Title

Diabetes Increases Apoptosis and Necrosis in the Ischemic and Non-ischemic Human Myocardium: Role of Caspases and Poly (ADP-ribose) Polymerase.

Short Title: Diabetes and Cardiac Cell Death

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ABSTRACT

Background: Diabetes is an important predictor of morbidity and mortality after cardiac surgery but the reason is unclear. Therefore the aims of these studies were to elucidate whether cell death is greater in the ischemic and non-ischemic diabetic human myocardium than in the non-diabetic and to investigate the underlying mechanism.

Methods: The right atrial appendages (n=8/group) of non-diabetics, non-insulin dependent diabetics (NIDDM) and insulin dependent diabetics (IDDM) were subjected to 90 minutes simulated ischemia/120 minutes reoxygenation (SI/R). Tissue injury was measured by the release of CK into the media, and cellular apoptosis and necrosis were assessed in tissue by the TUNEL assay and propidium iodide. Initiator and effector caspases activation was also measured.

Results: Apoptosis and necrosis were greater in the NIDDM and the IDDM groups than in the non-diabetic group both in fresh tissue and after SI/R. Activation of effector caspases was also higher in the NIDDM and IDDM groups than in the non-diabetic group after SI/R. Caspase-3 inhibition reduced apoptosis in all study groups without influencing necrosis; however, PARP inhibition resulted in a similar reduction in apoptosis and in necrosis in all groups and caspase-2 inhibition did not.

Conclusions: Diabetes increases both apoptosis and necrosis in the human myocardium in fresh and after being subjected to ischemia/reoxygenation, an effect that is mediated, at least in part, by caspase-3 and PARP activation. These results may explain the increased cardiac-related morbidity and mortality during cardiac surgery in patients with diabetes.
Ultramini-Abstract:

Diabetes increases both apoptosis and necrosis in the human myocardium in fresh and after being subjected to ischemia/reoxygenation, an effect that is mediated, at least in part, by caspase-3 and PARP activation. These results may explain the increased cardiac-related morbidity and mortality during cardiac surgery in patients with diabetes.
INTRODUCTION

Diabetes is associated to an increased risk of cardiac complications in patients with coronary atherosclerosis and is also an important predictor of morbidity and mortality after cardiac surgery. Myocardial injury caused by ischemia and reperfusion during cardiac surgery may induce cell death that is considered to be an important determinant in the development of left ventricular dysfunction and heart failure in ischemic cardiomyopathy. Animal studies have demonstrated that loss of myocytes secondary to necrosis leads to wall restructuring, side-to-side slippage of cells, mural thinning, chamber dilation, fibroblast activation and myocardial scarring, which in turn results in depression of ventricular function. It is possible that diabetes may exacerbate these changes by increasing apoptosis and necrosis. Although patients with this condition are at an increased risk of ischemic events, the susceptibility of the diabetic myocardium to ischemic injury is controversial in animal studies and the subject remains largely unexplored in man (for review see ref 10).

Caspases are a large protein family of cysteine proteases that have been specifically linked with apoptosis and their inhibition has been shown to attenuate apoptosis and myocardial ischemic injury in non-diabetic rats but the role of caspases in ischemic injury of the diabetic myocardium is unknown. More recently, Poly (ADP-ribose) Polymerase (PARP), a nuclear protein which plays an essential role in DNA damage and repair, has also been shown to be linked to tissue damage in various pathological conditions and associated with oxidant stress occurring in myocardial ischemic injury, stroke and circulatory shock. It may be expected that PARP inhibition will reduce myocardial ischemic injury, however, the role of PARP in the presence of pathological conditions such as diabetes needs to be clarified.
The aims of the present studies performed in the human myocardium were to elucidate the role of diabetes on the degree of cell death by apoptosis and necrosis in the non-ischemic and in the ischemic tissue. In addition, the role played by caspases and PARP activation in ischemia/reperfusion induced cell death was investigated.

MATERIALS AND METHODS

Patients

The right atrial appendage from patients undergoing elective coronary artery bypass graft surgery was retrieved at the time of the right atrial cannulation. For this, local ethical approval and patients’ informed consent was obtained (Leicestershire Research Ethics Committee reference no. 7805). Diabetic and non-diabetic patients (n=8/group) undergoing elective cardiac surgery were included in the study. Patients with atrial fibrillation, poor ejection fraction (EF<30%), mitral and/or tricuspid valve pathology, those taking potassium channel openers (nicorandil or diazoxide) and patients undergoing emergency cardiac surgery were excluded. Blood glucose levels were well controlled before surgery in the participants with insulin-dependent and insulin-independent diabetes and they were kept within the physiological range during surgery in all study patients with and without diabetes.

Experimental Preparation and Solutions

The sectioning of the atrial muscle and the simulated ischemia/reoxygenation preparation has been previously described.16 Briefly, the atrial muscles were transferred from the operating theatre to the laboratory immersed in cold (4°C) buffer solution in a time under 2 minutes. The appendages were then mounted onto a ground glass plate with the epicardial surface face down and then sliced freehand with the use of surgical skin graft blades (Swan-Morton, Sheffield, UK) to a thickness of between 300 and 500μm. The muscles (weight 30-50mg) were transferred to conical flasks
(25mL Erlenmeyer flasks Schott Glaswerk, Mainz, Germany) containing 10mL of oxygenated buffered solution (see below for composition), and the flasks were placed in a shaking water bath maintained at 37°C. Following this the muscles were equilibrated for 30 minutes in oxygenated (95% O₂ / 5% CO₂) Krebs Henseleit Hepes (KHH) buffered solution containing (in mM): NaCl (118), KCl (4.8), NaHCO₃ (27.2), MgCl₂ (1.2), KH₂PO₄ (1.0), CaCl₂ (1.20), glucose.H₂O (10), HEPES (20) at a pH of 7.4. The buffer was supplemented with 10% fetal calf serum (FCS: Harlanseralabs #S-0001A). For the induction of simulated ischemia, the media was bubbled with 95% N₂ / 5% CO₂ (pH 6.80-7.00) in the absence of glucose.

**Experimental Protocols**

**Study 1: Degree of apoptosis and necrosis in the non-ischemic fresh diabetic myocardium**

Apoptosis, necrosis and initiator (including caspases-2,-8,-9 and -10) and effector caspase (including caspases -3, -6 and -7) activation were assessed in non-ischemic fresh atrial muscles from patients without diabetes and with NIDDM and IDDM (n=8/group).

**Study 2: Effect of ischemia/reoxygenation in the diabetic myocardium**

The atrial muscles (n=8/group) from non-diabetic, NIDDM and IDDM patients were equilibrated for 30 minutes and then subjected to the following two experimental protocols: (i) aerobic perfusion for 210 minutes; (ii) 90 minutes of simulated ischemia followed by 120 minutes of reoxygenation (SI/R). CK release was measured in the incubation media during the 120 minutes of reoxygenation or during the last 120 minutes of aerobic incubation in the controls and the tissue was taken at the end of protocols for the assessment of tissue viability, cell apoptosis and necrosis and initiator and effector caspase activation.
**Study 3: The role of caspases and PARP in cell death in the diabetic myocardium**

Sections of atrial muscle (n=6) from non-diabetic, NIDDM and IDDM patients were equilibrated for 30 minutes and then subjected to the following protocols: (i) aerobic perfusion for 210 minutes; (ii) 90 minutes SI followed by 120 minutes R; (iii) SI/R with caspase-3 inhibitor z.DEVD.fmk (70nM); (iv) SI/R with different concentrations of the caspase-2 inhibitor z-VDVAD.fmk (0, 0.02, 0.2, and 2µM); (v) SI/R with different concentrations of the PARP inhibitor PJ-34 (0, 0.07, 0.7, and 7µM). The caspase and PARP inhibitors were incubated with the muscles for the entire experimental period. As in the previous protocol, CK release was measured in the incubation media and cell apoptosis and necrosis were assessed in the muscles at the end of the protocols.

**Assessment of tissue injury and viability**

CK release into the perfusate during the 120 minutes of reoxygenation was measured as an index of tissue injury. The enzyme activity was measured by a linked-enzyme kinetic assay according to the manufacturers instruction (DG147-K: Sigma Chemicals, Perth, Australia) and expressed as IU/g wet weight.

Tissue viability was assessed by the mitochondrial reduction of 3-[4, 5 Dimethylthiazol-2-y1]-2, 5 diphenyltetrazolium bromide (MTT) to an insoluble purple formazan dye (M2128-Sigma Chemicals, Perth, Australia). The absorbance of the blue formazan product was measured on a plate reader (Benchmark, Bio-Rad Laboratories, Hercules, CA, USA) at 550nm and the results were expressed as mM of formazan/g wet weight. A reduction in the MTT values would represent decreased tissue viability.
Assessment of apoptosis and necrosis

First, the muscles were incubated for 10 minutes on ice with 5µM propidium iodide (PI) in 0.1M tri-sodium citrate and 20mM phosphate buffered saline (PBS) at pH7.4 to identify the necrotic nuclei. Sections were then fixed twice, initially for 30 minutes with 4% paraformaldehyde in 30% sucrose and then with 20mM PBS overnight on ice and at pH7.4. Following this, serial sections of 10 µm were cut with a Bright cryomicrotome (model OTF) at −25°C in tissue embedding matrix (Tissue Tek® OCT compound). The cryopreserved tissue sections were washed with 20mM PBS at pH 7.4 for 2 minutes, then permeabilised in 0.02mg/ml proteinase-K for 10 minutes at 37°C, and pre-sensitised for 1 minute in a microwave oven at 800 watts in 0.1% Triton X-100 and 0.1M sodium citrate at pH 6.0. To assess apoptosis, the terminal deoxynucleotidyl transferase (TdT) was used to incorporate fluorescein (FITC) labelled dUTP oligonucleotides to DNA strand breaks at the 3’-OH termini in a template dependent manner (TUNEL technique) using a commercially available kit (Roche:1684795, Basel, Switzerland). Using this labelling procedure sequence the nuclei can either be stained with PI or TUNEL but not both. An 8µm section of the mirror specimens opened up the cell membrane of all the cells to enable all the nuclei to be stained. Prior to the TUNEL labelling of muscles, positive controls were treated with DNase-I and negative controls were obtained by adding the label solution of the kit without the enzyme solution. The FITC fluorescence emission (range 600-630nm) was measured using argon-ion fluorescence excitation at 488nm and detected by laser confocal epifluorescence microscopy with a x10 oil immersion objective. The PI labelled nuclei was excited with helium-neon laser light at 543nm and fluorescence was detected using an emission range of 680-730nm in order to abolish fluorescence ‘bleed-through’ from FITC labelled nuclei. Analysis was done using NIH Image software (Scion Corp, Frederick, Maryland, USA) with the Cavalieri-3 macro (G. MacDonald, University of Washington), that allows to place point-counting templates
over an image to perform stereological estimates. Fluorescent signals with areas greater than 16µm² were counted to ensure that only nuclei are taken into account and to avoid the inclusion of artefact. Absolute numbers of green fluorescent apoptotic (A) and necrotic (N) red fluorescent nuclei in any given image field were determined by dividing by the total number of PI labelled nuclei (M) in the next serial or mirror section. The absolute percentage of apoptotic cells was given by A/M *100% and the percentage of necrotic cells by N/M *100%. The automatic counting was combined with regular manual inspection to ascertain that artefacts were not represented in the data.

**Measurement of caspase activity**

The muscle sections stored at –80°C until analyses were thawed in 400µl of cell lysis buffer (in mM): Hepes, 10% sucrose, 0.1% Chaps and DTT (10), in the presence of a cocktail of enzyme inhibitors (P2850-Sigma Chemicals, Perth, Australia) at a pH of 7.0 to release the intracellular contents. The muscle was diced finely and then homogenised (Ultra-Turrax homogeniser: Janke and Kunkel GmbH, Staufen, Germany) at 13,000 rpm for 1 minute on ice. This was followed by centrifugation (PK121R-ALC International) at 14,000 rpm for 30 minutes. Subsequently, the protein concentration of the soluble supernatant (cell lysate) was measured using a detergent compatible Bio-Rad assay (23225-Pierce, Cheshire, UK). Aliquots of cell lysate were then tested for caspase activity by the addition of a caspase-specific peptide or substrate, DEVD that is conjugated to the chromophore (fluorescent reporter molecule) 7-amino-4-trifluoromethyl coumarin (AFC). The cleavage of the peptide DEVD from DEVD.AFC (final concentration 20µM; Alexis Chemicals, San Diego, CA, USA) releases AFC that when excited by light at 400nm emits fluorescence at 505nm. The level of caspase activity in the cell lysate was detected by fluorescence signal obtained with a fluorometer (Fluostar P401, BMG software, Longmont, CO,
USA). The amount of caspase-3 like activity was measured by using the effector caspase inhibitor z-DEVD.fmk at a final concentration of 10µM in the well of the reader plate and by subtracting the fluorescence obtained by the total fluorescence measured in the absence of the inhibitor. The results were expressed as arbitrary units fluorescence of activity/g wet weight.

### Chemicals

The caspase-2 inhibitor z-VDVAD.fmk (R&D Systems-FMK003) was used at different concentrations (0, 0.02, 0.2, and 2µM). The caspase-3 inhibitor z-DEVD.fmk (FMK004) was used at the concentration of 70nM which was shown to be the optimal dose by previous experiments in our laboratory. The PARP inhibitor PJ-34 (Alexis Biochemical L10210) was also used at different concentrations (0, 0.07, 0.7, and 7.0µM).

### Statistical analysis

All results were expressed as mean ± standard error of mean. To compare the overall statistical significance among ND, NIDDM and IDDM groups, the non-parametric analysis of variance (ANOVA, Kruskal-Wallis H) technique was used. The comparisons between the independent groups are based on non-parametric Mann-Whitney test. A p value of <0.05 was taken to be statistically significant.
RESULTS

Study 1: Degree of apoptosis and necrosis in the non-ischemic fresh diabetic myocardium

(i) Apoptosis and necrosis

Figure 1 shows that in the non-ischemic fresh myocardium, cell death was significantly higher in the NIDDM and IDDM groups than in the non-diabetic group. Thus, whereas apoptosis was 15.0±2.8 % and 12.5±2.9% in the NIDDM and IDDM groups, respectively, values were 3.7± 0.8 % (p<0.05) in the non-diabetic group. Similarly, the mean values for necrosis were 10.7±2.9 % in the NIDDM and 10.3±2.0% in IDDM groups but only 5.0± 1.1 % in the non-diabetic group (p<0.05). Figure E1 shows representative images for apoptosis and necrosis in fresh myocardium from the three study groups. It should be noted that, although some of the observed cell death in the atrial muscles could be attributed to the trauma of tissues induced by the procurement procedure, all muscles were treated identically and therefore the differences seen between the diabetic and non-diabetic groups are real.

(ii) Caspase activity

As shown in Figure 2, the activity of initiator caspases in fresh non-ischemic muscles was similar in non-diabetics and in NIDDM and IDDM groups. However, the activity of effector caspases was significantly increased in the NIDDM and IDDM groups as compared to the non-diabetic group.
Study 2: Effect of ischemia/reoxygenation in the diabetic myocardium

(i) Apoptosis and necrosis

Figures 3a and 3b show that apoptosis and necrosis were increased in time-matched aerobically incubated muscles for 210 minutes of the NIDDM and IDDM as compared to the values in the non-diabetic group, which confirms the results seen above in fresh (not incubated) tissue. The similar degree of apoptosis and necrosis seen in fresh and aerobically-incubated muscles rules out a significant effect of the tissue culture conditions on these two end-points. The figures also show that SI/R caused a significant increase of the two forms of cell death in the non-diabetic group and that values were even greater in the NIDDM and IDDM groups. Figure E2 shows representative images for apoptosis and necrosis following ischemia/reoxygenation for the three study groups. It is worth to note that the degree of apoptosis and necrosis in aerobically incubated and ischemic/reoxygenated muscles was similar in the two types of diabetes and because of this no separation between insulin-dependant and insulin-independent patients was made in study 3 (see below).

(ii) CK release

Table E1 demonstrates that the mean CK release values during the first two hours of reoxygenation and after 90 minutes of ischemia were similarly increased in the non-diabetic and diabetic groups.

(iii) MTT reduction

Table E2 shows that SI/R resulted in a significant decrease in MTT reduction in the non-diabetic and diabetic groups that, in contrast with the above results on apoptosis and necrosis, it was of similar degree in all the three groups.
(iv) *Caspase Activity*

Figure 2 shows that SI/R induced a significant increase in initiator caspase activity in atrial muscles from the three group of patients when compared to fresh tissue. However, SI/R did not significantly influence the activity of the effector caspases in the muscles from non-diabetics but this was greatly elevated in the NIDDM and IDDM groups.

**Study 3: The role of caspases and PARP in cell death in the diabetic myocardium.**

(i) *Apoptosis and necrosis*

Figures 4a and 4b show that in the presence of the caspase-3 inhibitor z.DEVD.fmk the apoptosis caused by SI/R was almost completely abolished in the non-diabetic group but necrosis was unaffected. In contrast, in the muscles from diabetics caspase-3 inhibition reduced apoptosis by only 50% and again did not affect necrosis.

Table E3 shows that the percentage of apoptosis and necrosis in atrial tissue subjected to SI/R from non-diabetic and diabetic patients was not affected by the presence of various concentrations of the caspase-2 inhibitor z-VDVAD.fmk.

Figures 5a and 5b show that the percentage of apoptosis and necrosis in atrial tissue subjected to SI/R was similarly reduced in non-diabetic and diabetic groups in a dose-dependent manner by the PARP inhibitor PJ-34. Significant reduction was obtained at 0.7µM concentration for apoptosis and at 7µM for necrosis.

(ii) *CK release*

Table E4 shows that the release of CK following SI/R was not significantly affected by the caspase-3 and caspase-2 inhibitors z.DEVD.fmk and z.VDVAD.fmk. By
contrast, PARP inhibition resulted in a significant reduction in CK release. These results taken together may suggest that CK release is a reflection of necrosis rather than apoptosis.

**DISCUSSION**

The present studies have demonstrated that cell death by apoptosis and necrosis is greater in the human fresh, non-ischemic myocardium of diabetics than of non-diabetics, which is associated to an increase in the expression of effector caspases. They have also shown that the diabetic myocardium is more susceptible to ischemia/reoxygenation injury than the non-diabetic tissue, an effect that is mediated, at least in part, by caspase-3 and PARP activation. The importance of these findings for the understanding of the pathophysiology of ischemia/reoxygenation injury in the diabetic myocardium and their clinical implications are discussed below.

**Cell death in the fresh, non-ischemic diabetic myocardium**

Cell death by apoptosis and necrosis is a feature of end-stage heart failure but also occurs in the healthy myocardium. Here we have demonstrated that the occurrence of apoptosis and necrosis in human atrial tissue is greater in patients with diabetes than without diabetes a finding that is supported by those of Frustaci et al in ventricular biopsies. Because myocytes rarely proliferate in adult cardiac muscles, the increase loss of cardiac muscles in the diabetic myocardium may lead to a reduction of cardiac mass and to elevated interstitial and per vascular fibrosis causing a decrease in myocardial performance and ventricular dilatation, a sequence of events that may be responsible for the increasing cardiovascular mortality and morbidity in diabetic patients.
A possible explanation for the increase in cell death in diabetes may be a greater oxidative stress observed with this condition\textsuperscript{23} which in turn may be responsible for the activation of effector caspases seen in our studies. There is experimental evidence that caspases are activated in diabetes\textsuperscript{11} and also that caspases are activated by oxidative stress in non-diabetics\textsuperscript{20} and in diabetics.\textsuperscript{24} Indeed high glucose causes oxidative stress that results in caspase-3 activation in human endothelial cells via SAPK/JNK activation.\textsuperscript{25} Activating of effector caspases in diabetes can also be induced by the abnormal accumulation of beta hydroxyl fatty acid, as seen in mice, which alters the permeability of the mitochondrial membrane, and causes the release of cytochrome c and the activation of the downstream caspases.\textsuperscript{26} Our findings that effector caspases are activated in the fresh, non-ischemic human atrial myocardium suggests a central role of these proteins in the increase in apoptosis seen in the heart of patients with diabetes, but it is clear that investigation is required to elucidate the precise molecular mechanism involved.

Susceptibility of the diabetic myocardium to ischemia/reoxygenation injury

The present studies have also shown that the diabetic myocardium is more susceptible to ischemia/reoxygenation injury than the non-diabetic myocardium when the propidium iodide and TUNEL technique read-out for apoptosis and necrosis are used. These results in the human myocardium are supported by experimental animal studies in rats\textsuperscript{27} and dogs\textsuperscript{28} showing that diabetes makes the heart more susceptible to ischemia/reoxygenation injury. However, other experimental studies\textsuperscript{29} have reported that the diabetic myocardium is in fact more tolerant to injury than the non-diabetic tissue. The reason for these divergent results is not clear but it is possible that the duration and the severity of the diabetic state and the differences in the experimental preparations used may play a role.\textsuperscript{10} Another potential explanation could be a lack of
correspondence between different end-points as shown by our results on CK release and MTT reduction.

The observation that the activity of the effector caspases is more elevated in the diabetic than in the non-diabetic myocardium when subjected to an ischemic insult may suggest that this class of enzymes are responsible for the greater occurrence of apoptosis in diabetes. However, caspase-3 inhibition reduced apoptosis in the diabetic to a lesser extent than in the non-diabetic myocardium and caspase-2 inhibition, an enzyme that shares sequence homology with initiator caspases like caspase-9 and -1\textsuperscript{30} but with cleavage specificity closer to the effector caspases caspase-3 and -7,\textsuperscript{31} did not effect apoptosis in either of the two groups, all suggesting that the increase of myocardial apoptosis in diabetes is not dependent on the greater activation of the effector caspases alone. Our finding that the induction of apoptosis by ischemia/reoxygenation in the human non-diabetic myocardium is caspase-3 dependent, is supported by \textit{in vivo} animal experimental studies;\textsuperscript{11} however, the finding that caspase-3 inhibition only partially reduced apoptosis in the diabetic myocardium suggests that a caspase-independent pathway causing apoptosis also exist in the diabetic myocardium.

In contrast with the results on inhibition of caspases-3 activity, the inhibition of PARP similarly reduced apoptosis in a dose-dependent manner in both the diabetic and the non-diabetic ischemic myocardium with almost complete abolition at the highest concentration of the inhibitor PJ-34 used (7µM). The above suggests that both caspase-dependent and –independent pathways of apoptosis converge in activation of PARP. It is possible that the reported increase activation of PARP seen in the diabetic rat myocardium\textsuperscript{32} is responsible for the greater susceptibility of this tissue to apoptosis. However, the role of PARP in ischemic injury has been disputed and whereas \textit{in vitro}\textsuperscript{33} and \textit{in vivo}\textsuperscript{13} studies have demonstrated a limitation in cellular
injury by PARP inhibition, other investigators have suggested that PARP activation is not indispensable for apoptosis.\textsuperscript{34}

**Clinical implications and limitations of the study**

The present studies have identified molecular mechanisms by which the diabetic myocardium is more susceptible to ischemic injury than the non-diabetic tissue. These findings may be of clinical relevance since they provide an explanation for the increased cardiac-related morbidity and mortality seen in diabetic patients during heart surgery and because ischemic injury can be reduced by pharmacological manipulation of the pathways involved. However, it should be clarified that our studies were performed *in vitro* using atrial myocardium and that any extrapolation to the clinical situation should be done with caution. An additional limitation of these studies is that because of methodological constraints, the cell type of each nucleus undergoing death could not be determined. Therefore, the observed cell death could be due to the demise of any cell type within the myocardium including the cardiomyocyte.

**Acknowledgements:**

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REFERENCES:


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**Figure 1:** Percent of apoptosis and necrosis (n=8/group) in non-ischemic fresh myocardial tissue from non-diabetics (ND), non-insulin dependent diabetes mellitus (NIDDM) and insulin-dependent diabetes mellitus (IDDM) patients. * p<0.05 vs. ND.

**Figure 2:** Caspase activation in fresh myocardial tissue and in muscles after ischemia/reoxygenation (n=8/group) from non-diabetics (ND), and from patients with non-insulin dependent diabetic mellitus (NIDDM) and insulin-dependent diabetes mellitus (IDDM). * p<0.05 vs ND and † p<0.05 vs the corresponding fresh tissue group.

**Figure 3:** (a) Percent of apoptosis and (b) necrosis after simulated ischemia/reoxygenation (SI/R) (n=8/group) in cardiac muscles from non-diabetics (ND), non-insulin dependent diabetic mellitus (NIDDM) and insulin-dependent diabetes mellitus (IDDM) patients. * p<0.05 vs ND and † p<0.05 vs the corresponding aerobic control group.

**Figure 4:** Percent of apoptosis (a) and necrosis (b) in myocardial tissue from non-diabetics and diabetics subjected to ischemia/reoxygenation (n=6/group) in the presence and absence of the caspase-3 inhibitor Z.DVAD.fmk (70nM). * p<0.05 vs without caspase-3 inhibition group.

**Figure 5:** Percent of apoptosis (a) and necrosis (b) in myocardial tissue from non-diabetics and diabetics subjected to ischemia/reoxygenation in the presence of various concentrations of the PARP inhibitor PJ-34 (n=6/group). * p<0.05 vs control group.
Chowdhry et al. Figure 2

Fluorescence (AU x 10^3/g wet wt)

- *: initiator caspases
- †: effector caspases

ND  NIDDM  IDDM  ND  NIDDM  IDDM

fresh tissue  after ischemia/reoxygenation
Figure 3

(a) Apoptosis (percent nuclei)

- ND
- NIDDM
- IDDM

(b) Necrosis (percent nuclei)

- ND
- NIDDM
- IDDM

Legend:
- *: Significant difference from aerobic control
- †: Significant difference from ischemia/reoxygenation
- **: Highly significant difference from aerobic control
- † †: Highly significant difference from ischemia/reoxygenation
Figure 4

(a) Apoptosis (percent nuclei)

(b) Necrosis (percent nuclei)

With C3 inhibition

Non-diabetic

Diabetic

Without C3 inhibition

Non-diabetic

Diabetic

* Indicates statistical significance.
Apoptosis (percent nuclei)

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Diabetic (■) vs. Non-diabetic (●)

Necrosis (percent nuclei)

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Diabetic (■) vs. Non-diabetic (●)

* indicates statistical significance.
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**Figure E1:** Representative images of apoptosis (green) and necrosis (red) in non-ischemic fresh myocardial tissue from non-diabetics (ND), non-insulin dependent diabetes mellitus (NIDDM) and insulin-dependent diabetes mellitus (IDDM).

**Figure E2:** Representative images of apoptosis (green) and necrosis (red) in myocardial tissue from non-diabetics (ND), non-insulin dependent diabetes mellitus (NIDDM) and insulin-dependent diabetes mellitus (IDDM) following 90 minutes ischemia and 120 minutes of reoxygenation.
Table E1: Mean values for CK release (n=5/group) expressed as IU/mg wet wt in time-matched aerobic controls and after 90 minutes of simulated ischemia and 120 minutes reoxygenation (SI/R) of cardiac muscles from non-diabetics and patients with non-insulin dependent diabetes mellitus (NIDDM) and insulin dependent diabetes mellitus (IDDM). *p<0.05 vs aerobic control.

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<td>IDDM</td>
<td>0.63±0.08</td>
<td>2.12±0.14*</td>
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Table E2: Mean values for MTT reduction (n=5/group) expressed as IU/mg wet wt in time matched aerobic controls and after 90 minutes of simulated ischemia and 120 minutes reoxygenation (SI/R) of cardiac muscles from non-diabetics and patients with non-insulin dependent diabetes mellitus (NIDDM) and insulin dependent diabetes mellitus (IDDM). *p<0.05 vs aerobic control.

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Table E3: Percent of apoptosis and necrosis in myocardial tissue from non-diabetic and diabetics subjected to 90 minutes of simulated ischemia and 120 minutes reoxygenation in the presence of various concentrations of the caspase-2 inhibitor Z.VDVAD.fmk (n=6/group).

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<tr>
<td><strong>Apoptosis (% of nuclei)</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Non-diabetic</td>
<td>14.5±4.3</td>
<td>15.1±4.8</td>
<td>10.5±3.1</td>
<td>11.3±4.3</td>
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<tr>
<td>Diabetic</td>
<td>34.0±5.2</td>
<td>27.0±2.5</td>
<td>30.7±7.6</td>
<td>30.2±8.0</td>
</tr>
<tr>
<td><strong>Necrosis (% of nuclei)</strong></td>
<td></td>
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<tr>
<td>Non-diabetic</td>
<td>18.6±1.7</td>
<td>19.7±6.4</td>
<td>23.4±2.9</td>
<td>23.3±6.3</td>
</tr>
<tr>
<td>Diabetic</td>
<td>36.7±5.8</td>
<td>25.7±3.5</td>
<td>36.2±6.3</td>
<td>34.4±5.3</td>
</tr>
</tbody>
</table>
Table E4: Effect of (a) the caspase -3 inhibitor Z.DEVD.fmk (70nM), (b) caspase -2 inhibitor Z.VDVAD.fmk (2μM), and (c) the PARP inhibitor PJ-34 (7μM) on CK release expressed as IU/mg wet wt after 90 minutes of simulated ischemia and 120 minutes reoxygenation (SI/R) of cardiac muscles from non-diabetics and diabetics. n=6/group. *p<0.05 vs SI/R alone.

(a)

<table>
<thead>
<tr>
<th></th>
<th>SI/R alone</th>
<th>SI/R plus Caspase -3 inhibitor</th>
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</thead>
<tbody>
<tr>
<td>Non-diabetic</td>
<td>2.92±0.72</td>
<td>2.51±0.96</td>
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<tr>
<td>Diabetic</td>
<td>2.67±0.59</td>
<td>2.79±0.32</td>
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</table>

(b)

<table>
<thead>
<tr>
<th></th>
<th>SI/R alone</th>
<th>SI/R plus Caspase -2 inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-diabetic</td>
<td>2.53±0.19</td>
<td>2.38±0.24</td>
</tr>
<tr>
<td>Diabetic</td>
<td>2.76±0.94</td>
<td>2.52±0.81</td>
</tr>
</tbody>
</table>

(c)

<table>
<thead>
<tr>
<th></th>
<th>SI/R alone</th>
<th>SI/R plus PARP inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-diabetic</td>
<td>2.88±0.25</td>
<td>1.48±0.15*</td>
</tr>
<tr>
<td>Diabetic</td>
<td>2.98±0.17</td>
<td>1.61±0.19*</td>
</tr>
</tbody>
</table>