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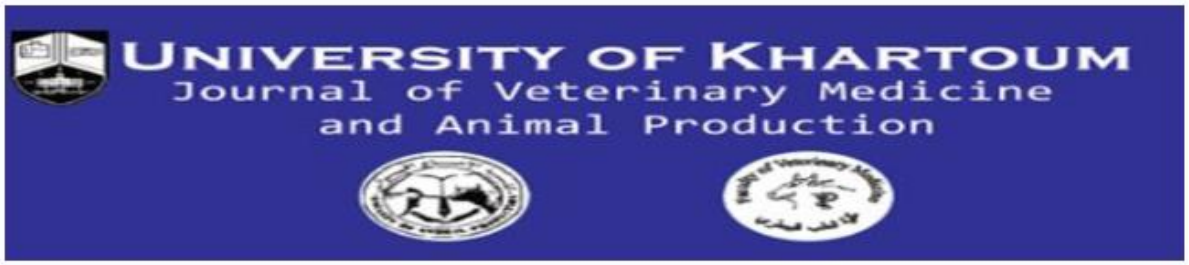

Hsp90α and *hsf* -1 is involved in cell damages and apoptosis after heat stress in primary myocardial cells of neonatal rat

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Abstract

This study investigated the effect of heat stress on myocardial cell expression levels of apoptosis signal transduction molecules in myocardial cells of neonatal rat by detecting the expressions of *hsp90α* mRNA and Hsp90α protein and Hsf- in response to heat stress in vitro. After incubated at 37℃ for 72 h in humidified atmosphere of 5% CO2 and 95% air, the primary myocardial cells were suddenly heat stressed for 10 min, 20 min, 40 min, 1 h, 2 h, 4 h, 6 h and 8 h respectively in another incubator with 95% air and 5% CO2 at 42℃. The results of flow cytometry showed that apoptotic levels at 40 min and 8 h of heat stress were significantly higher than those of the control ($P<0.01$ and $P<0.05$) respectively, indicating that the myocardial cells started some kind of self-protection mechanisms to adapt to changes in the environment. At 40 min heat stress, hsp90α show significant decrease and the level of hsf-1 was similar to that of hsp90α protein however, the transcription level of hsp90α mRNA increased significantly. These results indicate that apoptosis inhibit hsp90α by down regulating hsf-1.

Key Words: Heat stress, heat shock protein 90α, myocardial cells, Rat, apoptosis

المستخلص

أجريت هذه الدراسة لمعرفة تأثير الإجهاد الحراري على خلايا عضلة القلب ومستويات موت الخلايا المبرمج في خلايا عضلة القلب في الجرزان حديثي الولادة عن طريق كشف hsp90αmRNA والبروتين Hsp90α و hsf1 كرد فعل على الإجهاد الحراري في الزجاج. بعد وضعها فى حاضنة في 37 درجة مئوية لمدة 72 ساعة ونسبة رطوبة 5٪ ثانى اكسيد الكربون و 95٪ الهواء، عرضت خلايا عضلة القلب الأولية فجأة للإجهاد الحرارى لمدة 10 دقيقة، 20 دقيقة، 40 دقيقة، 1ساعة، 2 ساعة، 4 ساعات، 6 ساعات و 8 ساعات على التوالي في حاضنة أخرى مع الهواء 95٪ و 5٪ ثانى اكسيد الكربون و 42 درجة مئوية. وأظهرت نتائج قياس flow cytomytry أن مستويات موت الخلايا المبرمج في 40 دقيقة و 8 ساعات من الإجهاد الحراري كانت أعلى بكثير من تلك التي تم التحكم بها (P و $P<01.0$)على التوالي، مما يدل على أن خلايا عضلة القلب بدأت نوعا من الحماية الذاتية للتكيف مع التغيرات في البيئة. عند 40 دقيقة إجهاد حراري، أظهر hsp90α إنخفاض كبير ومستوى hsf-1 كان مشابها لذلك المستوى من hsp90α على الرغم من إرتفاع مستوى hsp90α mRNA بصورة معنوية .هذه النتائج تشير إلى أن موت الخلايا المبرمج يثبط hsp90α عن طريق تنظيم انخفاض hsf-1.

الكلمات المفتاحية: الاجهاد الحراري، بروتين الصدمة الحرارية 90α ، خلايا عضلة القلب، الجرزان، موت الخلايا المبرمج

Introduction

characterized by cellular shrinkage, nucleus fragmentation, chromatin condensation and lytic DNA degradation. It is also participating in various biological processes such as development, maintenance of tissue homeostasis and elimination of cancer cells (Jacobson et al., 1997). For cardiac dysfunction in different pathological cases, the adult cardiomyocytes loss by apoptosis has been described as an important event (Narula *et al*., 1996; Teiger et al., 1996; Haunstetter and Izumo, 1998; Elsasser *et al*., 2000). Apoptosis or programmed cell death is one of biochemical events occur in all tissues and The heat shock proteins (Hsps) are highly conserved proteins whose expression is induced in response to a wide variety of physiological and environmental insults, including heat exposure (McCormick et al., 2003; Ganter *et al*., 2006; Staib *et al*., 2007). Hsps has the ability to protect and repair cells and tissues. Hsps can inhibit or aid the apoptotic mechanism through their chaperone functions by affecting protein folding, ubiquitin degradation pathways, and protein translocation (Takayama *et al*., 2003). They can also protect the myocardium from the damaging effects of ischaemia and reperfusion.

The role of HSPs in survival is due to the suppression of apoptosis (Samali and Orrenius, 1998). The first observation of HSPs ability to protection was the cell exposing to a mild hyperthermia, thus reduce their susceptibility to apoptosis. This indicates a fundamental HSPmediated enhancement of cellular survival independent of functional interaction with the apoptotic pathway. Apoptotic pathway is mediated by a family of cysteine-dependent aspartate-specific proteases, known as caspases that have specificity for aspartate residues (Wolf and Green, 1999). Two pathways are known to be important for apoptosis. The extrinsic pathway involves activation of death receptors, such as Fas/CD95 or TNF receptor 1, by binding of their respective ligands and thereby recruitment of caspase-8 (Huang *et al*., 1999). Caspase-8 can activate effector caspases directly (Boldin et al., 1996; Muzio *et al*., 1996) or indirectly by cleaving Bid and inducing the release of mitochondrial cytochrome (Li *et al*., 1998; Luo *et al*., 1998; Gross *et al*., 1999). By contrast, the death receptor-independent, intrinsic, apoptotic pathway is activated directly by death stimuli and induces the release of mitochondrial cytochrome c into the cytosol (Liu *et al*., 1996; Kharbanda *et al*., 1997; Kim *et al*., 1997; Kluck *et al*., 1997; Yang *et al*., 1997; Bossy-Wetzel *et al*., 1998; Narula *et al*., 1999). Different apoptotic signals affect the permeability of the outer mitochondrial membrane thus, the release of cytochrome c one of the major pathway of apoptosis (Ferri and Kroemer, 2001). Cytochrome c, interacts with a protein known as Apaf-1 (apoptotic protease activation factor1), and trigger the ATP-dependent oligomerization of Apaf-1, while exposing itscaspase recruitment domain (CARD domain) (Li *et al*., 1997; Hu *et al*., 1999).

Hsp90 is the most expressed stress protein synthesized by normal unstressed cells and increased in response to stressful stimuli. The function of Hsp90 includes assisting in protein folding, cell signalling, and tumour repression (Prodromou *et al*., 2000). Hsp90α and Hsp90β are prominent members of the HSP90 family of protein (Csermely *et al*., 1998; Sreedhar *et al*., 2004). The two Hsp90 isoforms are essential for the viability of eukaryotic cells. Hsp90 seems to have different molecular partners depending on the apoptotic stimuli, the effect of the protein being predominantly antiapoptotic and most studies do not differentiate

between the α and β isoforms of Hsp90. Hsp90α, a main type of HSP90 (Romanucci *et al*., 2006), is a copious, well preserved cytosolic protein that accounts for 1%– 2% of all cellular proteins in nearly all cells (Csermely *et al*., 1998) under basal, non-stress conditions and the levels increase in response to heat stress and other proteotoxic insults (Bagatell *et al*., 2000). Hsp90α is able to maintain cell structure and maturation, therefore the activity of a set of specific key client proteins (Maloney and Workman, 2002; Neckers, 2002). This protein also has a major role when induced in response to cellular stress and is thought to be essential for the maintenance of cellular integrity, survival and also important in buffering the cell against the effects of mutation (Maloney and Workman, 2002; Ekambaram *et al*., 2008).

The transcriptional factor Hsf-1 plays a major role in Hsp synthesis regulation. Several cytoplasmic chaperones such as hsp90 and hsp70 have been shown to bind to hsf-1 and keep it inactive in unstressed cells. During stress both chaperones become occupied by misfolded proteins, which results in the dissociation, nuclear translocation, and activation of hsf-1. The dissociation of hsf-1 from the promoter regions of hsp genes also requires the action of several molecular chaperones including hsp90Depend on its highly conserved and inducible nature, this stress protein acts as a good mediator of cellular stress (Prohaszka and Fust, 2004). However, the protective mechanisms for Hsp90α are still unclear. The aim of this study was to investigate the effect of heat stress on myocardial cell expression levels of apoptosis signal transduction molecules in vitro by detecting the expression of hsp90α mRNA and Hsp90α protein and Hsf- in response to heat stress. The expression levels of apoptosis in myocardial cells of neonatal rat were also studied in present study.

Materials and Methods

Cell culture and experimental treatment

Primary myocardial cells of neonatal rat (Fu Meng biological technology limited., Shanghai, China) were grown on polylysine-coated coverslips (size of the plates 35 cm²) at a density of 2 ～8 × 104 cells/plate. The culturing myocardial cells were incubated at 37℃ in humidified atmosphere of 5% CO2 and 95% air for 72 h to allow the cells to adapt to the temperature. If a minimum of 85% of the cells in a culture were alive and viable after this period, the primary myocardial cells were suddenly heat stressed in vitro for 10 min, 20 min, 40 min, 1 h, 2 h, 4 h, 6 h and 8 h respectively in another incubator with 95% air and 5% CO2 at 42℃. The ambient temperature of the control group was kept at 37℃ in humidified atmosphere of 5%CO2 and 95% air.

Detection of hsp90α mRNA and HSF-1 mRNA by fluorescence quantitative real time PCR (FQ RT-PCR)

Isolation of total RNA and RT-PCR

After the heat exposure of the experimental culture plates to 42℃ for the respective periods, the plates were taken out of the incubator and washed with phosphate-buffered saline. Then, total RNA was isolated by using TRIZOL reagent (Invitrogen, USA) according to the manufacturer'sinstructions.The concentration of RNA was determined by a spectrophotometer (Mx3000P, USA) at 260 nm. Serial dilutions of RNA were prepared with ribonuclease-free water and 2 g of each sample was synthesized into DNA using the Transcript M-MLV kit (AM1710, Invitrogen, USA,) following the manufacturer's protocol, and finally stored at -80℃.

Design of primers for hsp90α mRNA and HSF-1 mRNA

Primer sets were specifically designed to anneal to each target mRNA. The sequences of hsp90α
mRNA, HSF-1 mRNA and β-actin mRNA were obtained from the National Center for Biotechnology Information's (NCBI) Genbank (accession NC_005105.2, NP_077369.1 and NC_005111.2, respectively. Using this sequence, primers were designed for hsp90α and βactin
by Primer premier5.0 software for real-time reverse transcription–polymerase chain reaction (RT-PCR) amplification. Primer sequences for these genes were:
hsp90α gene: forward primer, 5′-CCCGGTGCGGTTAGTCACGT-3 reverse primer, 5′ TCCAGAGCGTCTGAGGAGTTGGA-3
The expect width of PCR products was 214 bp
HSF-1 gene: forward primer, 5′-ACCCCAGCCTCTGCCTGCT-3
reverse primer, 5′-TTCCCACTCGGGCTCCAGCA-3
The expect width of PCR products was 153 bp
β-actin gene: forward primer, 5′-CCCATCTATGAGGGTTCA-3
reverse primer, 5′- TCACGCACGATTTCC -3
The expect width of PCR products was 143 bp

FQ RT-PCR
Each DNA sample (2 μL, 25 times dilution) was suspended in 2 × SYBR Premix Ex Taq™ (15218-019, Invitrogen, USA) with primer (25 pmol of sense and anti-sense primer, respectively), and double-distilled water to a total volume of 25 μL. Quantitative PCR was performed using an ABI 7300 RT-PCR thermocycler (Applied Biosystems, USA). The thermal profile was established according to the manufacturer's protocol. Briefly, this protocol was 95°C for 3 min for enzyme activation, followed by denaturing at 95°C for 5 s, and annealing and elongation at 52°C for 30 s, for a total of 45 cycles. For each run, a negative control tube without DNA was run along with the experimental samples. A 2-fold dilution series of the template was used in the FQ-PCR reactions. The hsp90α mRNA of all samples could be normalized using the following formula:

Relative quantity of hsp90α mRNA = $2^{-\Delta\Delta Ct}$ ΔΔCt = (Ct hsp90α mRNA - Ctβ-actin mRNA) control group - (Ct hsp90α mRNA - Ct β-actin mRNA) test group Relative quantity of HSF-1 mRNA = $2^{-\Delta\Delta Ct}$ ΔΔCt = (Ct HSF-1 mRNA - Ct β-actin mRNA) control group - (Ct HSF-1 mRNA -Ct β-actin mRNA) test group.

Western blot analysis
After heat stressed at 42°C, myocardial cells were washed with phosphate-buffered saline (PBS) and lysed with sodium dodecyl sulphate (SDS)-polyacrylamide gel Laemmli sample buffer. Cell lysates were collected and boiled for 5 min. Equal amounts of protein (10 μg) were subjected to SDS-10% polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane by electrotransfer. The membrane was blocked with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween 20 (TBS) for 1 h at room temperature (RT). The membranes were blocked with 5% skim milk in Tris-buffered saline (20 mM Tris–HCl, pH 7.6, 137 mM NaCl) containing 0.1% Tween 20 (TTBS), and incubated with anti-rat Hsp90α monoclonal antibody and anti-rat HSF-1 monoclonal antibody (ab79849 and ab 61382 respectively, Abcam, USA) for 16 h at 4°C. After washed with TTBS, the membrane was further incubated with horse radish peroxidase-conjugated goat anti-mouse IgG antibody (ab6789, Abcam, USA,) at RT for 1 h. Then, the antibody-antigen complexes were detected using western blotting luminal reagent. The bands on the developed film were quantified with Quantity one- 4.6.2

software (Bio-rad, USA). The density of each band was normalized to that of β-actin protein.

Flowrocytometry

Apoptotic cells were detected by an annexin V-FITC/PI apoptosis detection kit in a FACsort flow cytometer. After exposure to 42°C, the myocardial cell cultures were collected from each plate incl. the control group and digested by trypsin The cells were combining and treated according to the provided protocol and measured by FITC/PI flow cytometry to differentiate apoptotic cells (annexin-positive and propidium iodide-negative) from necrotic cells (annexin- positive and propidum iodide-positive).

Statistical analysis

Between each group the differences by statistical analysis were analyzed using a one-way analysis of variance (ANOVA) using the Statistical Package for Social Sciences (SPSS). Duncan test for multiple comparisons was carried out to compare the mean value of the control group with that of each experimental group. Differences were regarded as significant at $p<0.05$. Three experiments were performed for all the experiments above.

Result

The transcription levels of *hsp*90*α* mRNA in the heat-stressed myocardial cells of rat

The hsp90α mRNA transcription data of the rat myocardial cells at different time of heat stress
is displayed in Fig.1. The level of hsp90α mRNA increased after the beginning of heat stressing
($p < 0.05$) and showed significant induction over all heat stressing time ($p < 0.01$) compared to
control rat myocardial cells. After 20 min to 1 h of heat stress, the levels of hsp90α mRNA increased four times higher than control group. After 2 h to 8 h of heat stress, the levels of hsp90α mRNA increased five times higher than control group and hsp90α mRNA transcription reached its peak level ($p < 0.01$) at 4 h of heat stress.

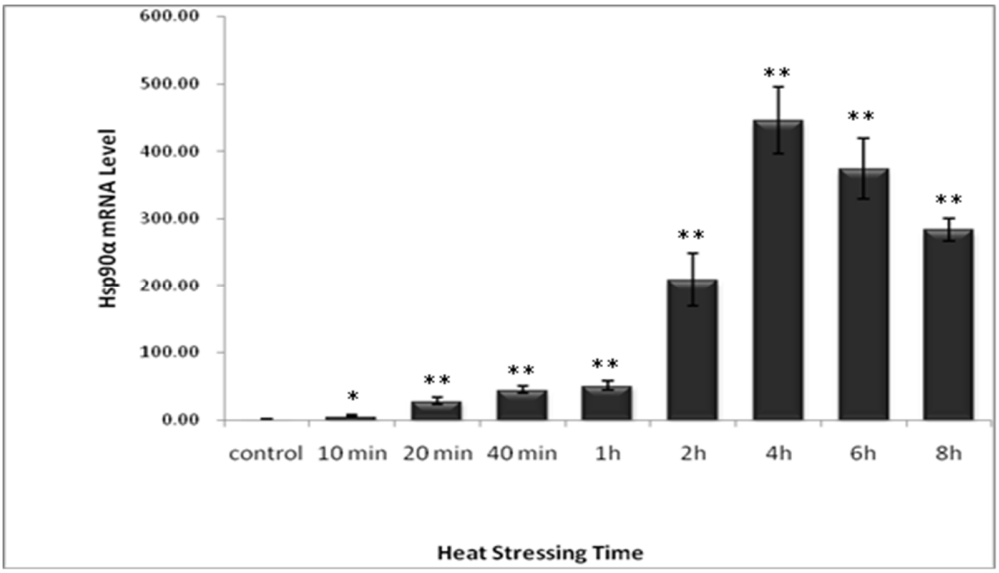

Fig.1. The transcription levels of hsp90α mRNA in primary cultures of rat neonatal myocardial cells exposed to various heat stressing time. *$P < 0.05$; **$P < 0.01$; values indicated are Mean ± SD; $n = 3$.

The expression levels of Hsp90 in the heat-stressed myocardial cells of rat by western blot detection The results of western blot analysis are shown in Fig.2. Hsp90α was consistently present in the rat myocardial cells in the heat-stressed and in control groups. However, within the heat stress duration, Hsp90α protein not expressed to a similar extent. After exposure to heat stressing, Hsp90α decreased immediately and significantly at 10 min ($p < 0.01$), 20 min ($p < 0.01$), 40 min ($p < 0.01$) and 1 h ($p < 0.05$) of heat stress when compared to the control. Although there were no significant differences between the heat-stressed cells and non-stressed cells, the tendency of Hsp90α expression levels in the rat myocardial cells could be detected gradually after 2 h and 4 h of heat stress. The levels of Hsp90α expression increased significantly at 6 h ($p < 0.01$) and 8 h ($p < 0.01$) of heat stress as compared to the control, And the levels of Hsp90α expression in the rat myocardial cells at 6 h and 8 h of heat stress increased 2 times higher than control group.

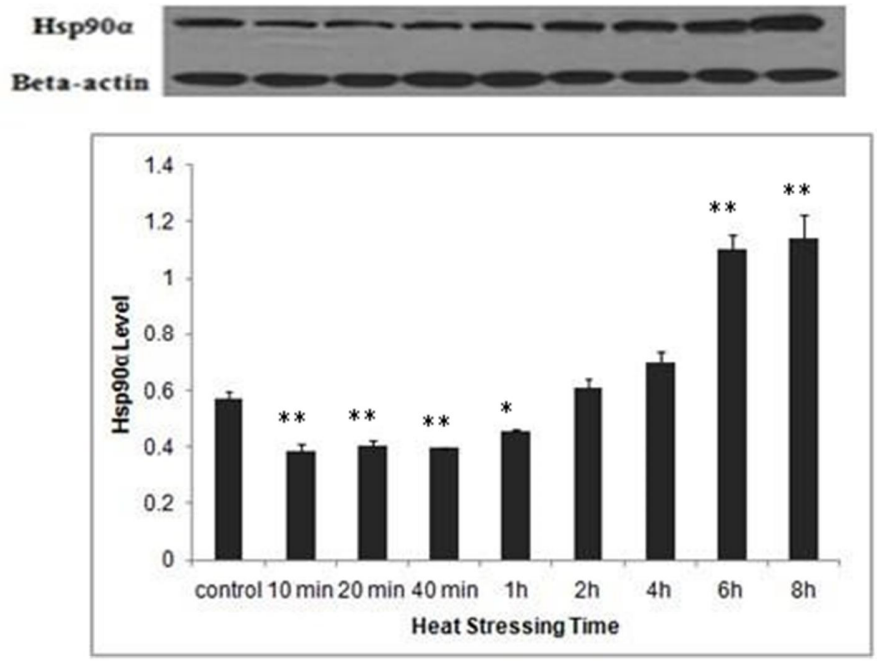

Fig.2. Levels of Hsp90α expression of heat stressed primary rat neonatal myocardial cells *P < 0.05; **P < 0.01; values indicated are Mean ± SD; n = 3.

The transcription levels of hsf-1 mRNA in the heat-stressed myocardial cells of rat

The hsf-1 mRNA detected in the rat myocardial cells at different time heat stress as well as the control Fig.3. At 10 min heat stress, hsf-1 mRNA level increase significantly ($p < 0.01$) and maintain this high level during the course heat shock. After 8 h heat stressing time, hsf-1 mRNA reaches the highest level.

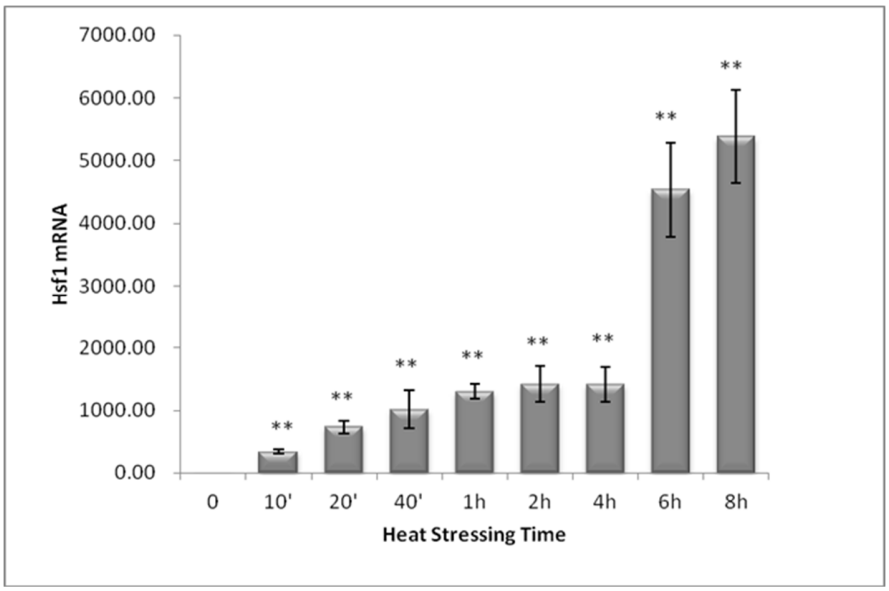

Fig.3. Levels of hsf1 mRNA in primary cultures of rat neonatal cardiac myocytes exposed to various heat stressing time. *P < 0.05; **P < 0.01; values indicated are Mean ± SD; n = 3.

The expression levels of hsf-1 protein in the heat-stressed myocardial cells of rat

Hsf1 protein expression data, normalized to heart tissue β-actin is shown in Fig.4. The level of hsf-1 was decreased significantly (p < 0.05) in the primary rat myocardial cells after 10 min 40 min, 1 h and 2 h of heat stressing time when compare to control. Compared to level of control myocardial cells group, the level of hsf-1 increased significantly (p < 0.01) in the heat stressed group by 4, 6 and 8h heat stress.

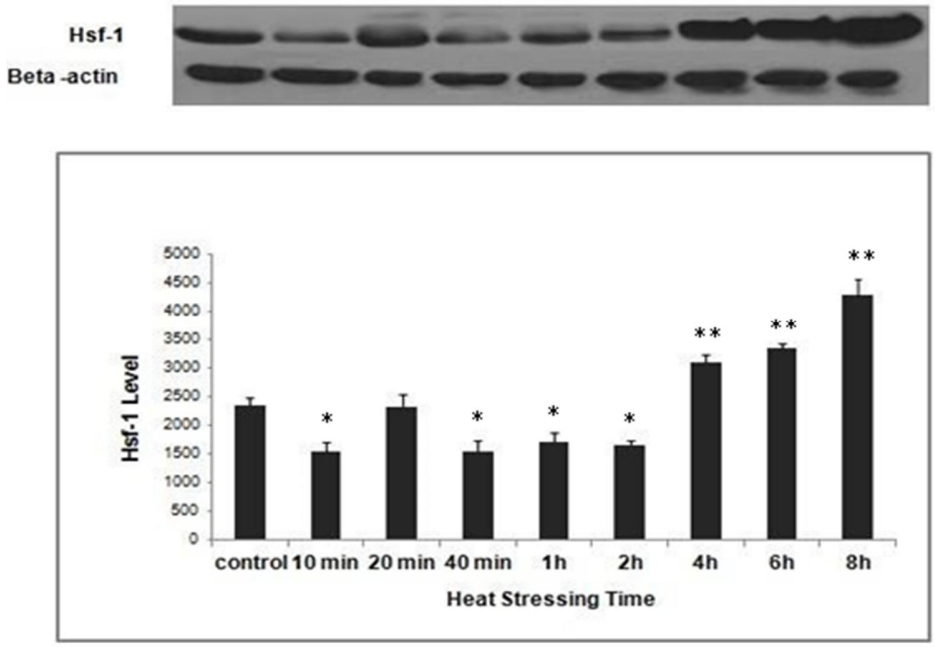

Fig.4. Levels of hsf1 expression of heat stressed primary rat neonatal myocardial cells*P < 0.05; **P < 0.01; values indicated are Mean ± SD; n = 3.

Flowrocytometry

Apoptotic rates in primary myocardial cells of neonatal rat were measured using annexin VFITC/PI assay are displayed in Fig.6. The difference level of apoptosis in the cardiac muscle cells was not significant between test groups stressed by 10min, 20min, 1h, 2hm 4h, and 6h and control groups. However, the cells stressed by 40 min and 8 h demonstrated significant apoptotic levels ($P < 0.01$ and $P < 0.05$) respectively.

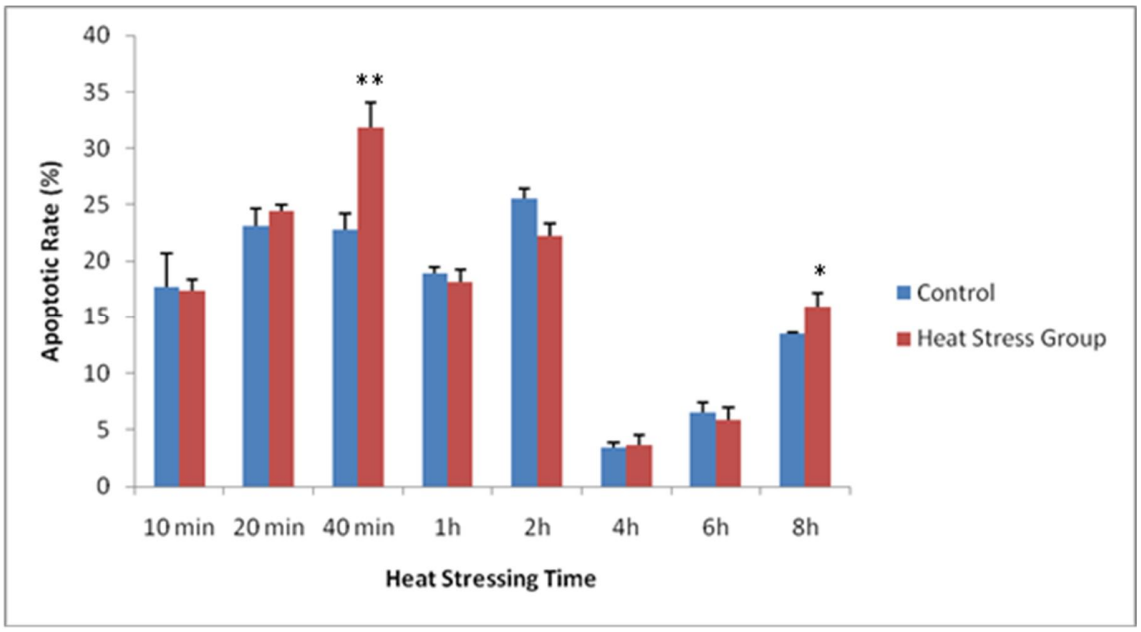

Fig.5. Flow cytometric analysis of apoptotic primary myocardial cells of neonatal rat (%). *P < 0.05; **P < 0.01; values indicated are Mean ± SD; n = 3.

Discussion

In this study, we designed a model of heat stressed myocardial cells indicate activation of apoptotic myocardial cells death. Previous studies have reported that HSp known to regulate cell survival, proliferation, apoptosis and cell death resulting from stress (Parsell and Lindquist, 1993). Apoptotic level increase significantly compared to control while the level of hsp90α also show significant decrease at 40 min heat shock. These results suggested that depletion hsp90α leads to apoptotic rate increment. By the meantime, the level of hsf-1 was similar to that of hsp90α protein however, the transcription level of hsp90α mRNA increased significantly in the primary heart cells. These results indicate that apoptosis inhibit hsp90α by down regulating hsf-1.

Although most studies indicated an anti-apoptotic function for Hsp90, some reports suggest a pro-apoptotic function for this chaperone as described by (Galea-Lauri *et al*., 1996; Wu *et al*., 2002). In our study, the flow cytometry result showed elevated apoptotic level at 8 h heat stressing time significantly. At the same time heat stress, the level of hsf-1 also increased significantly, while the levels of hsp90α and its corresponding mRNA reflect significant increase. These results suggest that the overexpression of Hsp90α and its corresponding mRNA transcription lead to

increase the rate of apoptosis. These results can be sustained by earlier reports demonstrated the overexpression of hsp90 was able to increase the rate of apoptosis in the monoblastoid cell line U937 following induction with TNFα and cycloheximide (Galea-Lauri *et al*., 1996). Furthermore, treatment of human embryonic fibroblasts with the Hsp90α inhibitor geldanamycin increases the resistance of these cells to nicotine which may induce apoptosis via Hsp90 alpha expression, in human cells tested (Wu *et al*., 2002).

Conclusion

This study indicate that apoptosis is inhibiting hsp90α by down regulating hsf-1. In addition, hsp90α may activates apoptotic myocardial cells death induced by heat stress. Hsp90α and its expression in response to high temperature and its association with apoptotic cell death and myocardial cells protection against heat stress still need further investigation.

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Legend

Table 1. List of oligonucleotide primers used in this study

Target gene	Primers	Sequence (5'→3')	Length of PCR products (bp)	Source/reference
16S rRNA gene	16S-F	ACCGCACTTTAGTGTGTGTG	816	Çetinkaya *et al.* (2002)
	16S-R	TCTCTACGCCGATCTTGTAT		
rpoB	C2700F	CGTATGAACATCGGCCAGGT	446	Khamis *et al.* (2004)
	C3130R	TCCATTTCGCCGAAGCGCTG		
pld	PLD-F	ATAAGCGTAAGCAGGGAGCA	203	Pacheco *et al.* (2007)
	PLD-R1	ATCAGCGGTGATTGTCTTCC		
	PLD-R2	ATCAGCGGTGATTGTCTTCCAGG		

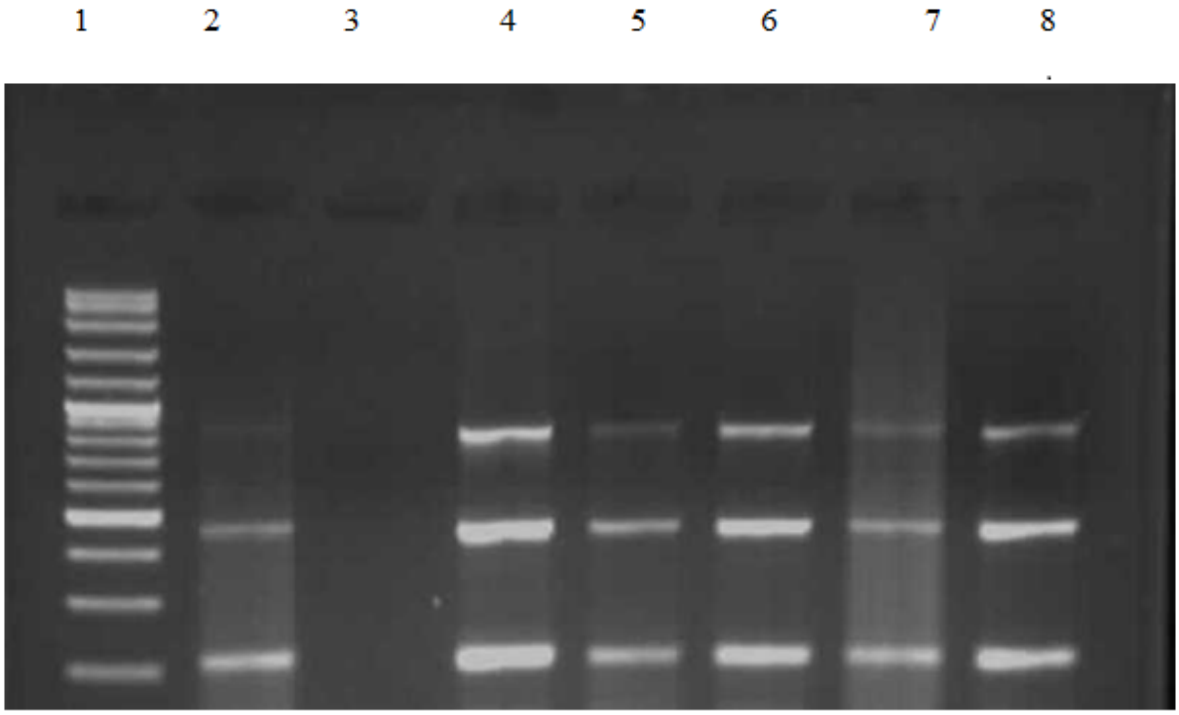

Fig. 6: PCR amplification of C. pseudotuberculosis 16S rDNA, rpoB and pld genes sequences on agarose gel electrophoresis for strains: CP201N (4), CP152N (5), CP41N (6) CP275N (7) and CP216N (8). Whereas DNA marker ladder is in lane 1, CP47W (2) positive control and D.W (3) negative control.