Growth-hormone-releasing peptide 6 (GHRP6) prevents oxidant cytotoxicity and reduces myocardial necrosis in a model of acute myocardial infarction


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ABSTRACT

Therapies aimed at enhancing cardiomyocyte survival following myocardial injury are urgently required. As GHRP6 [GH (growth hormone)-releasing peptide 6] has been shown to stimulate GH secretion and has beneficial cardiovascular effects, the aim of the present study was to determine whether GHRP6 administration reduces myocardial infarct size following acute coronary occlusion in vivo. Female Cuban Creole pigs were anaesthetized, monitored and instrumented to ensure a complete sudden left circumflex artery occlusion for 1 h, followed by a 72 h reperfusion/survival period. Animals were screened clinically before surgery and assigned randomly to receive either GHRP6 (400 µg/kg of body weight) or normal saline. Hearts were processed, and the area at risk and the infarct size were determined. CK-MB (creatine kinase MB) and CRP (C-reactive protein) levels and pathological Q-wave-affected leads were analysed and compared. Evaluation of the myocardial effect of GHRP6 also included quantitative histopathology, local IGF-I (insulin-growth factor-I) expression and oxidative stress markers. GHRP6 treatment did not have any influence on mortality during surgery associated with rhythm and conductance disturbances during ischaemia. Infarct mass and thickness were reduced by 78 % and 50 % respectively, by GHRP6 compared with saline (P < 0.01). More than 50 % of the GHRP6-treated pigs did not exhibit pathological Q waves in any of the ECG leads. Quantitative histopathology and CK-MB and CRP serum levels confirmed the reduction in GHRP6-mediated necrosis (all P < 0.05). Levels of oxidative stress markers suggested that GHRP6 prevented myocardial injury via a decrease in reactive oxygen species and by the preservation of antioxidant defence systems (all P < 0.05). Myocardial IGF-I transcription was not amplified by GHRP6 treatment compared with the increase induced by the ischaemic episode in relation to expression in intact hearts (P < 0.01). In conclusion, GHRP6 exhibits antioxidant effects which may partially contribute to reduce myocardial ischaemic damage.

Key words: cardioprotection, growth-hormone-releasing peptide 6 (GHRP6), infarction, ischaemia, myocardium, necrosis, oxidative stress.

Abbreviations: AAR, area at risk; AMI, acute myocardial infarction; BMS, biomodel standardization; CK-MB, creatine kinase-MB; CRP, C-reactive protein; GH, growth hormone; GHRP, GH-releasing peptide; I/R, ischaemia/reperfusion; IGF-I, insulin-like growth factor-I; LV, left ventricular; MDA, malondialdehyde; NBT, Nitroblue Tetrazolium; PI3K, phosphoinositide 3-kinase; ROS, reactive oxygen species; RT, reverse transcriptase; SODt, total superoxide dismutase; T0, basal pre-ischaemia; T1, at 30 min after the beginning of the reperfusion period; T2, the end of 72 h of reperfusion following ischaemia; THP, total hydroperoxides; WBC, white blood cell.

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INTRODUCTION
AMI (acute myocardial infarction) is the rapid onset of myocardial necrosis caused by a sudden critical imbalance between oxygen supply and its demand by the myocardium [1]. As cardiac myocytes are terminally differentiated cells, their ability to regenerate is very limited, thus the consequences of I/R (ischaemia/reperfusion) damage within the injured myocardium can only be partially repaired [2]. The preservation of myocardial and other tissue viabilities during and following an ischaemic episode is therefore a major goal for modern medicine.

GHRPs [GH (growth hormone)-releasing peptides] are a heterogeneous group of synthetic peptidic agents that act as potent GH secretagogues via specific G-protein-coupled receptors in the hypothalamus and the pituitary [3]. Different studies indicate that GHRP6 and hexarelin exert potent cardioprotective actions, including in models of myocardial injury by ischaemia or hypoxia [4–6]. Some of these experimental findings were substantiated further in clinical studies which demonstrated that LV (left ventricular) ejection fraction, cardiac index and cardiac output were improved upon hexarelin administration [7,8]. Furthermore, Iwase et al. [9] recently demonstrated that GHRP6 attenuates LV dysfunction and dilation in hamsters with cardiomyopathy. Fundamental to the present study is the finding that GHRP6 treatment prevented sudden death in dogs with cardiomyopathy subjected to AMI, without reducing infarct size or ameliorating ischaemia-associated electrical instability [10]. Previous observations from our group [11] have indicated that GHRP6 exerts potent cytoprotective effects by enhancing tissue viability in acute I/R episodes in different splanchnic organs (small bowel, liver and kidneys). However, the mechanisms whereby GHRP6 enhances cell tolerance to otherwise lethal insults such as hypoxia remain to be fully understood. Our own observations led us to suggest the hypothesis that GHRP6 may also assist in myocardial tissue survival during an ischaemic episode. The main aim of the present study was to determine whether pharmacological intervention with GHRP6 in a combined scheme of administration contributed to rescue cardiomyocytes from necrosis and thus reduce infarct size in a porcine model of acute coronary occlusion. The present findings suggest that the pro-survival effect of GHRP6 is related to the attenuation of I/R-associated oxidative cytotoxicity.

MATERIALS AND METHODS
Animals
Female inbred Cuban Creole pigs weighing 23–29 kg were used. Animals were provided by the National Swine Genetic Center, Havana, Cuba. Body weight during the study was ensured by the stepwise purchase of litters of approx. ten pigs. They were maintained and manipulated in accordance with the Guide for the Care and Use of Laboratory Animals of the National Swine Research Institute. Animals were fasted for 16 h before surgery.

GHRP6
The hexapeptide GHRP6 (His-d-Trp-Ala-Trp-d-Phe-Lys-NH₂) was purchased from BCN Peptides (pyrogen-free with 95% purity). The peptide was aliquoted into sterile vials in a laminar flow hood and kept at –20 °C until use. For in vivo administration, fresh solutions were always prepared by diluting the peptide in sterile normal saline.

Standardization of the animal model
For inclusion in the study, all of the pigs from each litter were subjected to a clinical assessment, which included body weight and temperature, heart and respiratory rates, 12-lead ECG, haemoglobin, total WBC (white blood cell) count, and basal levels of CK-MB (creatine kinase-MB) and CRP (C-reactive protein). These examinations were done during the acclimitization period approx. 7–9 days prior to surgery. A non-lethal infarct model was required in order to achieve an appropriate survival window for the study. Sixteen animals were assigned to the biomodel standardization (BMS) group in order to assess the feasibility of inducing a repeatable and reproducible AMI in the pig strain used. Left circumflex artery clamping was the preferred procedure. An ischaemic period of 60 min was used as reported in a previous study [10]. Animals were monitored electrocardiographically before surgery, during the acute I/R period and once a day until completing a 72 h reperfusion period. After this period, the pigs were killed. The pilot studies revealed that 72 h was an appropriate time window to determine myocardial changes. Myocardial injury was studied as described below.

Anaesthesia and surgery
Pigs were anaesthetized with a combination of ketamine (16 mg/kg of body weight), flunitrazepam (0.45 mg/kg of body weight) and pavulon (0.03 mg/kg of body weight) via a tube inserted into an ear marginal vein. Auffed endotracheal tube was inserted, and the animals were ventilated with a Mark 10 machine. Animals were monitored electrocardiographically using a 12-lead solid state CYS SG-electrocardiographic device (ICID) supplied with CARDIOCID PC (EICISOFT) software, allowing for continuous on-screen ECG and heart rate registers, and for automatic recordings every 20 s. A left thoracotomy was performed between the fourth and fifth intercostal spaces. Once the heart was exposed, a cavafix-indwelling catheter was inserted into the left atrium and its syringe connection tip was externalized between the scapulae. After opening the pericardium, the...
coronary branches were dissected and the left circumflex artery was exposed, suspended and totally occluded for 1 h with a vascular bulldog clamp (Fine Science Tools). Once the ischaemic period was completed, the clamp was removed and the chest was sutured in layers.

Allocation to the experimental groups, GHRP6 administration and doses

Once the baseline clinical assessment and the blood chemistry data were reviewed, 36 pigs were randomly assigned to one of the two groups with 18 animals in each: group I, saline placebo, or group II, GHRP6 treatment. Allocation was done using a computer-generated randomization list, matching the animal number to either group I or II. A balanced-group distribution was confirmed thereafter by reviewing the baseline data, and no pigs were excluded. Nine animals survived in each group until being killed at 72 h post-coronary occlusion. Pigs assigned to group I received 10 ml of sterile normal saline equilibrated at 37 °C. For group II, GHRP6 was administered at a dose of 400 µg/kg of body weight in an equal volume of normal saline. The treatments were infused via a cavafix catheter. The dosing regimen used was introduced to investigate the potential influence of the acute treatment on specific myocardial electrical derangements (detected by electrocardiography) associated with different conditions during I/R. GHRP6 interventions were as follows. (i) A pre-ischaemia bolus, given 10 min before circumflex artery occlusion, with acute ST-segment elevation being the parameter observed. (ii) A second bolus was infused 20 min after clamping, coinciding with the peak of arrhythmic beats and ventricular fibrillation, to examine whether GHRP6 ameliorated ischaemia-associated ventricular arrhythmias. (iii) A third bolus was infused 5 min before the beginning of the reperfusion period in order to characterize reperfusion arrhythmias. Treatments were continued thereafter twice a day at regular intervals. Animals were killed at 72 h after surgery. The GHRP6 dose used in this study derived from our liver I/R dose–response experiments in which 100 and 400 µg/kg of body weight exhibited similar hepatoprotective effects (D. Cibrian and J. Berlanga, unpublished work), which demonstrated a such a dose is supported by pharmacokinetic and bio-distribution data in male Wistar rats (J. Berlanga and D. Cibrian, unpublished work). The 400 µg dose was preferred because it was twice the dose reported to prevent sudden death in dogs with cardiomyopathy subjected to acute I/R with no resulting differences in infarct size [10]. Furthermore, such a dose is supported by pharmacokinetic and bio-distribution data in male Wistar rats (J. Berlanga and D. Cibrian, unpublished work), which demonstrated a half-life of 12 h (90% of the area under the curve) and a myocardial residence of 0.2% after 24 h.

Infarct size and weight

A few minutes after clamping the circumflex artery, 200 ml of 2.5% (w/v) Evans Blue solution was infused. The unstained epicardial territory was traced on a transparent sheet and digitally processed further to determine the AAR (area at risk). These data were used to calculate the percentage of the infarcted area in relation to the original myocardial AAR. After 72 h of reperfusion, the pigs were anaesthetized and a final 12-lead ECG recording was done. Again, 2.5% (w/v) Evans Blue solution was infused to assist in delimiting the necrotic area, particularly when it was small. Subsequently, the hearts were arrested with 0.5 mmol/l KCl, excised and processed to determine infarct size, as described previously [12,13]. Heart weights were recorded. Infarct thickness was measured with a caliper square along the affected ventricular wall after a longitudinal incision at three different points to obtain average values. Thus the average value of the wall necrosis thickness per group is reported. Transverse sections were taken on the LV wall from the apex upwards to the level just below the coronary sulcus. Serial sections were then incubated in 1% NBT (Nitroblue Tetrazolium; Sigma-Aldrich) for 20 min at 20 °C, and then immersed in 10% buffered formalin [12]. After NBT incubation, the area of viable perfused tissue was coloured blue, viable ischaemic tissue was coloured red (stunned myocardium) and necrotic tissue appeared pale or white. The necrotic territory was excised, weighed and its weight adjusted to the total heart weight to determine the percentage of the infarcted mass. In addition, the percentage of the infarcted area was also given adjusted to the AAR [13].

Myocardial tissue characterization

In order to characterize the impact of treatment on the biochemical and structural preservation of myocardial tissue, standard-sized transmural punch biopsies were harvested from macroscopically normal myocardium (Acupunch biotomes; Acuderm) adjacent to the necrotic core. Tissue fragments were used to examine myocardial expression of IGF-I (insulin-like growth factor-I), to measure oxidative damage and for histopathological analysis. Five additional hearts samples were obtained from sex- and age-matched healthy pigs of the same strain, which were used as a reference for constitutive expression of IGF-I and to gain information on basal myocardial redox status in an intact control group.

Myocardial transcriptional expression of IGF-I was examined using RT (reverse transcriptase)-PCR. Total RNA was isolated from intact hearts and from those subjected to I/R and receiving either saline or GHRP6 (n = 5 in each group), using TRIZol (Invitrogen). Total RNA was digested with RNase-free DNase I (Epিন迫切 Technologie), according to the manufacturer’s instructions. A total of 3 µg of RNA were reverse-transcribed using the GeneAmp® RNA PCR Core kit (Applied Biosystems) with an oligo(dT) primer. PCR was performed using the following porcine IGF-I-specific primers: 5′-CTGTGGGGGCTGAGCTGGTGGACG-3′.
(sense) and 5'-AAGGATCCGTGCGTAGCATGTC-3' (antisense) (GenBank® accession number M311775). A final product of 353 bp was generated after 30 cycles at 68 and 94 °C. IGF-I expression was normalized against porcine β-actin expression as a housekeeping gene using the following primers: 5'-GGAGATCTGGCAGCCTGATCAAGG-3' (sense) and 5'-GCGCGAATCTCCTGTACTCTCTGC-3' (antisense) (Genbank® accession number AY 550069). After 30 cycles at 68 and 94 °C, the amplification process produced a final product of 482 bp. The bands were detected in a 1 % (w/v) agarose gel and were quantified using the Kodak I D 3.6 software package.

For assessment of lipoperoxidative damage, tissue homogenates [1:10 (w/v)] were prepared by myocardial disruption in 50 mmol/l KCl/5 mmol/l histidine buffer (pH 7.4) at 4 °C using a tissue homogenizer (T25 Basic; IKA Labortechnik) for 3 min at 15 000 rev./min and were subsequently centrifuged at 5000 g at 4 °C for 20 min (Himac SCR 20B; Hitachi). Tissue accumulation of MDA (malondialdehyde) and THP (total hydroperoxides), as well as SODt (total superoxide dismutase) and catalase activities, were determined in the supernatants. All of the biochemical parameters were measured by spectrophotometric methods using an Ultrospec 2000 UV/Visible spectrophotometer (Pharmacia Biotech). MDA and THP were determined using the Bioxytech LPO-586 and Bioxytech H₂O₂-560 kits respectively, according to the manufacturers’ instructions (Bio-Rad Laboratories). SODt activity was estimated by determining the capacity of the enzyme to inhibit the auto-oxidation of pyrogallol at a rate of 50 % [14]. Catalase activity was based on the reduction of H₂O₂ to oxygen and water, as described previously [15]. All of the biochemical data were adjusted to total protein concentration determined in the tissue supernatants using a commercial kit (Bio-Rad Laboratories).

For histopathological analysis, normal and damaged myocardial samples were harvested and fixed in 10 % (v/v) formalin, paraffin-embedded and processed for haematoxylin/eosin and basic fuschin staining. LV wall integrity was determined qualitatively by two independent pathologists. In addition, quantitative parameters, such as the number of necrotic peninsulas within the tissue section and the total number of irreversibly damaged nuclei (exhibiting karyorrhexis, karyolysis and/or karyopyknosis) in 10–15 microscopic fields at ×10–20 magnification, were examined in serial sections, as described previously [16]. All of the pathological studies were performed in a blinded manner.

**ECG study**

The time points at which rhythmic and conductance disturbances started, including ST elevation, ventricular arrhythmic beats and ventricular fibrillation, were registered in each ECG during the ischaemic period.

### Table 1 Baseline clinical characterization of the study groups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group</th>
<th>BMS</th>
<th>Saline</th>
<th>GHRP6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (kg)</td>
<td></td>
<td>25.66 ± 1.97</td>
<td>27.62 ± 2.41</td>
<td>26.31 ± 2.25</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td></td>
<td>73 ± 4</td>
<td>75 ± 6</td>
<td>74 ± 7</td>
</tr>
<tr>
<td>Respiratory rate (breaths/min)</td>
<td></td>
<td>44 ± 3</td>
<td>42 ± 4</td>
<td>46 ± 6</td>
</tr>
<tr>
<td>Haemoglobin (g/l)</td>
<td></td>
<td>123.84 ± 7.73</td>
<td>120.56 ± 6.44</td>
<td>127.77 ± 7.03</td>
</tr>
<tr>
<td>WBC (× 10³/l)</td>
<td></td>
<td>8 ± 1.41</td>
<td>9 ± 2.17</td>
<td>8 ± 1.66</td>
</tr>
<tr>
<td>CK-MB (units/l)</td>
<td></td>
<td>1267.72 ± 6.44</td>
<td>1270.23 ± 43.25</td>
<td>1288.55 ± 81.97</td>
</tr>
<tr>
<td>CRP (µg/ml)</td>
<td></td>
<td>0.087 ± 0.004</td>
<td>0.09 ± 0.0026</td>
<td>0.093 ± 0.0041</td>
</tr>
</tbody>
</table>

Duration of reperfusion arrhythmia and the time point of the resumption of sinusal rhythm within the acute reperfusion phase were also determined individually. Both basal and final (pre-autopsy) ECG recordings were analysed and compared to determine the number of leads showing pathological Q waves in each animal.

### Circulating levels of CK-MB and CRP

Circulating CK-MB and CRP levels were determined in serum collected before surgery (T0), 30 min after the beginning of the reperfusion period (T1; at chest suturing) and at the end of 72 h of reperfusion (T2; before autopsy). Commercially available kits were used to determine CK-MB (Roche Diagnostics) and CRP (Diagnostics Automation), according to the manufacturers’ instructions. However, results should be interpreted with caution as the kits used are intended for human, rather than porcine, samples.

### Statistical analysis

All of the experimental data were initially evaluated for a normal distribution using the Kolmogorov-Smirnov test (P < 0.05). When a normal distribution was established, an unpaired Student’s t test was used, except for oxidative-stress-related parameters in which one-way ANOVA followed by the Student-Newman-Keuls multiple comparisons test was used. A P value < 0.05 was used to indicate a significant difference.

### RESULTS

### Animal model

The baseline clinical characterization of the animals is summarized in Table 1. The similarity of the values indicated that the experiment was done on healthy animals, that no clinical differences existed among the groups.
Heart weights and infarct dimensions in the study groups

Values are means ± S.D. No statistical differences were detected when heart weights were compared among the groups (P = 0.81), indicating an appropriate allocation of animals. **P < 0.01, ***P < 0.001 and ****P < 0.0001 compared with the saline group, as determined by an unpaired Student’s t test.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group</th>
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<th>Saline</th>
<th>GHRP6</th>
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</thead>
<tbody>
<tr>
<td>Heart weight (g)</td>
<td></td>
<td>158.23 ± 11.64</td>
<td>156.18 ± 15.45</td>
<td>153.92 ± 26.2</td>
</tr>
<tr>
<td>Infarct mass (absolute)</td>
<td></td>
<td>39.4 ± 10.55</td>
<td>37.4 ± 11.81</td>
<td>12.03 ± 10.09***</td>
</tr>
<tr>
<td>weight in g</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infarcted mass (%)</td>
<td></td>
<td>24.91 ± 4.78</td>
<td>24.0 ± 7.24</td>
<td>7.81 ± 5.63**</td>
</tr>
<tr>
<td>Infarcted area (cm²)</td>
<td></td>
<td>32.06 ± 12.27</td>
<td>30.31 ± 14.74</td>
<td>6.66 ± 6.25**</td>
</tr>
<tr>
<td>AAR (cm²)</td>
<td></td>
<td>40.44 ± 6.77</td>
<td>38.70 ± 8.03</td>
<td>39.67 ± 4.80</td>
</tr>
<tr>
<td>Infarcted area/AAR (%)</td>
<td></td>
<td>77.89 ± 18.17</td>
<td>74.51 ± 23.82</td>
<td>15.33 ± 14.78****</td>
</tr>
<tr>
<td>Infarct thickness (cm)</td>
<td></td>
<td>1.58 ± 0.52</td>
<td>1.56 ± 0.30</td>
<td>0.7 ± 0.61**</td>
</tr>
</tbody>
</table>

Table 3 Electrocardiographic changes during I/R in the study groups

Values are means ± S.D. of the time point at which the electrophysiological changes started and were registered along the continuous ECG automatic recording. No significant differences were detected (P > 0.05) between the groups, as determined by an unpaired Student’s t test.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group</th>
<th>BMS</th>
<th>Saline</th>
<th>GHRP6</th>
</tr>
</thead>
<tbody>
<tr>
<td>ST elevation (s)</td>
<td></td>
<td>54 ± 3.14</td>
<td>53 ± 2.61</td>
<td>58 ± 5.30</td>
</tr>
<tr>
<td>Ventricular arrhythmias</td>
<td></td>
<td>18.54 ± 11.15</td>
<td>17.36 ± 10.37</td>
<td>19.75 ± 13.82</td>
</tr>
<tr>
<td>(min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ventricular fibrillation/</td>
<td></td>
<td>17.24 ± 12.22</td>
<td>15.6 ± 13.16</td>
<td>20.8 ± 5.43</td>
</tr>
<tr>
<td>sudden death (min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sinusical rhythm resuming</td>
<td></td>
<td>5.68 ± 4.97</td>
<td>5.89 ± 5.69</td>
<td>4.56 ± 5.18</td>
</tr>
</tbody>
</table>

and that the experimental arms of the study were balanced. Ventricular fibrillation/sudden death, appearing during the ischaemic phase, led to the death of 50% of the pigs used during surgical procedure in the BMS group as in the study groups. Fibrillation frequently started after 15–20 min of clamping the left circumflex artery and could be readily anticipated by extrasystolic ventricular beats. The remaining 50% of animals that tolerated the ischaemic impact had uneventful post-surgical events. The infarcts appeared limited to the lateral side of the left ventricle and were largely similar in terms of size and depth (Table 2). Hearts were also largely similar in terms of electrocardiographic changes (Table 3).

Effect of GHRP6 on infarct dimensions

As shown in Table 2, mean heart weights were very similar between the two groups involved in the study (P = 0.81). Treatment with GHRP6 significantly reduced the absolute weight of the infarct territory compared with the saline controls (P = 0.001). Consequently, the infarct mass adjusted to the total heart weight was reduced by 78% by GHRP6 treatment (P = 0.005). There were no statistical differences in the AAR between the three groups (P = 0.78), indicating that our results were not influenced by collateral networks. This may explain why, when the infarcted area was adjusted to the value of the myocardial AAR, infarct reduction remained close to 80% (P < 0.0001). Accordingly, infarct size and thickness appeared significantly reduced (P = 0.005) in the GHRP6-treated animals.

Effect of GHRP6 on myocardial electrical instability

GHRP6 treatment did not prevent or delay the onset of acute rhythmic and conductance disturbances during the ischaemic episode or those associated with the early reperfusion period. Consequently, mortality during the surgical procedure associated with ventricular fibrillation was not reduced by GHRP6 intervention. As shown in Table 3, the time points at which ST elevation, arrhythmic ventricular beats and ventricular fibrillation/sudden death started were very similar between the groups (all P > 0.05). In the reperfusion phase, GHRP6 treatment did not prevent or reduce the duration of reperfusion arrhythmias (P > 0.05). Irrespective of the fact that GHRP6 treatment did not ameliorate myocardial electrical instability, the ECG study confirmed its benefits in terms of a reduction in myocardial necrosis, as judged by the number of ECG leads showing abnormal Q waves. Figure 1 shows that five out of nine animals treated with GHRP6 did not exhibit pathological Q waves in any of the 12 leads studied. In contrast, pathological Q
waves appeared in all of the saline-treated animals, mostly involving four or five ECG leads ($P < 0.001$).

**Myocardial morphological characterization**

Necrosis was the most prominent form of myocardial death within the damaged area, as determined by NBT staining and histopathology. Areas suggesting myocardial stunning were rarely detected. The necrotic core appeared as a white well-delimited zone (Figure 2A), predominantly involving the lateral side, in agreement with the AAR following Evans Blue staining. Treatment with GHRP6 resulted in gross differences in morphology compared with the controls (Figure 2B). Histological examination of the transmural biopsies demonstrated that substantial differences existed in relation to fibre lysis, inflammatory infiltrate intensity, haemorrhage extension and nuclear viability in areas close to the infarcted core between the control (Figure 2C) and GHRP6 (Figure 2D) groups. As shown in Figure 2(D), preservation of fibre integrity was a major hallmark of the effect of GHRP6 treatment compared with the predominant lytic pattern found in saline-treated animals (Figure 2C). Inflammatory infiltrate and haemorrhagic events appeared to be attenuated more in the samples from the GHRP6 group. Furthermore, myocardial necrotic peninsulas and the amount of lethally injured nuclei appeared significantly reduced ($P < 0.001$) in the GHRP6-treated animals compared with the controls (Table 4).

**Serum CK-MB and CRP levels**

The changes observed in CK-MB levels suggested that GHRP6 preserved myocardial viability. As shown in Figure 3(A), a significant increase was detected in the control group at T1 (at 30 min after the beginning of the reperfusion period) compared with T0 (basal pre-ischaemic values; $P < 0.01$). A 5-fold increase was observed in the control group at T2 (the end of 72 h of reperfusion following ischaemia) compared with the levels at T0 and T1 ($P < 0.001$). The BMS group behaved in a similar manner to the saline group (Figure 3A). In contrast, CK-MB levels in the GHRP6 group were not significantly different when the data at T0 and T1 were compared. At T2, a lower increase (2-fold) was detected compared with T0 and T1 ($P < 0.01$). Comparisons of the results from
Table 4  Myocardial microscopic assessment in the study groups

Values are means ± S.D. Lethally damaged nuclei were those showing karyorrhexis-, karyolysis- and/or karyopyknosis-typical morphology, averaged from serial sections in 10–15 microscopic fields. Peninsulas of necrotic fibres were also quantified. Tissue analysis was done on full-thickness left ventricular wall punch biopsies collected from normally appearing areas adjacent to the necrotic core. ***P < 0.001 compared with the saline control group, as determined by an unpaired Student’s t test.

<table>
<thead>
<tr>
<th>Group</th>
<th>BMS</th>
<th>Saline</th>
<th>GHRP6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Necrotic fibre peninsulas (n)</td>
<td>23.36 ± 6.11</td>
<td>25.8 ± 4.27</td>
<td>6.18 ± 2.22***</td>
</tr>
<tr>
<td>Nuclei with lethal changes (n)</td>
<td>95.33 ± 18.22</td>
<td>90.07 ± 21.8</td>
<td>27.39 ± 11.6***</td>
</tr>
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</table>

Figure 3  Effect of GHRP6 on circulating CK-MB (A) and CRP (B) levels

(A) Serum CK-MB levels were determined as a marker of myocardial fibre damage. Acute elevation of CK-MB was detected at T1 (** P < 0.01) compared with T0 in the saline group. A similar observation was found with the BMS group. A progressive increase was detected in saline and BMS groups in T2 compared with T1 and T0 (** P < 0.001). However, no increase in CK-MB levels were detected at T1 in the GHRP6 group (P = 1.00) compared with T0. At T2, a significant increase was detected in GHRP6-treated animals compared with T1 and T0 (** P < 0.01). This increase at T2 was significantly lower (** P < 0.001) than that observed in the control group. All statistical analyses were performed using an unpaired Student’s t test. No significant difference was observed between the saline and GHRP6 groups (P = 0.5476).

(B) CRP levels were not increased at T1 in any of the groups (Figure 3B). The highest CRP levels were detected at T2 compared with those at T0 and T1 in the saline and BMS groups (P < 0.001). In contrast, no significant difference was observed in CRP values at T2 compared with those at T0 and T1 in GHRP6-treated animals (Figure 3B).

Figure 4  Effect of GHRP6 on transcriptional expression of myocardial IGF-I

Constitutive myocardial IGF-I mRNA expression was determined from samples from intact healthy hearts, and hearts from the saline-treated and GHRP6-treated groups. Upper panel, a representative agarose gel of RT-PCR-amplified IGF-I and β-actin. Lower panel, the amount of mRNA quantified by densitometry and expressed relative to β-actin. ** P < 0.01 compared with the intact group, as determined by an unpaired Student’s t test. No significant difference was observed between the saline and GHRP6 groups (P = 0.5476).

Myocardial expression of IGF-I

Five individual RT-PCR products from each experimental group are shown in Figure 4. A significant difference in constitutive myocardial IGF-I expression levels was observed when samples from intact healthy hearts were compared with those receiving the ischaemic insult (saline and GHRP6 groups; P < 0.01). No differences were noted, however, when the saline and GHRP6 groups were compared.

Assessment of myocardial redox status

Assessment of some critical redox parameters within the myocardial areas adjacent to the necrotic core provided important insights into the effect of GHRP6 treatment (Table 5). Intramyocardial MDA levels were elevated over 14-fold in the saline group compared with intact healthy hearts, whereas this increase was only 2-fold in the GHRP6 group. Correspondingly, a similar effect...
was observed for intramyocardial THP levels. A 9-fold increase was observed in the saline group compared with the values in intact healthy hearts, and only a 5-fold elevation was detected in the GHRP6 group. A substantial increase in catalase activity was observed in the saline group compared with the intact hearts. GHRP6 blocked the 10-fold increase in catalase activity. A 53 % decrease in total SODt activity was demonstrated in the hearts from the saline group compared with the healthy hearts. With GHRP6, the decrease in SODt activity was only 36 %.

**DISCUSSION**

Although one of the limitations of the present study is the lack of characterization of ventricular function upon the induction of the infarction and during the reperfusion phase, the fact that the treatment with GHRP6 was able to rescue myocardial mass by more than 70 % is a remarkable finding. The present study has the merit of being performed following a careful pre-surgical clinical examination of the animals to ensure that an equal allocation of healthy animals was obtained in each study arm. Although previous studies on the cardioprotective effects of GHRP6 in different experimental cardiopathy settings [4–10] are confirmed and extended in the present study, our study appears to provide the first evidence of a reduction in myocardial necrosis by GHRP6 administration in a model of acute arterial occlusion in otherwise healthy animals.

More than 50 % of the GHRP6-treated animals had no macroscopic evidence of epicardial necrosis. In these pigs (five out of nine), a negligible well-delimited damaged territory (basically haemorrhagic) was detected in the mesocardium upon dissection of the ventricle. Coincidentally, no pathological Q waves were found in any of the 12 ECG leads in the pre-autopsy recordings in these animals. The convergence of morphological, electrophysiological and biochemical findings in the present study emphasizes the capability of the hexapeptide to rescue myocardial cells during I/R and its ensuing pathological cascade.

As stated above, the effect of a multi-dose GHRP6 regimen was examined in the present study and was intentionally planned to study the impact on specific ventricular electrical disturbances derived from an I/R episode. Although this dosing schedule meant that it was difficult to define the contribution of each intervention to the overall clinical effect of infarct reduction, it did allow us to conclude that GHRP6 did not ameliorate ECG-registered rhythmic and conductance failures linked to I/R that eventually accounted for mortality during the surgical period (ventricular fibrillation during the ischaemic phase). This observation is in agreement with previous findings [10].

The time course of changes in serum CK-MB and CRP levels were both similar. Both parameters were found to be elevated in all of the groups at 72 h after infarction when compared with their respective baseline and acute reperfusion values. Although differences exist between CK-MB and CRP in terms of the site of production and specific clinical significance, it is noteworthy that the increase in both markers was much lower in the GHRP6-treated pigs at 72 h after coronary occlusion compared with the saline controls. This appears to confirm the ability of GHRP6 to reduce myocardial damage, even when the analytical systems used is intended for human, rather than porcine, samples.

The mechanism whereby GHRP6 enhances tissue protection during ischaemia remains unclear to date. Previous findings from our group [11] identified for the first time that GHRP6 possessed extracardiac multi-organ protection capabilities against prolonged ischaemia when administered as a single prophylactic bolus. Indeed, these effects appeared to be conferred by a putative control of cellular oxidative stress and its ensuing pro-inflammatory status [11]. Following this line of evidence and taking into account that ischaemic myocardial damage is, at least in part, associated with the local generation of ROS (reactive oxygen species) [17,18], components of the myocardial redox status were studied in macroscopically healthy fragments obtained close to the infarct core. A positive oxidative stress balance was confirmed in the GHRP6 group as judged by the following findings: (i) a significant reduction in the intramyocardial accumulation

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**Table 5  Myocardial redox assessment in the study groups**

Values are means ± S.D. Statistically significant differences (P < 0.05) were obtained between the groups for each parameter, as determined by one-way ANOVA and Student–Newman–Keuls test.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Intact</th>
<th>Saline</th>
<th>GHRP6</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDS (nmol/mg of protein)</td>
<td>0.054 ± 0.007</td>
<td>0.766 ± 0.068</td>
<td>0.1207 ± 0.013</td>
</tr>
<tr>
<td>THP (µmol/mg of protein)</td>
<td>31.78 ± 0.45</td>
<td>295.61 ± 8.47</td>
<td>56.27 ± 1.01</td>
</tr>
<tr>
<td>SODt (units · mg⁻¹ of protein · min⁻¹)</td>
<td>190.81 ± 249</td>
<td>9033 ± 229</td>
<td>12195 ± 706</td>
</tr>
<tr>
<td>Catalase activity (units · mg⁻¹ of protein · min⁻¹)</td>
<td>11.34 ± 0.26</td>
<td>374.86 ± 10.53</td>
<td>34.11 ± 0.55</td>
</tr>
</tbody>
</table>
of THP and MDA, indicating that GHRP6 attenuated ROS accumulation; and (ii) a preservation of basal antioxidant enzyme defence levels (catalase and SODt) otherwise altered by ROS generation [19,20]. These positive findings may justify the contribution of GHRP6 in rescuing myocardial mass [21]. Other mechanisms for the cardioprotective action of GHRP6 during the I/R episode include (i) modification of myocardial regional blood flow; (ii) acute establishment of collateral microcirculatory circuits near the AAR; and/or (iii) improvement in general haemodynamic performance. These have been examined previously in a similar experimental setting with negative results [10].

GH stimulates myocardial IGF-I synthesis, which locally promotes cardiomyocytes division, differentiation and survival [22]. Thus myocardial IGF-I expression level was examined as a candidate effector distal to peptide-mediated GH release [23]. RT-PCR experiments indicated that, in the groups receiving ischemia (saline and GHRP6 groups), higher IGF-I expression was observed compared with levels detected in intact hearts. This possibly indicates a hypoxia-associated response or, alternatively, an ongoing local repair process. Under the sampling schedule used in the present study, GHRP6 did not amplify myocardial IGF-I expression. This observation appears to substantiate in vivo [10] and in vitro [24,25] observations that show that GHRP6 and other peptidyl GH secretagogues may have direct cardioprotective effects independent of the GH/IGF-I system. However, this does not exclude GHRP6-mediated GH released in a pulsatile fashion having beneficial cardiac effects.

Activation of ERK1/2 (extracellular-signal-regulated kinase 1/2) and PI3K (phosphoinositide 3-kinase)/Akt pathways by ghrelin and other cytoprotective agents play a critical role in preventing cells death under different stressful conditions [24]. Cell survival against pro-oxidant cytotoxicity also requires the involvement of PI3K/Akt [21]. Other hypothetical mechanisms for GHRP6-mediated cardiomyocytes protection include an improvement in myocardial metabolic efficiency by activation of glycolysis and reduction in reperfusion-induced hypermetabolism. Previous studies have shown that cell survival via Akt activation is largely dependent on glycolytic metabolism and energy optimization [26,27].

In summary, GHRP6 treatment substantially reduced myocardial infarct size following an acute and sudden ischemic event. The replication of this finding in the clinical arena may improve the prognosis of individuals affected by myocardial infarction.

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