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1 **Cardioprotective effect of fingolimod against calcium paradox-induced**
2 **myocardial injury in the isolated rat heart**

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18

19 **Abstract:** Fingolimod (FTY720) inhibits Ca^{2+} -permeable, Mg^{2+} -sensitive channels called
20 transient receptor potential melastatin 7 (TRPM7), but its effects on Ca^{2+} paradox (CP)-
21 induced myocardial damage have not been evaluated. We studied the effect of FTY720 on
22 CP-induced myocardial damage, and used other TRPM7 channel inhibitors
23 nordihydroguaiaretic acid (NDGA) and Mg^{2+} to test if any effect of FTY720 was via TRPM7
24 inhibition. Langendorff-perfused Wistar rat hearts were treated with FTY720 or NDGA and
25 subjected to a CP protocol consisting of Ca^{2+} depletion followed by Ca^{2+} repletion. Hearts of
26 rats pre-treated with MgSO_4 were also subjected to CP. Hemodynamic parameters were
27 measured using an intraventricular balloon, and myocardial infarct size was quantified using
28 triphenyltetrazolium chloride stain. TRPM7 proteins in ventricular tissue were detected using
29 immunoblot analysis. FTY720, but not NDGA, decreased CP-induced infarct size. Both
30 FTY720 and NDGA minimized the CP-induced elevation of left ventricular end-diastolic
31 pressure, but only FTY720 ultimately improved ventricular developed pressure. Mg^{2+} pre-
32 treatment had effect neither on CP-induced infarct size, hemodynamic parameters during CP,
33 nor the level TRPM7 protein expression in ventricular tissue. Overall, FTY720 attenuated
34 CP-induced myocardial damage, with potential therapeutic implications on Ca^{2+} -mediated
35 cardiotoxicity. However, the cardioprotective mechanism of FTY720 seems to be unrelated
36 to TRPM7 channel modulation.

37

38 Keywords

39 cardiac; calcium paradox; fingolimod; ion channels; TRPM7

40 Introduction

41 Abnormalities of calcium (Ca^{2+}) homeostasis underlie the pathophysiology of life-
42 threatening cardiovascular conditions such as myocardial injury and malignant arrhythmias
43 (Wagner et al. 2015). Ca^{2+} paradox (CP) is a form of Ca^{2+} -mediated myocardial injury that
44 may occur perioperatively in hearts temporarily subjected to Ca^{2+} -deficient conditions such
45 as perfusion with Ca^{2+} -free cardioplegic solutions (Zimmerman 2000). In other non-
46 perioperative situations, cardiac Ca^{2+} paradox could occur in a subtle form and remain
47 relatively undetected in conditions of temporary Ca^{2+} deficits. Unlike the myocardium
48 damage due to ischemia/reperfusion injury, CP can occur even with continuous tissue
49 perfusion (Mani et al. 2015, Piper 2000). During CP, the removal of extracellular Ca^{2+}
50 induces cardiac intracellular- and intercellular molecular changes that make cardiomyocytes
51 susceptible to damage upon the re-introduction of extracellular Ca^{2+} ions (Aggeli et al. 2013,
52 Hearse et al. 1978, Zimmerman 2000). Unfortunately, preventative options for CP remain
53 limited because the underlying mechanisms are not fully understood. The known
54 abnormalities in CP include myocardial ultra-structural damage mainly due to Ca^{2+} overload
55 as well as due to cell-cell separation and ATP depletion, even in the absence of ischemia
56 (Kovacs et al. 2017, Mani et al. 2015, Piper 2000). The Ca^{2+} overload in CP may occur via
57 Ca^{2+} -selective channels such as L-type Ca^{2+} channels or secondary to Na^{+} overload via the
58 action of the reverse-mode $\text{Na}^{+}/\text{Ca}^{2+}$ exchanger (Guppy et al. 1999, Karmazyn et al. 1993,
59 Piper 2000). It has also been suggested to occur via Ca^{2+} -permeable channels such as
60 transient receptor potential (TRP) channels (Bosteels et al. 1999, Kojima et al. 2010).

61 Fingolimod (FTY720) is a drug used in the treatment of multiple sclerosis, but some
62 studies have also demonstrated its ability to protect against cardiovascular conditions such as
63 ischemia/reperfusion myocardial injury (Hofmann et al. 2009, Santos-Gallego et al. 2016,
64 Vessey et al. 2013) and arrhythmias (Egom et al. 2010, Egom et al. 2015). Nonetheless, even

65 in the context of ischemia/reperfusion injury, the effect of FTY720 is still not fully known
66 since both pro-arrhythmic and anti-arrhythmic effects of FTY720 have been observed in the
67 same disease model, depending on the timing of drug administration (Hofmann et al. 2010).
68 Furthermore, in ischemia/reperfusion injury models, FTY720 has been shown either to
69 reduce infarct size (van Vuuren et al. 2016), to have no effect on infarct size, despite
70 hemodynamic improvements (Hofmann et al. 2009, Hofmann et al. 2010), or to have a dose-
71 dependent differential effect on functional recovery (van Vuuren et al. 2016).

72 The pathophysiological mechanisms of disease conditions such as
73 ischemia/reperfusion myocardial injury are distinctly different from that of CP (Piper 2000),
74 and as such the effect of FTY720 on CP-induced myocardial damage is unknown. FTY720 is
75 a sphingosine analogue. When protecting the heart against damage caused by
76 ischemia/reperfusion injury or arrhythmias, FTY720 has been shown to act through S1P
77 receptor-dependent mechanisms and activation of pro-survival kinases to protect the heart
78 (Ahmed et al. 2019, Egom et al. 2010, Hofmann et al. 2009, Hofmann et al. 2010, Santos-
79 Gallego et al. 2016). Interestingly, FTY720 also acts via S1P receptor-independent pathways
80 to protect the heart (Vessey et al. 2013). One of these mechanisms is via its inhibition of the
81 enzyme S1P lyase (Bandhuvula et al. 2005), the inhibition of which is linked to the
82 cardioprotection against ischemia/reperfusion injury (Bandhuvula et al. 2011). However,
83 FTY720 can also inhibit the Mg²⁺-sensitive, Ca²⁺-permeable TRP melastatin 7 (TRPM7)
84 channels (Qin et al. 2013). The TRPM7 channels mediate cellular entry of Ca²⁺ and other
85 divalent cations (Gwanyanya et al. 2004, Monteilh-Zoller et al. 2003, Nadler et al. 2001).
86 Considering the major changes in Ca²⁺ homeostasis that occur in several cardiac pathologies,
87 it is possible that FTY720 may also protect the heart via its modulation of these channels.

88 In this study, we therefore investigated the effect of FTY720 on CP-induced
89 myocardial damage. We also tested whether FTY720 may act via inhibiting TRPM7 channels

90 by using another chemically-unrelated TRPM7 inhibitor nordihydroguaiaretic acid (NDGA)
91 (Chen et al. 2010) as well as Mg^{2+} , given its intracellular inhibition of TRPM7 channels
92 (Gwanyanya et al. 2004, Monteilh-Zoller et al. 2003). TRPM7 channels were targeted
93 because they are a relatively new type of Ca^{2+} -permeable channels that are known to be
94 inhibited by fingolimod, and therefore can become therapeutic drug targets. As for Mg^{2+} , its
95 extracellular application is already known to protect against Ca^{2+} paradox in guinea pig hearts
96 (Suleiman et al. 1993), so we tested for a different, intracellular effect of Mg^{2+} on CP via
97 TRPM7 inhibition.

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98 **Materials and methods**

99 **Animals**

100 The study was approved by the Faculty of Health Sciences Animal Research Ethics
101 Committee of the University of Cape Town (AEC Protocol 014-014), and all research
102 involving animals was conducted according to the Canadian Council on Animal Care
103 (CCAC) guidelines and the Guide for the Care and Use of Laboratory Animals (8th edition,
104 National Academies Press). Adult male Wistar rats (250–300 g) were housed under
105 standardized conditions (12-hour light/dark cycle and temperature of 23°C) and had
106 unlimited access to rat chow and drinking water. Rats used to test Mg²⁺ effects were injected
107 intraperitoneally (i.p.) with either MgSO₄ (270 mg/kg) or an equivalent volume of saline
108 daily for 7 consecutive days (Amoni et al. 2017b, Sameshima et al. 1999). The MgSO₄ salt
109 was chosen as it is more often clinically used than say MgCl₂ (Durlach et al. 2005), and also
110 offsets any enteral-route related toxicity when administered intraperitoneally as was done in
111 this study. For enteral administration, MgCl₂ would have been a better choice, given its lower
112 enteral toxicity profile (Durlach et al. 2005). In addition, the MgSO₄ pharmacokinetic profile
113 in rats has been determined in other tissue-protection studies (Sameshima et al. 1999). Mg²⁺
114 was administered as pre-treatment *in vivo*, rather than acutely on isolated hearts in order to
115 allow the Mg²⁺ to enter cells and exert its TRPM7 channel inhibitory effects from the
116 intracellular compartment (Gwanyanya et al. 2004, Monteilh-Zoller et al. 2003, Nadler et al.
117 2001). The experiments on Mg²⁺-treated rats were performed 24 hours after the final MgSO₄
118 or saline injection to exclude direct extracellular effects of Mg²⁺.

119

120 **Heart isolation and perfusion protocols**

121 Unless stated otherwise, chemicals were obtained from Sigma (Sigma-Aldrich, SA).
122 Hearts were removed for Langendorff perfusion as previously described (Araibi et al. 2020).
123 Briefly, rats were anticoagulated with heparin (500 I.U./kg, i.p.) and anesthetized with
124 sodium pentobarbital (70 mg/kg i.p., Vertserv, SA). Hearts were rapidly removed and placed
125 in cold (4°C), filtered (Whatman filter paper, Sigma-Aldrich, SA), modified Krebs-Henseleit
126 (K-H) solution containing (in mmol/L): 118.5 NaCl, 4.7 KCl, 25 NaHCO₃, 1.2 MgSO₄,
127 1.8 CaCl₂, 1.2 KH₂PO₄ and 11 glucose; pH 7.4, gassed with 95%O₂ and 5%CO₂). For
128 perfusion studies, the aorta was cannulated for retrograde perfusion with K-H solution
129 (maintained at 37°C) on a constant-pressure (74 mmHg) Langendorff perfusion apparatus
130 (Fig. 1A). To induce Ca²⁺ paradox, a parallel Langendorff apparatus was used to deliver
131 Ca²⁺-free K-H solution, and this system converged with the main perfusion system via a 3-
132 way stop cock positioned above the aortic cannula (Fig. 1A). The Ca²⁺-free K-H solution was
133 made by excluding CaCl₂ from K-H solution and adding 0.5 mmol/L EGTA.

134 Hearts used to test the effects of fingolimod (FTY720) and NDGA were randomly
135 assigned to 6 groups (Fig. 1B), whereas those used to test the effect of Mg²⁺ were assigned to
136 4 groups (Fig. 4A), with each group named according to the perfusion- and drug
137 administration protocol used. Hearts were stabilized on the perfusion system for 20 minutes
138 prior to drug treatments. The CP protocol consisted of heart perfusion with Ca²⁺-free K-H
139 solution for 3 minutes, followed by the re-introduction of Ca²⁺-containing solution for 30
140 minutes (Bi et al. 2012). FTY720 (1 µmol/L) and NDGA (10 µmol/L) were dissolved in
141 DMSO (final concentration < 0.01%) and administered for 5 minutes before the
142 commencement of the CP protocol. The concentrations of these drugs used are adequate to
143 inhibit TRPM7 channels (Chen et al. 2010, Qin et al. 2013). The drugs were delivered into
144 the perfusate column through a 3-way stop cock using a syringe pump (Graseby 2100, Smith
145 Medical, SA; Fig 1A) set to run at 10% of the coronary flow rate in order to minimize

146 disturbances in perfusion pressure. The concentration of the drug in the syringe was 10 times
147 higher than the final concentration to cater for the dilution by the main perfusion column. The
148 coronary flow rate was measured by timed collection of coronary effluent. At the end of
149 perfusion, hearts were stored at -20°C for infarct size measurements.

150

151 **Hemodynamic parameter measurements**

152 Left ventricular (LV) pressure was measured using a water-filled, intraventricular
153 balloon (Fig. 1A) mounted at the tip of a catheter connected to a blood pressure transducer
154 (MLT1199) and amplifier (Bridge Amp ML221, ADInstruments, Australia). The balloon was
155 inflated to a LV end-diastolic pressure (LVEDP) of 5–10 mmHg, and the balloon volume
156 was not altered thereafter. Hemodynamic parameters were recorded using the PowerLab data-
157 acquisition system and LabChart 7 software (ADInstruments, Australia) and were analyzed
158 using the LabChart 7 Pro BP module (ADInstruments, Australia). The LV developed pressure
159 (LVDP) was obtained as the difference between peak systolic pressure and LVEDP.

160

161 **Infarct size measurements**

162 Ventricles of frozen hearts were cut transversely into a series of 2-mm slices from
163 apex to base and thawed for 2,3,5-triphenyltetrazolium chloride (TTC) staining as previously
164 described (Amoni et al. 2017b, Araibi et al. 2020). Infarct size was measured as TTC-
165 negative area on the slices from each heart using ImageJ software (NIH, USA) and was
166 expressed as a percentage of the total ventricular area (Araibi et al. 2020).

167

168 **Western immunoblotting**

169 Western blot analysis was performed as previously described (Aboalgasm et al.
170 2021a, Aboalgasm et al. 2021b). Sections of frozen LV tissue (approximately 0.05 g) were

171 homogenized on ice by sonication in a modified radioimmunoprecipitation assay (RIPA)
172 lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate,
173 0.1% sodium dodecyl sulphate, pH 7.4) containing a protease/phosphatase inhibitor cocktail
174 (HALT, Thermo Fisher Scientific, Rockford, USA). The samples were mixed on a vortex and
175 centrifuged at 15 000 g for 30 minutes at 4°C. The supernatant was decanted, and the protein
176 concentration was quantified using a BCA protein assay kit (Thermo Fisher Scientific,
177 Rockford, USA). Lysates were denatured at 95°C for 5 minutes in a mixture of Laemmli
178 buffer (Bio-Rad, SA), RIPA buffer, and diethyltritol. Protein samples (40 µg) were
179 electrophoresed on 12% SDS-PAGE gels using a Mini-PROTEAN Tetra Cell system
180 (Bio-Rad, SA) and transferred to nitrocellulose membrane using a Trans-Blot turbo transfer
181 system (Bio-Rad, SA). Gel electrophoresis and protein transfer to membrane were confirmed
182 by Ponceau S staining. The membrane was blocked with 5% milk in 0.1% Tween-20
183 phosphate-buffered saline (PBS-T) at 4°C for 3 hours, and incubated with anti-TRPM7
184 mouse monoclonal primary antibody (1:8000, Abcam 85016, USA) in 5% milk PBS-T
185 overnight at 4°C. In the negative control, anti-TRPM7 antibody was excluded to rule out non-
186 specific binding of secondary antibody. The membrane was then washed in PBS-T and
187 incubated with horseradish peroxidase-linked goat anti-mouse secondary antibody (1:20 000,
188 Abcam 205719, USA) in 5% milk PBS-T for 1 hour at room temperature. Blots were
189 developed by adding an enhanced chemiluminescence substrate (Bio-Rad, SA) and exposure
190 to X-ray film. The film images were scanned and analysed using ImageJ software (NIH,
191 USA). The membrane was thereafter washed in water, stripped with 8% NaOH, blocked for 3
192 hours with 5% BSA in PBS-T, and re-probed with anti-β-actin mouse monoclonal primary
193 antibody (1:15000, Abcam 8226, USA) and goat anti-mouse secondary antibody (1:20 000,
194 Abcam 205719, USA).

195

196 **Plasma Mg²⁺ measurements**

197 Blood used for Mg²⁺ measurements was collected at the time of excision of the heart
198 (24 hours after the final MgSO₄ or saline injection). The blood was centrifuged to obtain
199 plasma, from which ionized Mg²⁺ concentration was measured using photometric assays
200 (Beckman AU, PathCare, SA) as previously described (Amoni et al. 2017a).

201

202 **Data analysis**

203 Data are expressed as mean and standard error of mean (SEM), with *n* indicating the
204 number of rats studied under each condition. Statistical analysis was conducted using
205 Statistica (v.13). Differences among multiple groups were evaluated using analysis of
206 variance (ANOVA) and Tukey's *post-hoc* test or repeated-measures ANOVA. Differences
207 between two independent groups were compared using unpaired *t*-test. *P* < 0.05 was
208 considered statistically significant.

209 **Results**

210 **Effects of FTY720 and NDGA on CP-induced infarct size**

211 Representative images of TTC-stained ventricular slices (Fig. 2A) show a
212 prominent TTC-negative ventricular area in the CP heart compared to that in the control
213 heart, which is indicative of CP-induced myocardial damage. The application of FTY720
214 (1 $\mu\text{mol/L}$), but not NDGA (10 $\mu\text{mol/L}$), significantly decreased CP-induced infarct size from
215 $64.6 \pm 5.3\%$ to $39.0 \pm 6.8\%$ ($P = 0.001$; Fig. 2B). The sizes of background infarcts due to
216 handling artefacts in non-CP hearts (i.e., control and drug only treated hearts) were not
217 significantly different from each other ($P = 0.78$) but were significantly smaller than the
218 infarcts in CP hearts ($P < 0.001$; Fig. 2B).

219

220 **Effects of FTY720 and NDGA on CP-induced hemodynamic parameters**

221 A typical CP protocol (Fig. 3A) produced a complete loss of ventricular force of
222 contraction and an elevation of LVEDP during the periods of Ca^{2+} depletion and Ca^{2+}
223 repletion (LVEDP: $P < 0.001$ for CP vs. control; Fig. 3B), whereas the ventricular force of
224 contraction and LVEDP in control, non-CP hearts remained relatively stable over the
225 duration of perfusion. Although there was still an elevation of LVEDP due to CP, both
226 FTY720 and NDGA decreased the extent of CP-induced elevation of LVEDP compared to
227 CP alone ($P = 0.039$ for FTY720 + CP vs. CP and $P = 0.020$ for NDGA + CP vs. CP;
228 Fig. 3B). However, only FTY720 significantly improved LVDP during CP ($P = 0.029$,
229 FTY720 + CP vs. CP; Fig. 3C). In the absence of CP, neither FTY720 nor NDGA altered
230 LVDP compared to control ($P = 0.90$; Fig. 3C). However, FTY720 alone increased LVEDP
231 in the absence of CP compared to control ($P = 0.003$), but this increase was still significantly
232 lower than the LVEDP in CP (Fig. 3B). Neither CP nor the application of drugs (FTY720 or

233 NDGA) had statistically significant effect on coronary flow rate compared to control
234 ($P = 0.40$; Fig. 3D).

235

236 **Effect of Mg^{2+} on CP**

237 We also tested effects of Mg^{2+} as an additional type of TRPM7 channel inhibitor. This
238 ion has a different inhibitory action since it acts intracellularly as compared to the
239 extracellular effects of FTY720 and NDGA. Mg^{2+} pre-treatment did not significantly alter
240 CP-induced infarct size ($50.7 \pm 3.7\%$ for CP vs. $50.2 \pm 4.1\%$ for Mg^{2+} + CP, $P = 0.98$;
241 Fig. 4B and Fig. 4C). The sizes of background infarcts in non-CP hearts were not
242 significantly different from each other (infarct size: $7.4 \pm 0.5\%$ for control vs. $6.4 \pm 0.5\%$ for
243 Mg^{2+} pre-treated, $P = 0.90$), but were significantly smaller than the infarcts in CP hearts
244 ($P < 0.001$, CP vs. control or Mg^{2+} ; Fig. 4B and Fig. 4C). Mg^{2+} pre-treatment also did not
245 reverse the CP-induced changes in LVEDP or LVDP (Fig. 4D and Fig. 4E). In the absence of
246 CP, pre-treatment with Mg^{2+} had no significant effect on LVDP or LVEDP (Fig. 4D and Fig.
247 4E). Furthermore, Mg^{2+} concentration was measured to verify plasma normomagnesemia at
248 the time of CP experiments. The plasma Mg^{2+} concentration was not significantly different
249 between Mg^{2+} pre-treated rats and controls (0.87 ± 0.03 mmol/L for control vs. 0.93 ± 0.04
250 mmol/L for Mg^{2+} pre-treated; $P = 0.62$, $n = 5$ rats per group).

251

252 **TRPM7 immunoblotting**

253 In order to verify the presence of TRPM7 channels and the possible modulation by
254 Mg^{2+} pre-treatment in the rat hearts used in our experiments, TRPM7 immunoblotting was
255 performed in ventricular tissue. Images of western blot analysis (Fig. 5A) showed the
256 immune-detection of both TRPM7- and the loading control β -actin proteins in cardiac LV
257 tissue. In contrast, the TRPM7 signal was virtually undetectable in the negative controls in

258 which the anti-TRPM7 primary antibody was omitted ($P = 0.003$ vs. TRPM7; $n = 6$ rats per
259 group; result not illustrated). The intensity of the TRPM7 bands on films appeared similar in
260 ventricular tissue of control rats and Mg^{2+} pre-treated rats (Fig. 5A). As such, there was no
261 statistically significant difference in the level of TRPM7 protein expression between
262 ventricular tissue of control- and Mg^{2+} pre-treated rats ($P = 0.43$; Fig. 5B).

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263 Discussion

264 CP represents a unique, Ca^{2+} -related threat to perioperative cardiovascular tissue
265 survival, even in the absence of ischemia (Piper 2000, Zimmerman 2000). The results of the
266 present study showed that FTY720, but not NDGA nor Mg^{2+} , decreased CP-induced infarct
267 size and improved LV functional recovery.

268 In this study, FTY720 was partially effective at rescuing contractile function of the
269 heart during CP as was evidenced by improved LVDP; minimized cardiac tissue death from
270 CP-induced myocardial injury as was evidenced by the reduction in infarct size; and reduced
271 the extent of dysfunctional contractures as was indicated by decreased LVEDP during CP.
272 Although some studies have demonstrated cardioprotective effects of FTY720 in models of
273 ischemia/reperfusion injury and cardiac arrhythmia, this cardioprotective effect of FTY720
274 during CP seems to be a novel finding since there were no previous reports that we could find
275 that specifically addressed the effect of FTY720 on CP. However, we also noticed that
276 FTY720, on its own, increased LVEDP by the end of perfusion compared to control hearts.
277 The reason for this increase was not clear, but other studies showed that the infusion of
278 FTY720 in conscious rodents increased mean arterial pressure (Forrest et al. 2004), and that
279 FTY720, when orally administered, induced hypertension in rodents (Fryer et al. 2012). In
280 contrast, in healthy human subjects, FTY720 induced transient decreases in mean arterial
281 pressure (Schmouder et al. 2006). Such effects of FTY720 on blood pressure as well as the
282 induction of bradycardia (Faber et al. 2013) therefore constitute important side effects to be
283 recognized in the utility of the drug in cardioprotection. Nonetheless, the increase in LVEDP
284 by FTY720 observed in the present study did not undermine the overall improvement of
285 LVDP during CP.

286 The mechanism underlying the cardioprotective effect of FTY720 during CP is
287 unclear since the inhibition of TRPM7 channel did not seem to mediate the effect. In our

288 study, there was a lack of cardioprotection against CP by NDGA, a chemical which, similar
289 to FTY720, inhibits the Ca^{2+} -permeable TRPM7 channels at the doses administered (Chen et
290 al. 2010, Qin et al. 2013). The lack of NDGA effect was despite the presence of TRPM7
291 protein in the rat hearts used in our experiments as was confirmed by the positive TRPM7
292 immunoblots in LV tissue. Consistent with the findings from another study (Murphy et al.
293 1995), in our study, NDGA, on its own, had no detrimental effects on cardiac hemodynamic
294 function, but rather stabilized LVEDP during CP. However, NDGA was also shown to impair
295 protective effects of cardiac preconditioning via the lipoxygenase pathway (Murphy et al.
296 1995) but such a mechanism may not be relevant to CP since the inhibition of TRPM7
297 channels by NDGA is lipoxygenase-independent (Chen et al. 2010). Furthermore, in our
298 study, Mg^{2+} pre-treatment did not alter CP, suggesting that the intracellular inhibitory effect of
299 Mg^{2+} on TRPM7 channels did not modulate CP. The protective effect of acute extracellular
300 Mg^{2+} against CP observed in guinea pig hearts (Suleiman et al. 1993) is different from an
301 intracellular inhibition of TRPM7 channels, and therefore would not be applicable in the
302 present study since the plasma Mg^{2+} concentration had reverted to normal at the time of CP
303 experiments as was verified by Mg^{2+} assays. Taken together, our results suggest that the
304 cardioprotective effect of FTY720 during CP may be unrelated to the inhibition of TRPM7
305 channels. The possible contribution to CP of other TRP channels that belong to the same
306 melastatin subfamily as TRPM7 channels such as TRPM4 is not known. TRPM4 channels
307 are highly selective for monovalent cations such as Na^+ (Launay et al. 2002), and it has been
308 proposed that the monovalent cation influx through them could induce membrane
309 depolarisation that may, in turn, cause Ca^{2+} influx via Ca^{2+} -permeable channels, thereby
310 modulating cardiac activity (Alonso-Carbajo et al. 2017). However, there is no evidence yet
311 to support this possibility for Ca^{2+} influx through TRPM7 channels, which would otherwise
312 be relevant in CP. In addition, such an effect would most likely affect voltage-gated Ca^{2+} -

313 permeable channels, rather than TRPM7 channels, for which the gating is voltage-
314 independent (Gwanyanya et al. 2004, Monteilh-Zoller et al. 2003, Nadler et al. 2001).

315 Most of what is currently known about the cardiac effects of FTY720 relates to the
316 models of ischemia/reperfusion injury. Although Ca^{2+} dysregulation may be involved in both
317 ischemia/reperfusion injury and Ca^{2+} paradox, these conditions are different. Ca^{2+} paradox is
318 not often easily recognised as it presents with some features similar to those of
319 ischemia/reperfusion injury (Hearse et al. 1978), yet it is a detrimental clinical entity that
320 occurs even in the absence of an ischemic insult (Aggeli et al. 2013, Hearse et al. 1978,
321 Zimmerman 2000). Furthermore, given that Ca^{2+} paradox represents a complication of
322 temporary Ca^{2+} deficit, but, at the same time, Ca^{2+} abnormalities are known to affect a
323 plethora of cellular functions, its manifestation could be missed and attributed to other well-
324 known Ca^{2+} -related cellular events. However, an important limitation in studying Ca^{2+}
325 paradox (as was done in the present study) is that the standard experimental protocol of
326 extracellular Ca^{2+} exclusion is extreme and unphysiological, but at the same time, it has the
327 advantage that it amplifies and quickens the subtle tissue damage that would otherwise occur
328 undetected whenever there is a temporary Ca^{2+} deficit (Piper 2000). Our results, therefore,
329 show the potential of FTY720 as a cardioprotective agent against Ca^{2+} paradox, a novelty that
330 has not been described in Ca^{2+} paradox. This FTY720 effect on CP is therefore different from
331 the FTY720 modulation of other cardiac conditions such as ischaemia/reperfusion injury.
332 However, the exact mechanism underlying the protective effect of FTY720 against CP was
333 not established in the present study.

334 In conclusion, the results of this study showed that FTY720, but not NDGA or Mg^{2+} ,
335 partially reduced CP-induced myocardial damage and improved cardiac contractile function.
336 Clinically, given that FTY720 is already being used in the treatment of multiple sclerosis, this
337 cardioprotective effect may represent a novel therapeutic role of the drug (or in combination

338 with other protective drugs) in attenuating CP-induced myocardial damage during
339 perioperative cardiac perfusion. The results suggest that the cardioprotective action of
340 FTY720 during CP may be unrelated to the inhibition of TRPM7 channels, but the exact
341 underlying mechanism still requires further investigation.

Draft

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346 of interest.

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347 References

- 348 Aboalgasm, H., Ballo, R., Mkatzo, T. & Gwanyanya, A. 2021a. Hyperglycaemia-Induced
349 Contractile Dysfunction and Apoptosis in Cardiomyocyte-Like Pulsatile Cells Derived
350 from Mouse Embryonic Stem Cells. *Cardiovasc Toxicol.* **21**(9): 695-709.
351 doi:10.1007/s12012-021-09660-3. PMID:33983555.
- 352 Aboalgasm, H., Petersen, M. & Gwanyanya, A. 2021b. Improvement of cardiac ventricular
353 function by magnesium treatment in chronic streptozotocin-induced diabetic rat heart.
354 *Cardiovasc J Afr.* **32**(3): 141-148. doi:10.5830/CVJA-2020-054. PMID:33300932.
- 355 Aggeli, I.K., Zacharias, T., Papapavlou, G., Gaitanaki, C. & Beis, I. 2013. Calcium paradox
356 induces apoptosis in the isolated perfused *Rana ridibunda* heart: involvement of p38-
357 MAPK and calpain. *Can J Physiol Pharmacol.* **91**(12): 1095-1106. doi:10.1139/cjpp-
358 2013-0081. PMID:24289081.
- 359 Ahmed, N., Mehmood, A., Linardi, D., Sadiq, S., Tessari, M., Meo, S.A., Rehman, R.,
360 Hajjar, W.M., et al. 2019. Cardioprotective Effects of Sphingosine-1-Phosphate
361 Receptor Immunomodulator FTY720 in a Clinically Relevant Model of Cardioplegic
362 Arrest and Cardiopulmonary Bypass. *Front. Pharmacol.* **10**: 802.
363 doi:10.3389/fphar.2019.00802. PMID:31379576.
- 364 Alonso-Carbajo, L., Kecskes, M., Jacobs, G., Pironet, A., Syam, N., Talavera, K. &
365 Vennekens, R. 2017. Muscling in on TRP channels in vascular smooth muscle cells and
366 cardiomyocytes. *Cell Calcium.* **66**: 48-61. doi:10.1016/j.ceca.2017.06.004.
367 PMID:28807149.
- 368 Amoni, M., Kelly-Laubscher, R., Blackhurst, D. & Gwanyanya, A. 2017a. Beneficial Effects
369 of Magnesium Treatment on Heart Rate Variability and Cardiac Ventricular Function in
370 Diabetic Rats. *J Cardiovasc Pharmacol Ther.* **22**(2): 169-178.
371 doi:10.1177/1074248416653831. PMID:27276916.

- 372 Amoni, M., Kelly-Laubscher, R., Petersen, M. & Gwanyanya, A. 2017b. Cardioprotective
373 and Anti-arrhythmic Effects of Magnesium Pretreatment Against
374 Ischaemia/Reperfusion Injury in Isoprenaline-Induced Hypertrophic Rat Heart.
375 *Cardiovasc Toxicol.* **17**(1): 49-57. doi:10.1007/s12012-015-9355-6. PMID:26696240.
- 376 Araibi, H., Van Der Merwe, E., Gwanyanya, A. & Kelly-Laubscher, R. 2020. The effect of
377 sphingosine-1-phosphate on the endothelial glycocalyx during ischemia-reperfusion
378 injury in the isolated rat heart. *Microcirculation.* **27**(5): e12612.
379 doi:10.1111/micc.12612. PMID:32017300.
- 380 Bandhuvula, P., Honbo, N., Wang, G.Y., Jin, Z.Q., Fyrst, H., Zhang, M., Borowsky, A.D.,
381 Dillard, L., et al. 2011. S1P lyase: a novel therapeutic target for ischemia-reperfusion
382 injury of the heart. *Am J Physiol Heart Circ Physiol.* **300**(5): H1753-1761.
383 doi:10.1152/ajpheart.00946.2010. PMID:21335477.
- 384 Bandhuvula, P., Tam, Y.Y., Oskouian, B. & Saba, J.D. 2005. The immune modulator
385 FTY720 inhibits sphingosine-1-phosphate lyase activity. *J. Biol. Chem.* **280**(40):
386 33697-33700. doi:10.1074/jbc.C500294200. PMID:16118221.
- 387 Bi, S.H., Jin, Z.X., Zhang, J.Y., Chen, T., Zhang, S.L., Yang, Y., Duan, W.X., Yi, D.H., et al.
388 2012. Calpain inhibitor MDL 28170 protects against the Ca²⁺ paradox in rat hearts. *Clin*
389 *Exp Pharmacol Physiol.* **39**(4): 385-392. doi:10.1111/j.1440-1681.2012.05683.x.
390 PMID:22356295.
- 391 Bosteels, S., Matejovic, P., Flameng, W. & Mubagwa, K. 1999. Sodium influx via a non-
392 selective pathway activated by the removal of extracellular divalent cations: possible
393 role in the calcium paradox. *Cardiovasc Res.* **43**(2): 417-425. doi:10.1016/s0008-
394 6363(99)00098-x. PMID:10536672.

- 395 Chen, H.C., Xie, J., Zhang, Z., Su, L.T., Yue, L. & Runnels, L.W. 2010. Blockade of TRPM7
396 channel activity and cell death by inhibitors of 5-lipoxygenase. *PLoS One*. **5**(6):
397 e11161. doi:10.1371/journal.pone.0011161. PMID:20567598.
- 398 Durlach, J., Guiet-Bara, A., Pages, N., Bac, P. & Bara, M. 2005. Magnesium chloride or
399 magnesium sulfate: a genuine question. *Magnes. Res.* **18**(3): 187-192. PMID:16259379.
- 400 Egom, E.E., Ke, Y., Musa, H., Mohamed, T.M., Wang, T., Cartwright, E., Solaro, R.J. & Lei,
401 M. 2010. FTY720 prevents ischemia/reperfusion injury-associated arrhythmias in an ex
402 vivo rat heart model via activation of Pak1/Akt signaling. *J. Mol. Cell. Cardiol.* **48**(2):
403 406-414. doi:10.1016/j.yjmcc.2009.10.009. PMID:19852968.
- 404 Egom, E.E., Kruzliak, P., Rotrekl, V. & Lei, M. 2015. The effect of the sphingosine-1-
405 phosphate analogue FTY720 on atrioventricular nodal tissue. *J Cell Mol Med.* **19**(7):
406 1729-1734. doi:10.1111/jcmm.12549. PMID:25864579.
- 407 Faber, H., Fischer, H.J. & Weber, F. 2013. Prolonged and symptomatic bradycardia
408 following a single dose of fingolimod. *Mult. Scler.* **19**(1): 126-128.
409 doi:10.1177/1352458512447596. PMID:22729989.
- 410 Forrest, M., Sun, S.Y., Hajdu, R., Bergstrom, J., Card, D., Doherty, G., Hale, J., Keohane, C.,
411 et al. 2004. Immune cell regulation and cardiovascular effects of sphingosine 1-
412 phosphate receptor agonists in rodents are mediated via distinct receptor subtypes. *J*
413 *Pharmacol Exp Ther.* **309**(2): 758-768. doi:10.1124/jpet.103.062828. PMID:14747617.
- 414 Fryer, R.M., Muthukumarana, A., Harrison, P.C., Nodop Mazurek, S., Chen, R.R.,
415 Harrington, K.E., Dinallo, R.M., Horan, J.C., et al. 2012. The clinically-tested S1P
416 receptor agonists, FTY720 and BAF312, demonstrate subtype-specific bradycardia
417 (S1P(1)) and hypertension (S1P(3)) in rat. *PLoS One.* **7**(12): e52985.
418 doi:10.1371/journal.pone.0052985. PMID:23285242.

- 419 Guppy, L.J. & Littleton, J.M. 1999. Damaging effects of the calcium paradox are reduced in
420 isolated hearts from ethanol-dependent rats: paradoxical effects of dihydropyridine drugs.
421 *J. Cardiovasc. Pharmacol.* **34**(6): 765-771. doi:10.1097/00005344-199912000-00001.
422 PMID:10598118.
- 423 Gwanyanya, A., Amuzescu, B., Zakharov, S.I., Macianskiene, R., Sipido, K.R., Bolotina,
424 V.M., Vereecke, J. & Mubagwa, K. 2004. Magnesium-inhibited, TRPM6/7-like
425 channel in cardiac myocytes: permeation of divalent cations and pH-mediated
426 regulation. *J Physiol.* **559**(Pt 3): 761-776. doi:10.1113/jphysiol.2004.067637.
427 PMID:15272039.
- 428 Hearse, D.J., Humphrey, S.M., Boink, A.B. & Ruigrok, T.J. 1978. The calcium paradox:
429 metabolic, electrophysiological, contractile and ultrastructural characteristics in four
430 species. *Eur. J. Cardiol.* **7**(4): 241-256. PMID:689059.
- 431 Hofmann, U., Burkard, N., Vogt, C., Thoma, A., Frantz, S., Ertl, G., Ritter, O. & Bonz, A.
432 2009. Protective effects of sphingosine-1-phosphate receptor agonist treatment after
433 myocardial ischaemia-reperfusion. *Cardiovasc Res.* **83**(2): 285-293.
434 doi:10.1093/cvr/cvp137. PMID:19416991.
- 435 Hofmann, U., Hu, K., Walter, F., Burkard, N., Ertl, G., Bauersachs, J., Ritter, O., Frantz, S.,
436 et al. 2010. Pharmacological pre- and post-conditioning with the sphingosine-1-
437 phosphate receptor modulator FTY720 after myocardial ischaemia-reperfusion. *Br J*
438 *Pharmacol.* **160**(5): 1243-1251. doi:10.1111/j.1476-5381.2010.00767.x.
439 PMID:20590616.
- 440 Karmazyn, M., Ray, M. & Haist, J.V. 1993. Comparative effects of Na⁺/H⁺ exchange
441 inhibitors against cardiac injury produced by ischemia/reperfusion,
442 hypoxia/reoxygenation, and the calcium paradox. *J. Cardiovasc. Pharmacol.* **21**(1): 172-
443 178. doi:10.1097/00005344-199301000-00025. PMID:7678674.

- 444 Kojima, A., Kitagawa, H., Omatsu-Kanbe, M., Matsuura, H. & Nosaka, S. 2010. Ca²⁺
445 paradox injury mediated through TRPC channels in mouse ventricular myocytes. *Br J*
446 *Pharmacol.* **161**(8): 1734-1750. doi:10.1111/j.1476-5381.2010.00986.x.
447 PMID:20718730.
- 448 Kovacs, A., Kalasz, J., Pasztor, E.T., Toth, A., Papp, Z., Dhalla, N.S. & Barta, J. 2017.
449 Myosin heavy chain and cardiac troponin T damage is associated with impaired
450 myofibrillar ATPase activity contributing to sarcomeric dysfunction in Ca²⁺-paradox rat
451 hearts. *Mol Cell Biochem.* **430**(1-2): 57-68. doi:10.1007/s11010-017-2954-8.
452 PMID:28213770.
- 453 Launay, P., Fleig, A., Perraud, A.L., Scharenberg, A.M., Penner, R. & Kinet, J.P. 2002.
454 TRPM4 is a Ca²⁺-activated nonselective cation channel mediating cell membrane
455 depolarization. *Cell.* **109**(3): 397-407. doi:10.1016/s0092-8674(02)00719-5.
456 PMID:12015988.
- 457 Mani, H., Tanaka, H., Adachi, T., Ikegawa, M., Dai, P., Fujita, N. & Takamatsu, T. 2015.
458 How Does the Ca²⁺-paradox Injury Induce Contracture in the Heart? - A Combined
459 Study of the Intracellular Ca²⁺ Dynamics and Cell Structures in Perfused Rat Hearts.
460 *Acta Histochem. Cytochem.* **48**(1): 1-8. doi:10.1267/ahc.14059. PMID:25861132.
- 461 Monteilh-Zoller, M.K., Hermosura, M.C., Nadler, M.J., Scharenberg, A.M., Penner, R. &
462 Fleig, A. 2003. TRPM7 provides an ion channel mechanism for cellular entry of trace
463 metal ions. *J. Gen. Physiol.* **121**(1): 49-60. doi:10.1085/jgp.20028740.
464 PMID:12508053.
- 465 Murphy, E., Glasgow, W., Fralix, T. & Steenbergen, C. 1995. Role of lipoxygenase
466 metabolites in ischemic preconditioning. *Circ Res.* **76**(3): 457-467.
467 doi:10.1161/01.res.76.3.457. PMID:7859391.

- 468 Nadler, M.J., Hermosura, M.C., Inabe, K., Perraud, A.L., Zhu, Q., Stokes, A.J., Kurosaki, T.,
469 Kinet, J.P., et al. 2001. LTRPC7 is a Mg.ATP-regulated divalent cation channel
470 required for cell viability. *Nature*. **411**(6837): 590-595. doi:10.1038/35079092.
471 PMID:11385574.
- 472 Piper, H.M. 2000. The calcium paradox revisited: an artefact of great heuristic value.
473 *Cardiovasc Res*. **45**(1): 123-127. doi:10.1016/s0008-6363(99)00304-1.
474 PMID:10728324.
- 475 Qin, X., Yue, Z., Sun, B., Yang, W., Xie, J., Ni, E., Feng, Y., Mahmood, R., et al. 2013.
476 Sphingosine and FTY720 are potent inhibitors of the transient receptor potential
477 melastatin 7 (TRPM7) channels. *Br J Pharmacol*. **168**(6): 1294-1312.
478 doi:10.1111/bph.12012. PMID:23145923.
- 479 Sameshima, H., Ota, A. & Ikenoue, T. 1999. Pretreatment with magnesium sulfate protects
480 against hypoxic-ischemic brain injury but postasphyxial treatment worsens brain
481 damage in seven-day-old rats. *Am. J. Obstet. Gynecol*. **180**(3 Pt 1): 725-730.
482 doi:10.1016/s0002-9378(99)70279-6. PMID:10076154.
- 483 Santos-Gallego, C.G., Vahl, T.P., Goliash, G., Picatoste, B., Arias, T., Ishikawa, K., Njerve,
484 I.U., Sanz, J., et al. 2016. Sphingosine-1-Phosphate Receptor Agonist Fingolimod
485 Increases Myocardial Salvage and Decreases Adverse Postinfarction Left Ventricular
486 Remodeling in a Porcine Model of Ischemia/Reperfusion. *Circulation*. **133**(10): 954-
487 966. doi:10.1161/CIRCULATIONAHA.115.012427. PMID:26826180.
- 488 Schmouder, R., Serra, D., Wang, Y., Kovarik, J.M., Dimarco, J., Hunt, T.L. & Bastien, M.C.
489 2006. FTY720: placebo-controlled study of the effect on cardiac rate and rhythm in
490 healthy subjects. *J. Clin. Pharmacol*. **46**(8): 895-904. doi:10.1177/0091270006289853.
491 PMID:16855074.

- 492 Suleiman, M.S. & Chapman, R.A. 1993. Calcium paradox in newborn and adult guinea-pig
493 hearts: changes in intracellular taurine and the effects of extracellular magnesium. *Exp.*
494 *Physiol.* **78**(4): 503-516. doi:10.1113/expphysiol.1993.sp003702. PMID:8398104.
- 495 Van Vuuren, D., Marais, E., Genade, S. & Lochner, A. 2016. The differential effects of
496 FTY720 on functional recovery and infarct size following myocardial
497 ischaemia/reperfusion. *Cardiovasc J Afr.* **27**(6): 375-386. doi:10.5830/CVJA-2016-039.
498 PMID:27966000.
- 499 Vessey, D.A., Li, L., Imhof, I., Honbo, N. & Karlner, J.S. 2013. FTY720 postconditions
500 isolated perfused heart by a mechanism independent of sphingosine kinase 2 and
501 different from S1P or ischemic postconditioning. *Med Sci Monit Basic Res.* **19**: 126-
502 132. doi:10.12659/MSMBR.883877. PMID:23567658.
- 503 Wagner, S., Maier, L.S. & Bers, D.M. 2015. Role of sodium and calcium dysregulation in
504 tachyarrhythmias in sudden cardiac death. *Circ Res.* **116**(12): 1956-1970.
505 doi:10.1161/CIRCRESAHA.116.304678. PMID:26044250.
- 506 Zimmerman, A.N. 2000. The calcium paradox. *Cardiovasc Res.* **45**(1): 119-121.
507 doi:10.1016/s0008-6363(99)00323-5. PMID:10728322.

508 **Legends to figures**

509 **Fig. 1.** Heart perfusion set up and experimental groups. Schematic diagram of the perfusion
510 apparatus and drug administration set up (A). Experimental groups as determined by the
511 cardiac perfusion protocols (B). Each drug, nordihydroguaiaretic acid (NDGA), FTY720 or
512 vehicle (DMSO) was administered at steady-state, after a period of stabilization.

513 Abbreviation: CP, Ca²⁺ paradox.

514

515 **Fig. 2.** Effects of NDGA and FTY720 on Ca²⁺ paradox-induced infarcts. Representative
516 images of 2,3,5-triphenyltetrazolium chloride (TTC) stained mid-ventricular slices of hearts
517 subjected to various drug treatment- and perfusion protocols (A). Viable myocardium appears
518 darker (TTC-positive) compared to the myocardium with irreversible infarcts that appears
519 pale (TTC-negative). Infarct size, expressed as % of total the ventricular area (B). Values are
520 presented as mean ± SEM; n = 6 rats per group; ****P* < 0.001 vs. control; ***P* = 0.003 vs.
521 control; ##*P* = 0.001 vs. CP. Abbreviations: FTY, FTY720; CP, Ca²⁺ paradox.

522

523 **Fig. 3.** Effects of NDGA and FTY720 on Ca²⁺ paradox-induced hemodynamic changes.
524 Screenshot images of typical left ventricular (LV) pressure recordings in control- and Ca²⁺
525 paradox hearts (A). Summary data of LV end-diastolic pressure (LVEDP), LV developed
526 pressure (LVDP), and coronary flow rate (B-D). The parameters were measured at the
527 beginning (baseline) and at the end of the perfusion protocols from different hearts [○,
528 control; ●, Ca²⁺ paradox (CP); □, nordihydroguaiaretic acid (NDGA); ■, NDGA + CP; Δ,
529 FTY720 (FTY); ▲, FTY720 + CP]. Values are presented as mean ± SEM; n = 6 rats per
530 group; ****P* < 0.001 for CP vs. control; ***P* = 0.008 for FTY720 + CP vs. control or
531 *P* = 0.006 for NDGA + CP vs. control; #*P* = 0.039 for FTY720 + CP vs. CP or *P* = 0.020 for

532 NDGA + CP vs. CP; § $P = 0.029$ for FTY720 + CP vs. CP; n.s., not statistically significant
533 ($P = 0.40$).

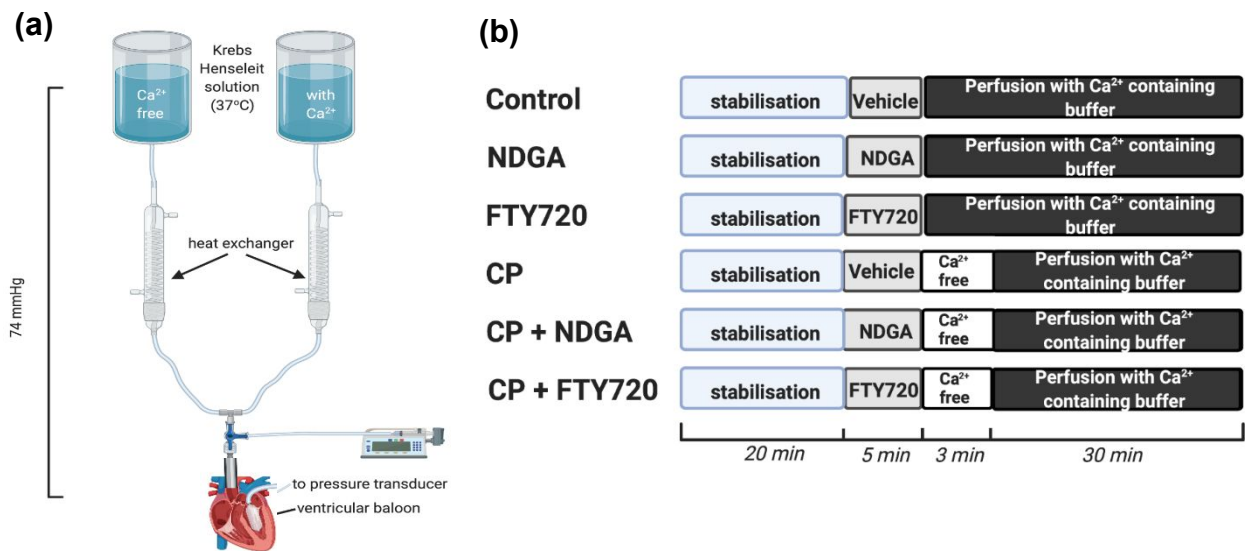
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535 **Fig. 4.** Effect of Mg^{2+} pre-treatment on Ca^{2+} paradox. Experimental groups as determined by
536 the type of pre-treatment and cardiac perfusion protocols (A). Abbreviations: Mg, Mg^{2+} ; i.p.,
537 intraperitoneal; CP, Ca^{2+} paradox. Representative images of TTC-stained mid-ventricular
538 slices of hearts from different experimental protocols (B). Infarct size, expressed as % of the
539 ventricular area (C). Left ventricular (LV) hemodynamic parameters [LV end-diastolic
540 pressure (LVEDP) and LV developed pressure (LVDP)] measured at baseline and at the end
541 of the perfusion protocol in the various groups of hearts [\circ , control; \square , Mg^{2+} ; \bullet , CP; \blacksquare , Mg^{2+}
542 + CP] (D-E). Values are presented as mean \pm SEM; $n \geq 6$ rats per group; *** $P < 0.001$ for
543 Mg + CP or CP vs. control; ** $P = 0.001$ for Mg + CP vs. control.

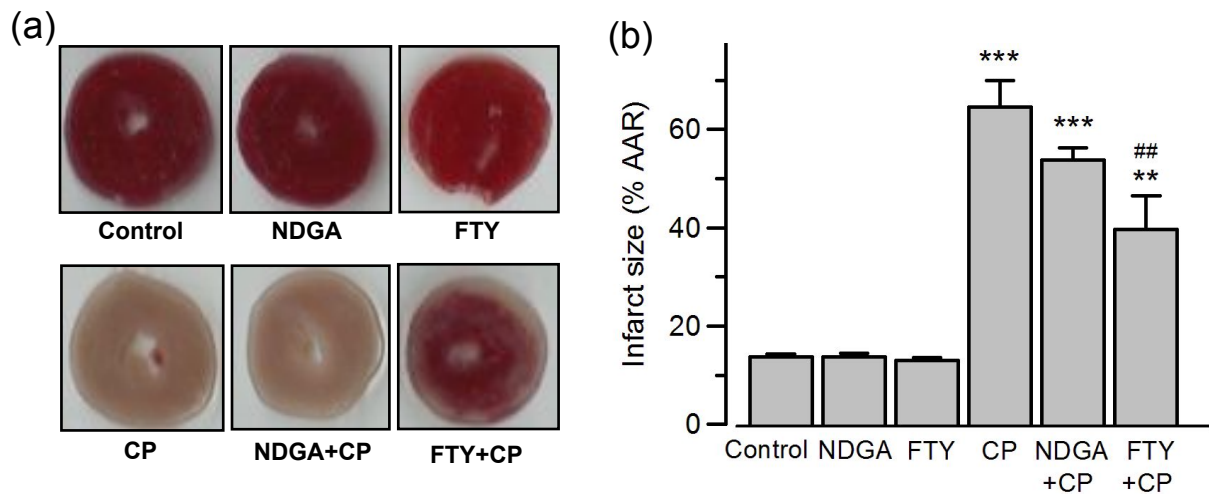
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545 **Fig. 5.** TRPM7 protein expression in ventricular tissue. Representative Western blot film
546 images of TRPM7 and β -actin in left ventricular tissue of control (untreated) rats and Mg^{2+} -
547 pre-treated rats (A). Summary data of TRPM7 protein expression, relative to that of β -actin
548 (B). Values are presented as mean \pm SEM; $n = 6$ rats per group; n.s., not statistically
549 significant ($P = 0.43$). Abbreviation: TRPM7, transient receptor potential melastatin 7.

FIGURE 1.



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FIGURE 2.

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FIGURE 3.

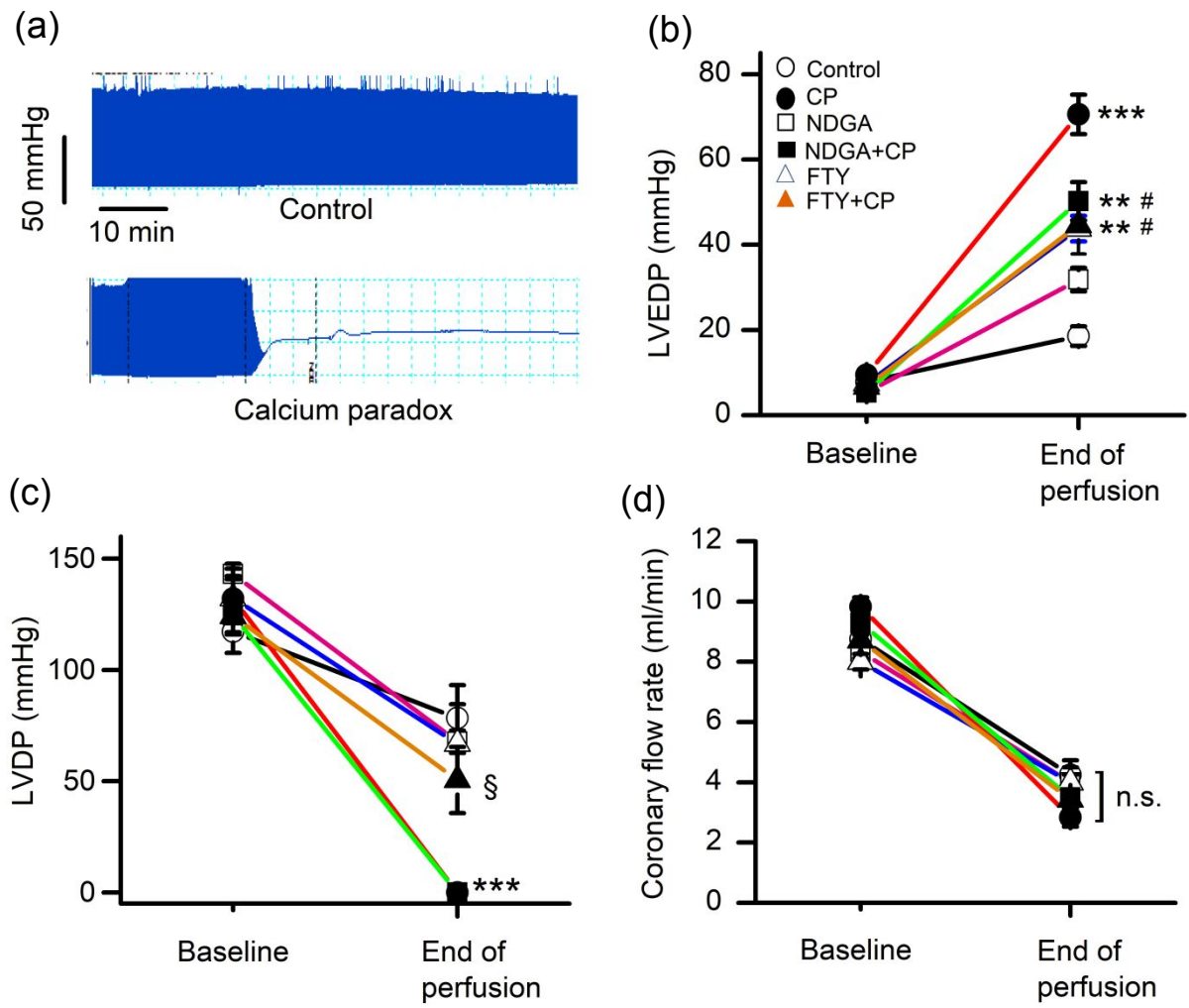


FIGURE 4.

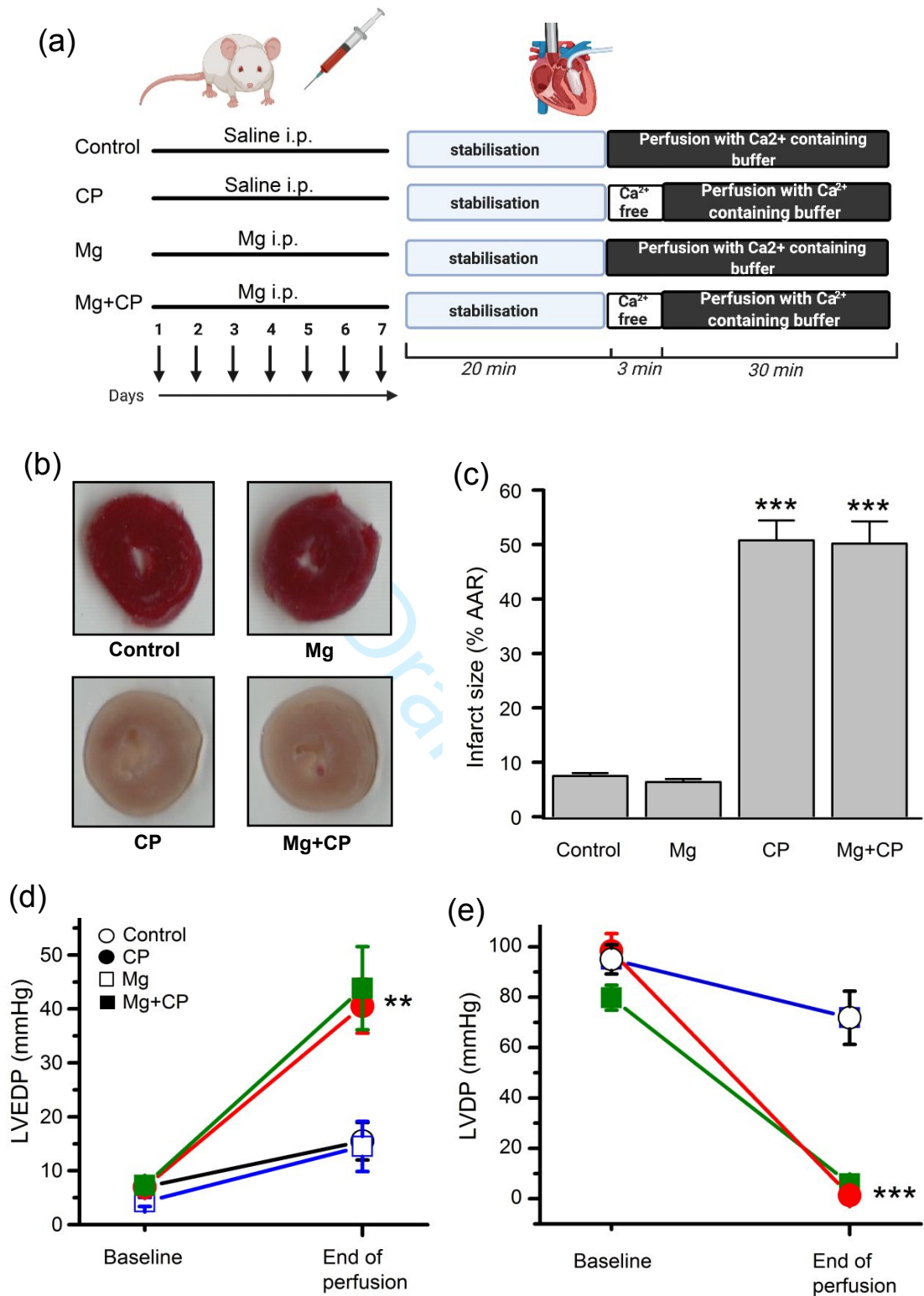
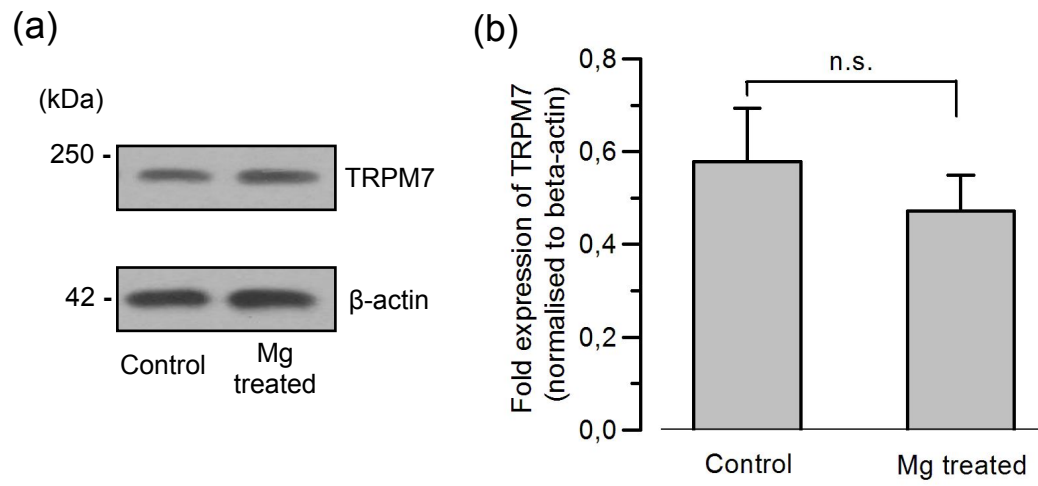


FIGURE. 5



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