusion (I/R) is a pivotal mechanism of organ injury during clinical setting for example for cardiopulmonary bypasses. The generation of reactive oxygen species (ROS) during I/R induces oxidative stress that promotes endothelial dysfunction, DNA dissociation and local inflammation. In turn, those processes induce cytokine release, resulting in damage to cellular structures and cell death. One of the major psychoactive compounds of *Cannabis* is delta-9-tetrahydrocannabinol ( $\Delta^9$ -THC), which is known as an anti-inflammatory mediator. Our research aimed to test if  $\Delta^9$ -THC may be protective in the treatment of cardiovascular system dysfunction arising from I/R heart injury. **Methods:** Two experimental models were used: isolated rat hearts perfused with the Langendorff method and human cardiac myocytes (HCM) culture. Rat hearts and HCM underwent *ex vivo*/chemical *in vitro* I/R protocol with/without  $\Delta^9$ -THC treatment. The following parameters were measured: cell metabolic activity, morphology changes, cell damage as lactate dehydrogenase (LDH) activity, ceramide kinase (CERK) activity, ROS level, total antioxidant capacity (TAC) and heart hemodynamic parameters. **Results:**  $\Delta^9$ -THC protected the heart, as evidenced by the improved recovery of cardiac function ( $p < 0.05$ ,  $N = 3-6$ ). Cells subjected to I/R showed lower cytoplasmic LDH activity, and 10  $\mu$ M  $\Delta^9$ -THC treatment reduced cell injury and increased LDH content ( $p = 0.019$ ,  $N = 6-9$ ). Morphology changes of HCM-spherical shape, vacuolisation of cytoplasm and swollen mitochondria—were inhibited due to  $\Delta^9$ -THC treatment. I/R condition affected cell viability, but 10  $\mu$ M  $\Delta^9$ -THC decreased the number of dead cells ( $p = 0.005$ ,  $N = 6-9$ ). The total level of CERK was lower in the I/R group, reflecting oxidative/nitrosative stress changes. The administration of  $\Delta^9$ -THC effectively increased the production of CERK to the level of aerobic control ( $p = 0.028$ ,  $N = 6-9$ ). ROS level was significantly decreased in I/R cells ( $p = 0.007$ ,  $N = 6-8$ ), confirming oxidative stress, while administration of 10  $\mu$ M  $\Delta^9$ -THC enhanced TAC in cardiomyocytes subjected to I/R ( $p = 0.010$ ,  $N = 6-8$ ). **Conclusions:**  $\Delta^9$ -THC promotes the viability of cardiomyocytes, improves their metabolic activity, decreases cell damage and restores heart mechanical function, serving as a cardioprotective. We proposed the use of  $\Delta^9$ -THC as a cardioprotective drug to be, administered before onset of I/R protocol.

**Keywords:** ischemia-reperfusion injury; cardioprotection; 9-tetrahydrocannabinol; oxidants; antioxidants; matrix metalloproteinases; contractility; inflammation

## 1. Introduction

Ischemia–reperfusion (I/R) is a pivotal mechanism of organ injury during surgery for cardiopulmonary bypasses and pharmacological or surgical treatment of myocardial infarction [1], and still remains a therapeutic challenge for physicians. Heart ischemia-reperfusion injury (IRI) induces metabolic, morphological and contractile disorders, leading to irreversible microvascular damage, manifested by myocardial hibernation, acute heart failure, cerebral and gastrointestinal dysfunction, systemic inflamma-

tory response syndrome, and multiple organ dysfunction syndrome [2,3].

At least several mechanisms arising from ischemia/hypoxia and reperfusion lead to cell injury. One element of this complex process is the generation of reactive oxygen species (ROS) which further induce oxidative stress that promotes endothelial dysfunction, DNA dissociation, and local inflammation. In turn, the inflammatory process and oxidative stress induce cytokine release, resulting in damage to cellular structures and cell



death [4–6]. For this reason, obtaining evidence about the immunological mechanisms of IRI may provide a strong foundation for novel therapeutic and cardiac event prevention strategies.

*Cannabis* is a complex plant, divided into the drug-type- *C. indica* Lam. rich in psychoactive substances and used for medicinal or recreational purposes, and the fibre-type *C. sativa* L., which is used for textile or food production [7]. The major psychoactive compounds are delta-9-tetrahydrocannabinol ( $\Delta^9$ -THC) and cannabidiol, which cause opposing effects [8]. It is well known that cannabis is a widely used drug, already legalized in some countries, and its use has been associated with a variety of mental health problems, particularly among the young [9–11]. However, it is also widely documented that cannabinoids are an important group of substances serving as anti-inflammatory mediators, since endocannabinoids modulate immune function in an autocrine and paracrine manner [12].

$\Delta^9$ -THC is a partial agonist of the two cannabinoid (CB1 and CB2) receptors in the endogenous cannabinoid system [13–15]. CB1 and CB2 receptors (CB1R/CB2R) are present in the heart and vascular smooth muscles and numerous studies have suggested that CB1R/CB2R are up-regulated in response to tissue injury and may have an anti-inflammatory effect in these settings [16–18]. Therefore we aimed to test if  $\Delta^9$ -THC proves effective in the treatment of cardiovascular system dysfunction such as ischemia/reperfusion injury.

## 2. Materials and Methods

### 2.1 Rats and Ex Vivo I/R Model

Male Sprague-Dawley rats weighing 200–250 g (Charles River, Burlington, NJ, USA) were used in this study. The rats were adapted for one week in conditions appropriate to their species and ensuring their welfare. The animals were housed in cages (two rats per cage) and kept at controlled temperature ( $22 \pm 2$  °C), humidity ( $55 \pm 5\%$ ) and light/dark (12/12 hours) cycle. An ad libitum access to a diet of standard laboratory chow and water was provided. All rat experiments were conducted according to the Guide to the Care and Use of Experimental Animals published by the Canadian Council on Animal Care and reviewed by the Animal Research Ethics Board, University of Saskatchewan, Canada (Resolution no. 20060054).

Before heart surgery, rats were desensitised with buprenorphine (0.05 mg/kg, i.p.), and anaesthetised with sodium pentobarbital (0.5 mL/kg i.p.). Then, the chest was opened and the heart was rapidly excised from the animal. The spontaneously beating hearts were rinsed by immersion in an ice-cold Krebs-Henseleit Buffer (118 mmol/L NaCl, 4.7 mmol/L KCl, 1.2 mmol/L  $\text{KH}_2\text{PO}_4$ , 1.2 mmol/L  $\text{MgSO}_4$ , 3.0 mmol/L  $\text{CaCl}_2$ , 25 mmol/L  $\text{NaHCO}_3$ , 11 mmol/L glucose, and 0.5 mmol/L EDTA, pH 7.4), and cannulated in the Langendorff system (EMKA Technologies, Paris, France) by the aorta. This procedure took no

more than 30 sec to reduce the time of uncontrolled ischemia. Then, hearts were perfused at constant pressure (60 mmHg) with a Krebs-Henseleit Buffer (pH 7.4, 37 °C) and gassed continuously (5%  $\text{CO}_2$ /95%  $\text{O}_2$ ). The following haemodynamic parameters were monitored using an EMKA recording system with IOX2 software (EMKA Technologies, Paris, France): coronary flow (CF), heart rate (HR), left ventricular developed pressure (LVDP). LVDP and HR were measured with the help of a water-filled latex balloon connected to a pressure transducer and inserted through an incision in the left atrium into the left ventricle via the mitral valve. The volume was adjusted at the beginning of the perfusion period to achieve an end-diastolic pressure of 8–10 mmHg. LVDP was calculated as the difference between peak systolic and diastolic pressures.

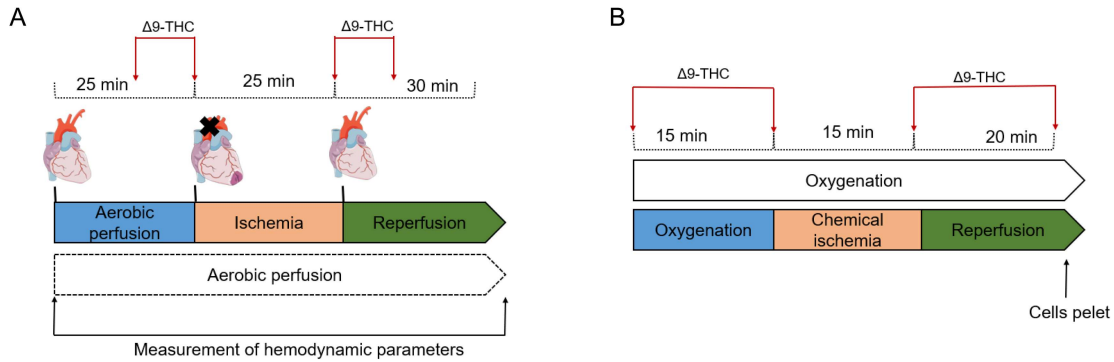
Exclusion criteria: hearts that present atrial or ventricular fibrillation during *ex vivo* procedure as well as hearts that showed CF  $>28$  mL/min or  $<10$  mL/min when cannulated in EMKA system were excluded from the study.

The experimental rats were randomly allocated to the following groups: the aerobic control group, I/R group and I/R with  $\Delta^9$ -THC 0.1–1.0  $\mu\text{M}$  groups.

Hearts from the I/R group were subjected to 25 min of aerobic stabilisation, 25 min of global no-flow ischemia (by cessation of the buffer flow) and 30 min of reperfusion (by buffer flow restoration), according to previous protocols [19–21]. The hearts from the aerobic control were perfused aerobically for 80 min. THC (Sigma Aldrich cat.no. T-005, Sigma-Aldrich, St. Louis, MO, USA) was administered with the Krebs-Henseleit buffer into the hearts during the last 10 min of aerobic stabilisation and in the first 10 min of reperfusion (after global ischemia) [19,22] (Fig. 1A). Methyl alcohol (MetOH) was used as a vehicle for THC. Maximal MetOH infused  $<0.001\%$  (v/v). To determine cardiac mechanical function, the recovery of rate pressure product (RPP) was expressed as the product of HR and LVDP and evaluated at experiment's 25 min (the end of aerobic perfusion) and 80 min (the end of reperfusion) marks [19,22]. After the experimental protocol, isolated hearts were immediately immersed in liquid nitrogen and stored at  $-80$  °C for further investigations.

### 2.2 Cell Culture, Chemical Ischemia/Reperfusion Injury and Study Design In Vitro

Human Cardiac Myocytes (HCM) were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA). The cells were grown in flasks covered with polylysine (final concentration at 0.014 mg/mL) at 37 °C in a water-saturated, 5%  $\text{CO}_2$  atmosphere in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, St. Louis, MO, USA) containing Cardiac Myocyte Growth Supplement (ScienCell Research Laboratories, Carlsbad, CA, USA), 5% fetal bovine serum, 100 U/mL penicillin, 100  $\mu\text{g/mL}$  streptomycin (all from Sigma-Aldrich, St. Louis, MO, USA) to 90% confluence.



**Fig. 1. Experimental protocol schemes.** Experimental protocol scheme for heart I/R injury (A) and isolated cardiomyocytes chemical I/R injury (B).

HCM were subjected to *in vitro* chemical I/R in accordance with the guidelines for experimental models of myocardial ischemia [23]. Briefly, the cells underwent 15 min oxygenation, 15 min *in vitro* chemical ischemia and 20 min reperfusion [19], in the presence and absence of 10  $\mu\text{M}$  THC (Sigma Aldrich cat.no. T-005, Sigma-Aldrich, St. Louis, MO, USA) (Fig. 1B). The aerobic stabilisation and reperfusion were performed in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (5.5 mmol/L HEPES, 63.7 mmol/L  $\text{CaCl}_2$ , 5 mmol/L KCl, 2.1 mmol/L  $\text{MgCl}_2$ , 5.5 mmol/L glucose, 10 mmol/L taurine) containing an additional 55  $\mu\text{mol/L}$   $\text{CaCl}_2$  and 0.75 mg/mL BSA. The chemical ischemia group was incubated in a HEPES buffer containing 4.4 mmol/L 2-deoxyglucose (to inhibit glycolysis) and 4.0 mmol/L sodium cyanide (an inhibitor of cellular respiration) [19]. The optimal duration of ischemia (15 min) was previously established experimentally (data not shown). In the I/R group, after 15 min of aerobic stabilisation in the HEPES buffer at RT, the buffer was removed by centrifugation (1 min at  $1500 \times g$ ) and the cell pellet was resuspended in the ischemia buffer and incubated for 15 min at RT. Then, the cells were centrifuged for 1 min at  $1500 \times g$ , the buffer was removed and cells were incubated in aerobic conditions with the HEPES buffer for 20 min at RT (reperfusion). After reperfusion, the buffer was removed by centrifugation at  $1500 \times g$  for 5 min, and the pellet was homogenised. The cells from the aerobic control group were incubated aerobically for 50 min in HEPES buffer at RT; however, they were centrifuge like the I/R group, and a new portion of an oxygenated HEPES buffer was administered as well. In the I/R+THC group,  $\Delta^9$ -THC (0.1–10  $\mu\text{M}$  final concentration depending on the experiment) was added into the HEPES buffer.

### 2.3 Isolation of Ventricular Cardiomyocytes, Induction of Chemical I/R and Contractility Measurement

Rat hearts were rapidly excised from rats anaesthetised with sodium pentobarbital (40 mg/kg, i.p.) as described above. The spontaneously beating hearts were immersed in an ice-cold Myocyte Isolation Buffer (MIB) con-

taining 120 nM NaCl, 5 mM KCl, 2 mM NaAc, 2 mM  $\text{MgCl}_2$ , 1 mM  $\text{Na}_2\text{HPO}_4$ , 20 mM  $\text{NaHCO}_3$ , 5 mM glucose, 9 mM taurine and 10 mM  $\text{CaCl}_2$  at pH 7.4 immediately after removal. Then, hearts were suspended on a blunt-end needle of the Langendorff system by the aorta, perfused at a constant flow of 10 mL/min with an MIB buffer containing 10 mM  $\text{CaCl}_2$ , pH 7.4, at 37  $^\circ\text{C}$ , and gassed continuously with 5% carbogen for 5 min in the Langendorff system's water-jacketed chamber.

After 5 min of stabilisation, the buffer was replaced with an MIB containing 5  $\mu\text{M}$   $\text{CaCl}_2$  and the hearts were perfused for 5 more minutes. The low concentration of  $\text{CaCl}_2$  resulted in the loss of cell contractility. After mild swelling of the myocardium with a HEPES buffer (120 mM NaCl 140, 5 mM KCl, 2 mM  $\text{MgCl}_2$ , 5 mM glucose, 9 mM taurine, 5 mM HEPES) containing 40  $\mu\text{M}$   $\text{CaCl}_2$ , 25 mg of collagenase and 2 mg of protease at pH 7.4, the right ventricle was excised from the heart, rinsed with a HEPES buffer containing 100  $\mu\text{M}$   $\text{CaCl}_2$ , 150 mg bovine serum albumin (BSA), and then minced into small pieces in the digestion solution (a HEPES buffer containing 100  $\mu\text{M}$   $\text{CaCl}_2$ , 150 mg BSA, 15 mg collagenase and 1 mg protease). The minced tissue was repeatedly digested (6 times for 20 and 10 min in a water bath (37  $^\circ\text{C}$ )), and the 3rd–6th fraction was used for further experiments.

Chemical ischemia was induced after 15 min of  $\Delta^9$ -THC treatment in a HEPES buffer containing 100  $\mu\text{M}$   $\text{CaCl}_2$ , 150 mg BSA, by covering the cell pellets with a HEPES buffer containing 4 mM 2-deoxyglucose and 40 mM sodium cyanide (2.5  $\mu\text{M}$ ). The optimal ischemia duration of 3 min was established in previous studies [20]. After 3 min chemical ischemia, the buffer containing sodium cyanide was removed by centrifugation (1 min at  $1500 \times g$ ) and the cells pellet was suspended in the fresh portion of the HEPES buffer containing 100  $\mu\text{M}$   $\text{CaCl}_2$ , 150 mg BSA and 10  $\mu\text{M}$   $\Delta^9$ -THC. After a 20 min reperfusion, the cells were centrifuged at  $1500 \times g$  for 5 min and the cell pellet suspended in HEPES buffer (100  $\mu\text{M}$   $\text{CaCl}_2$ , 150 mg BSA) was used for contractility measurement. The aerobic

control group was kept exposed to atmospheric air for 38 min, and the chemical ischemia control cardiomyocytes underwent the same experimental protocol without  $\Delta^9$ -THC treatment.

Next, the contractility of cardiomyocytes was measured. A 100  $\mu$ L aliquot of cell suspension was placed in the rapid change stimulation chamber of the IonOptix Contractility System (IonOptix, Milton, MA, USA). After 3 min of stabilisation, cardiomyocytes were perfused with an oxygenated HEPES buffer containing 2 mM  $\text{CaCl}_2$  (4 mL/min) at 37 °C. Cells were continuously paced with 1 Hz and 5 V (IonOptix MyoPacer, Milton, MA, USA), and the contractility—expressed as a per cent of peak shortening in comparison to the length of the diastolic cell—was measured on an average of 5 cells per sample. At least 5 samples per one experimental condition were evaluated.

#### 2.4 Cell Homogenisation

Three cycles of freezing (in liquid nitrogen) and thawing (at 37 °C) in the homogenisation buffer (50 mmol/L Tris-HCl (pH 7.4) containing 3.1 mmol/L sucrose, 1 mmol/L DTT, 10  $\mu$ g/mL leupeptin, 10  $\mu$ g/mL soybean trypsin inhibitor, 2  $\mu$ g/mL aprotinin and 0.1% Triton X-100) were used to disorganise the cell pellet. Then, myocytes were homogenised mechanically on ice (three times for 30 seconds) with a hand-held homogeniser. Supernatants for further analysis (stored at -80 °C) were obtained by a 5 min centrifugation at 10,000  $\times$  g 4 °C.

#### 2.5 Determination of Total Protein Concentration

The Bradford method was used to determine protein concentration in cell homogenates and supernatants. BSA (heat shock fraction,  $\geq 98\%$ , Sigma-Aldrich) at a final concentration of 1.0–0.0062  $\mu$ g/ $\mu$ L served as the protein standard to create the standard curve. A Bio-Rad Protein Assay Dye Reagent (BioRad, Hercules, CA, USA) and a Spark multimode microplate reader (Tecan Trading AG, Switzerland) were used to measure the total protein concentration.

#### 2.6 Measurement of Cells Metabolic Activity

Fluorescein diacetate (FDA) and DAPI were used as the vital inclusion and vital exclusion dyes, respectively [24]. Viable and metabolically active cells can incorporate the nonpolar, nonfluorescent FDA and rapidly hydrolyse it by acetyl esterase to fluorescein, a polar, fluorescent compound retained in the cell. Damaged cells release esterified fluorescein to liquid medium, rendering cell visualisation impossible. Nonviable cell nuclei were stained with DAPI [25,26]. Briefly, myocytes were seeded in a 96-well plate at a density of  $2 \times 10^4$  cells/well for 24 h and then subjected to *in vitro* chemical I/R injury in the presence and absence of  $\Delta^9$ -THC, according to the above protocol. The cells were washed three times with PBS and stained with 5  $\mu$ g/mL FDA (cat. no. F7378, Sigma-Aldrich, St. Louis, MO, USA) and 1:1000 DAPI (cat.no. D8417-

5MG, Sigma-Aldrich, St. Louis, MO, USA) for 15 min in the dark. ZOE Fluorescent Cell Imager (BioRad, Hercules, CA, USA) was used to estimate the bright green fluorescence for FDA and blue fluorescence for DAPI. Cells were considered metabolically active when stained only with FDA (FDA-positive, DAPI-negative), whereas cells that excluded FDA and stained only with DAPI were considered necrotic (FDA-negative, DAPI-positive) [22]. Image J 1.52a software (NIH, Bethesda, Maryland) was used to analyse the area of fluorescence of each image. To determine cell metabolic activity, green fluorescence (live cells) was normalised to the total number of cells (green + blue fluorescence) in each experiment [24]. Data were collected from three independent image trials for each experiment and shown as AU. The metabolic activity was compared in cells that were exposed to aerobic conditions and cells subjected to I/R and I/R with the addition of  $\Delta^9$ -THC.

#### 2.7 Cell Morphology Assessment

The influence of  $\Delta^9$ -THC on cardiomyocyte morphology in chemical I/R was assessed in 9-well plates. HCM ( $4.8 \times 10^4$  cells/well) were cultured for 24 h and subjected to chemical I/R, I/R + 10  $\mu$ M  $\Delta^9$ -THC or maintained in aerobic conditions, as described above and shown in Fig. 1B. The structural changes in cardiomyocytes were photographed using a Live Cell Imaging Microscope-3D Cell Explorer (Nanolive, Tolochenaz, Switzerland). The cells were analysed for cytoplasm vacuolisation, the size and the number of mitochondria, as well as the degree of degradation of the nucleus and cytoplasm.

#### 2.8 LDH Assessment

To determine the activity of LDH in coronary effluents, a Lactate Dehydrogenase Activity Assay Kit (cat.no. MAK066-1KT, Sigma-Aldrich, St. Louis, MO, USA) was used, according to the manufacturer's instruction. Since LDH is a stable cytosolic enzyme, it is released upon membrane damage/increased permeability or cell lysis and commonly serves as a marker of cell damage [19,27,28]. LDH activity in cultured cells was normalised to total protein.

#### 2.9 MMP-2 Activity

Gelatine zymography was performed based on the Heussen and Dowdle protocol with our modifications, as previously described [22,29]. 40  $\mu$ g of heart homogenates were placed in a sample loading buffer and applied to polyacrylamide gel co-polymerised with gelatine. After electrophoresis, gels were rinsed in 2.5% Triton X-100 to remove SDS. Gels were incubated in an incubation buffer at 37 °C overnight, stained in a staining solution and destained in a destaining solution. Zymograms were scanned with a VersaDoc 5000 (BioRad, Hercules, CA, USA) and the band intensities were analysed using Quantity One software v. 4.6.6 (BioRad, Hercules, CA, USA). MMP-2 activity was expressed as activity per microgram of protein.

### 2.10 Assessment of CERK Activity

A Human CERK (Ceramide kinase) ELISA Kit (cat.no. EH1538, Fine Test, Wuhan, Hubei, China) was used to evaluate the potential mechanism of  $\Delta^9$ -THC cardioprotection in I/R cardiomyocytes. The assay was used to quantitatively assesses ceramide kinase (CERK) in cell homogenates with the ELISA method, as per the manufacturer's instructions. The level of CERK in cell homogenates was normalised to total protein concentration and expressed in pg/ $\mu$ g protein.

### 2.11 Assessment of Oxidative Stress

An OxiSelect™ *In Vitro* ROS/RNS Assay Kit (Cell Biolabs, San Diego, CA, USA) was used to explore the influence of  $\Delta^9$ -THC on the level of total reactive oxygen and nitrogen species (ROS/RNS) in cardiomyocytes. The assay is designed to measure the total ROS and RNS, including hydrogen peroxide, nitric oxide, peroxy radicals, and peroxynitrite anions, with the use of a proprietary fluorogenic probe—dichlorodihydrofluorescein DiOxyQ (DCFH-DiOxyQ). DCFH-DiOxyQ is primed with a dequenching reagent to the highly reactive DCFH form. In the presence of ROS and RNS, the DCFH is quickly oxidised to the highly fluorescent 2, 7-dichlorodihydrofluorescein (DCF). Fluorescence intensity is proportional to the total ROS/RNS level within the sample. The total ROS/RNS level was assessed in cell homogenates and supernatants.

### 2.12 Total Antioxidant Capacity in Cells

An OxiSelect™ Total Antioxidant Capacity (TAC) Assay Kit (cat.no. STA-360, Cell Biolabs, San Diego, CA, USA) was used to examine the influence of  $\Delta^9$ -THC on resistance to oxidative stress during I/R. Measurement of the total non-enzymatic antioxidant capacity (TAC) indicates the ability of cells to counteract induced oxidative stress. The TAC Assay is based on the reduction of copper (II) to copper (I) by the antioxidants present in the sample. After reduction, the copper (I) ion further reacts with the coupling chromogenic reagent which produces a color with a maximum absorbance of 490 nm. The absorbance values were proportional to the total reducing capacity of the cardiomyocytes. TAC levels were measured in cell homogenates and normalised to total protein concentration in each sample.

### 2.13 Measurement of Interleukin 6 (IL-6) Concentration in Cells

IL-6 concentration in cardiomyocytes homogenates was determined using Human IL-6 DuoSet ELISA Kit (cat.no. DY206-05, R&D Systems, a bio-technique brand, Minneapolis, Minnesota, USA), according to the manufacturer's instructions. The minimum detectable concentration of IL-6 was 9.4 pg/mL. IL-6 concentration in cell homogenates was expressed as pg per  $\mu$ g of total protein.

### 2.14 Statistical Analysis

GraphPad Prism 8.0 software (GraphPad Software, San Diego, CA, USA) was used for statistical analysis of the results. To assess normality of variance changes, the Shapiro-Wilk normality test or the Kolmogorov-Smirnov test was used. The Student *t*-test or the Mann-Whitney *U* test was used for data comparison of two groups. For multiple groups, ANOVA or nonparametric Kruskal-Wallis test with appropriate post hoc tests (Tukey-Kramer Multiple Comparisons test/Dunn test) were used. The correlation was assessed using the Spearman test. The results were expressed as mean  $\pm$  SEM and a value of  $p < 0.05$  was regarded as statistically significant. Different sizes of animal groups (3–6) used in experiments depended on the exclusion criteria (presented above) and the Power Analysis test (alpha = 0.05, power = 0.80,  $\pm 10\%$ ) as well as the principles of the 3Rs (replacement, reduction, refinement). The difference in N numbers between experiments also followed from the fact that some part of HCM died during cell culturing, and as such, the N number for selected tests had to be reduced. Some results had to be removed from the analysis as well, as they were significant outliers according to the Grubbs test.

## 3. Results

### 3.1 $\Delta^9$ -THC Improves the Mechanical Function of the Heart During I/R

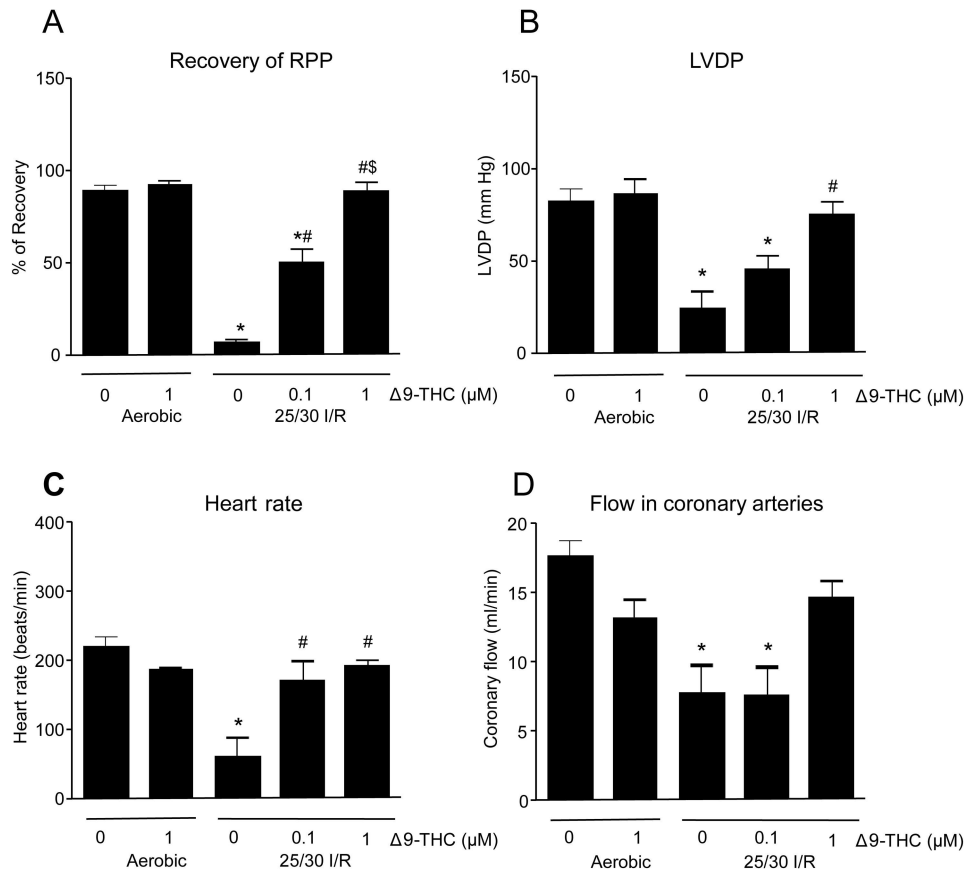
To study the effect of  $\Delta^9$ -THC in hearts *ex vivo*, we used well established model of global ischemia with use of isolated rat hearts by inducing global, no-flow ischemia for 25 min, followed by 30 min reperfusion. The  $\Delta^9$ -THC was administered 10 min before ischemia and during the first 10 min of reperfusion (Fig. 1A).

Data showed that  $\Delta^9$ -THC 0.1–1  $\mu$ M protected the heart, as evidenced by the improved recovery of cardiac function ( $p < 0.0001$ ), HR ( $p < 0.0001$ ) and LVDP ( $p = 0.001$ ) analysis (Fig. 2A–C). We also noticed increased coronary flow in comparison to I/R hearts when 1  $\mu$ M of  $\Delta^9$ -THC was used (Fig. 2D).

### 3.2 $\Delta^9$ -THC Improves Cardiomyocyte Contractility Through Reduced Cell Damage

As shown in Fig. 2, we constructed a stable model in which  $\Delta^9$ -THC did not affect hearts maintained in aerobic conditions. Hence, to study the mechanism of  $\Delta^9$ -THC action in cardiomyocytes, we constructed the model of chemical I/R, as shown in the scheme in Fig. 1B.

Since LDH is a cellular protein that can be easily released from cells due to cell injury or increased permeability of the membrane [30], cells subjected to chemical I/R showed lower cytoplasmic LDH activity—as more of it was released into the extracellular space (here supernatants), while a 10  $\mu$ M  $\Delta^9$ -THC treatment reduced cell injury and increased LDH content in cell homogenates to the level observed in the aerobic control (less LDH was released into



**Fig. 2. Protection of mechanical function of isolated rat hearts subjected to ischemia/reperfusion (I/R) injury and perfused with  $\Delta^9$ -tetrahydrocannabinol ( $\Delta^9$ -THC).** (A) Recovery of heart rate pressure product (RPP). Recovery is expressed as a % of the pre-I/R mechanical function. (B) Left ventricular developed pressure (LVDP). (C) Heart rate. (D) Coronary flow. Results were expressed as mean  $\pm$  SEM and  $p < 0.05$  was considered as statistically significant. \* vs. Aerobic; # vs. 25/30 I/R; \$ vs. 25/30 I/R + 0.1  $\mu$ M THC. Aerobic, N = 6; Aerobic + 1  $\mu$ M THC, N = 3; 25/30 I/R, N = 6; 25/30 I/R + 0.1  $\mu$ M THC, N = 3; 25/30 I/R + 1  $\mu$ M THC, N = 6. Methyl alcohol (MetOH) was used as a vehicle for THC. Maximal MetOH infused  $<0.001\%$  (v/v).

supernatants) ( $p = 0.019$ ) (Fig. 3A). At this point, we determined that 0.1 and 1.0  $\mu$ M of  $\Delta^9$ -THC did not bring improvement in cardiomyocytes injury and thus assumed that the working dose of  $\Delta^9$ -THC was 10  $\mu$ M, and the latter dose was used in further experiments.

To demonstrate the effect of  $\Delta^9$ -THC on impaired contractility, we measured the contractility of cardiomyocytes. Data showed that I/R attenuates cardiomyocytes contractility, but the treatment of the cells with 10  $\mu$ M  $\Delta^9$ -THC ameliorated cell contractility,  $p = 0.024$  (Fig. 3B).

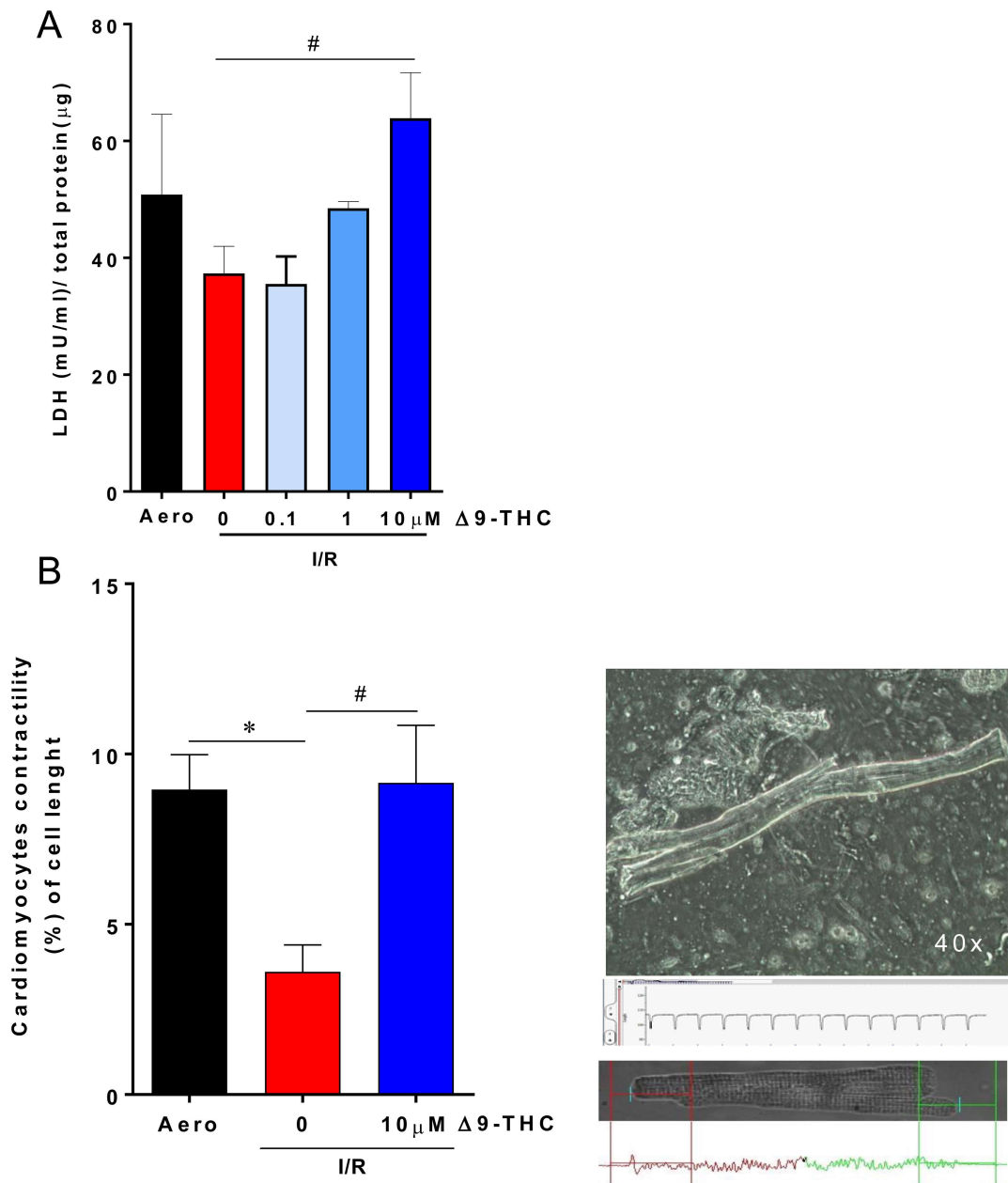
### 3.3 $\Delta^9$ -THC Alleviates Cell Morphology Changes Due to I/R

To further confirm that 10  $\mu$ M  $\Delta^9$ -THC protected cardiomyocytes from I/R induced injury and then from apoptosis/necrosis, cell morphological changes have been analysed. In aerobic conditions, cells showed a morphologically normal state as it was expressed by a large surface area, spread out over the plate surface, with no signs of

swelling and almost imperceptible mitochondria (about 0.3  $\mu$ m in diameter) (Fig. 4A–C). In the I/R control group, the cells changed their shape into spherical due to oedema. A strong vacuolisation of the cytoplasm and many enlarged and swollen mitochondria (4.2  $\mu$ m–3.0  $\mu$ m) were observed. The cytoplasm was often fragmented, and the cell nucleus had a blurred structure and destructive features (Fig. 4D–F). In the group subjected to I/R in the presence of  $\Delta^9$ -THC at a dose of 10  $\mu$ M, I/R lesions were also present, as in the I/R control, but they were less severe. The cells did not change their shape in any significant way. The swelling was smaller (2.3  $\mu$ m–1.0  $\mu$ m) and occurred in fewer mitochondria. The cytoplasm did not show any fragmentation features (Fig. 4G–I).

### 3.4 $\Delta^9$ -THC Increases Viability and Supports the Metabolic Function of Cardiomyocytes Exposed to I/R

Cell viability was evaluated in cardiomyocytes subjected to chemical I/R, I/R with  $\Delta^9$ -THC, as well as in the



**Fig. 3. Cardiomyocyte contractility and damage after I/R.** (A) Cell injury and (B) cardiomyocyte contractility during I/R. I/R, ischemia/reperfusion;  $N = 6-9$  per group. Results were expressed as mean  $\pm$  SEM and  $p < 0.05$  was considered as statistically significant. \* Aero vs. I/R; # I/R vs. I/R+10  $\mu\text{M}$  THC. LDH, lactate dehydrogenase; Aero, aerobic; I/R, ischemia/reperfusion.

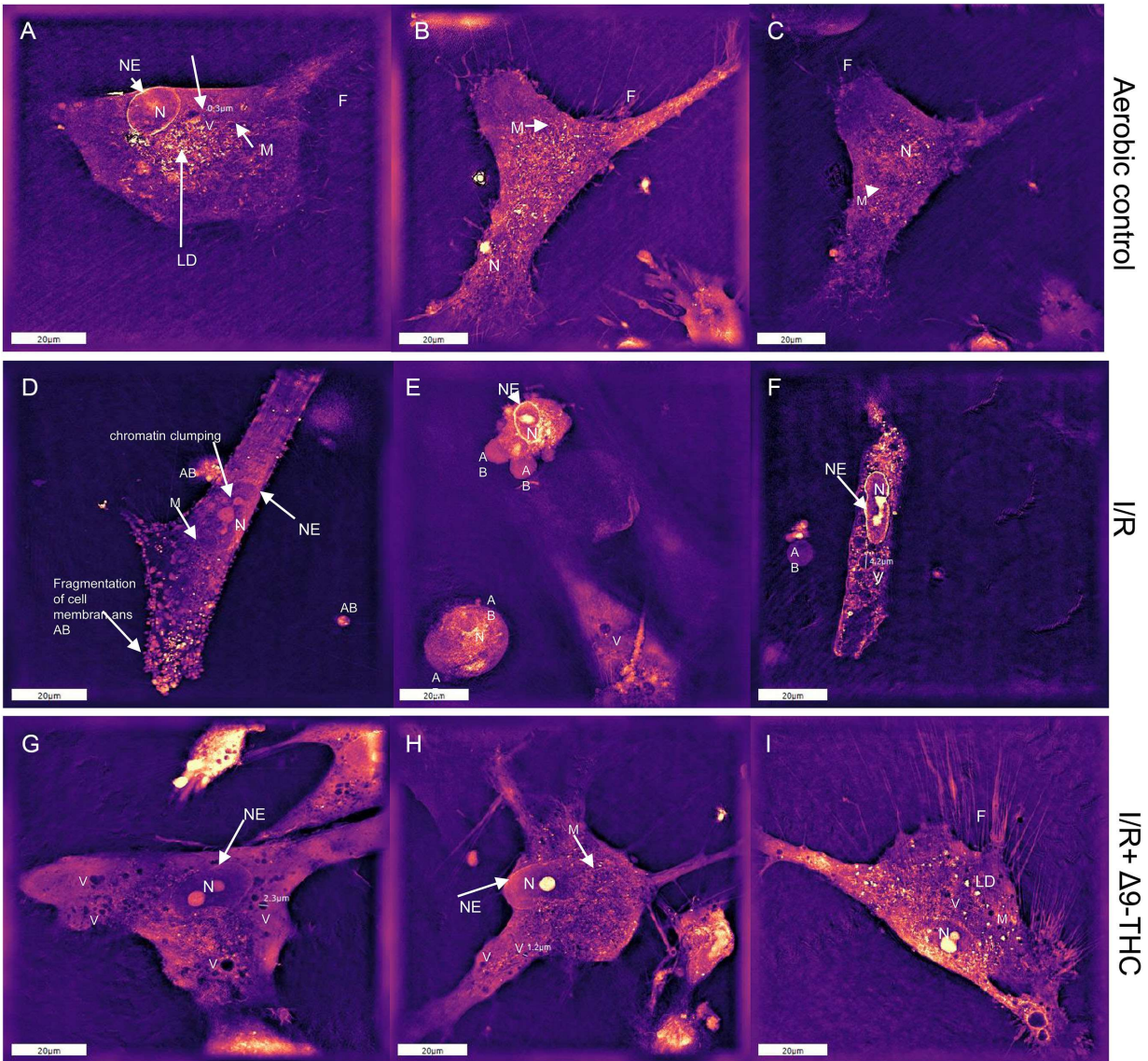
aerobic control group, by vital staining with DAPI. I/R condition affected cell viability ( $p = 0.024$ ) but 10  $\mu\text{M}$   $\Delta^9\text{-THC}$  decreased the number of dead cells ( $p = 0.005$ ) (Fig. 5). The above results indicated that  $\Delta^9\text{-THC}$  exerts protection in the I/R damage of cardiomyocytes.

In addition to increased cardiomyocyte viability, 10  $\mu\text{M}$   $\Delta^9\text{-THC}$  also enhanced the activity of intracellular acetyl esterase ( $p = 0.035$ ) (Fig. 6A–D), which was able to rapidly hydrolyse fluorescein diacetate into polar fluorescein. This confirmed the cells' ability to preserve their

metabolic function. Increased metabolic activity of the cells positively correlated with cell contractility (Fig. 6E), providing evidence of a cardioprotective role of  $\Delta^9\text{-THC}$ .

### 3.5 MMP-2 as a Potential Target of $\Delta^9\text{-THC}$ in I/R Cardiomyocytes

To assess the potential mechanism of  $\Delta^9\text{-THC}$  cardioprotection, we analysed changes in MMP-2 activity using gelatine zymography. Data confirmed the decreased activity of MMP-2 (Fig. 7) in cells subjected to I/R ( $p = 0.019$ )



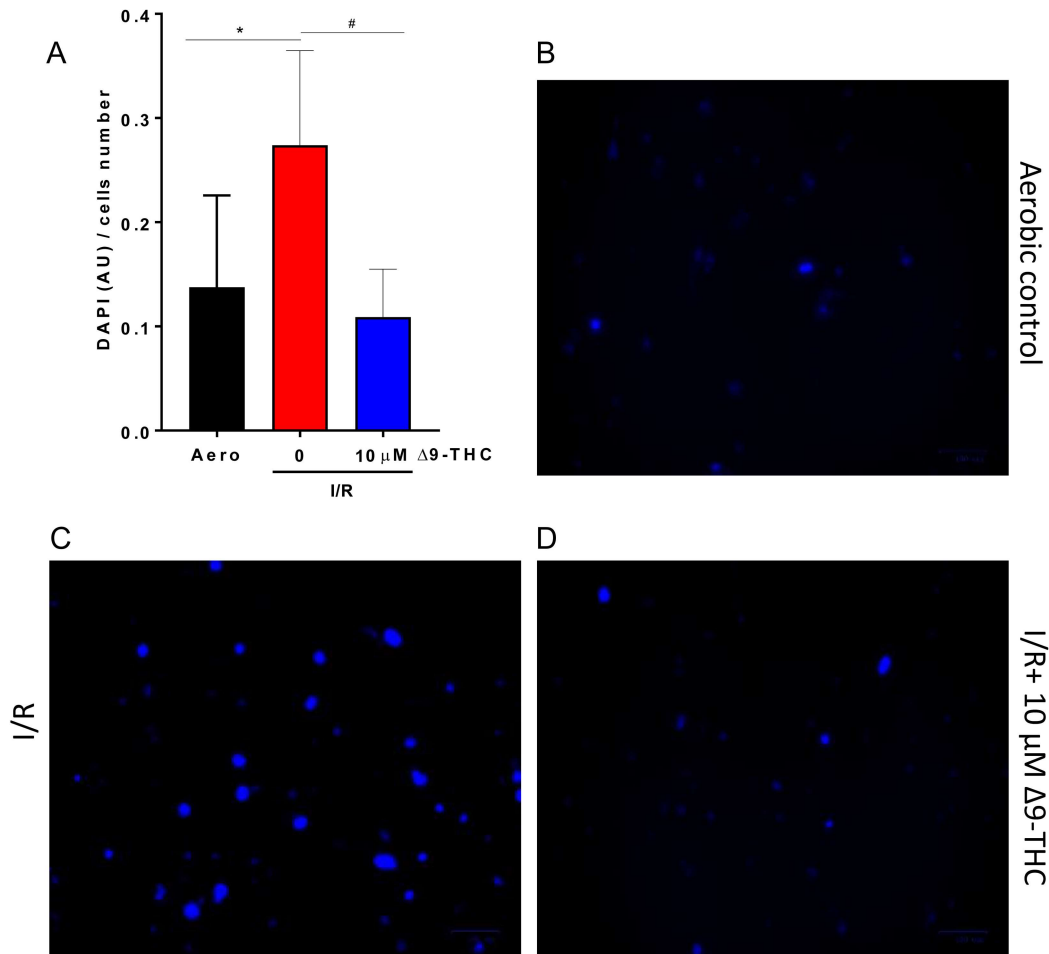
**Fig. 4. Cell morphology of I/R injured cardiomyocytes.** (A–C) Aerobic control. Morphologically normal cells, with a large surface area, spread out over the plate surface, did not show signs of swelling, almost imperceptible mitochondria and vacuoles (mean  $\sigma$  0.3  $\mu\text{m}$  in diameter). (D–F) I/R cells. Cell shape changed into spherical due to oedema. A strong vacuolisation of the cytoplasm (mean  $\sigma$  3.6  $\mu\text{m}$ ); numerous enlarged and swollen mitochondria with amorphous densities. The cytoplasm was fragmented, and the cell nucleus has a blurred structure and destructive features. (G–I) No significant change of shape. Swelling occurred in fewer mitochondria, a lower number of vacuoles (mean  $\sigma$  1.6  $\mu\text{m}$ ) was observed. The cytoplasm does not show any fragmentation features. Live Cell Imaging Microscope-3D Cell Explorer (Nanolive, Tolothenaz, Switzerland). N, nucleus; M, mitochondria; V, vacuoles; F, filopodium; LD, lipid droplets.

compared to the Aero group. Moreover, the administration of 10  $\mu\text{M}$   $\Delta^9$ -THC into cells subjected to I/R ( $p = 0.040$ ) restored MMP-2 activity to the level of the aerobic control. Decreased MMP-2 activity in cardiomyocytes was accompanied by an increased synthesis of IL-6,  $r = 0.998$   $p = 0.023$ .

### 3.6 CERK Activity in Cardiomyocytes

The total level of CERK was lower in the I/R group, reflecting oxidative/nitrosative stress changes. The administration of 10  $\mu\text{M}$   $\Delta^9$ -THC effectively increased the production of CERK (Fig. 8A) to the level of the aerobic control. Since CERK induces cell survival [31], we showed a negative correlation between CERK and the number of dead cells,  $r = -0.53$   $p = 0.040$  (Fig. 8B).





**Fig. 5. Cell viability after I/R.**  $\Delta^9$ -THC affects cell viability: graphical presentation of the result (A); microscopic photos (B–D). Nuclei *in vivo* staining with DAPI were considered necrotic. I/R, ischemia/reperfusion; N = 6–9 per group, \* $p = 0.024$ ; # $p = 0.005$ .

### 3.7 Oxidative Status of Cells

The total ROS/RNS level was significantly decreased in I/R cells ( $p = 0.007$ ) and increased in I/R supernatants ( $p = 0.013$ ) (Fig. 9A–B), confirming oxidative/nitrosative stress. The administration of 10  $\mu$ M  $\Delta^9$ -THC significantly enhanced the total antioxidant capacity (TAC) in cardiomyocytes subjected to I/R ( $p = 0.010$ ) (Fig. 9C).

### 3.8 Changes in IL-6 Concentration Under the Influence of $\Delta^9$ -THC

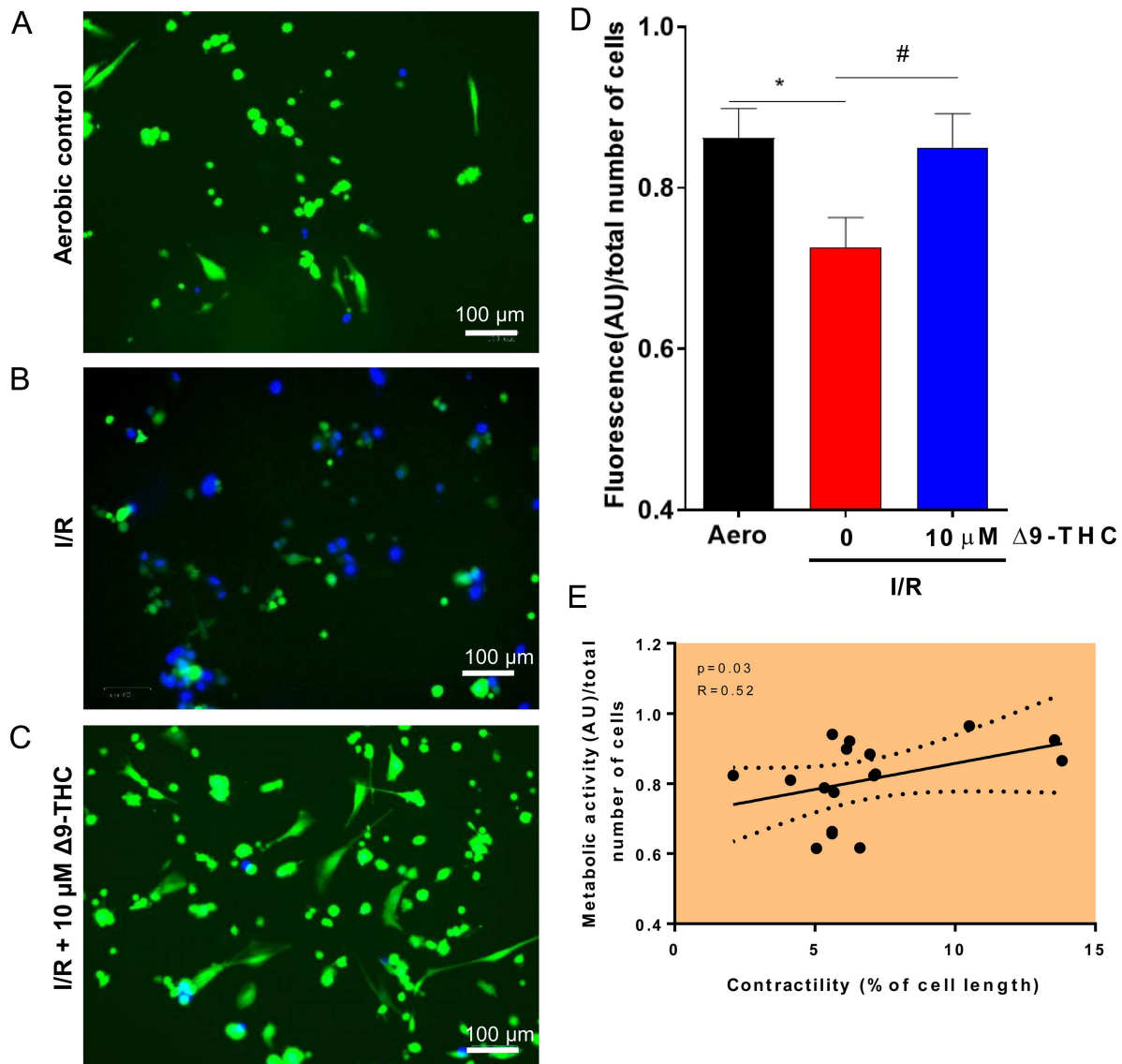
The IL-6 level in cells subjected to I/R as well as in those treated with  $\Delta^9$ -THC was increased, but this difference was not statistically significant (Fig. 10).

## 4. Discussion

Ischemia-reperfusion injury is a critical condition and remains challenging for physicians. Coronary artery bypass grafting (CABG) is associated with I/R and there is a well-known notion that complete revascularization is an important goal in the treatment [32,33]. However, it has also be-

come clear over the decades that manipulation of the myocardial response to I/R, based on both preconditioning or protection during CABG and reperfusion by administering drugs to tissue, can reduce or delay injury as well [34,35].

Numerous basic studies suggested that the introduction of cardioprotective drugs or strategies at the very onset of reperfusion can significantly reduce infarct size. In our *ex vivo* model of I/R, we used  $\Delta^9$ -THC 10 min before ischemia, as a prevention, and within the 10 first minutes of reperfusion to extend the THC treatment duration.  $\Delta^9$ -THC restored heart mechanical function to values observed in the aerobic control, suggesting the possibility of using  $\Delta^9$ -THC in future supportive therapy to PCI. Since Murphy *et al.* [34] reported data about cardioprotective strategies during I/R and showed that it is important to administer cardioprotective agents as soon as possible, in this study we proposed the use of THC as a cardioprotective drug to be administered before ischemia and during first minutes of reperfusion.

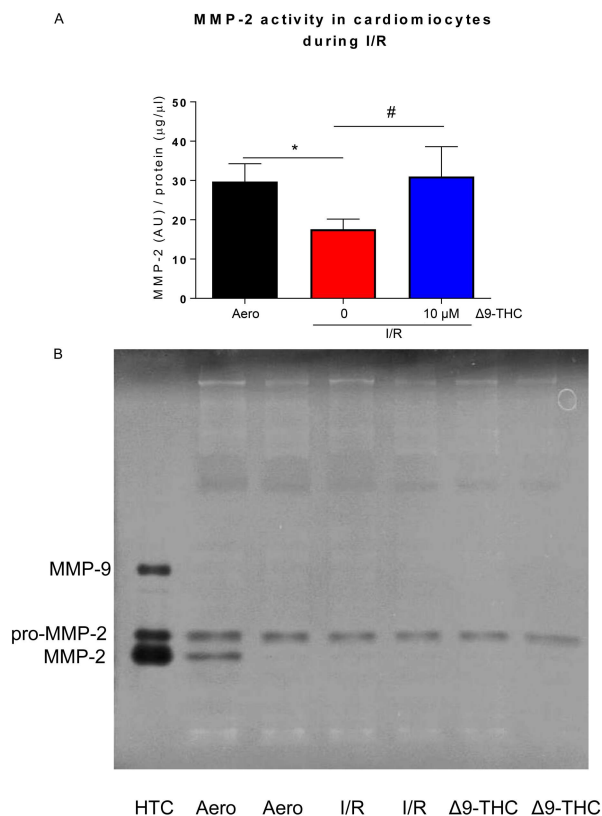


**Fig. 6. The influence of  $\Delta^9\text{-THC}$  on the metabolic activity of cardiomyocytes.** Microscopic photos (A–C), graphical presentation of the result (D); Correlation of cardiomyocytes contractility and metabolic activity (E). I/R, ischemia/reperfusion; N = 6–9 per group, \* $p = 0.025$ , # $p = 0.035$ .

Numerous previous studies suggested causal roles for increased reactive oxygen species (ROS) in the development of contractile dysfunction following reperfusion and pressure overload [36–38]. They indicated a burst in ROS generation both during ischemia and at the onset of reperfusion [34,39,40]. It was proposed that ROS, particularly ROS generated during early reperfusion, would lead to extensive oxidative damage to the cell resulting in loss of cell viability. Our current study supports this hypothesis since *in vitro* chemical ischemia and reperfusion of human cardiac myocytes led to an increased ROS level in cell supernatants, significant cell damage, reduced contractile capacity, decrease in cell vitality, and depressed metabolic

function compared to cells maintained in aerobic conditions. Moreover, *in vitro* studies revealed significant morphological signs of apoptosis/necrosis in cells subjected to I/R.

Going further, numerous studies found that antioxidants or ROS scavengers reduced infarct size [41,42]. Others have suggested that antioxidants can delay but not prevent manifestations of global ischemia [43] or do not affect heart necrosis [44–46]. For this reason, the need to continue studies on pharmacological agents preventing and/or treating IRI remains evident. There has also long been interest in the use of cannabis products in the treatment of various medical conditions, including pain [47], multiple sclerosis



**Fig. 7. MMP-2 activity in I/R cardiomyocytes.** (A) MMP-2 activity in HCM normalised to the protein content in each sample (N = 6–8 per group). (B) Representative zymogram of MMP-2 activity in HCM. Data are presented as the mean  $\pm$  SEM. HCM—human cardiomyocytes; Aero, aerobic control; I/R, chemical ischemia and reperfusion; AU, arbitrary units. 10  $\mu$ M  $\Delta^9$ -THC was used. \* $p$  = 0.019; # $p$  = 0.040.

[48], Huntington’s disease [49], stroke [50], atherosclerosis [51,52], inflammatory bowel diseases [53], renal fibrosis [54] and cancer [55]. On the other hand, chronic inflammatory disease of the artery wall and endothelial dysfunction has been proposed as a key mechanism of CV risk [1,6,56,57], and the endocannabinoid system is also thought to be an important signalling pathway in the cardiovascular system [58]. CB1 receptors have been documented in vasculature [59] and heart [60]. Apart from CB2 receptors being found in the brain [61], immune and haematopoietic cells [62], more recent studies have shown CBR2 in the myocardium [63], cardiomyoblasts [64] and endothelial cells [65]. Furthermore, Maresz *et al.* [66] and Hsieh *et al.* [67] described a 100-fold increase in CB2 mRNA levels following nerve injury or inflammation. Considering that increased mRNA expression leads to a corresponding increase in functional receptor protein, activation of the receptor with THC may be therapeutically beneficial. Therefore, the pharmacological regulation of the endocannabinoid system might be a new strategy in inflammatory and

cardiovascular disorders [reviewed in [68]]. Also worth adding that Rungatscher *et al.* [69] revealed that  $\Delta^9$ -THC have a role in reproducing “pharmacological induced hypothermia” and exert cardioprotective effect activating pro-survival signalling pathways with ERK and Akt kinases involved. It is well established that Akt is able to regulate the death receptors as well as mitochondrial apoptosis cascades [69].

Based on the anti-inflammatory and antioxidative properties of cannabis, we tested  $\Delta^9$ -THC as a potentially cardioprotective agent. Analysis of tissue injury, expressed by a decreased intracellular activity of LDH (widely used as a marker of tissue injury) [19] in the *in vitro* model of chemical ischemia and reperfusion showed that 10  $\mu$ M  $\Delta^9$ -THC reduced the damage caused by oxidative stress in the I/R group. Moreover, decreased cardiomyocyte injury affected cell contraction. As shown in Fig. 3B, 10  $\mu$ M  $\Delta^9$ -THC improved cardiomyocyte contractility and restored contractility positively correlated with cell metabolic function, confirming that THC has a cardioprotective effect. Numerous studies also indicated ROS as a causal agent in the development of contractile dysfunction following MI [36–38]. It was proposed that ROS, particularly those generated during early reperfusion, would lead to extensive oxidative damage to the cell resulting in loss of cell viability [34,39,40]. In this study, data showed an enhanced total antioxidant capacity of cardiomyocytes protected by  $\Delta^9$ -THC, confirming their antioxidative properties during ischemia/reperfusion heart injury.

Morphological analysis of cardiomyocytes subjected to chemical ischemia and reperfusion showed typical signs of homeostasis perturbations. Testing cells showed acute cell swelling, which might be a morphologic change in reversible injury or an early change of irreversible cell injury, since it results from adenosine triphosphate depletion. It also might arise from direct cell membrane damage due to ROS-induced lipid peroxidation [70]. Cells from Aerobic control were morphologically typical, with large surface area, spread out over the plate surface, without signs of swelling, almost imperceptible mitochondria and vacuoles. I/R cells changed their shape into spherical due to oedema [70]. Strong vacuolisation of the cytoplasm and numerous enlarged and swollen mitochondria with amorphous densities were also observed. The cytoplasm was fragmented while the cell nucleus had a blurred structure and destructive features. As previously described, these morphological changes might arise from the influx of water in hydropic degeneration dilutes the cytosol, separation of organelles, and distends the cells, giving them a pale, swollen and finely vacuolated appearance [3,70]. Treatment with  $\Delta^9$ -THC protected cells against the change of shape and swelling of mitochondria and lowered the number of vacuoles. The cytoplasm also did not show any features of fragmentation. All these confirmed the protective features of  $\Delta^9$ -tetrahydrocannabinol.

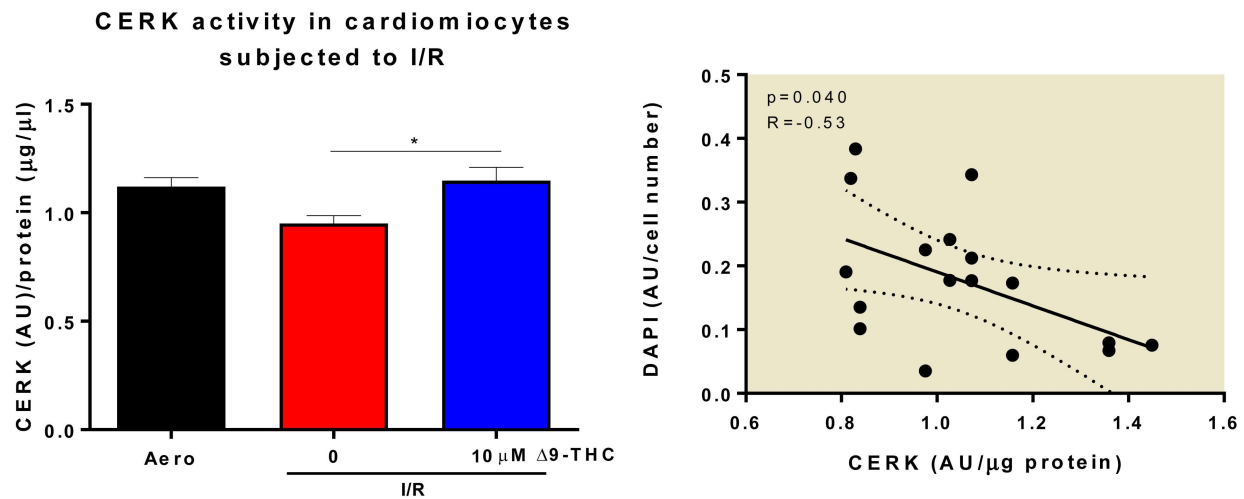


Fig. 8. CERK activity in I/R cardiomyocytes. CERK, ceramide kinase; I/R, ischemia/reperfusion; N = 6–9 per group, \* $p = 0.028$ .

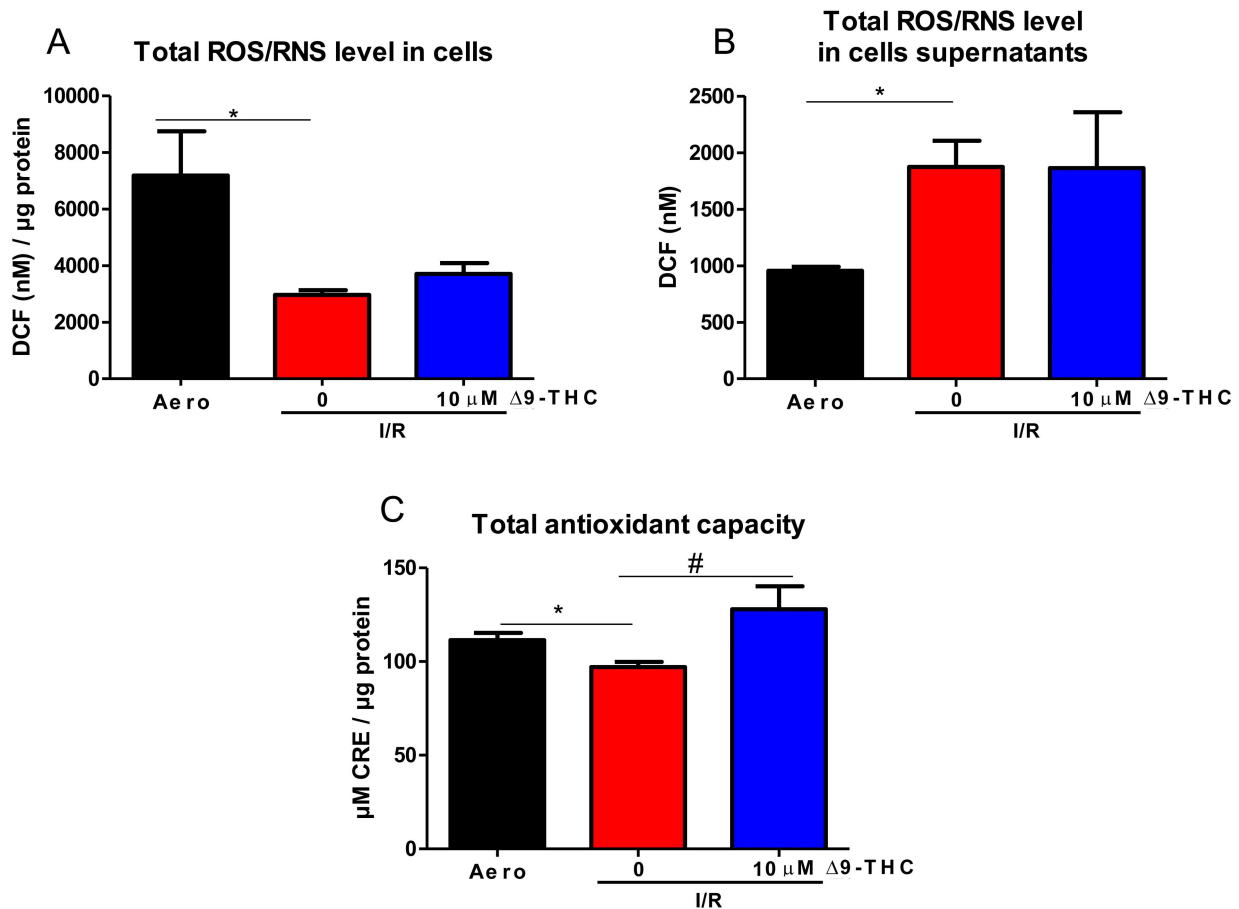
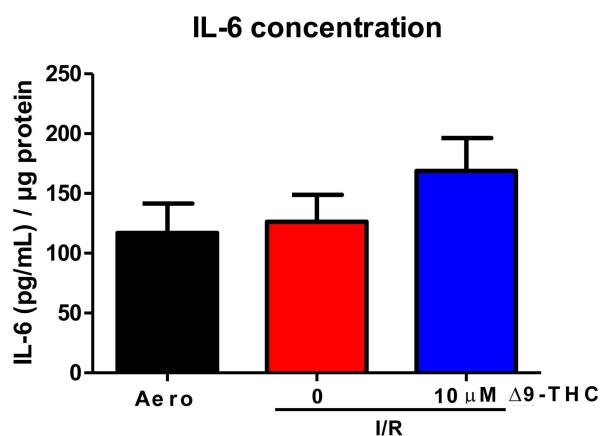


Fig. 9. Oxidative status in cardiomyocytes. (A) Total ROS/RNS level in cells expressed as nM of DCF normalised to  $\mu\text{g}$  protein, \* $p = 0.007$ . (B) total ROS/RNS level in cell supernatants expressed as nM of DCF, \* $p = 0.013$ . Supernatants were collected from the same number of cells. (C) Total antioxidant capacity of cells, \* $p = 0.010$ , # $p = 0.010$ . TAC was expressed as  $\mu\text{M}$  of CRE and normalised to the total protein concentration. Aero, aerobic control group; I/R, ischemia/reperfusion; CRE, copper reducing equivalents; DCF, 2', 7'-dichlorodihydrofluorescein; RNS, reactive nitrogen species; ROS, reactive oxygen species; TAC, total antioxidant capacity, N = 6–8 per group.



**Fig. 10. IL-6 concentration in cardiomyocytes.** Aero—aerobic control group, I/R, ischemia/reperfusion; IL-6, interleukin 6. N = 5–8 per group.

In exploring the mechanistic foundations of the cardioprotective role of  $\Delta^9$ -THC, we focused on I/R-injured cardiomyocytes. The data show that the administration of  $\Delta^9$ -THC into cardiomyocytes subjected to I/R revealed decreased mortality and increased metabolic activity of cells. The maintaining of metabolism at the level of cells maintained in aerobic conditions made it possible to support proper cell contractility, the main physiological function of the heart muscle.

MMPs play a role in cell differentiation, proliferation, wound healing, apoptosis, and angiogenesis [71]. They also contribute to the pathogenesis of such diseases as inflammation, atherosclerosis and myocardial infarction [68,72,73]. It has also previously been documented that prolonged ischemia led to the alteration of the left ventricular (LV) remodelling, an underlying cause of ischemic heart failure [74]. For optimal wound healing, balance among the inflammatory, proliferation, and maturation phases is crucial. Since matrix metalloproteinases play a major role in all phases of MI cardiac repair and remodeling, we looked at MMP-2 in the I/R cell model. Here, data showed dysregulation of MMP-2 activity in cells exposed to anaerobic conditions, however, early administration of  $\Delta^9$ -THC restored the activity to the level of aerobic control [75]. These data are consistent with previous studies, in which oxidative stress suppressed MMP-2 gene expression followed by decreased protein level and activity in favour of increased activity in extracellular space, due to damage-induced release [30,76,77]. In 2021, Euler *et al.* [78] demonstrated that MMP upregulation was not found under hypertrophic growth stimulation. Instead, some MMP mRNAs were downregulated under prohypertrophic conditions, hence downregulation could be functionally involved in the hypertrophic growth process of cardiomyocytes. This confirms that the presence of MMPs at the proper level reduces the hypertrophic growth of cardiomyocytes, for ex-

ample during heart remodeling after I/R injury.

It was documented that extracellular MMPs are strongly associated with the development and regulation of inflammation through the proteolytic regulation of inflammatory cytokines and chemokines [79]. Recent studies highlighted the role of intracellular MMPs in mediating either anti- or pro-inflammatory processes. Through the cleavage of different metabolic regulators, it modulates intracellular inflammatory pathways and lipid metabolism. MMP-2 deficiency in humans and transgenic mice was reported to induce inflammation and affect cardiac metabolism [80]. In this study we reported that a decreased level of MMP-2 in I/R cells correlated with slightly increased production of IL-6, the main proinflammatory cytokine. A similar effect was shown in the liver where MMP-2 deficiency was reported to induce hepatic dysfunction due to the enhanced inflammatory response [81]. On the other hand, cardiac-specific overexpression of MMP-2NTT76 induces an innate immune response and enhanced level of pro-inflammatory cytokines, further associated with apoptosis and inflammatory cell infiltration [82]. This is consistent with our results in which we showed that  $\Delta^9$ -THC restored MMP-2 level in I/R cardiomyocytes to the level of aerobic control, and then positively affected cell apoptosis and necrosis. These data are consistent with further results, showing that  $\Delta^9$ -THC increased CERK activity in I/R cardiomyocytes. Since the increased activity of CERK induces cell survival, it may be one of the numerous pathways through which THC can act as a cardioprotective compound.

## 5. Conclusions

In conclusion, in this study we found that  $\Delta^9$ -THC promotes the viability and improves the metabolic activity of cardiomyocytes, as well as decreasing cell damage and restoring heart mechanical function, proving that it has a cardioprotective function at the pre-treatment level. We propose the use of THC as a potentially cardioprotective compound to be, administered before ischemia and during the first minutes of reperfusion. We hope that this cannabis product may be included in future clinical investigations using similar models of ischemia-reperfusion injury. We also recommend the presented data to be considered alongside clinical observations of patient outcomes with history of cannabis use.

## 6. Limitations

In current article we used the Langendorff model, which allows only for global ischemia. Global ischemia corresponds more closely with heart transplantation and cardiopulmonary bypasses. We are aware that to discuss about myocardial infarction, the regional ischemic model, e.x. LAD occlusion would be more relevant model. Our studies present the effects of drug administration before ischemia and during reperfusion, they are not post-treatment

studies. N number was also small, this was due to following the principles of the 3Rs.

### Author Contributions

IBL, GS designed the research study. MB, PT, AKZ, RL, AO performed the research. RL, GS, ANN provided help and advice on clinical relevance. IBL, GS, MB, AKZ analysed the data. IBL, GS, MB wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors have read and approved the final manuscript.

### Ethics Approval and Consent to Participate

All rat experiments were conducted according to the Guide to the Care and Use of Experimental Animals published by the Canadian Council on Animal Care and reviewed by the Animal Research Ethics Board, University of Saskatchewan, Canada (Resolution no. 20060054).

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### Conflict of Interest

The authors declare no conflict of interest. IBL is serving as one of the Guest Editor of this journal. We declare that IBL had no involvement in the peer review of this article and has no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to JJ.

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