World Journal of Medical Sciences 4 (2): 85-88, 2009
ISSN 1817-3055

Role of Time as a Variable in the Apoptotic Changes Following Ischemia-Reperfusion in the Isolated Heart of Rat

[1]M. Hashemi, [2]Y. Dostar, [3]A.R. Azizi Saraji, [3]S.R. Rohani and [4]Mansour Bayat

[1]Department of Genetics, Islamic Azad University, Tehran medical Branch, Tehran, Iran
[2]Department of Pathology, Islamic Azad University, Tabriz Branch, Tabriz, Iran
[3]Faculty of specialized veterinary sciences,
Science and Research Branch, Islamic Azad University, Tehran, Iran
[4]Department of medical and Veterinay Mycology, Faculty of Specialized Veterinary Sciences,
Science and Research Branch, Islamic Azad University, Tehran, Iran

Abstract: the present study was carried out to study the time-dependent effects of ischemia-reperfusion on the myocardial cells. in this experimental study, male rats of strain SD, 270-330 gr in weight were divided into four groups of 10; they anesthetized with sodium pentobarbital (50-60 IP-kg/mg) with the heart of treatment group isolated immediately and connected to crebs solution langendroph set with constant pressure and temperature of 37°C and while stabilization, applied by 30 min ischemia and reperfusion for 60, 90 and 120 min. Heart of control group remained intact. Apoptotic cells were immunohistochemically diagnosed by TUNEL POD kit and positive Tunel myocardium cells of each member was counted in 5 microscopic fields and their results measured as mean±standard deviation. results indicated that in the control group, apoptotic cells counted as 1±0.4 while in treatment group called T/60 min, T/90min and T/120 min were counted as 2±0.5, 3±0.3 and 6±0.3 respectively. there was no significant relations between groups (T/120min and T/90min) and (T/60 min and Control) but there was significant relation between groups T/120 min and T/60 min($P<0.001$) and T/120 min andand T/90 min($P<0.01$).in conclusion, this study indicated that ischemia-reperfusion time can be effective in apoptotic changes of myocardial cells.

Key words: Ischemia-Reperfusion · Apoptosis · Infarction-heart-rats

INTRODUCTION

Necrosis and apoptosis are two main separated pathways for cell death in the muscle and myocardium cells accompanying with Ischemia (I) and Reperfusion (R). Cardiocyts under necrosis and apoptosis have morphological and biological close properties. Necrosis, often called accidental cell death or pathological one, diagnosed by inflation or strict cell division, denaturation of cytoplasmic proteins, breaking out intra cell organelles and strict inflation response. Apoptosis is a planned and genetically controlled cell death, its main morphological aspect includes cell leakage, chromatin condensation, forming cytoplasmic bubbles and apoptotic bodies occurring without losing cell membrane entity and inflammatory response. Although apoptosis and necrosis possess different mechanisms, but there are common attributes under pathological conditions [1-5]. Different studies indicated that ischemia-reperfusion can induce apoptosis of myocardium cells *in vivo*. However, it is controversy if apoptosis begins in I or R state. Gottlieb *et al* [6] found that apoptosis Lydian stone, i.e. nucleosomal fragments of DNA has been traced in the ischemic myocardium of rabbit 30 min after infarction and 4 h after reperfusion; but this is not shown in myocardium only affected by ischemia. Based on studies, researchers then suggested that apoptosis can be only made by reperfusion [6].

Briefly studying texts about pathology, one could make clear the role of out-regulated apoptosis in most disorders and diseases. Now we can not find a disease with no interference of apoptosis in it. So, we can justify the main role played by apoptosis in diseases by brief details. Other studies indicated that brief periods of experimental ischemia-reperfusion in right kidney make apoptosis while prolonged ischemia make necrosis.

Corresponding Author: Hashemi, M, Department of Genetics, Islamic Azad University,Tehran medical Branch, Tehran, Iran

Gottlieb *et al*. [6] indicated that ischemia-reperfusion result in apoptosis in rat myocytes and described the role of pH and ATPase in apoptosis after reperfusion. stated that Ischemia-reperfusion in rat heart can indicate occurrence of apoptosis. They could later indicated this after prolonged reperfusion of coronary blood flow. Although they could explain apoptotic changes following the ischemia-reperfusion, they couldn't indicate how much apoptosis occurs during any ischemic period for brief pre-conditioning [6,8]. Studying the possible effects of ischemia-reperfusion in coronary angioplasty, stated that increased reperfusion period can be effective in the changes occur after surgery [6,7]. Pathology of heart infraction includes various situations but gradually losing the myocardium cells accounts is the most important pathological discussions. Over several years, there are more information about the role of apoptosis following the infraction and ischemia-reperfusion; each one can separately indicate its importance. For this reason, for treating infraction and observing ischemia-reperfusion period, it is important to prevent apoptosis potentially [8-9]. Based on other findings, In this study, we decided to examine the role of time, as variable, in ischemia-perfusion of rat myocardium.

MATERIAL AND METHODS

In this experimental study, male rats of strain SD, 250-330 gr in weight were divided into four groups of 10; they were anesthetized with sodium pentobarbital (50-60 IP-kg/mg) with the heart of treatment group isolated immediately and connected to crebs solution langendroph set containing carbogene gas (95% oxygen, 5% di oxide carbon) with constant pressure and temperature of 37°C while stabilization, applied by 30 min ischemia and reperfusion for 60, 90 and 120 min. Above mentioned groups called based on ischemia-reperfusion period as T/60 min, T/90 min and T/120 min, respectively. Control groups didn't receive reperfusion of kerebs solution or ischemia-reperfusion. In treatment group, after reperfusion finished, their heart located in fixator and put in the autotechnium set and fixed by formalin, ethanol, xylelol and paraffin. After cross sectioning, then, they specially diagnosed for apoptosis (Tunel assay) and counted for apoptotic cells using light microscopy in 5 random fields for treatments and controls [1]. To analyze data statistically, one way ANOVA was used and Bartlett's test with results indicated as mean ±standard deviation.

Diagnostic Tunel Technique:

- Initially, sections, before hydration were added by Proteinase K and incubated for about 30 min in 37°C and then washed by PBS.
- Sections then were added by Tunel reaction mixture to 50µl and left about 60 min in 37°C and washed by PBS.
- After incubation, sections were washed with Converter-POD (50µl), 30min in 37°C and then washed with PBS and incubated in 25°C again.
- Finally, they washed with PBS and stained with Tuloidin-blue [1,10].

RESULTS

Results of microscopic studies and Tunel technique in controls and treatments were as follows: microscopic studies indicated increased chromatin density and fragmentation of cell nucleus and occasionally formation of chromatin crescent. In Tunel staining, because of DNA fragmentation, often multiply by 180-200bps, apoptotic cells was shown in light and dark brown in

Table 1: mean, standard deviation and error of apoptotic cell numbers in treatments and controls

Groups	Mean	Mean±standard deviation	Mean±standard error
T/60min	2.4	2.4±1.1	2.4±0.5
T/90min	3.8	3.8±0.8	3.8±0.3
T/120min	6.0	6±0.707	6±0.316
Control	1.6	1.6±80	1.6±0.4

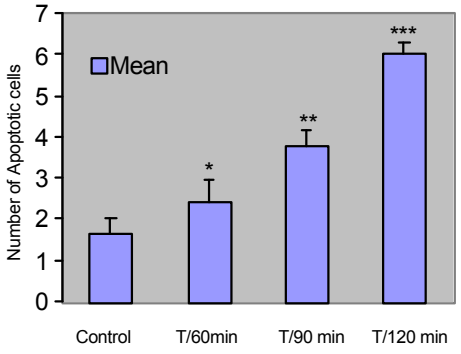

Chart 1: Mean number of apoptotic cells in 15 microscopic field obtained from myocardium of treatment and control by Tunel technique in rats (n=10). Data indicated as Mean±SEM. P>0.05*, p<0. 01**, p<0.001*** comparing with control group.

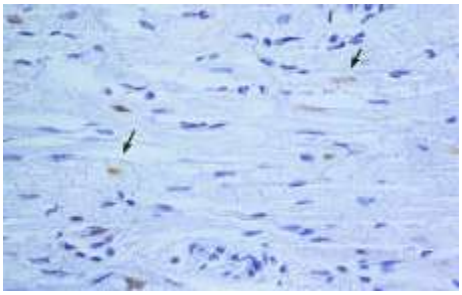

Fig. 1: Sectional photomicrograph of myocardium tissue of treatment rat with 120min of perfusion period. Arrows indicate Positive Tunel cells (Tunel staining, ×100).

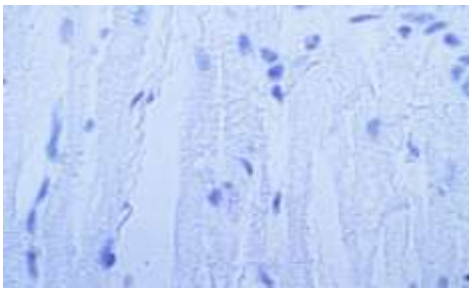

Fig. 2: Sectional photomicrograph of myocardium tissue of control rat. Arrows indicate Positive Tunel cells (Tunel staining, ×100)

the myocardium, such that the number of apoptotic cells were counted in controls to 1.6±0.4 and in treatment groups to 2.4±1.14, 3.8±0.836 and 6±0.707 respectively.

There is no significant difference between (T/120min and T/90 min) and (T/60 minand Control) but it was significant between T/120min and T/60min ($P<0.001$) and T/120min and T/90min ($P<0.01$) Table 1 indicates Figures obtained from statistical analysis of data.

DISCUSSION

In pathophysiology of cardiomyocyts apoptosis, various factors play role as proapoptotic factors to induce the apoptosis; one of these factors include tissue ischemia-reperfusion with several apoptotic pathways. Basically, after reperfusion of blood, cytosolic calcium will be increased and so it will increase the permeability potential of mitochondrion membrane. At the same time, more calcium will enter the mitochondrion and so cytochrom C (Apaf-2) will leak from membrane channels and it will make a cascade of caspase enzymes accompanying with Apaf-1, dATP and Apaf-3. On the other hand, increased cytoplasmic Ca^{2+} following the tissue reperfusion activates endonuclease and DNA fragmentation with nucleosoms multiplied of 180-200bps and result in apoptotic cell death [11]. There is another pathway in cardiomyocyts apoptosis following the reperfusion, independent from caspase enzymes and that is the role of AIF[4] or apoptosis inducer factor by which increased cytosolic calcium and its entrance to mitochondrion results in releasing this factor and inducing apoptosis by this factor. Here, the role of time in ischemia-reperfusion of heart was examined and results indicated a significant difference in the number of apoptotic cells in treatment and control groups such that minimum numbers of apoptotic cells indicated in controls and maximum numbers of apoptotic cells in T/120min; this is accompanying with results of other studies, as indicated in Albercht Elsasser *et al* [2001] who indicated maximum numbers of apoptotic cells in 120min of reperfusion [2,5,6]. But in his study, Albercht indicated that there are also apoptotic changes after 18h reperfusion. There are several factors my play role in cell death changes: 1-Cytosolic calcium attain its maximum for periods higher than 60min reperfusion and result in increased permeability potential of mitochondrion membrane and facilitate cytocrom C leakage and AIF, but the rate of AIF is usually more than cytpcrom C and that is because cytocrom C has been linked to anionic phospholipids like cardiolipin usually in intra-membrane space while AIF can not link to any material in intra-membrane space and thus AIF can go out of the membrane [4,13].2-Expression of genes inducing apoptosis like BAX which are important for formation of membrane channels of mitochondrion and cytochrom C leakage and in long periods, presence of active Protein, BAX is more prominent [12].3-Kim *et al* [2003] found that caspase 3 activity, the most effective factor in apoptosis, will be increased after 2h reperfusion and so increased changes in 120min may be due to presence of enough caspase enzymes, specially caspase 3 [11-13].

CONCLUSION

Anyway, the role of time, as variable and acknowledging of reperfusion time of tissue can be effective in attempt to study for attaining the decreased apoptotic changes in tissue reperfusion materials. Using anti-apoptotic drugs in tissue reperfusion especially allograft (heart and kidney) in a proper time will have a specific effect on inhibition of tissue damage. Regarding to the results of this study, it is suggested to use anti-apoptotic drugs before tissue reperfusion despite increased apoptotic cells by time.

REFERENCES

1. Doustari, experimental study of apoptosis induced by the virus infecting burse fabrisios of chickens using diagnostic TUNEL technique and microscopy. Thesis for PhD, Tehran University, venetiary college, 2005.
2. Ambrosio, G. and I. Tritto, 1999. Reperfusion injury: experimental evidence and clinical implications. Am. Heart J., 138: 569-75.
3. Deodato, B., D. Altavilla, G. Squadrito, G.M. Campo, M. Arlotta, L. Minutoli, *et al*. 1999. Cardioprotection by the phyto-estrogen genistein in experimental myocardial ischaemia-reperfusion injury. Br J. Pharmacol, 128: 1683-90.
4. Eefting, F., B. Rensing, J. Wigman, W.J. Pannekoek, W.M. Liu, M.J. Cramer, *et al*. 2004. Role of apoptosis in reperfusion injury. Cardiovasc Res., 61: 414-26.
5. Gao, W.D., Y. Liu, R. Mellgren, and E. Marban, 1996. Intrinsic myofilament alterations underlying the decreased contractility of stunned myocardium: a consequence of Ca^{2+}-dependent proteolysis? Circ Res., 78: 455-65.
6. Gottlieb, R.A., K.O. Burieson, R.A. Kloner, and B.M. Babior, 1994. Reperfusion injury induces apoptosis in rabbit myocardiocytes. J Clin Invest, 94: 1625-9.
7. Harper, I.S., J.M. Bond, E. Chacon, J.M. Reece, B. Herman and J.J. Lemasters, 1993. Inhibition of Na^+/H^+ exchange preserves viability, restores mechanical function and prevents the pH paradox in reperfusion injury to rat neonatal myocytes. Basic Res. Cardiol., 88: 430-42.
8. Kang, P.M. and I. Seigo, 2000. Apoptosis and heart failure. Circ. Res., 86: 1107.
9. James, T.N., 1999. Apoptosis in cardiac disease. Am. J. Med., 107(6): 606-20.
10. Elsässer, A., K. Suzuki, S. Lorenz-Meyer, C. Bode, and J. Schaper, 2001. The role of apoptosis in myocardial ischemia. Basic Res. Cardial., 96: 219-20.
11. Khoynezhad, A., Z. Jalili, and J. Anthony, 2004. Apoptosis: pathophisiology and therapeutic implications for the cardiac surgeon. Ann Thorac Surg., 78: 1109-18.
12. Kim, G.T., Y.S. Chun, J.W. Park and M.S. Kim, 2003. Role of apoptosis-inducing factor in myocardial cell death by ischemia-reperfusion. Biochem Biophys Res. Commun., 209: 619-24.
13. Zhao, Z.Q., *et al*, 2000. Reperfusion induces myocardial apoptosis cell death. Cardiovascular Res., 45: 651-60.