Influence of Fasting on Functional Recovery of the Isolated Heart Exposed to Ischemia Reperfusion in Young and Aged Male Albino Rats

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Abstract

Mortality due to ischemic cardiovascular diseases is significantly higher in elderly than in young adults.

In both cardiac surgical and cardiological settings, clinical interventions used to reperfuse the ischemic heart are associated with myocardial damage that is likely to influence long-term functional recovery.

The aim of this study was to investigate the effect of fasting on functional recovery of isolated heart exposed to ischemia reperfusion in young and old male rats and to study some mechanisms of protection of fasting against ischemia reperfusion.

48 male albino rats were divided according to age and diet type into young and old control rats fed on standard rat chow, ad-libitum fed group (ALG), and young and old fasted groups (FG) which included rats fasted for 3 days (3 days water only fasting). The rats were subjected to 25min of myocardial ischemia followed by 30min of reperfusion. Myocardial performance parameters were done before ischemia (baseline) and 10, 20 and 30min reperfusion.

The results showed that left ventricular developed pressure LVDP & the peak rate of maximum left ventricular pressure rise (dP/dt), as an index of contractility, increased significantly \( p<0.05 \) (after 30min reperfusion) in young compared to old ALG and in young and old FG compared to ALG of the same age. Also cardiac expression of AMPK showed a significant increase \( p<0.05 \) and LDH in perfusate showed a significant decrease \( p<0.05 \) in young and old FG when compared to ALG of the same age. Histological examination of cardiac tissues showed that inflammation and degeneration were less in FG in comparison to ALG in cardiac tissues 30 minutes after reperfusion.

We concluded that senescent hearts are more susceptible to myocardial ischemia reperfusion injury than adult hearts. Slightly longer periods of fasting, prior to surgery may have protective effect against certain types of acute organ stress in young and old rats. The protective mechanism of fasting is partly mediated by increased cardiac expression of AMPK.

Key Words: Fasting – Age – Heart – Ischemia reperfusion – Rats.

Introduction

THE rapid growth of the world elderly population has heightened awareness of age-related diseases, including interest in the study of the aging human heart. Cardiovascular diseases are the most leading causes of death in the elderly, as those older than 65 years account for greater than 80% of patients with ischemic heart disease [1] cardiac aging itself may be a major risk factor for cardiovascular pathology such as ischemic heart disease. Myocardial ischemia-reperfusion (MI/R) can induce oxidative stress and an inflammatory response [2].

Dietary restriction (DR) encompasses a variety of interventions resulting in reduced nutrient and energy intake without malnutrition. DR is best known for its ability to extend lifespan in a wide variety of organisms [3,4]. Effects of DR on human longevity are not known, but prospective studies show a favorable impact on markers of aging and predictors of long-term health, including improved cardiovascular fitness, body mass index and insulin sensitivity [5]. DR increased resistance to multiple forms of acute stress [6].

Shorter periods of more severe restriction have also proven effective in protecting against acute stress. Mitchel et al. [7] stated that brief periods of water-only fasting were similarly effective at protecting against ischemic damage as four weeks DR. Also, fasting for 3-4 days improves organ and animal survival in rodent models of liver transplantation [8]. Finally, 2 days of water-only fasting protects mice against the toxic effects of chemotherapeutic agents [9].
Nakano et al. [10] demonstrated that fasting attenuates myocardial stunning (reversible change) but not myocardial infarction (irreversible damage). However Little is known about the effect of fasting on the resistance of the myocardium against ischemic stress.

AMP-activated protein kinase, AMPK, is a protein kinase that has taken center stage in metabolic regulation over the last decade. Protein kinases are specialized enzymes that transfer phosphate groups from ATP to amino acids on specific target proteins. The AMPK pathway has received a great deal of attention because of its potential importance in the ischemic heart, diabetes and cancer [11,12]. DR might induce mild shortage of energy substrates. It seems reasonable to assume that short-term DR switches off ATP-consuming pathways and switches on ATP-generating mechanisms [13]. Alterations in cardiac AMPK activity are reportedly associated with cardiac hypertrophy, cardiomyopathy, and Wolf-Parkinson-White syndrome [14].

The aim of the present research was to study the possible effect of fasting on functional recovery of isolated heart exposed to ischemia reperfusion. The effect of fasting was compared between young and old rats. Lactate dehydrogenase (LDH) in perfusate during cardiac ischemia reperfusion was measured as LDH levels are affected by tissue injury. Also, myocardial expression of AMPK was studied.

Material and Methods

Animals:
Forty eight male albino rats were used in this study. Twenty four were adult (6 months) weighing 180-200 grams and Twenty four were aged (18 months) weighing 310-330 grams.

The animals were kept in the animal house of Physiology Department, Kasr Al-Aini Faculty of Medicine, Cairo University from May 2011-July 2011, with normal daily light-dark cycle. Animals were allowed to acclimatize to their environment and received standard rat chow ad libitum for one week, then young & old rats were randomly divided according to the diet type into two groups of 12 rats each, ad-libitum fed group (ALG), fed on standard rat chow & fasted group (FG) which included rats fasted for 3 days (water only fasting). So, 4 groups were included in this study, young ALG, old ALG, young FG and old FG.

Each group was further subdivided into two subgroups. So, the hearts of the animals in the first subgroup were exposed to ischemia for 25min then reperfusion for 30min and cardiac performance and LDH estimation were done while the hearts of the other subgroup were subjected to AMPK measurements.

Experimental methods:
Rats were anesthetized by intraperitoneal injection with thiopental sodium (30mg/kg) then left thoracotomy was performed to expose the heart. Surgical procedures were conducted between 8.30 and 12.00 am. Exposed hearts from 6 rats in each group were used to measure AMPK & the hearts from the other 6 rats were quickly placed in ice cold modified kreb-henseleit solution, transferred & attached to modified langendorff apparatus by the aortic root through the perfusion cannula for measurements of myocardial performance as previously described [15].

The time between extraction of the hearts and attachment to the langendorff apparatus did not exceed two minutes. Hearts were then perfused retrogradely with non-recirculating modified oxygenated kreb-henseleit solution of the following concentration in mM 118 Nacl, 25 NaHCO, 1.2 KHPO, 4.7 Kcl, 1.2 MgSO, 2 Cacl and 10 dextrose in 1000ml distilled water. The perfusate was maintained at 38°C. The hearts were also maintained at 38°C using a water reservoir surrounding the hearts in which the open end was covered to maintain temperature and humidity. LDH was estimated in perfusate after 30 minutes of reperfusion.

To record intra-ventricular pressure, a saline filled latex balloon connected to a catheter was inserted into the left ventricle. The catheter was then connected to a pressure transducer (Goldstatum). Pressure changes were then analyzed and displayed on an electronic polygraph (NEC-Sanei, 2238). The intraventricular balloon was then filled with saline to adjust the baseline end diastolic pressure (EDP) to 10mmHg. Hearts were then left to stabilize for 30 minutes.

The measured parameters of myocardial performance:
- Heart rate.
- Left ventricular systolic pressure.
- Left ventricular diastolic pressure.
- Left ventricular developed pressure LVDP designed as the difference between systolic & diastolic left ventricular pressure.
- The peak rate of maximum left ventricular pressure rise (dP/dt) as an index of contractility.
- All measures were done before ischemia and at 10,20 & 30min reperfusion [16].
Biochemical measurements:

**Measurement of LDH:**

The LDH activity in perfusate was measured by an automatic biochemistry analyzer (Roche, 04744934001, CH-4070 Basel, Switzerland) according to the manufacturer’s instructions; namely, 50uL medium and 50uL mix of reagent A and B were co-incubated for 30min and then the absorbance was detected at 492nm with use of a spectrophotometer (Bekman, Germany) [17].

**Detection of gene expression of AMPK in heart:**

AMPK gene expression was detected in heart tissue by reverse transcription-polymerase chain reaction (RT-PCR).

Total RNA was extracted with Trizol reagent (Gibco- BRL). In brief, 30mg of heart tissues were lysed in an adequate volume of Trizol reagent according to the manufacturer’s instructions. After chloroform extraction and high-speed centrifugation (12 000g, 15min), RNA was purified and stored at −70 C until required for use [18].

RNA (1 µg) was then reverse transcribed into single-stranded DNA with 200U of SUPERSCRIPT TMII RNaše Reverse Transcriptase (Life Technologies, Gibco BRL, Gaithersburg, MD, USA), and oligo (dT) 15 primer (Promega) at 37 °C for 45 minutes, 42 °C for 15 minutes, and 99 °C for 5 minutes. PCR amplification was performed on 1/10th of the cDNA solution with 0.5U of Taq DNA polymerase (Sigma) at a final volume of 50 µL.

The PCR conditions and primers sequence were as follows: AMPK Forward primer- 5’-CACCATTCAAGAGAGATCCGAGAG- 3’, Reverse primer: 5’-TCAAATCTTTCTCACAACCC ACC TCC- 3’, β-actin primers sequence: Forward primer: 5’-GAGACCTTCAAACCCCAAGC-3’, Reverse primer: 5’GCTCATGTGCAATGGTGATG-3’, PCR program 30 cycles of 94 °C, for 30 seconds, 60.3 °C for 30 seconds, and 72 °C for 30 seconds. RT-PCR products were separated on 1.5% agarose (Sigma).

**Histopathological examinations:**

The hearts were kept in 10% formaline for histopathological examinations, dehydrated, cleared in zylol and embedded in parabalst. Paraffin sections were cut serially at 6 µm thickness and stained by Hematoxylin and Eosin (Hx & E) as described by Drury and Wallington (1980) [19].

Histological examination of all tissues was evaluated per section in at least 10 randomly selected non-overlapping fields at x 200 & x 400 magnification.

**Statistical analysis:**

Data were coded and entered using the statistical package SPSS. Data were summarized using mean and standard deviation for quantitative variables. Comparisons between groups were done using analysis of variance (ANOVA) and multiple comparisons (Post Hoc test) for quantitative variables while nonparametrical (Kruskal-Wallis test) and (Mann-Whitney test) were used for quantitative variables not normally distributed.

**Results**

**Myocardial performance parameters:**

Table (1) shows details of cardiac function parameters recorded in the four study groups, before ischemia at baseline (0min) and after 10, 20 and 30min of reperfusion. All measured parameters (HR, LVDP, dp/dt) were comparable among the studied groups. At baseline all parameters showed insignificant changes between different groups.

After 30min reperfusion:

HR showed insignificant changes between different groups.

Regarding, the effect of age, LVDP and dP/dt showed a significant increase (p<0.05) in young ALG compared to old ALG (59% & 65% versus 53% & 60% of corresponding baselin values respectively). Also there was a significant increase (p<0.05) in dP/dt in fasting young compared to fasting old rats (79% versus 72% of corresponding baseline values respectively).

Regarding the effect of fasting, LVDP and Dp/dt showed a significant (p<0.05) improvement in young FG (76% & 79%) and old FG (68% & 72%) compared to their corresponding young ALG (59% & 65%) and old ALG (53% & 60% of corresponding baseline values respectively) (Fig. 1).

**Results of biochemical analysis:**

Table (2) showed levels of gene expression of AMPK in cardiac tissues and LDH in perfusate in different study groups. AMPK and LDH showed insignificant change when comparing their levels in young (FG & ALG) to old (FG & ALG). AMPK showed a significant increase (p<0.05) in young FG when compared to young ALG and in old FG versus old ALG. LDH showed a significant decrease (p<0.05) in young FG when compared versus young ALG and in old FG versus old ALG (Fig. 2).

**Histopathological study:**

To study the effect of fasting on the cardiac tissues, H&E stained sections from young and old...
### Table (1): Myocardial performance parameters of isolated hearts before and after ischemia reperfusion (10, 20 & 30 minutes reperfusion).

<table>
<thead>
<tr>
<th>Group</th>
<th>Base line Heart Rate (beats/min)</th>
<th>Left ventricular developed pressure LVDP (mmHg)</th>
<th>The peak rate of maximum left ventricular pressure rise (dP/dt, mmHg/sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10min. rep.</td>
<td>20min. rep.</td>
<td>30min. rep.</td>
</tr>
<tr>
<td>Control young (ALG)</td>
<td>115.00±</td>
<td>85.00±</td>
<td>93.67±</td>
</tr>
<tr>
<td>Control Aged (ALG)</td>
<td>106.83±</td>
<td>82.33±</td>
<td>90.50±</td>
</tr>
<tr>
<td></td>
<td>15.03±</td>
<td>12.18±</td>
<td>11.04±</td>
</tr>
<tr>
<td>Fasting young (FG)</td>
<td>105.5±</td>
<td>90.17±</td>
<td>86.33±</td>
</tr>
<tr>
<td></td>
<td>11.98±</td>
<td>7.60±</td>
<td>7.20±</td>
</tr>
<tr>
<td>Fasting Aged (FG)</td>
<td>98.00±</td>
<td>89.00±</td>
<td>85.17±</td>
</tr>
<tr>
<td></td>
<td>9.38±</td>
<td>7.48±</td>
<td>7.70±</td>
</tr>
</tbody>
</table>

The values are expressed as means± standard deviation.

ALG: ad-libitum fed group.

FG: 3 days water only fasting group.

Significant difference when comparing (young & aged FG) versus their corresponding (young & aged ALG) (p<0.05).

Significant difference when comparing (young FG) versus (aged FG) (p<0.05).

Significant difference when comparing (young ALG) versus aged (ALG) (p<0.05).

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### Table (2): Measurement of AMPK in cardiac tissues and LDH in perfusate after myocardial ischemia reperfusion.

<table>
<thead>
<tr>
<th>Group</th>
<th>AMPK</th>
<th>LDH u/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control young</td>
<td>0.102±0.046</td>
<td>78.078±12.388</td>
</tr>
<tr>
<td>Control Aged</td>
<td>0.123±0.0592</td>
<td>88.367±16.112</td>
</tr>
<tr>
<td>Fasting young</td>
<td>0.353±0.089a</td>
<td>59.467±14.649a</td>
</tr>
<tr>
<td>Fasting Aged</td>
<td>0.331±0.109b</td>
<td>62.683±15.833b</td>
</tr>
</tbody>
</table>

The values are expressed as means± standard deviation.

Significant difference when comparing fasting young versus control young rats (p<0.05).

Significant difference when comparing fasting aged versus control aged rats (p<0.05).
Fig. (1): Left ventricular developed pressure LVDP (A) and the peak rate of maximum left ventricular pressure rise $dP/dt$, (B) at baseline & 30 minutes reperfusion after myocardial ischemia in control (fed-ad-libitum) and fasting young and old male rats.

Fig. (2): Lactate dehydrogenase LDH and cardiac expression of AMPK in control (fed ad-libitum) and fasting young and old male rats.
Fig. (3): H&E stained sections of cardiac tissues showed normal tissues in young (A) and old (B) control rats, hydropic degeneration (C) in young ad libitum fed group ALG, severe inflammation (D) & apoptosis (D2) in old ALG, mild inflammation (E) in young & degeneration (F) in old fasted groups.
Discussion

Myocardial ischemia results in damage to heart cells and an inflammatory response that can impair heart function. Mortality due to ischemic cardiovascular diseases is significantly higher in elderly than in young adults [2].

de Lorgeril et al. [20] have shown that dietary factors can attenuate the extent of heart damage and impaired function in animal models of myocardial infarction [20].

Mitchel et al. [7] suggested that brief periods of fasting induced functional changes similar to those induced by short-term dietary restriction in mice, and these changes include protection from renal ischemia/reperfusion injury and protection against ischemic damage to the liver.

So, this work was aimed to study the possible effect of short period of fasting (3 days only water fasting) on functional recovery of isolated heart exposed to ischemia reperfusion in young and aged rats.

The results of the present study showed that after 30min reperfusion there was a significant improvement in LVDP & contractility index dP/dt in young ALG group (6 months) compared to old ALG group (18 months). Also young FG showed a significant increase in dP/dt compared to old FG.

In accordance with these results, Boys et al. [21] have reported that hearts from senescent animals are more susceptible to ischemia than those from young animals.

Other investigations have concluded that myocardial ischemic tolerance decreases with age, [22]. In addition, several experimental and clinical investigations have addressed the issue of whether ischemic preconditioning IPC occurs in aged hearts. The efficacy of IPC has been reported to be decreased or lost in senescent patients [23] and in aged animals [24], also aging exacerbated ischemia/reperfusion-induced myocardial necrosis [25]. Moreover, Shi et al. [26] have shown that advanced age is a major risk factor for heart failure development.

Ischemia can rapidly deplete myocardial cell pools of ATP and causes cell necrosis. Reperfusion of the ischemic tissue is necessary to increase tissue levels of ATP and to improve heart function. Although blood flow is restored, tissue injury may be enhanced by the acute inflammatory response and increased oxidative stress induced by reperfusion [27].

The results of the present study showed that fasting animals (both young and aged) had a significant improvement in LVDP and contractility index (dp/dt) after 30min of reperfusion compared to ad libitum fed animals.

These results are in accordance with other studies which investigated the effect of DR on cardiac function after ischemia reperfusion.

Shinmura et al. [28] showed that short-term (2 weeks) DR is capable of improving myocardial ischemic tolerance in both young and old Fischer 344 rats. Abete and colleagues [29] found that IPC reduces postischemic dysfunction in the hearts from adult and food-restricted but not in the ad libitum-fed senescent rats.

Snorek et al. [30] investigated whether the three-day fasting affects the incidence and severity of ventricular arrhythmias occurring during myocardial ischemia and reperfusion in adult male Wistar rats. They reported that the number of premature ventricular complexes during early reperfusion phase was significantly reduced in fasting rats compared to controls. Ventricular tachycardia was absent in fasting animals while controls exhibited total ventricular tachycardia duration of 18 ±4s. They supposed that fasting decreases reductive stress in reperfusion & subsequent reduction of the free radical damage could provide its antiarrhythmic effect.

Moreover, Yamagishi et al. [31] have found that, following ischemia reperfusion, cardiac function, high-energy phosphate content, and intracellular pH, had recovered to a much greater degree in the food restricted rats for 11 days than in the ad libitum fed group.

DR increases acute stress resistance in most organisms tested, including mammals. For example, DR lasting between 3 months and 1 year mitigates injury in rodent models of cardiac and cerebral ischemia [32]. However, the length of restriction required for the onset of such benefits is not known.

Shorter periods of more severe restriction can be effective in protecting against acute stress. For example, fasting for 3-4 days improves organ and animal survival in rodent models of heterotopic heart transplantation [33].

There is a potential overlap between mechanisms of protection by fasting, short-term and long-term DR [34]. Evidence from animal models and preliminary studies in humans indicates that DR delays cardiac aging and prevents cardiovascular disease. DR seems to confer vasoprotection through
In the setting of short-term DR, it has been reported a role of the activation of adiponectin-AMPK signaling in DR-induced cardioprotection [41]. Adiponectin is one of the most abundant adipocyte-derived hormones (adipokines) and increases significantly with DR [40]. AMPK activation has a number of important physiological effects that appear to prevent myocardial injury during ischemia. Hearts from mice with genetically inactivated AMPK show impaired recovery of left ventricular contractile function after ischemia and reperfusion [42]. These hearts also demonstrate significantly increased myocardial necrosis after ischemia and reperfusion, indicating that AMPK protects against cell death [16]. Although these findings support the contention that AMPK is cardioprotective in isolated hearts, many additional factors are operative in the intact organism that can influence the action of AMPK [42].

When AMPK activation is impaired, apoptosis is substantially increased in hearts subjected to ischemia/reperfusion [43]. AMPK activation also contributes to the antiapoptotic effects of the hormone adiponectin [44]. Thus, it appears that activated AMPK is also protective in the heart by limiting apoptosis during ischemia/reperfusion.

More recent evidence suggests that AMPK might also protect against injury by promoting ischemic preconditioning. Ischemic preconditioning is an interesting phenomenon through which short periods of ischemia render the heart less susceptible to injury during subsequent more prolonged ischemic insults. AMPK is activated by short durations of ischemia [45] as well as by experimental preconditioning [46] AMPK has been shown to induce preconditioning in isolated cardiomyocytes and to prevent hypoxic injury [47]. The degree to which AMPK activation is either required or sufficient to induce preconditioning in the intact heart is uncertain but warrants further investigation because of its potential clinical application in preventing ischemic myocardial injury.

The molecular mechanisms responsible for ischemic preconditioning are complex. However, one mechanism through which activated AMPK might induce ischemic preconditioning is by activating ATP-sensitive potassium channels [48].

AMPK stimulates the movement of these channels from storage membranes to the cell surface membranes, where they are physiologically active [48]. These channels shorten the action potential and prevent calcium overload during reperfusion. Activated AMPK also stimulates other molecular

attenuation of oxidative stress and antiinflammatory effects in aged animals. DR also increases bioavailability of anti-atherogenic NO and improves endothelial function [35]. In addition, DR exerts beneficial effects on a range of systemic cardiovascular risk factors [36]. AMPK plays an important role in regulating the energy balance in the myocardium; the activation of AMPK during ischemia-reperfusion can reduce ischemia-induced necrosis and apoptosis [37].

In the present study, we investigated some possible mechanisms of protection by fasting. So, assessment of LDH & myocardial expression of AMPK and histological examination were done.

In the present work, fasting rats showed increased myocardial expression of AMPK and decreased LDH levels in perfusate when compared to ALG of the same age groups.

It has been reported that LDH levels was affected by cellular damage [7] so, we measure LDH in perfusate after 30min reperfusion as a marker of acute tissue injury.

In this work we found that LDH is significantly decreased in FG compared to ALG. In accordance with our results, Shinmura et al. [28] found that DR significantly attenuated total CK and LDH release during reperfusion compared with the corresponding AL group. Mitchel et al. [7] also reported that DR and fasted groups showed improvement in LDH level after renal ischemia reperfusion compared to their control groups.

Histopathological examination of cardiac tissues by H&E showed more inflammation and apoptosis in old ALG when compared to young ALG, young FG and old FG.

In agreement with these results, Liu et al. [38] found that aging increased ischemia/reperfusion-induced myocardial apoptosis in rats. They reported that age aggravated MI/R injury via gene expression changes in Bax and Bel-2, ultimately increasing the ischemia/reperfusion-induced myocardial apoptotic ratio.

Furthermore, more recent evidence indicates that myocardial apoptosis might be a major cause of post-ischemic heart failure development [39].

In this study we found that myocardial expression of AMPK is significantly increased in fasting groups compared to ad-libitum fed groups.

These results are in agreement with previous finding that phosphorylated AMPK was significantly increased with short-term DR [40].
signaling pathways, including endothelial nitric oxide synthetase [48]. Thus, activation of AMPK before ischemia has a number of downstream actions that may serve to protect the heart against subsequent myocardial injury [49].

In conclusion, senescent hearts are more susceptible to ischemia reperfusion injury than adult hearts during myocardial ischemia reperfusion. Slightly longer periods of fasting, prior to surgery may be beneficial for protection against certain types of acute organ stress including ischemia reperfusion injuries unavoidably encountered in elective surgeries. It remains to be seen if the benefits of fasting observed in young and old rats will translate to humans. Ongoing research should confirm the relation between fasting and AMPK and clarify whether AMPK has a protective effect in clinically relevant animal models of regional ischemia.

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