IOSR Journal Of Pharmacy
(e)-ISSN: 2250-3013, (p)-ISSN: 2319-4219
www.iosrphr.org Volume 5, Issue 12 (December 2015), PP. 09-15

Transition from Reversible to Irreversible Myocardial Cell Injury in Isolated Rat Heart Preparation.

Rashmi Arora[1]
[1]Sri Aurobindo Institute of Pharmacy, Indore, Madhya Pradesh,-453555, India.

Abstract : Myocardial ischaemia reperfusion is associated with activation of intracellular death proteases known as calpains. Ischaemia and reperfusion was induced in isolated rat heart to evaluate the effect of calpain inhibitors SJA-7019 and SJA 7029(10µM and 15µM) and calpastatin, against myocardial injury assessed in terms of infract size, release of LDH and CK. Agarose gel electrophoresis was used to assess extent of necrosis in terms of DNA smearing. TUNEL staining was done to investigate the apoptotic index. Calpain inhibitors SJA-7019,SJA 7029 and calpastatin has attenuated ischaemia reperfusion induced increase in LDH,CK, myocardial infarct size ,DNA smearing and apoptotic index. It is concluded that inhibition of intracellular death proteases prevented ischaemia reperfusion induced apoptosis induced necrotic cell death.

Keywords - *Apoptosis , Calpain inhibitors, Ischaemia , Myocardial injury, Necrosis.*

I. INTRODUCTION

Cell death during cardiac ischaemia and reperfusion is reported to occur through necrosis [1]. However there is increasing evidence to suggest that myocardial cell death may also occur due to apoptosis [2,3,4]. Apoptosis may lead to necrotic cell death[5,6,7,8]. The transition from reversible to irreversible injury is characterized by the development of a severe membrane permeability defects that allows the unregulated influx of divalent and trivalent cations including calcium [9,10,1]. Reperfusion is associated with brisk drop in intracellular pH and an associated rise in calcium[11,12]which leads to activation of calpain which is a calcium dependent membrane protease implicated in necrotic cell death. Calpain expression is noted to increase during ischaemia and reperfusion [13] and is involved in signal transduction of apoptosis [14]. The rise in Ca^{2+} in cardiomyocytes during ischaemia and reperfusion is considered to be a pivotal event in cell death [15]. An increase in Ca^{2+} influx may activate dormant Ca^{2+}dependent intracellular death proteases – calpains, causing damage to myocardial structural proteins leading to membrane breakdown and eventually cell death [16]. The transition from reversible to irreversible injury is characterized by the development of severe membrane permeability defects (1,9) and degradation of membranous proteins [17]. Reperfusion results in sodium ion influx followed by Ca^{2+} accumulation which may activate calpain and consequently produce Bid [11],Bax[18,19] and Bcl-xl cleavage. The cleaved activated fragments acts on mitochondria causing its dysfunction and release of pro-apoptotic factors resulting in DNA fragmentation and cell death. On the other hand release of cytochrome –c produces depletion of ATP stores [11] and lead to necrotic cell death [20]. The mode of cell death shifts from apoptotic to necrotic due to use of ATP stores by apoptosis [6]. Therefore, apoptosis may lead to necrotic cell death [5,6,7,8]. Also during ischaemia and reperfusion, calpain activities increased through both an increase in the expression of calpains and a decrease in the expression of its endogenous inhibitor Calpastatin [13, 21]. Calpastatin suppresses conformational changes in calpain molecule during the initial stages of its activation and inhibits binding to its cell membrane and degradation of membrane proteins [17, 22]. The SJA 7019 and SJA 7029 inhibits extra cellular influx of Ca^{2+} leading to down regulation of calpain activity [23,24].

In the present study the selective inhibition of calpain activity by calpastatin and compounds like SJA 7019 and SJA 7029 has improved function of heart which were declined as a result of apoptosis and necrosis induced by ishaemia and reperfusion.

II. MATERIAL AND METHODS

Wistar albino rats of either sex were used to carry out studies.

Rats were heparinised and sacrificed by stunning; heart rapidly excised and immediately mounted on Langendroff's apparatus [25].The preparation was perfused with Krebs-Heinslet (K-H) solution pH 7.4, maintained at 37^{o}C and bubled with 95% O_2 and 5% CO_2. Coronary flow rate was maintained 6-9 ml/min and perfusion pressure was kept constant at 70mm Hg. Global ischaemia was produced for 30 min by closing the inflow of physiological solution and it was followed by reperfusion for 120 min. Two thin electrodes fixed at ventricular apex and origin of aorta were employed to record ECG(BPL, MK 801, Banglore, India) for monitoring heart rate.

2.1 Infarct size Mearsurement

Infarct size was measured and expressed as percentage of total left ventricular volume (%LVV) and left ventricular weight(%LVW) respectively by Volume and Weight method.

2.2 DNA extraction and Gel electrophoresis

It was carried out for measuring the extent of necrosis of myocardium [26]. The concentration of DNA was determined spectrophotometrically at 260nm. Protein contamination of DNA was accessed by determining the ratio of absorbance at 260nm and 280nm which should not be more than 1.75. To detect the internucleosomal cleavage, 10-12 µg of extracted DNA was added to equal volume of loading dye and it was loaded in the well. Electrophoresis was carried out using 1.8% agrose gel in 1X TBE buffer for 1.5 hrs at 400mA and 3W in submarine electrophoresis unit(Pharmacia Biotech, Freibury, Germany). Ethidium bromide was added to gel for DNA detection.

2.3 TUNEL Staining

TUNEL positive cardiomyocytes were counted and apoptotic index was calculated(1) using the formula.

$$\text{Apoptotic Index} = \frac{\text{Number of TUNEL positive cell nuclei}}{\text{Number of total cell}} \times 100 \qquad (1)$$

2.4 Estimation of LDH

LDH was estimated in coronary effluent by 2,4-DNPH method[27] spectrophotometrically at 440nm.
Optical density of Test($O.D._T$) and control($O.D._C$) was measured (2) against distilled water.

$$\text{Net optical density of test } (O.D._{Tn}) = O.D._T - O.D._C \qquad (2)$$

Enzyme activity was calculated from standard plot by making $O.D._{Tn}$ on Y-axis and extrapolating it to corresponding enzyme activity on X-axis.

2.5 Estimation of CK

CK was estimated in coronary effluent by modified method of Hughes [28].
Optical density of test($O.D._T$), standard($O.D._S$) and blank($O.D._B$) was measured (3)spectrophotometrically against distilled water at 520nm.

$$CK(1U/L) = \frac{O.D._T - O.D._B}{O.D._S - O.D._B} \times \frac{10^3 \times \text{Creatinine taken } (\mu M)}{\text{Incubation time} \times \text{volume of coronary effluent}} \qquad (3)$$

2.6 Drugs

SJA 7019, SJA 7029 (Senju Pharmaceutical Co., Kobe, Japan) and Calpastatin (Calbiochem, CA, USA), Proteinase K(Sigma-Aldrich, St. Louis, USA) and RNAse(Hi Media, Mumbai, India) were used to carry out the study.

The experimental protocol has been designed and followed to carry out the study (Fig.1)

III. RESULTS AND DISCUSSION

Lactate dehydrogenase(LDH) is a biochemical index of myocardial injury. Maximum increase in the release of LDH is noted immediately and 30 min after reperfusion. It may be due to sustained ischaemia and late spurt in the release of LDH noted after 30 min of reperfusion may be as a result of reperfusion(Fig 2). Release of creatine kinase(CK) during reperfusion is used as a measure of lethal cell injury. Peak increase of CK noted after 5 min of reperfusion (Fig 3).

Apoptosis generate high molecular weight fragments(Susin et al., 1999) which cannot be detected by agarose gel electrophoresis. TUNEL staining uses the divalent cations Co^{2+} and enzyme terminal deoxynucleotidyl transferase (Tdt) to add flourescein isothiocyanate(FITC) labeled deoxyuridine triphosphate(dUTP) to 3'OH ends of DNA which are detected by fluorescent microscope. TUNEL assay labels apoptosis in tissues sections on a single cell level making it much more sensitive than agarose gel electrophoresis. TUNEL (terminal deoxynucleotidyl transferase mediated UTP nick end labeling) technique has been employed in the present study to determine the apoptotic cell death.

Calpain activation due to ischaemia and reperfusion consequently produce apoptotic cell death through release of proapoptotic factors. That's why perhaps global ischaemia for 30 min followed by reperfusion for 120 min has significantly increased apoptotic index estimated by TUNEL staining in the present study. Calpain is specifically inhibited by calpastatin a peptide inhibitor and SJA 7019, SJA 7029 are nonpeptide inhibitors. The calpastatin suppresses the conformational changes in the calpain structure necessary during the initial stages of

its activation and binding to the cellular membranes and consequent degradation of membrane proteins. The SJA 7019 and SJA 7029 inhibit extracellular influx of Ca^{2+} leading to down regulation of calpain activity. Therefore, the specific inhibition of calpain activity by calpastatin and compounds like SJA7019 and SJA 7029 are responsible for the noted decrease in apoptotic index (Fig4-7,8).

The mechanism underlying the reperfusion induced necrotic cell death involves onset of mitochondrial permeability transition(MPT). Opening of permeability transition pores in mitochondria causes release of cytochrome c along with depletion of ATP and ultimately leads to necrotic (energetic) cell death. Thus the specific inhibition of calpain activity by calpain inhibitors are responsible for the noted decrease in infarct size(Fig 9) and release of LDH and CK.

Apoptosis and necrosis are reported to coexist in acute ischaemia and reperfusion. Myocardium subjected to ischaemia and reperfusion has often shown gel electrophoresis pattern that do not demonstrate clear cut or pure DNA laddering and appear to represent mixtures of DNA smearing. Moreover the mode of cell death shifts from apoptotic to necrotic because of use of ATP during apoptosis and consequent depletion of ATP stores. Therefore it is possible to suggest that DNA smearing(Fig 10) noted in present study may reflect apoptosis induced necrotic cell death. The caplain inhibition ultimately block apoptosis induced necrotic cell death and is responsible for the noted decrease in DNA smear formation. The compound SJA 7019 is more effective and selective than compound SJA 7029 to inhibit calpains because of the presence of methoxy group in phenyl ring of compound SJA 7019. That's why perhaps compound SJA 7019 has been demonstrated to be more effective than compound SJA 7029 to attenuate ischaemia reperfusion induced increase in apoptotic index, DNA smearing and myocardial injury. Moreover calpastatin has been found to be the most potent in present study to decrease ischaemia reperfusion induced myocardial injury, DNA smearing and apoptotic index. It may be because of its peptide nature and consequent better membrane permeability and availability in the cytoplasm.

IV. CONCLUSION

On the basis of results obtained in the present study, it may be concluded that

1. Ischaemia of 30 min followed by reperfusion for 120 min produced significant increase in myocardial injury measured in terms of infarct size and release of LDH and CK. It produced marked increase in apoptotic index and DNA smearing. It suggests that ischaemia and reperfusion has produced apoptotic and apoptosis induced necrotic cell death in isolated rat heart.
2. The peptide inhibitor of calpain i.e. calpastatin and nonpeptide (SJA-7019 and SJA-7029) inhibitors of calpain significantly decreased ischaemia-reperfusion-induced apoptotic index. It indicates that peptide and nonpeptide inhibitors of calpain has attenuated ischaemia and reperfusion induced apoptotic cell death.
3. Calpastatin, SJA-7019 and SJA-7029 have prevented ischaemia-reperfusion-induced increase in ventricular DNA smearing, which has occurred as a result of apoptotic-induced-necrotic cell death.
4. The results suggests that ischaemia and reperfusion induced necrotic injury has been prevented by calpain inhibitors.
5. The compound SJA-7019 is more effective than compound SJA-7029 and calpastatin is most effective to attenuate ischaemia-reperfusion-induced increase in apoptotic index, DNA smearing and myocardial injury. It may be due to presence of methoxy group in the phenyl ring of compound SJA-7019 and peptide nature of calpastatin.

V. ACKNOWLEDGEMENT

Author is thankful to Late Dr. Manjeet Singh for his supreme guidance in performing the study.

REFERENCES

[1]. Buja, M.L. and Entmon, M.L. (1998). Modes of myocardial cell injury and cell death in ischaemic heart disease. *Circulation.* **14**: 1355-1361.

[2]. Yaoita, H., Ogawa, K., Maechara, K. and Matujama, Y. (1998). Attenuation of I/R injury in rats by a caspase inhibitor. *Circulation*, **97**: 276-281.

[3]. Holly, T.A., Drincic, A., Byun, Y., Nakarmuna, S., Harris, K., Kolocke, F.J. and Cryns, V.L. (1999). Caspase inhibition reduces myocyte cell death induced by myocardial I and R. in vivo *J. Mol. Cell Cardiol*, **31**: 1707-1715.

[4]. Ma, X.L., Kumar, S., Gao, F., Lauden, C.S., Lopen, B.L., Christopher, T.A., Wang, C., Lee, J.C., Feuerstein, G.Z. and Yue, T.L. (1999). Inhibition of p38 mitogen activated protein kinase decreases cardiomyocyte apoptosis and improves cardiac function after myocardial T and R *Circulation*, **99**: 1685-1691.

[5]. Daemen, M.A., Van't Veer, C., Denecker, G. Heemskerk, V.H., Wolfs, T.G., Clauss, M., Vandenabeele, P. and Buurman, W.A. (1999). Inhibition of apoptosis induced by ischaemic-reperfusion prevents inflammation, *J. Clin. Invest.*, **104**: 541-549.

[6]. Nicotera, P. and Leist, M. (1997). Energy supply and the shape of death in neurons and lymphoid cells. *Cell Death Differ.*, **4**: 435-442.

[7]. McCarthy, N.J., Whyte, M.K.B., Gilbert, C.S. and Evan, G.I. (1997). Inhibition of Ced3/ICE related proteases doesnot prevent cell death induced by oncogenes, DNA damage or the Bcl-2 homologue Bak. *J. Cell. Bio.l*, **136**: 215-227.

[8]. Ferrer, I., Martin, F., Serrano, T., Reirig, J., Perez-Navarro, E., Alberch, J., Macaya, A. and Planas, A.M. (1995). Both apoptosis and necrosis occur following intrastrail and administration of excitotoxins. *Acta. Neuropathol. (Berl).*, **90(5)**: 504-510.

[9]. Buja, M.L., Eigenbrodt, M.L. and Eigenbrodt, E.H. (1993). Apoptosis and necrosis: basic types and mechanism of cell death. *Arch. Pathol. Lab. Med.*, **117**: 1208-1214.

[10]. Christofferson,D.E., Yuan, J.,(2010).Necroptosis as an alternative form of programmed cell death. *Curr. Opin. Cell Biol*., **22** : 263–268.

[11]. Chen, M., He, H., Zhan, S., Krajewaki, S., Reed, J.C. and Gottlieb, R.A. (2001). Bid is cleaned by calpain to an active fragment *in vitro* and during myocardial I/K. *J Biol Chem*, **276(33)**: 30724-30728.

[12]. Chen, M., Won, D.J., Krajowski, S. and Gottlieb, R.A. (2002). Calpain and mitochondria in ischaemia-reperfusion injury. *J. Biol. Chem.,* **277(32)**: 29181-29186.

[13]. Kakkar, R., Seitz, D.P., Kantham, R., Rajala, R.V.S., Radhi, J.M., Wang, X., Pasha, M.K., Ruiwang and Sharma, R.K. (2002). Calmodulin – dependent cyclic nuclrotide phorphodiesterase in an experimental rat model of cardiac ischaemia – reperfusion. *Can J Physiol Pharmacol*, **89**: 59-66.

[14]. Goll, D.E., Thompson, V.F., Li, H., Wei, W. and Cong, J. (2003). The calpain system. *Physiol Rev*, **83**: 731-801.

[15]. Goll, D.E., Kluse, W.C., Okitani, A., Kumamoto, T., Cong, J. and Kapprell, H-P. (1990). Historical background and current status of the Ca^{2+} dependent protease system. In: Mellgren, R. and Murachi, T. (eds.), *Intracellular Calcium Dependent Proteolysis, CRC Press Inc, Boca Raton, Florida*, 3-24.

[16]. Yoshida,K.,Sorimachi,Y.,Fujiwara,M and Hironaka,K(1995). Calpain is implicated in rat myocardial injury after ischaemia or reperfusion.*Jpn.Circ*, **59:**40-48

[17]. Tatiana G. Sanzontova, Alexey A. Matskevich and Yuriy V. Arkhipenko (1999). Calpains: physiological & pathological significance. *Pathology*, **6**: 91-102.

[18]. Gao, G. and Dou, O.P. (2000). N-terminal cleavage of bax by calpain generates a potent proapoptotic 18-kDa fragment that promote 2-independent cytochrome C release and apoptotic cell death. *J Cell Biochem*, **80(1)**: 53-72.

[19]. Wood, D.E. and Newcomb, E.W. (2000). Cleavage of bax enhances its cell death function. *Exp Cell Res*, **256(2)**: 375-382.

[20]. Kim, J.S., He, L., Qian T and Lemasters, J.J. (2003). Role of the mitochondrial permeability transition in apoptotic and necrotic death after ischaemia/reperfusion injury to hepatocytes. *Curr. Mol. Med.*, **3(6)**: 527-535.

[21]. Singh Nisha., Srivatava. Anurag., Olson.Doug., Lakshmikuttyamma. Ashakumary.,Ross. Andrew RS. and Parr. Tim (2008). Cardiac high molecular weight calmodulin-binding protein is homologous to calpastatin I and calpastatin II. *Biochemical and Biophysical Research Communications*, **373(3)**:387-91.

[22]. Zimmermann, U.J. and Schlaepfer, W.W. (1991). Two stage autolysis of the catalytic subunit initiates activation of calpain I. B*iochem. Biophys. Acta.*, **1078**: 192-198.

[23]. Liu, X., Harriman, J.F. and Schnellmann, R.G. (2002). Cytoprotective properties of novel nonpeptide calpain inhibitors in renal cells. *Journal of Pharmacology and Experimental Therapeutics*, **302**: 88-94.

[24]. Donkor, Isaac O (2012 - 2014). An updated patent review of calpain inhibitors *Expert Opinion on Therapeutic Patents.*, **25(1)**: 17-3

[25]. Langendorff, O. (1895). Untersuchungen am uberlebender saugethierherzen. Pflungers. Archiv. Fur. Die. Gesmate. *Physiologie.*, **61**: 291-332.

[26]. Ausubel, F., Brent, R., Kingston, R.E., Moore, D.D. and Struhl, K.S. (1995). Preparation and analysis of DNA. In: Short Protocol in Molecular Biology. John Wiley & Sons, Canada, pp. 28-29.

[27]. King,J.(1959).A routine method for the estimation of lactic dehydrogenase activity. *J. Med. Lab. Tech*., **16**: 265-272.

[28]. Hughes, B. (1961). A method for the estimation of serum creatin kinase and aldose activity in normal and pathological sera. *Clin. Chim. Acta.,* **7**: 597-603.

[29].

Experimental Protocol

Figure:1 Diagrammatic representation of experimental protocol
S-stabilization; K-H perfusion with K-H solution; GI- global ischaemia; REP- reperfusion with K-H solution.

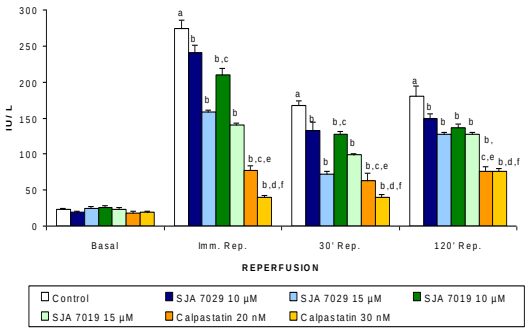

Figure 2 Effect of Calpain inhibitors on LDH release

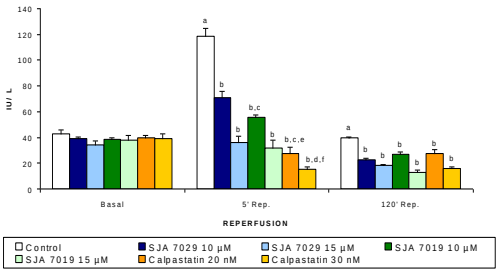

Figure 3 Effect of Calpain Inhibitors on CK Release

Figure 4 TUNEL +ve nuclei sham control

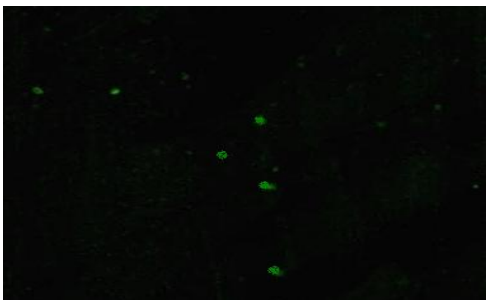

Figure 5 TUNEL +ve nuclei control

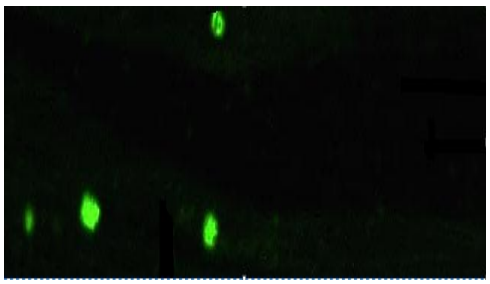

Figure 6 TUNEL +ve nuclei (SJA 7019, 15 uM)

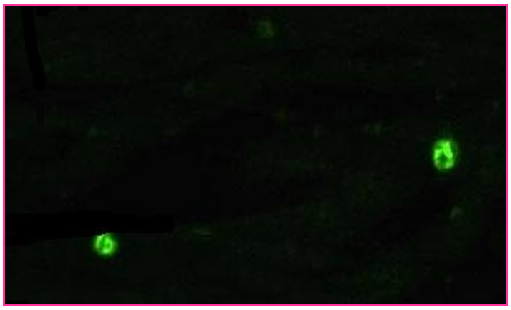

Figure 7 TUNEL +ve nuclei (Calpastatin, 30 nM)

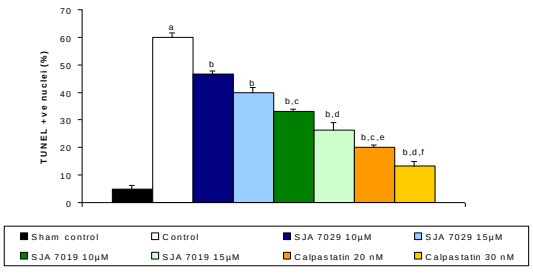

Figure 8 Effect of Calpain inhibitors on Apoptotic Index

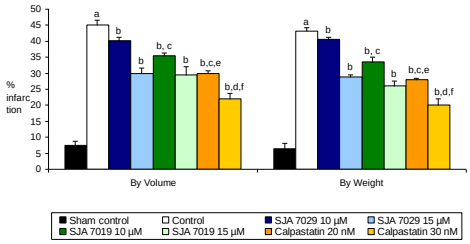

Figure 9 Effect of Calpain inhibitors on infarct size

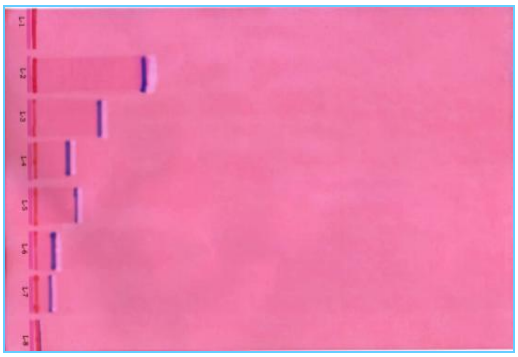

Figure 10 Electrophoresis of DNA