

**INVESTIGATION OF ISCHEMIA-REPERFUSION EFFECTS ON DUSP1
AND CXCL2 GENE EXPRESSION IN RAT KIDNEY TISSUES USING
RT-PCR AND REAL-TIME Q-PCR**

**M.Sc. Thesis by
Şeyma Hande TEKARSLAN**

Department : Advanced Technologies

Programme : Molecular Biology – Genetics and Biotechnology

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**M.Sc. Thesis by
Şeyma Hande TEKARSLAN
(521071047)**

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**Supervisors (Chairman) : Assoc. Prof. Z. Petek ÇAKAR (ITU)
Prof. Dr. Ziya AKÇETİN (NKU)
Members of the Examining Committee : Assist. Prof. Dr. Fatma Neşe KÖK
(ITU)
Assist. Prof. Dr. Rıfat BİRCAN (NKU)
Assist. Prof. Dr. Alper Tunga
AKARSUBAŞI (ITU)**

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

**RT-PCR VE GERÇEK ZAMANLI PCR KULLANILARAK SIÇAN BÖBREK
DOKULARINDA DUSP1 VE CXCL2 GENLERİ ÜZERİNDEKİ İSKEMİ
REPERFÜZYON ETKİLERİNİN İNCELENMESİ**

**YÜKSEK LİSANS TEZİ
Şeyma Hande TEKARSLAN
(521071047)**

Tezin Enstitüye Verildiği Tarih : 04 Mayıs 2009

Tezin Savunulduğu Tarih : 04 Haziran 2009

**Tez Danışmanları : Doç. Dr. Zeynep Petek ÇAKAR (İTÜ)
Prof. Dr. Ziya AKÇETİN (NKÜ)
Diğer Jüri Üyeleri : Yrd. Doç. Dr. Fatma Neşe KÖK (İTÜ)
Yrd. Doç. Dr. Rıfat BİRCAN (NKÜ)
Yrd. Doç. Dr. Alper Tunga
AKARSUBAŞI (İTÜ)**

HAZİRAN 2009

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Şeyma Hande TEKARSLAN

Molecular Biologist

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ABBREVIATIONS

IRI	:Ischemia Reperfusion Injury
DCD	:Donation by cardiac death
IPC	:Ischemic Preconditioning
Hsp	:Heat Stress Proteins
NO	:Nitric Oxide
ARF	:Acute Renal Failure
DUSP1	:Dual Specificity of Phosphatase
MKB	:MAPK binding domain
PP2A	:Protein phosphatase 2 A
PP2C	:Protein phosphatase 2 C
PTPN5	:protein-tyrosine phosphatase nonreceptor-type 5
PTPN7	:protein-tyrosine phosphatase nonreceptor-type 7
PTPRR	:Protein tyrosine phosphatase, receptor type, R
JNK	:c-Jun N-terminal kinase
HIF-1	:Hypoxia-Inducible Factor 1
ERK1/2	:extracellular signal-regulated kinases 1/2
mRNA	:Messenger Ribonucleic Acid
cAMP	:Complementary DNA
AP-1	:Activator Protein 1
CRE	:Cyclization Recombination
iNOS	:Inducible nitric oxide synthase
NOS	:nitric oxide synthase
DNA	:Deoxyribonucleic Acid
eNOS	:endothelial nitric oxide synthase
NAD	:Nicotinamide adenine dinucleotide
ROS	:reactive oxygen species
ATP	:Adenosine-5'-triphosphate
NF-kB	:nuclear factor kappa
SOD	:superoxide dismutase
MPT	:Mitochondrial permeability transition
HP	:heat preconditioning
MAPK	:Mitogen-activated protein (MAP) kinases
BH3	:Bcl-2 homology domain 3
Bcl-2	:B-cell lymphoma 2
COX-2	:cyclooxygenase-2
ecSOD	:extracellular SOD
HO-1	:heme oxygenase
TLR	:Toll-like receptors
CXCL2	:Chemokine (C-X-C motif) ligand 2
MKP-1	:MAP kinase phosphatase-1
MIP-2	:Macrophage-inflammatory protein-2
ICAM-1	:Inter-Cellular Adhesion Molecule 1
IL-8	:Interleukin-8
KC	:Keratinocyte-derived chemokine
PCR	:Polymerase chain Reaction

RT-PCR	:Reverse transcription-polymerase chain reaction
CT	:Threshold cycle
LNA	:Locked Nucleic Acids
UPL	:Universal Probe Library

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INVESTIGATION OF ISCHEMIA-REPERFUSION EFFECTS ON DUSP1 AND CXCL2 GENE EXPRESSION IN RAT KIDNEY TISSUES USING RT-PCR AND REAL-TIME PCR

SUMMARY

Ischemia is a restriction in blood supply, due to factors in the blood vessel with damage or dysfunction of tissue. Cellular metabolism prefers anaerobic pathways due to insufficient oxygen. Reperfusion injury refers to damage to tissue caused when blood supply returns to the tissue after a period of ischemia. Restoration of blood flow triggers the production of Reactive oxygen species (ROS) which can initiate a wide range of toxic oxidative reactions. ROS can react directly with cellular lipids, proteins and DNA leading to cell injury/death. Inflammation plays an important role in ischemia reperfusion injury.

In recent studies, ischemic preconditioning and heat preconditioning have been used as a therapy. Ischemic preconditioning (IP) is describing a condition that prior ischemic stress renders the organ resistant to a subsequent ischemic insult. Heat preconditioning was the first stress shown to induce the synthesis of heat shock proteins (Hsps) and has an anti-inflammatory, antiapoptotic effect. The described forms of preconditioning provide protective effect on kidney.

Dual specificity of phosphatases (DUSPs) inactivate MAPKs which are important players in signal transduction pathways activated by a range of stimuli and mediate a number of physiological and pathological changes in cell function via dephosphorylation of these phosphothreonine and phosphotyrosine residues. CXCL2 which is a chemokine that is in CXC family plays a crucial role in neutrophil recruitment in the post-ischemic kidney mediating tissue injury via cytokines, free radical intermediates, and proteases. In a previous study with microarray analysis, it was observed that DUSP1 and CXCL2 genes were upregulated when rat kidney tissues were studied for gene expression upon ischemia-reperfusion, ischemia-reperfusion with heat preconditioning and with ischemic preconditioning.

The aim of this study was to investigate the effects of ischemia reperfusion, IP, HP on DUSP1 and CXCL2 gene expression in rat kidney tissues using reverse transcriptase PCR and real-time q-PCR methods and compare the results with previous microarray data.

The results showed that DUSP1 gene expression was highest when reperfusion time was 60 min and also highest in IP group among all conditions tested. IP generally increases CXCL2 gene expression. However, in HP and IRI groups, CXCL2 gene expression increases as a function of reperfusion time. Real-time PCR results were in good agreement with the previously obtained microarray results. The ranges of relative quantification values were higher in real-time PCR results. Friedman test proved that the result of the three experimental methods were not significantly different from each other.

The early preconditioning effects on DUSP1 and CXCL2 gene expression levels were investigated in this study. New experiments could be designed to investigate late preconditioning effects by extending reperfusion time beyond 60 min.

RT-PCR VE GERÇEK ZAMANLI PCR KULLANILARAK SIÇAN BÖBREK DOKULARINDA DUSP1 VE CXCL2 GENLERİ ÜZERİNDEKİ İSKEMİ REPERFÜZYON ETKİLERİNİN İNCELENMESİ

ÖZET

İskemi damarlardaki hasara veya dokulardaki işlev bozukluğuna bağlı olarak kan akışındaki kısıtlamadır. Hücre metabolizması kısıtlı oksijen varlığında anaerobik yolizlerini tercih eder. Reperfüzyon ise belli bir süre iskemiden sonra dokulara kan akışının tekrar sağlanması sonucu dokuya verilen hasardır. Kan akışının geri kazanımı geniş bir alanda toksik oksidatif reaksiyon başlatabilen reaktif oksijen türlerinin oluşumunu tetiklemektedir. Reaktif oksijen türleri hücre lipidleri, protein ve DNA ile direkt reaksiyona girerek hücre hasarı ve/veya hücre ölümüne sebep olmaktadır. Enflamasyon iskemik reperfüzyon hasarında önemli rol oynamaktadır.

Son senelerde yapılan çalışmalarda, iskemik önkoşullaması ve ısı önkoşullaması terapi olarak kullanılmaktadır. Isı önkoşullaması önceki iskemik stresin sonra oluşacak iskemiye karşı daha dirençli olmasını sağlayan bir olgudur. Isı önkoşullaması ısı şoku proteinlerinin indüklendiğini gösteren ilk stresir ve antiinflamatuvar ve hücre ölümünü engelleyen özelliklere sahiptir. Bu önkoşullamalar böbreğe koruyucu etki sağlamaktadır.

DUSPs sinyal iletim yolizinde çeşitli uyarıcılar tarafından aktive olan MAPKleri inaktive ederek hücre fonksiyonlarındaki fizyolojik ve patolojik değişimleri kontrol etmektedir. CXCL2 iskemik sonrası böbrekte sitokin, serbest radikal araçları ve proteazları sayesinde oluşan doku hasarında nötrofil toplanmasında çok önemli rol oynamaktadır.

Mikroarray analizi yapılan daha önceki bir çalışmada, sıçan böbrek dokularında iskemik-reperfüzyon, ısı önkoşullamalı iskemik reperfüzyon ve iskemik önkoşullamalı iskemik reperfüzyon üzerinde gen ekspresyonu çalışıldığı zaman DUSP1 ve CXCL2 gen anlatımının arttığı gözlenmiştir.

Bu çalışmanın amacı revers transkriptaz Polimeraz Zincir Reaksiyonu ve Gerçek-Zamanlı Polimeraz Zincir Reaksiyonu metotlarını kullanarak sıçan böbrek dokularında iskemik reperfüzyon, iskemik önkoşullaması ve ısı önkoşullamasının DUSP1 ve CXCL2 gen ekspresyonu üzerindeki etkilerinin araştırılması ve bu sonuçların önceki mikroarray verileri ile karşılaştırılmasıdır.

Sonuçlar, DUSP1 gen ekspresyonunun reperfüzyon süresi 60 dakika olduğunda en yüksek olduğunu ve test edilen tüm koşullar arasında en yüksek ekspresyonun iskemik önkoşullaması grubunda olduğunu göstermiştir. İskemik önkoşullaması CXCL2 geninin ekspresyonunu arttırmaktadır. Fakat ısı önkoşullaması ve IRI gruplarında CXCL2 geninin ekspresyonu reperfüzyon süresine bağlı olarak artış göstermektedir. Real-time PCR sonuçları, önceki mikroarray sonuçları ile uyumludur. Göreceli ölçüm değerleri, gerçek zamanlı PCR sonuçlarında diğer metotlara kıyasla daha yüksektir. Friedman testi, her üç deneysel metot sonuçlarının birbirinden ciddi ölçüde farklı olmadığını kanıtlamıştır.

Bu alıřmada, erken iskemi nkořullamasının DUSP1 ve CXCL2 genlerinin ekspresyonu zerine etkisi arařtırılmıřtır. Ge iskemi nkořullama etkisinin arařtırılması iin reperfzyon sresi 60 dakika zerine ıkarılarak yeni deneyler tasarlanabilir.

1. INTRODUCTION

1.1 Ischemia Reperfusion Injury

Ischemia/reperfusion injury (IRI) is a complex phenomenon and an unavoidable consequence of organ transplantation that occurs during a number of stages in the transplantation procedure. IRI may begin in the donor as a result of brain death, continue during the process of organ procurement and cold ischemic storage and further it will continue during warm ischemia. It is not only an invariable consequence of transplantation but also results from circumstances such as cross clamping and resuscitation following systemic hypotension (Koo and Fuggle, 2000; De Groot and Rauen, 2007; Weight et al., 1996). It is associated with increased morbidity, prolonged hospitalizations and increased mortality (Thurman, 2007).

1.1.1 Ischemia

Ischemia is a restriction in blood supply, due to factors in the blood vessel with damage or dysfunction of tissue. Insufficient oxygen intake and conversion of cellular metabolism to anaerobic pathways are the characteristics of ischemia (Siemionow and Arslan, 2004). Ischemic tissue is depleted of oxygen and nutrients (Koo and Fuggle, 2000). Anoxic injury of O₂-dependent cells in ischemia is clearly the predominant injury process (De Groot and Rauen, 2007). Long periods of ischemia can alter the electron transport complexes in mitochondria (Siemionow and Arslan, 2004).

Mitochondrial energy production decreases and ATP content also decreases. ATP is dephosphorylated to adenosine diphosphate, adenosine monophosphate, and purines (Koo and Fuggle, 2000; De Groot and Rauen, 2007). In the kidney, 95% of ATP is lost within 4 hours of ischemia (Koo and Fuggle, 2000).

Dysregulation of cellular ion homeostasis resulting in ischemia-induced acidosis and increase in intracellular calcium concentration is a crucial feature of ischemia (Siemionow and Arslan, 2004; Dorweiler et al., 2007).

The sodium-(Na⁺)-potassium (K⁺)-ATPase, that removes sodium from the cytosol, is inhibited during ischemia-induced ATP-depletion so that the sodium (Na⁺)-

calcium (Ca^{++})-exchanger moves sodium out of the cell. Extracellular calcium is consequently transported into the cytosol and results in intracellular calcium overload (Dorweiler et al., 2007). Acting as a second messenger, calcium triggers the activation of several enzymes such as phospholipases (especially phospholipase A2) and proteases (calpains and others) and proinflammatory mediators accumulate (Siemionow and Arslan, 2004; Bohmova and Vicklicky, 2001). Cellular influx of water and swelling occurred as a consequence of an intracellular accumulation of sodium and calcium ions (Bohmova and Vicklicky, 2001).

Under physiological conditions, hypoxanthine is converted to xanthine by the enzyme xanthine dehydrogenase with consumption of nicotinamide adenine dinucleotide (NAD). However, in ischemia phase, xanthine dehydrogenase (D-form) undergoes a conformational change to xanthine oxidase (O-form) by intracellular Ca^{++} increase, and xanthine oxidase can generate reactive oxygen species (ROS) (Dorweiler et al., 2007). Akçetin et al.(1999) demonstrated that xanthine oxidase was the main oxygen radical generator in the very early reperfusion period after ischemia. The duration of ischemia determines the severity of tissue damage (Siemionow and Arslan, 2004).

1.1.2 Reperfusion

Reperfusion injury refers to damage to tissue caused when blood supply returns to the tissue after a period of ischemia. Restoration of blood flow initiates a cascade of events that may lead to additional cell injury, beyond that caused by the ischemia (Siemionow and Arslan, 2004). The return of oxygenated blood on reperfusion may lead to a chain of damaging events that results in the production of reactive oxygen species (ROS) an endothelial cell damage. Reactive oxygen and nitrogen species are important mediators and modulators of postischemic tissue injury in a number of different organ systems including kidney (Abe et al., 2009).

Reactive oxygen species can initiate a wide range of toxic oxidative reactions. These reactions are the initiation of lipid peroxidation, direct inhibition of mitochondrial respiratory chain enzymes, inactivation of glyceraldehyde-3 phosphate dehydrogenase, inhibition of membrane sodium-potassium ATPase activity, inactivation of membrane sodium channels, and other oxidative modifications of proteins (Cuzzocrea and Reiter, 2001).

ROS can react directly with cellular lipids, proteins and DNA leading to cell injury/death and activation of NF- κ B (Dorweiler et al., 2007).

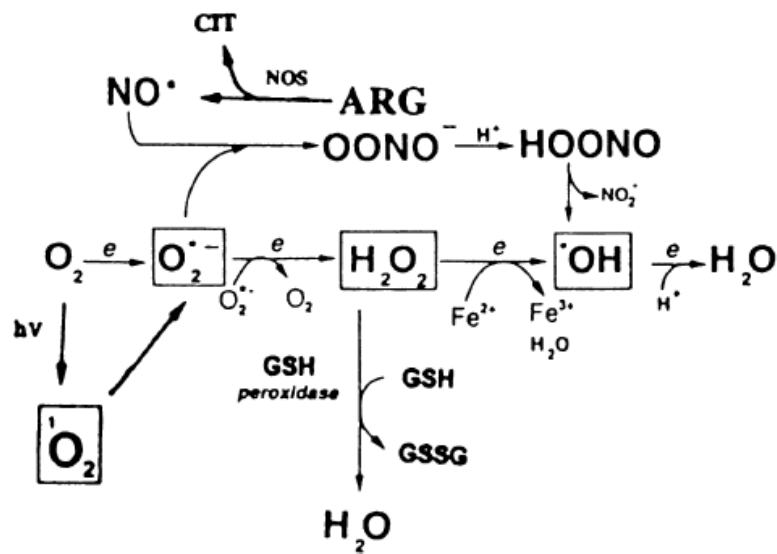


Figure 1.1: Free radicals and reactive oxygen intermediates (Cuzzocrea and Reiter, 2001)

O_2 is enzymatically reduced to hydrogen peroxide (H_2O_2) in the presence of a ubiquitously distributed enzyme, superoxide dismutase (SOD) which can be classified as an antioxidative enzyme that affords protection against free radical damage. In some cases, it can also be associated with increased oxidative stress (Cuzzocrea and Reiter, 2001). H_2O_2 does not possess an unpaired electron and is not a free radical. It is usually classified as a reactive oxygen intermediate or species. It can diffuse through membranes and it has a half-life much longer than that of O_2 . But in the presence of the transition metals Fe^{+2} or Cu^{+1} , it is reduced to $\text{OH}^\bullet$. $\text{OH}^\bullet$ is extremely reactive and highly toxic. It indiscriminately reacts with any molecule it encounters. It is classified as the radical's radical. Because of its large size and electrophilicity, it is not uncommon for $\text{OH}^\bullet$ to interact and produce damage to macromolecules such as DNA, proteins, carbohydrates, and lipids. Oxidative damage to macromolecules is especially noticeable because compared to the smaller molecules in cells, they are present in limited numbers (Figure 1.1) (Cuzzocrea and Reiter, 2001). In the kidney, ROS are produced in significant amounts in renal proximal tubules rather than distal tubular epithelium under ischemia reperfusion (Chiang-Ting et al., 2005).

Inflammation plays an important role with involvement of leukocytes, adhesion molecules, endothelial activation and dysfunction, adherence and activation of neutrophils and platelets and the activation of complement and T cells chemokines and cytokines (Huang et al., 2007; Foley and Chari, 2007). The accumulation of reactive oxygen species (ROS) initiate tissue injury and stimulate a cellular cascade leading to inflammation. The proinflammatory process that follows results in cell death and in severe cases leads ultimately to organ failure (Foley and Chari, 2007).

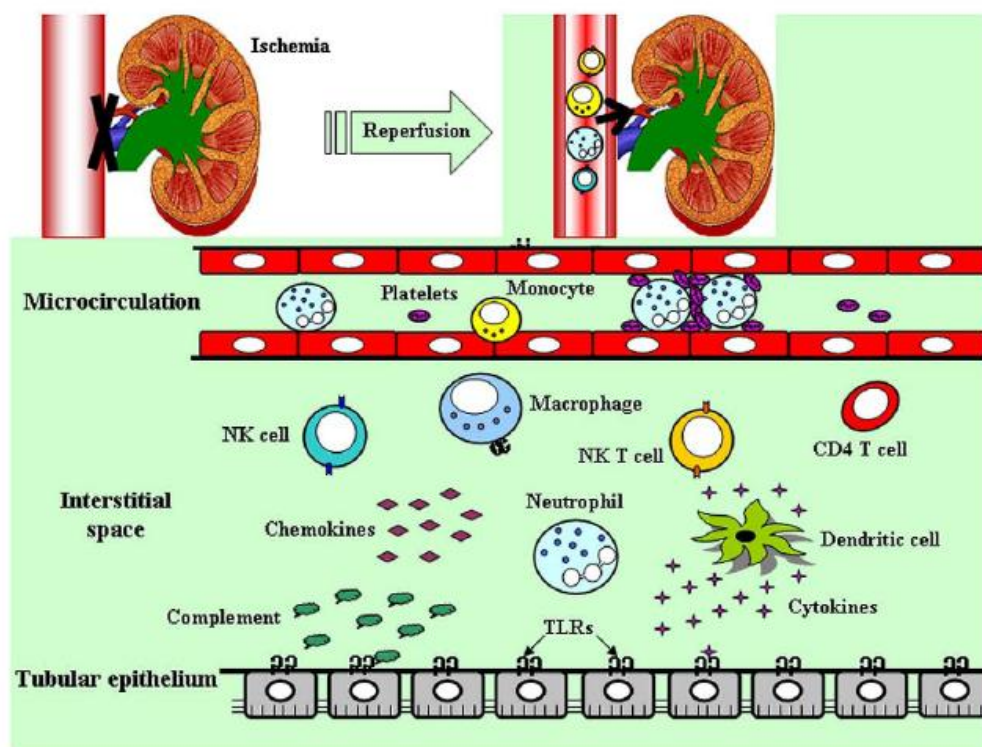


Figure 1.2: Overview of early immune response occurring in post-ischemic kidneys (Jang and Rabb, 2008)

In experimental ischemia reperfusion-induced acute kidney injury models, after ischemic insult occurs, reperfusion initiates inflammation in post-ischemic kidneys with entry of blood containing major cellular components of innate immunity, plus lymphocytes. Leukocytes including neutrophils, macrophages and lymphocytes gather in post-ischemic kidneys. Cytokines and complement system also cause renal injury. Early injury events are observed at the level of the microvasculature first and then in the tubular interstitial space. Leukocytes and platelets plug renal microcirculation and each immune component cause robust inflammatory responses in the tubular interstitial space (Figure 1.2) (Jang and Rabb, 2008).

NO is recognized as an important mediator of physiological and pathological processes of renal I/R injury (Chen et al., 2007). The endothelial barrier between the circulation and surrounding tissues secretes vasoconstrictors (eg, endothelin) and vasodilatory molecules (eg, nitric oxide [NO]) to regulate blood pressure and flow. NO is expressed constitutively at basal levels on the endothelial surface by the calcium-/calmodulin-dependent enzyme, NO synthase (eNOS), and an inducible isoform (iNOS). eNOS is expressed in the renal vasculature and tubular epithelium, whereas iNOS may be expressed by the proximal tubules, glomeruli, and inner medullary collecting ducts (Koo and Fuggle, 2000). The role of NO in I/R is still controversial. NO can induce cellular cytotoxicity and tissue injury via lipid peroxidation, DNA damage, and pro-apoptotic effects, which are included in I/R injury (Chen et al., 2007). On the other hand, many studies have demonstrated that the increased activity of NOS was associated with reduced I/R-induced injury by acting in a tissue-protective manner through the physiological regulation of vascular tone, inhibition of platelet aggregation, attenuation of leukocyte adherence to the endothelium, scavenging of oxygen-derived free radicals, maintenance of normal vascular permeability, inhibition of smooth muscle proliferation, immune defense, and stimulation of endothelial cell regeneration (Chen et al., 2007; Siemionow and Arslan, 2004).

Cell death occurs both via necrosis and apoptosis in ischemia reperfusion injury. During the degenerative process of necrosis, cellular integrity is lost and the release of cytosolic contents elicits an inflammatory response whereby the extent of necrotic cell loss is a function of the duration of ischemia (Dorweiler et al., 2007). Apoptosis can be defined as the morphologically defined form of programmed cell death. This differs from tissue necrosis, which is another major form of cell death (Siemionow and Arslan, 2004).

Apoptosis is a highly regulated, energy (ATP) dependent mechanism that leads to cellular degradation into membrane-covered apoptotic bodies that are removed by macrophages without provoking an inflammatory response. Ischemia itself triggers apoptosis whereas reperfusion accelerates the process and may lead to an enhancement of apoptosis as reperfusion restores the energy required for the completion of apoptosis (Dorweiler et al., 2007).

Cell-cell interactions are possible mechanisms underlying the development of apoptosis during reperfusion. Interactions between blood cells and vascular endothelial cells and the release of cytokines and generation of reactive oxygen species from activated neutrophils, endothelial cells, and myocytes during reperfusion were triggers for the induction of apoptosis. These interactions are initiated within the early moments of reperfusion, and may continue during the following hours and days (Siemionow and Arslan, 2004).

The pathway of necrosis and apoptosis in ischemia-reperfusion is activation of intrinsic and death receptor-dependent (extrinsic) pathways assemble to mitochondria to induce membrane permeabilization. Bax/Bak dependent permeabilization of the outer membrane is caused by BH3 only Bcl-2 family members, including tBid formed by death receptor-linked caspase 8 activation. Formation of channels in the outer membrane or induction of mitochondrial permeability transition (MPT) followed by mitochondrial swelling and outer membrane rupture may be triggered by permeabilization. After membrane permeabilization, cytochrome c is released to the cytosol and first activates caspase 9 and then caspase 3 in a reaction requiring ATP. If MPT onset is sudden and involves most mitochondria, ATP becomes profoundly depleted and depletion of ATP blocks caspase activation. Instead, plasma membrane rupture and the onset of necrotic cell death are culminated by ATP depletion (Casillas-Ramirez et al., 2006).

1.2 Preconditioning

1.2.1 Ischemic preconditioning

Ischemic preconditioning (IPC) is the phenomenon that a prior ischemic stress renders the organ resistant to a subsequent ischemic insult (Liu et al., 2007). The phenomenon of IPC was described in 1986 by Murry et al. using a canine heart ischemia model.

In that study, a group of open-chest dogs were exposed to a four cyclic episode of 5-min coronary occlusions followed by 5 min reperfusion and then subjected to a prolonged ischemic insult (40 min coronary occlusion followed by 4 days of reperfusion). Control dogs received only 40-min coronary occlusion. Those group of dogs that received four cyclic episodes of 5 min occlusion, followed by 5 min reperfusion before prolonged ischemia, had much smaller myocardial infarct size

compared with control group, although that group received additional 20 min ischemia. The term “ischemic PC” was introduced to describe this phenomenon (Murry et al., 1986).

After this first discovery in 1986, it was found that ischemic preconditioning had two phases called early preconditioning (lasts for 2–3 h) and (ii) late preconditioning (starting at 24 h lasting until 72–96 h after initial ischemia) (Bolli, 2000).

Early preconditioning depends on adenosine, opioids and to a lesser degree, on bradykinin and prostaglandins, released during ischemia. These molecules activate G-protein-coupled receptor, initiate activation of K_{ATP} channel and generate oxygen free radicals, and stimulate a series of protein kinases, which include protein kinase C, tyrosine kinase, and members of MAP kinase family. Late preconditioning is triggered by a similar sequence of events, but in addition essentially depends on newly synthesized proteins, which comprise iNOS, COX-2, manganese superoxide dismutase, and possibly heat shock proteins (Figure 1.3) (Das and Das., 2008).

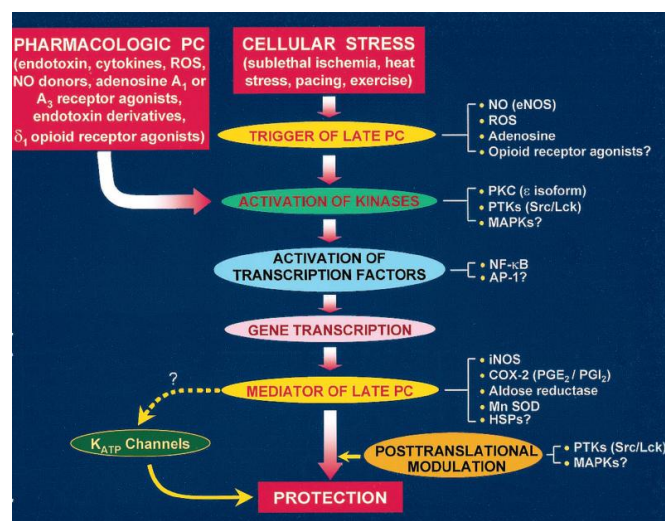


Figure 1.3: Schematic representation of the cellular mechanisms underlying late PC (Bolli, 2000).

One of the earlier genes identified as the mediator of late PC was the inducible isoform of NOS (iNOS); subsequently, other genes have been identified, including cyclooxygenase-2 (COX- 2), heme oxygenase-1 (HO-1), antioxidant enzymes such as extracellular SOD (ecSOD) and aldose reductase. Briefly, the possible mechanism by which NO enhances the resistance of myocardium to ischemia reperfusion injury include inhibition of calcium influx, reduction of myocardial oxygen demands, and opening of mitochondrial K_{ATP} channel. It was found that after a standard ischemic

PC protocol, there was robust upregulation of COX-2 protein and increase in the levels of COX-2 byproduct, prostanoids (PGI₂ and PGE₂), well known for their cardioprotective action (Das and Das, 2008).

The most widespread method used in experimental procedure of preconditioning is using four-cycle preconditioning schedule. This procedure may be confusing and complex and results have not always been conclusive in the organs especially in the kidney (Torras et al., 2002)

The beneficial effects of using multiple cycles over single cycle IPC, that is, cycles of multiple brief IPC, is correlative to its protective effect and is poorly understood (Li et al., 2005). Li et al found that IPC with 3 cycles is more protective compared to single or 2 cycles of IPC in dog kidney. Torras et al indicate that the protective effect of renal ischemic preconditioning is related to the local production of NO and found that 15 min of warm ischemia and 10 min of reperfusion in the kidney was the most suitable one-cycle schedule for preconditioning since it protected from both warm and cold ischemia.

Some of agents that simulate ischemic preconditioning without inducing ischemia have been studied in humans and adenosine, adenosine agonists, nicorandil, volatile anaesthetics, and nitroglycerin are used for pharmacological ischemia preconditioning (Casillas-Ramirez et al., 2006).

The use of IPC in clinical solid organ transplantation has its limitations. Inducing a period of ischemia before cold preservation to the donor organ has the potential to cause vascular injury or worsen posttransplant function. In addition, it cannot be used in the recovery of organs from DCD donors observing that the patient sustains cardiopulmonary arrest before organ procurement. No clinical trials have been published to date that examine the role of IPC in human kidney transplantation (Foley and Chari, 2007).

1.2.2 Heat preconditioning

It was the first stress shown to induce the synthesis of heat stress proteins (Hsps), in particular the inducible form Hsp70, in the heart and other tissues. Currie et al. (1988) were the first to show that 24 h after heat stress (HS), isolated rat hearts exhibited improved contractile recovery upon reperfusion compared to control

hearts. Since then, this observation has been extended to the rabbit and the dog (Joyeux-Faure et al., 2003).

The components of the mechanism of the heat preconditioning are i) Triggers of HS preconditioning which helps the generation of a wide variety of metabolites and ligands to trigger the development of protection, ii) Signalling aspects of HS preconditioning which trigger HS preconditioning by activating a complex cascade of signalling events that ultimately result in increased transcription of cardioprotective genes , iii) Mediators of HS preconditioning.

Triggers of heat preconditioning are: catecholamines, nitric oxide, reactive oxygen species, cytokines, heme oxygenase-1 pathway, opioids. Signalling pathways that are signaling aspects of HS preconditioning are phospholipase C and protein kinase C, protein tyrosine kinases, and mitogen-activated protein kinases. At the end the mediators that increase the effect of protection are heat stress proteins, nitric oxide, cyclooxygenase-2, endogenous cannabinoids, KATP channels, antioxidant enzymes and calcium homeostasis (Figure 1.4) (Joyeux-Faure et al., 2003).

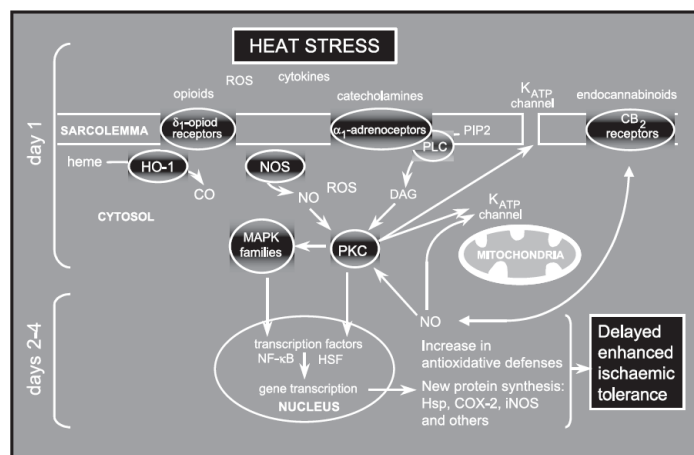


Figure 1.4: Hypothetical representation of cellular events thought to occur following heat stress preconditioning (Joyeux-Faure et al., 2003).

Heat preconditioning is known to be protective in various types of injury. Heat shock has an anti-inflammatory, antiapoptotic effect. Heat preconditioning provided marked functional protection and also reduced histological evidence of tubular necrosis. It had effects on ischemic acute renal failure (ARF). It suppressed ischemia/reperfusion induced NF-κB activation and decreased tubular cell apoptosis and caspase 3 activation. HSP-70 was induced only among several heat shock proteins (HSP) by heat preconditioning (Jo et al., 2006).

HSP-70 has been demonstrated to be protective in renal tubular cell apoptosis that is induced by inflammatory cytokine or ATP depletion (Jo et al., 2006).

HSP70 was induced by heat preconditioning and it protected renal tubular epithelia cells from Ca^{+2} mediated injury which was induced by ischemic damage (Kuhlmann et al., 1997).

The two genes - HSP70-1 gene and HSP70-2 gene -that regulates HSP70i were induced after ischemia. HSP70-2 is more sensitive to injury, thus it is activated immediately. However, HSP70-1 gene controls heat shock protein induction after severe injury (Akçetin et al., 1999).

Heat-shock preconditioning protects liver from microcirculatory failure which occurred after ischemia/reperfusion (I/R) by inducing expression of heat shock protein 72 (HSP72) and heme oxygenase (HO-1) (Yamagami et al., 2003).

1.3 DUSP1 Gene

Innate immune cells such as macrophages and dendritic cells localized in the affected tissues detect the pathogen-associated molecular patterns through their specialized receptors including Toll-like receptors (TLR) (Wang and Liu, 2007). Engagement of Toll-like receptors (TLRs) on macrophages leads to activation of the mitogen-activated protein kinases (MAPKs), which contribute to innate immune responses (Chi et al., 2005). NF- κ B is also upregulated to bind to the promoter regions of many pro-inflammatory cytokine and chemokine genes and activate their transcription (Wang and Liu, 2007). Mitogen-activated protein (MAP) kinases are important players in signal transduction pathways activated by a range of stimuli and mediate a number of physiological and pathological changes in cell function (Camps et al., 2000).

MAPKs are activated by phosphorylation on both the threonine and tyrosine residues of a conserved signature T-X-Y motif within the activation loop of the kinase (Keyse, 2008).

The reversible nature of MAPK phosphorylation suggests that the phosphatases play a key role in regulating MAPK activity. A growing family of dual specificity of phosphatases have been identified that inactivate MAPKs via dephosphorylation of these phosphothreonine and phosphotyrosine residues (Franklin et al.,1998). MKP

include serine-threonine phosphatases (PP2A and PP2C), the tyrosine phosphatases PTPN5, PTPN7, and PTPRR , and members of the DUSP family. Of the 30 protein-coding DUSP genes found in the human genome, 11 are bona fide MKP because in addition to the DUSP domain they contain a MAPK binding domain (MKB). The 19 atypical DUSP are much smaller proteins that lack such aMKB, but may still function as MKP (Lang et al., 2006). In 1992, the first mammalian DUSP was identified as the mouse immediate early gene 3CH134 (later also cloned as Erp) or its human orthologue CL100, or its rat ortolog DUSP1 which is induced rapidly after exposure to growth factors, heat shock, or oxidative stress (Camps et al., 2000).

The three major subfamilies of MAPK that are expressed in the immune system are p38, ERK, and JNK (Lang et al., 2006).

MKP-1 localizes to the nucleus through its N terminus and preferentially dephosphorylates activated p38 MAPK and c-Jun N-terminal kinase (JNK) relative to extracellular signal regulated kinase (ERK) in vitro (Table 1.1) (Chi et al., 2005).

Table 1.1 : General features of mammalian DUSPs (Camps et al., 2000)

Human gene	Species orthologue	Tissue distribution	Subcellular localization	Transcriptional induction	Chromosomal localization	MAPK specificity
hVH1/ CL100	MKP1/ 3CH134/ Erp (mouse)	Heart Skelatal muscle Pancreas Placenta liver Testes Stomach Brain lung	Nuclear	Mitogens Growth factors Oxidative stress Heat shock Brain ischemia Brain seixure activity	5q35	p38> JNK/SAPK> ERK

Although group of phosphatases exhibit differential substrate specificities toward different MAP kinases, many of the phosphatases also share substrates. For example, MKP-1 has been shown to prefer p38 and JNK as substrates, although it was originally characterized as an ERK phosphatase. In macrophages, at least four MKPs are expressed: MKP-1, MKP-2, phosphatase of activated cells 1, and MKP-5/MKP-M. It is possible that multiple MKPs act cooperatively to control the MAP kinase cascades. In the absence of MKP-1 protein, JNK and p38 will be eventually

inactivated by other MKPs, albeit at a much slower rate (Figure 1.5) (Zhao et al., 2005).

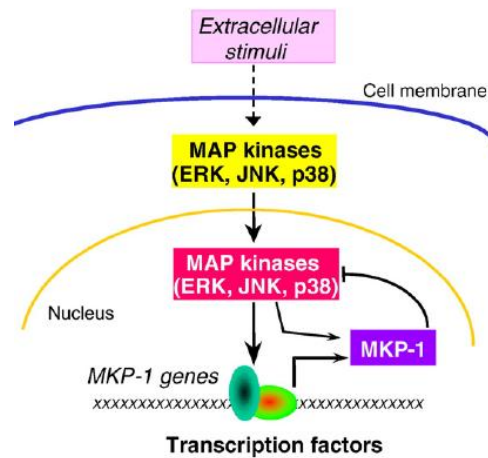


Figure 1.5: Feedback control of MAP kinases by MKP-1 (Wang and Liu, 2007)

Since the initial cloning of CL100/MKP-1, eight additional mammalian DSP gene family members have been identified and characterized. Another interesting feature of DUSPs is their tight and rapid transcriptional induction by growth factors and/or cellular stresses. Indeed, CL100/MKP-1 was identified originally as an immediate early gene induced rapidly within 30–120 min in cultured cells by mitogens, heat shock, or oxidative stress (Camps et al., 2000). MKP-1 regulates both proinflammatory and anti-inflammatory cytokines in LPS-stimulated macrophages (Chi et al., 2005).

DUSP1/MKP-1 gene is a transcriptional target of the p53 tumour suppressor and is also up-regulated in response to a variety of cellular stress conditions including oxidative stress and DNA-damaging agents. Interestingly, DUSP1/MKP-1 is also up-regulated in response to hypoxia at levels found in solid tumours, suggesting that it may play a key role in the regulation of MAPK activities within the tumour microenvironment (Keyse, 2008).

Liu et al. (2005) mentioned that hypoxia induces MKP-1 expression and hypoxia induced MKP-1 protects overactivation of HIF-1 activation through inhibiting ERK kinase activity.

Seo et al. (2006) mentioned about interaction between HSP25 or HSP70i and MKP1 dephosphorylated ERK1/2. Interaction of MKP1 with HSP25 or HSP70i activates MKP1 phosphorylation and then ERK1/2 inactivation, resulting in HSF1

dephosphorylation on serine 307. It shows that DUSP1 gene has interaction with heat shock proteins. It is known that the promoter sequence of the MKP-1 gene contains AP-1 and CRE sites, which respond to protein kinase C and Ca^{2+} /cAMP signaling pathways. Indeed, stimuli which activate protein kinase C, increase the intracellular cAMP content or raise the concentrations of Ca^{2+} , also increase the expression of mRNA for MKP-1 (Engelbrecht et al., 2005). Thus, upregulation during ischemia is possible.

It is demonstrated that MKP-1 is a critical negative regulator in the innate immune response to LPS. It is found that MKP-1 is induced by LPS and plays a critical role in the attenuation of both JNK and p38 in peritoneal macrophages (Zhao et al., 2005).

MKP-1 provide cytoprotection against UV-induced apoptosis. However, this was not observed with other DUSPs. Thus, this finding demonstrates that the survival advantage provided by MKP-1 is not a universal feature of all dual specificity phosphatases (Franklin et al., 1998).

1.4 CXCL2 Gene

The biological purpose of inflammation is to bring fluids, proteins and inflammatory cells such as neutrophils and monocytes from the blood into the damaged tissues to eliminate the injuring agents and trigger the healing and repairing processes. Development of inflammatory reactions is controlled by a number of cellular and molecular components, including proinflammatory cytokines (Yamamoto et al., 2008).

The mammalian immune system has two major components called the innate immunity and the adaptive immunity. The innate immune system serves as a first line of defense against pathogenic organisms. The innate immune system is comprised of a number of cell types, including dendritic cells, macrophages and monocytes, polymorphonuclear cells, natural killer cells, $\gamma\delta$ T cells, and natural killer T cells. The adaptive immunity is a pathogen-specific host defense based on B- and T-lymphocytes (Wang and Liu, 2007). Major function of the innate immune cell is the production of inflammatory mediators such as cytokines and chemokines.

Cytokines are low-molecular-weight (approximately 25 kDa) regulatory proteins or glycoproteins released from various cells, mainly from leukocytes, usually by various activating stimuli, and regulate the development and effector functions of immune cells. Cytokines clearly play an important role in both local and distant organ effects of acute kidney injury (Jang and Rabb., 2008). Chemokines are cytokines with chemoattractant properties for leukocytes. Cells respond to chemokines by their expression of specific membrane-spanning G-protein coupled receptors (Tarzami et al., 2001). Chemokines have three subtypes according to the number of amino acids between the first two cysteines; CC, CXC and CX3C families (Jang and Rabb, 2008). CC chemokines are the most numerous and diverse family, including at least 25 ligands in humans. CXC chemokines are further classified according to the presence of the tripeptide motif glutamic acid-leucine-arginine (ELR) in the NH2 terminal region (Frangogiannis, 2004).

Chemokines can be divided broadly into two categories: homeostatic chemokines are constitutively expressed in certain tissues and may be responsible for basal leukocyte trafficking and formation of the fundamental architecture of lymphoid organs, and inducible chemokines which are strongly upregulated by inflammatory or immune stimuli, actively participating in the inflammatory reactions by inducing leukocyte recruitment (Frangogiannis, 2004).

Cytokines and chemokines are essential for the mobilization of the leukocytes to the site of infection, the initiation of the adaptive immune response, and the initiation of acute phase response; the reactive nitrogen and oxygen species are crucial for the killing of the invading pathogens (Wang and Liu, 2007).

Mononuclear cells play a key role in the synthesis of proinflammatory cytokines. These proinflammatory cytokines can in turn trigger secondary inflammatory cascades, including the production of cytokines, lipid mediators, and reactive oxygen species, as well as the up-regulation of cell adhesion molecules that facilitate the migration of inflammatory cells into tissues. By inducing the expression of inducible nitric oxide synthase and augmenting the production of nitric oxide, proinflammatory cytokines can decrease systemic vascular resistance, resulting in profound hypotension. Moreover, these cytokines also stimulate the procoagulation pathway, leading to thrombosis of microvasculature and impaired tissue perfusion. The

combination of hypotension and microvascular occlusion results in tissue ischemia and ultimately leads to multiple organ failure (Zhao et al., 2005).

Among four subfamilies of chemokines, CXC chemokines such as CXCL8 and its functional homolog CXCL2 are known to show potent neutrophil chemotactic activity (Sonoda et al., 1998).

At inflamed sites, neutrophils deploy a potent antimicrobial arsenal that includes proteinases, antimicrobial peptides and ROS. Although ROS are extremely antimicrobial by virtue of their ability to kill microbial pathogens, in chronic inflammation, the continued production of ROS by neutrophils causes extensive tissue damage. Traditionally, this has been considered as random damage to cellular components (Yamamoto et al., 2008).

Recently, ROS have emerged as signal transduction molecules. In inflammatory cells, ROS contribute to the expression of a variety of different inflammatory cytokines, adhesion molecules and enzymes by activating redox-sensitive transcription factors such as nuclear factor- κ B (NF- κ B) (Droge, 2002).

Once monocytes adhere to endothelial cells from the bloodstream and migrate toward tissues, they differentiate into macrophages. During homeostasis, monocytes and macrophages phagocytose and remove senescent and apoptotic cells, whereas during inflammation they are the main effectors of innate immunity because of their antimicrobial activity and production of proinflammatory cytokines (Yamamoto et al., 2008). In human monocytes, CXCL8 production is induced by ROS, including by H_2O_2 (Josse et al., 2001) via Erk-activated NF- κ B (Zeng et al., 2003).

CXC chemokines are also up-regulated in remote organs, including lung, and play an important role in the development of remote organ injury after liver ischemia/reperfusion (Lentsch et al., 2000)

Chemokine induction during the inflammatory response after ischemia reperfusion injury has been reported in several organs such as brain, heart, liver, and kidney (Jang and Rabb, 2008).

CXCL2 (MIP-2) is a chemokine that is in CXC family and chemotactic for neutrophils (Tarzami et al., 2001). MIP-2 is known to amplify inflammation in the kidney. Such chemokine activation would initiate leukocyte migration into renal

tissue and activate ICAM-1 to facilitate leukocyte adherence and infiltration (Behrends et al., 2007).

The expression of MIP-2 upon exposure of cells to ischemia/hypoxia has recently been reported. MIP-2 expression has been shown to be induced in human microglia, cerebromicrovascular endothelial cells and astrocytes exposed to hypoxia. In renal ischemia reperfusion injury MIP-2 expression is upregulated (Tarzami et al., 2001).

In post-ischemic tissues, chemokines are induced by reactive oxygen species (ROS), cytokines complement activation, TLR-mediated pathways, and the IL-8/CXCL8, human analog of rat CXCL2, plays a crucial role in neutrophil recruitment in the post-ischemic kidney mediating tissue injury via cytokines, free radical intermediates, and proteases. IL-8 expression was reported to be increased during reperfusion of living and cadaveric donor kidney allografts in a clinical study and its expression correlated with the ischemic time imposed on the allograft. Growth-related oncogene (GRO)- α /keratinocytederived chemokine (KC, a mouse analog of human IL-8) was induced in a murine ischemic acute kidney injury model. Neutralization of GRO- α /KC and macrophage inflammatory protein (MIP)-2 was reported to attenuate acute kidney injury. KC, the analog of rat CXCL2 is also an attractive early biomarker of acute kidney injury based on both murine and human studies (Jang and Rabb, 2008).

CXC chemokines (i.e., IL-8 and homologues) are intricately involved in the process of neutrophil recruitment with the functions of cellular adhesion molecules (Lentsch et al., 2000).

Neutrophil accumulation was shown to be mainly mediated by the C-X-C chemokine MIP-2. Chronic hypoxia induces elevated levels of MIP-2 mRNA as part of a pronounced inflammatory response.

Hypoxic MIP-2 induction occurs exclusively via the NF- κ B pathway. NF κ B plays a pivotal role in the induction of a variety of genes involved in immune and inflammatory responses. Increased NF- κ B binding was observed under hypoxia (Zampetaki et al., 2004).

1.5 Reverse Transcriptase PCR (RT-PCR)

In RT-PCR method, mRNAs are reverse transcribed into complementary DNA (cDNA) which is then amplified by PCR, and analyzed by agarose gel electrophoresis. RT-PCR (reverse transcription-polymerase chain reaction) is one of the available techniques for mRNA detection and quantification. Compared to Northern blot analysis, RT-PCR can be used to quantify mRNA levels from much smaller samples (Dharmaraj, 2007).

However, this technique is semi-quantitative. After agarose gel electrophoresis, the PCR products become visible and can be quantified.

The application of RT-PCR involves cloning mRNA sequences in the form of complementary DNA, and allowing libraries of cDNA (cDNA libraries) to be created which contain all the mRNA sequences of genes expressed in a cell. Furthermore, this method allows the creation of cDNA constructs cloned by RT-PCR and enables the expression of genes at the RNA and protein level for further study. (“Reverse Transcription Polymerase Chain Reaction”, 25 March 2009).

1.5.1 Touch Down PCR

Touchdown PCR is modification of conventional PCR that may result in a reduction of nonspecific amplification (Prilusky, 2004). The annealing temperature is decreased in increments for every subsequent set of cycles. The primer will anneal at the highest temperature. The amplification at high temperatures involves the most specific binding. Thus, this fragment will be amplified at lower temperatures. It will avoid the nonspecific binding at lower temperatures.

1.5.2 Gradient PCR

Gradient PCR can be used for the determination of optimum annealing temperature. It is a technique that allows the empirical determination of an optimal annealing temperature using the least number of steps. This optimization can often be achieved in one experiment (Prezioso et al., 2000).

1.5.3 Multiplex PCR

Multiplex polymerase chain reaction (PCR) is a variant of PCR in which two or more target sequences can be amplified by including more than one pair of primers in the same reaction. Since it was first described in 1988, this method has been successfully

applied in many areas of DNA testing, including gene deletion analysis , mutation and polymorphism analysis, quantitative analysis, and reverse- Transcriptase (RT)-PCR (Markoulatos et al., 2002). The internal control gene and the gene of interest can be amplified in the same tube. Control gene products are required for the maintenance of the basal cellular function and are constitutively found in all human cells. These genes are also called housekeeping genes. Housekeeping genes can be used to calibrate measurements of gene expression (Eisenberg and Levanon, 2003). The conditions will be the same for internal control and gene of interest in multiplex reaction.

1.6 Real-Time PCR

Real Time PCR has established itself as the most sensitive and specific quantitative PCR method (Dorak, 2006). It allows quantification of rare transcripts and small changes in gene expression. Generally two quantification types in real-time RT-PCR are possible. (i) a relative quantification based on the relative expression of a target gene versus a reference gene. To investigate the physiological changes in gene expression, the relative expression ratio is adequate for the most purposes (Pfaffl, 2001). (ii) absolute quantification calculates the copy number of the gene usually by relating the PCR signal to a standard curve (Schmittgen and Livak, 2008). Real-Time PCR applications are gene expression analysis, microarray result validation, gene knock-down result validation, specific analysis of oncological or virological research parameters, food pathogens and spoilage test, screening for genetically modified organisms, SNP genotyping and mutation detection (Roche Diagnostics Corporation, 26 march 2009).

Real-time PCR is a powerful tool to quantify gene expression. Some examples of quantitative gene expression studies include: as a validation of protein levels; as a validation of the extent of transcription of a gene; to study the difference in expression of a gene in the diseased state compared to the normal state; change in gene expression during cell differentiation or development; change in expression for cells that are exposed to a chemical substance (e.g., drug, toxin, hormone or cytokine) ; quantification of noncoding RNA gene expression; to validate the effectiveness of small interfering RNA or antisense oligonucleotides; and as a diagnostic tool (Schmittgen and Livak, 2008).

Real-time PCR is a very promising quantitative method based on the concept of monitoring the PCR reaction in the thermal cycler as it progresses (Marone et al., 2001). However, this technique requires a relatively large amount of cDNA when compared with semi quantitative RT-PCR.

The quantitative endpoint for real-time PCR is the threshold cycle (C_T). The C_T is defined as the PCR cycle at which the fluorescent signal of the reporter dye crosses an arbitrarily placed threshold. By presenting data as the C_T , one ensures that the PCR is in the exponential phase of amplification. The numerical value of the C_T is inversely related to the amount of amplicon in the reaction (i.e., the lower the C_T , the greater the amount of amplicon) (Schmittgen and Livak, 2008). Error and efficiency are also important for Real-time PCR applications. Error must be below 0.2 and efficiency must be between 1.8 and 2.2. Efficiencies and errors of genes and internal control are calculated by standard curves with dilution series of undiluted, 1/10, 1/100 and 1/1000 diluted samples. $\Delta\Delta C_t$ is a method used for relative quantification. The advantage of using the comparative C_T method is that the need for a standard curve is eliminated in each run. This increases throughput because wells no longer need to be used for the standard curve samples. It also eliminates the adverse effect of any dilution errors made in creating the standard curve samples (Pfaffl, 2005).

1.7 Aim of the Study

The aim of the study was to investigate the effects of ischemia reperfusion, IP and HP on DUSP1 and CXCL2 gene expression in rat kidney tissues using rt-PCR and real-time q-PCR. Gene expression analysis was performed using RT-PCR and quantitative real-time PCR. These two methods were compared with previously obtained microarray results. Particularly the expression changes upon heat preconditioning, ischemic preconditioning and only ischemia reperfusion injury were analyzed. For all three groups, the duration of ischemia was 45 min but reperfusion time was varied between 0 and 60 min. Thus, the effect of reperfusion time on gene expression was also studied and compared for each group.

2. MATERIALS AND METHODS

2.1 Materials and Laboratory Equipment Used

2.1.1 Equipments Used

Balances	Precisa BJ 610C (Switzerland) Precisa 620C SCS (Switzerland)
Centrifuges	Beckman [®] Coulter Microfuge (USA)
Deep Freezers and Refrigerators	-80°C Heto Ultrafreeze 4410 (Denmark) -20°C Arçelik (Turkey) +4°C Arçelik (Turkey)
Electrophoresis equipments	E-C Minicell [®] primo EC320(USA) E-C Midicell [®] Primo EC330 (USA)
Gel Documentation System	UVI PhotoMW Version 99.05 for Windows
Magnetic Stirrer	Magnetic stirrer standard unit (Germany)
Ultrapure Water System	USF-Elga UHQ (USA)
PCR	Techne TC-412 (England)
Micropipettes	Eppendorf (Germany)
Real time PCR equipment	Roche Lightcycler 2.0
Transilluminator	Biorad UV Transilluminator 2000
Vortex	Heidolph Reax top

2.1.2 Chemicals, Enzymes, Markers and Buffers Used

2.1.2.1 Chemicals

Agarose	AppliChem
Boric acid	Amresco
EDTA	AppliChem
EtBr	Amresco
NaOH	Riedel-de Haën
Primers(Real-Time PCR)	IDT DNA
Primers (RT-PCR)	Genova

2.1.2.2 Enzymes

Taq DNA Polymerase

Fermentas

2.1.2.3 Markers

Gene Ruler™ 100bp plus DNA Ladder(SM 0323)

Fermentas

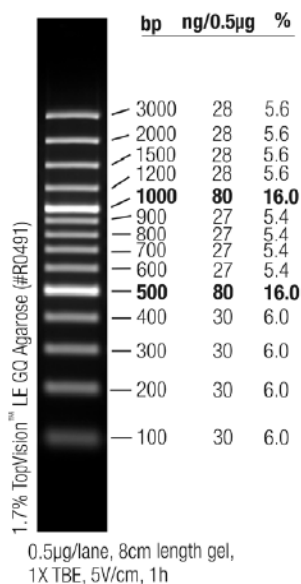


Figure 2.1: Information on Gene Ruler™ 100bp plus DNA Ladder (Fermentas).

GeneRuler™ DNA Ladder Mix(SM 0333)

Fermentas

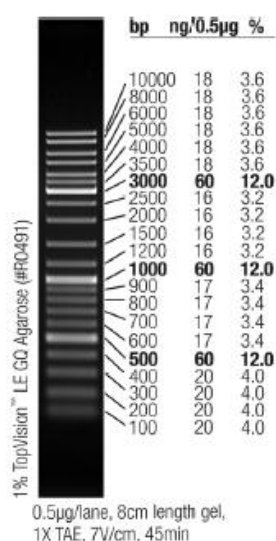
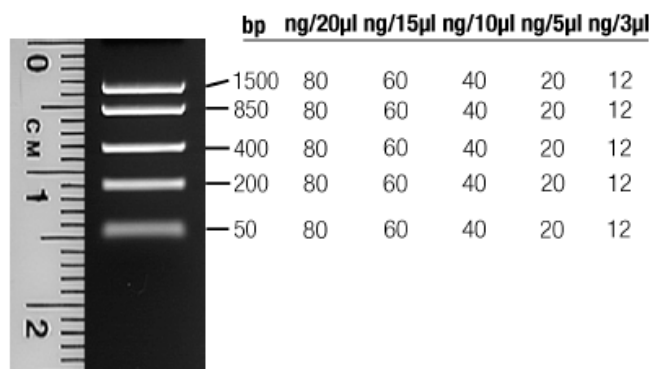


Figure 2.2: Information on GeneRuler™ DNA Ladder Mix (Fermentas).



Electrophoresis conditions:
2% agarose gel, 1X TBE buffer, 7V/cm, 14min.

Figure 2.3: Information on FastRuler™ DNA Ladder Low Range (Fermentas).

2.1.2.4 Buffers

TBE (Tris-Borate-EDTA) Buffer (10X)

Tris (hidroxymetyly) aminomethane	121.14 g
Boric Acid	61.83 g
EDTA	7.44 g

Add ddH₂O to 1 liter

Mini Agarose Gel (1,5%)

Agarose	0.9 g
TBE buffer (1X)	60 mL

Add 3 µL EtBr before pouring the gel into tray.

Midi Agarose Gel (1%)

Agarose	2.25 g
TBE buffer (1X)	150 mL

Add 7.5 µL EtBr before pouring the gel into tray.

2.1.3 Molecular Biology Kits Used

cDNA synthesis kit	Roche – Transcriptor First Strand cDNA synthesis kit
Real-Time PCR	Roche – LightCycler® TaqMan Master Kit
RT-PCR	Invitrogen-SuperScript™ III One-Step RT-PCR System with Platinum® Taq DNA Polymerase

2.1.4 RNA samples used in this study

Male Wistar rats, 30 weeks old and 220-358 g in weight were used for analysis of gene expression patterns of CXCL2 and DUSP1 genes upon ischemia/reperfusion (I/R), with ischemic preconditioning (IP) and heat preconditioning (HP). The rats were anesthetized by intraperitoneal atropine, ketamin and rompun. The retroperitonium was visualized following abdominal dissection. Ischemia was performed by clamping the left kidney. Rats were subjected to ischemia for 45 min followed by reperfusion. Heat shock was performed 24 hours before the ischemia by placing the rats at 42 °C for 5 min. Ischemic preconditioning was applied by 5 min of ischemia and 10 min of reperfusion for 3 cycles (Table 2.1). These procedures were done in Erlangen University. In this thesis work, RNA samples isolated from those rat kidney tissues previously were used.

Eleven samples were chosen according to their RNA concentration values. 9 samples were ischemia reperfusion subjected rats and 2 samples were sham operated rats which were subjected to surgical manipulation, without the induction of renal ischemia, thus serving as control groups.

Table 2.1: RNA samples used in the study

Sample No	Samples name	Explanation
1	HP_I45_R0_A	After heat preconditioning, rats were subjected to left renal ischemia for 45 min.
3	HP_I45_R15_A	After heat preconditioning, rats were subjected to left renal ischemia for 45 min followed by 15 min reperfusion
5	HP_I45_R60_A	After heat preconditioning, rats were subjected to left renal ischemia for 45 min followed by 60 min reperfusion
14	I45_R0_B	Rats were subjected to left renal ischemia for 45 min
16	I45_R15_B	Rats were subjected to left renal ischemia for 45 min followed by 15 min reperfusion
18	I45_R60_B	Rats were subjected to left renal ischemia for 45 min followed by 60 min reperfusion

Table 2.1 (contd) : RNA samples used in the study

Sample No	Samples name	Explanation
26	IP_I45_R0_B	After ischemic preconditioning was applied, rats were subjected to left renal ischemia for 45 min.
28	IP_I45_R15_B	After ischemic preconditioning was applied, rats were subjected to left renal ischemia for 45 min followed by 15 min reperfusion.
29	IP_I45_R60_A	After ischemic preconditioning was applied, rats were subjected to left renal ischemia for 45 min followed by 60 min reperfusion.
31	Sham A	Rats were subjected to surgical manipulation without the induction of renal ischemia
32	Sham B	Rats were subjected to surgical manipulation without the induction of renal ischemia

2.1.5 RNA isolation

RNA isolation and microarray experiments were applied in Erlangen University as follows: RNA isolation was done with with peqGOLD TriFast™. After treatment, total cellular RNA was extracted and pooled. RNA quality and quantity was assessed

using a Bioanalyzer 2100 (Agilent, Santa Clara, CA) and 6000NanoAssay (Agilent, Santa Clara, CA) (Table 2.2). High throughput gene expression profiling was performed using cDNA-microarrays (Affymetrix, Santa Clara, CA). Differentially expressed genes were further characterized in silico (Table 2.3).

Table 2.2: Properties of RNAs

Nr	Samples	Probe1	Probe2	Mixture (µg)	Concentr. in (µg/µl)	A 260/280	µl
1	HP_I45_R0_A	A 53 a	A 78	ca. 30	1.61	2.3	3
3	HP_I45_R15_A	A 73 b	A 74 b	ca. 30	1.57	2.32	21
5	HP_I45_R60_A	A 44 a	A 43	ca. 30	1.97	2.27	7
14	I45_R0_B	A 14	A 18	ca. 30	1.55	1.42	4.5
16	I45_R15_B	A 35	A 36 a	ca. 30	1.85	1.47	3.5
18	I45_R60_B	A 19	A 67 a	ca. 30	2.32	1.62	5.5
26	IP_I45_R0_B	A 65	A 96	30	1.87	1.96	12
28	IP_I45_R15_B	A 60 a	A 62 a	30	2.52	1.81	7
29	IP_I45_R60_A	B 55	B 56	30	1.80	1.64	7.5
31	Sham_A	A 39 a	A 30	30	2.07	1.96	9
32	Sham_B	A 29 a	A 42	30	2.25	2.27	10

Table 2.3: Microarray results of DUSP1 and CXCL2 gene (Tektaş, 2006 and Yılmaz, 2009).

SAMPLES	DUSP1/shamA ratio	DUSP1/shamB ratio	CXCL2/shamA ratio	CXCL2/shamB ratio
HP-I45-R0-A (1)	-1.27	-1.51	-0.18	0.14
HP-I45-R15-A (3)	-1.15	-1.2	-0.31	0.24
HP-I45-R60-A (5)	1.6	1.47	1.66	1.52
I45-R0-B (14)	-0.97	-1.24	0.03	-0.04
I45-R15-B (16)	-0.79	-1.01	-0.35	-0.16
I45-R60-B (18)	1.14	1.18	1.92	1.94
IP-I45-R0-B (26)	1.06	1.19	1.49	1.53
IP-I45-R15-B (28)	1.2	1.1	0.91	1.01
IP-I45-R60-A (29)	1.05	1	1.44	1.52

2.2 RT-PCR

PCR was used to amplify the target DNA sequences on CXCL2 and DUSP1 gene. RNAs were used as templates. Reverse transcription of mRNA samples and amplification steps were performed by SuperScriptTM III One-Step RT-PCR System involving SuperScriptTM III RT/ Platinum[®] Taq DNA polymerase enzyme complex. A standard PCR mixture was used to amplify sequences of CXCL2 and DUSP1 gene. RNA sample concentrations were determined previously as ($\mu\text{g}/\mu\text{l}$). RNA template concentrations that were used for PCR were determined in ng range and 1:10 dilutions were applied (Table 2.4, 2.5, 2.6 and 2.7). The template amounts of CXCL2 multiplex experiment were determined as twice of the other experiments.

Table 2.4: PCR mix of heat preconditioning samples for rt-PCR

Heat Preconditioning			
Ingredients	Volume (μl)		
	Sample No 1	Sample No 3	Sample No 5
dH ₂ O	20.48	20.47	20.59
Reaction mix ¹	25	25	25
Primer of gene	1	1	1
Primer of β -actin	1	1	1
Template	0.62	0.63	0.51
Superscript III RT/Platinum Taq Mix	1.9	1.9	1.9
FINAL Volume	50 μl	50 μl	50 μl

Table 2.5: PCR mix of Ischemia Reperfusion samples for rt-PCR

Ischemia Reperfusion			
Ingredients	Volume (μl)		
	Sample No 14	Sample No 16	Sample No 18
dH ₂ O	20.56	20.67	20.57
Reaction mix	25	25	25
Primer of gene	1	1	1
Primer of β -actin	1	1	1
Template	0.54	0.43	0.53
Superscript III RT/Platinum Taq Mix	1.9	1.9	1.9
FINAL Volume	50 μl	50 μl	50 μl

¹ The 2X Reaction Mix included in the kit consists of a proprietary buffer system that has been optimized for reverse transcription and PCR, Mg²⁺, dNTPs, and stabilizers.

Table 2.6: PCR mix of Ischemic preconditioning and Sham samples for rt-PCR

Ingredients	Ischemic Preconditioning			Sham	
	Volume(μ l)				
	Sample No 26	Sample No 28	Sample No 29	Sham A	Sham B
dH ₂ O	20.57	20.70	20.54	20.62	20.65
Reaction mix	25	25	25	25	25
Primer of gene	1	1	1	1	1
Primer of β -actin	1	1	1	1	1
Template	0.53	0.40	0.56	0.48	0.45
Superscript III RT/Platinum Taq Mix	1.9	1.9	1.9	1.9	1.9
FINAL Volume	50 μ l	50 μ l	50 μ l	50 μ l	50 μ l

Table 2.7: PCR mix of Sham A, Sham B and Sample 28 with only β -actin primers for rt-PCR

Controls			
Ingredients	Volume(μ l)		
	ShamA (with only β -actin)	ShamB (with only β -actin)	Sample 28 (with only β -actin)
dH ₂ O	21.62	21.65	21.70
Reaction mix	25	25	25
Primer of β -actin	1	1	1
Template	0.48	0.45	0.40
SuperscriptIII	1.9	1.9	1.9
RT/Platinum Taq Mix			
FINAL VOLUME	50 μ l	50 μ l	50 μ l

2.2.1 Oligonucleotide primers

Complementary DNA sequences of CXCL2 and DUSP1 genes were used for primer design via “Primer 3” and “Amplify 3” softwares. The primer sets were analyzed for dimer formation, non-specific binding and hairpin formation.

Table 2.8: Oligonucleotide primers used in the RT-PCR experiments in this study

Gene	Primer	Primer Sequence	T _m (°C)	GC%	Product Size (bp)
DUSP1	PrimerF	TGAAGCAGAGGCGGAGTATT	60.0	50.0	222 bp
	PrimerR	TGATGGGGCTTTGAAGGTAG	60.1	50.0	
CXCL2	Primer3F	AGGGTACAGGGGTTGTTGTG	59.7	55.0	179 bp
	Primer3R	GGCTTCAGGGTTGAGACAAA	60.2	50.0	
B-actin	FPrimer	TTGCTGACAGGATGCAGAAG	60.14	50.0	122 bp
	RPrimer	CAGTGAGGCCAGGATAGAGC	59.97	60.0	
CXCL2 (second primer)	Primer4F	CAGGGTACAGGGGTTGTTGT	59.7	55.0	180 bp
	Primer4R	GGCTTCAGGGTTGAGACAAA	60.2	50.0	

2.2.2 PCR conditions

The PCR condition for DUSP1 gene are shown in Table 2.9.

Table 2.9: PCR conditions for DUSP1 gene

Phase	Temperature (°C)	Time	Cycle Number
cDNA synthesis	55	30 min	1
Initial Denaturation	94	2 min	1
Denaturation	94	15 sec	32
Annealing	53	90 sec	
Extension	68	50 sec	
Final Extension	68	5 min	1

The PCR condition for CXCL2 gene are shown in Table 2.10.

Table 2.10: PCR conditions for CXCL2 gene

Phase	Temperature (°C)	Time	Cycle Number
cDNA synthesis	55	30 min	1
Initial Denaturation	94	2 min	1
Denaturation	94	15 sec	35
Annealing	58	30 sec	
Extension	68	30 sec	
Final Extension	68	5 min	1

2.2.3 PCR cycle conditions

Optimum PCR cycle conditions were determined both for CXCL2 and DUSP1 genes. HP_I45_R15_A sample was used for determining the optimal PCR cycle number. The optimum cycle number was determined by comparing the band values of 23., 26., 29., 32., 35., 38., and 40th cycles. Ten µl PCR mix was withdrawn by stopping reaction at 23., 26., 29., 32., 35., 38., and 40th cycles. Bands were observed in 1.5% agarose gels. Densitometric analysis of the PCR products of these cycles were performed by using “Biocapt Software” (Vilber-Lourmat, Marne LaValle, France).

2.2.4 PCR optimization

PCR reactions generally need to be optimized. The optimization can be done by determination of appropriate annealing temperature, annealing time, extension temperature and extension time. Amplification of correct sequence, correct primer sets and appropriate cycle numbers and Mg concentrations are also important parameters for optimization. Different primer sets; different annealing temperatures

and time; different extension temperatures and time; gradient PCR and touch-down PCR were used for PCR optimization throughout this study (Table 2.11 and 2.12)

DUSP1 gene optimization was performed with sample number 3, 18, 26 by comparing 23., 26., 29., 32., 35., 38., and 40th cycles. PCR amplification was conducted as follows: 55 °C (30 min) for cDNA synthesis, 94 °C (2 min) for initial denaturation and then 32 cycles at 94 °C (15 sec) / 53 °C (90 sec) / 68 °C (50 sec) and a final extension at 68 °C (5 min).

CXCL2 gene optimization was performed with sample number 3. DNA contamination experiment and control of primer dimers were firstly done. DNA contamination experiment was carried out with 0.4 µl Taq DNA polymerase (2 units) instead of Superscript III RT/Platinum Taq Mix. All of the samples were controlled. Control of dimers was done again with 0.4 µl Taq DNA polymerase (2 units). PCR reaction mixtures containing only one primer of each primer pairs and both primers of the primer pairs for CXCL2 were prepared. Each forward and reverse primers were diluted at the ratio of 1:10. All primers had a final concentration of 10 µM.

Table 2.11: Temperature and time optimizations of CXCL2 for rt- PCR

	PCR type	Annealing		Extension		Sample that is used (Number)
		Temperature °C	Time (sec)	Temperature °C	Time (sec)	
1.primer	RT-PCR	56	30	68	30	HP_I45_R15_A(3)
	RT-PCR	60	30	68	30	HP_I45_R15_A(3)
	Gradient PCR	55 -65	30	68	30	HP_I45_R15_A(3)
	RT-PCR	58	30	68	30	HP_I45_R15_A(3)
	RT-PCR	62	20	68	50	HP_I45_R15_A(3)
	RT-PCR	62	10	68	50	HP_I45_R15_A(3)
	RT-PCR	60	40	68	10	HP_I45_R15_A(3)
	RT-PCR	62	30	68	11	HP_I45_R15_A(3)
	1,8 mM and	62	30	68	50	HP_I45_R15_A(3)
	2 mM	62	20	68	30	HP_I45_R15_A(3)
	MgSO ₄	62	20	68	50	HP_I45_R15_A(3)

Table 2.12: Temperature and time optimizations of CXCL2 for rt- PCR

	PCR type	Annealing		Extension		Sample that is used (Number)
		Temperature (°C)	Time (sec)	Temperature (°C)	Time (sec)	
2. Primer	RT-PCR	53	90	68	50	HP_I45_R15_A(3)
	RT-PCR	56	30	68	50	HP_I45_R15_A(3)
	Gradient PCR	55-65	30	68	30	HP_I45_R15_A(3)
	RT-PCR	64	30	68	30	HP_I45_R15_A(3)
	Touch-down PCR	63-48	30	68	30	HP_I45_R15_A(3)
	Touch-down PCR	70-64	30	68	30	HP_I45_R15_A(3)& I45_R60_B(18)
	Touch-down PCR	70-64	30	68	20	HP_I45_R15_A(3)
	1,8 mM and 2 mM MgSO ₄	64	30	68	50	HP_I45_R15_A(3)

2.2.5 Touchdown PCR

Three different touchdown PCR optimization experiments were performed using the second primer set of CXCL2 gene.

The first touchdown PCR program involved decreasing the annealing temperature by 1 °C at every cycle from 63 °C to a touchdown of 48 °C (30 sec) at which 11 cycles were carried out.

The second Touch Down PCR experiment was carried out according to the following program: 55 °C (30 min) for cDNA synthesis, 94 °C (2 min) for initial denaturation and then 40 cycles at 94 °C (15 sec), the annealing temperature was decreased by 1 °C at every cycle from 70 °C to a touchdown of 64 °C (30 sec) at which 35 cycles were carried out; 68 °C (30 sec) and a final extension at 68 °C (5 min).

The third Touch Down PCR experiment was different from the second touchdown PCR only by extension time. Extension time was reduced from 30 seconds to 20 seconds.

2.2.6 Gradient PCR

Eleven tubes of the same PCR were run using a thermocycler with different annealing temperatures. The total reaction volume in each tube was 25 µl.

Gradient PCR was used to optimize the annealing temperature of CXCL2 gene by a gradient from 65 °C to 55 °C with first and second primer sets.

2.2.7 Multiplex PCR

The PCR reactions of DUSP1 and CXCL2 gene were performed with two primer sets. One set belonged to the gene of interest, either DUSP1 or CXCL2, and the other to the internal control gene, which was chosen as beta actin throughout this study as a housekeeping gene in eukaryotes (Thellin et al., 1999).

2.3 Agarose Gel Electrophoresis of PCR Products

The DNA bands belonging to the PCR products were visualized on a 1.5 % agarose gel. 1.5 % mini or midi gels were prepared. Mini gels were prepared with 0.9 g agarose and 3 µl ethidium bromide added into 60 mL of 1X TBE buffer, which was diluted from 10X stock TBE solution. Midi gels (1.5 %) were prepared with 2.25 g agarose and 7.5 µL ethidium bromide added into 150 mL of 1X TBE buffer. 10 µL of PCR product was mixed with 5 µL 1X loading dye and loaded into the wells. GeneRuler™ 100bp Plus marker (Fermentas) was used as DNA ladder. The gels were run in 1X TBE buffer, at 100V for 30 min, and observed under UV light using a transilluminator. Gel images were obtained using UV PhotoMW software.

2.4 Real Time PCR

2.4.1 cDNA synthesis

cDNA was synthesized from isolated total RNA by using Transcriptor First Strand cDNA Synthesis Kit (Roche).

The protocol used for Transcriptor First Strand cDNA synthesis Kit from Roche is as follows: template-Primer Mix components were prepared firstly using the components shown in Table 2.13. Reverse transcription can be done using either anchored-oligo (dT)₁₈ primer or random hexamer primer. Random hexamer primer was chosen.

Table 2.13 : Template-Primer Mix components

Template-PCR mix		
Component	Volume (μl)	Final Concentration
Total RNA (ng)	1	1 ng up to 4 μg
Anchored oligo (dT) ₁₈ primer, 50 pmol/μL	-	2.5 μM
or random hexamer primer, 600 pmol/μL	2	60 μM
Water, PCR grade	8.4	
Total Volume	11.4	

After preparing template-primer mix, the template-primer mixture was denatured by heating the tube at 65°C for 10 min in a thermal cycler with a heated lid to minimize evaporation. This step ensured denaturation of RNA secondary structures. After this step, the mixture was immediately transferred onto ice.

Remaining components of RT mix were added as in the order given in Table 2.14.

Table 2.14 : The volumes of the remaining components of the RT mix

Component	Volume (μl)	Final concentration
Transcriptor High Fidelity Reverse Transcriptase Reaction Buffer, 5× concentrated	4	1× (8 mM MgCl ₂)
Deoxynucleotide Mix, 10 mM each	2	1 mM each
Protector RNase Inhibitor, 40 U/μl	0.5	20 U
DTT	1	5 mM
Transcriptor High Fidelity Reverse Transcriptase	1.1	10 U

After preparing the second mixture by adding these five components, the template-primer mixture was removed from the thermal cycler and the RT mixture was added to it to make the total volume 20 μl. The components were mixed well by pipetting

and the tubes were spun in a microfuge. Thereafter, the tubes were placed into thermal cycler again. The mixture was incubated at 50 °C for 30 min. Transcriptor High Fidelity Reverse Transcriptase was inactivated by heating to 85 °C for 5 min. The reaction was then stopped by placing the tubes on ice. The synthesized cDNAs were stored at -20 °C for later use. cDNAs of 11 samples were synthesized according to this protocol.

2.4.2 Real-Time PCR conditions

LightCycler TaqMan Master Kit (Roche) was used for Real-Time PCR. The reaction was performed using Roche LightCycler® 2.0 equipment. Master mix was prepared according to LightCycler TaqMan Master Kit instructions (Roche). Total volume of 10 µl from FastStart Taq DNA Polymerase was pipetted into Reaction Mix. This mixture was called LightCycler® TaqMan® Master. Twenty µM solutions of the PCR primers were prepared. Twenty µl was withdrawn from 100 µM primer solutions and 80 µl water was added. The order of adding components is indicated in the Table 2.15.

Table 2.15: Real-Time PCR Mix for a 20 µl reaction volume

Component	Concentration	Volume (µl)	Final Concentration
Water, PCR-grade		9	
Primer Mix (UPL Ref. Gene Assay)	20 µM	0.4	200 nM
Primer (Gene of Interest-GOI) forward	20µM	0.4	200 nM
Primer (GOI) reverse	20µM	0.4	200 nM
Probe (UPL Ref. Gene Assay)	10µM	0.4	400 nM
UPL probe (GOI)	10µM	0.4	400 nM
LightCycler® TaqMan® Master	5X	4	1X

The components were mixed gently by pipetting up and down and were not vortexed. Fifteen µl PCR mix was pipetted into each precooled LightCycler® Capillary and 5

μ l of the cDNA template was added. At the end, each capillary was sealed with a stopper. Five tubes for the standard curve: undiluted; 1:10 diluted; 1:100 diluted; 1:1000 diluted; and negative control were prepared. Two μ l cDNA was used for undiluted sample. Dilutions were made both for β -actin and for the samples. Before placing the tubes into the real-time PCR instrument, they were centrifuged at 2000 rpm for 1 min in a benchtop centrifuge.

2.4.3 Selection of Primers and probes

Probes and primers that were used in this thesis were selected from Universal Probe Library (Roche) according to manufacturer's instructions. Probe 85 was used for CXCL2 gene and Probe 17 was used for DUSP1 gene (Table 2.16).

Table 2.16: Real-time PCR primers selected from Universal Probe Library (Roche) according to probes

Primer	Length	Position	T _m (°C)	%GC	Sequence	Amplicon
CXCL2 Left	23	333-355	59	43	ggatcgtccaaaagataactgaac	72 nt
CXCL2 Right	21	384-404	60	52	ggtacaggagcccatgttctt	
DUSP1 Left	20	597-616	59	55	gtgcctgacagtgcagaatc	94 nt
DUSP1 Right	20	671-690	59	60	ccaggtacaggaaggacag	

Water had to be added to the primers and Integrated DNA Technologies homepage was used to calculate the water volume to be added. Water resuspension volumes of DUSP1 and CXCL2 primers were calculated according to manufacturer's instructions provided with the primers (Table 2.17).

Table 2.17: The volume of water that has to be added to each primer to reach 100 μ M concentration

Gene	Water volume to be added (μ l)
DUSP1 R	668
DUSP1 L	650.1
CXCL2 R	714.6
CXCL2 F	933

Primer solutions of 100 μ M were prepared. Reverse and forward primers were not mixed together to avoid dimer formation. Primers were vortexed very shortly (four times), but probes were not vortexed and they were stored in dark.

The package inserts files were downloaded from Roche's homepage. Real-time PCR Protocol for use with LightCycler® TaqMan® Master protocol was obtained from the package inserts files. Table 2.18 shows the PCR parameters that must be programmed for a LightCycler® Carousel-based System PCR run with the LightCycler® Taq- Man® Master.

Table 2.18: PCR parameters for a LightCycler® Carousel-based System PCR run with the LightCycler® Taq- Man® Master.

Analysis Mode	Cycles	Target Temperature (°C)	Hold Time	Acquisition Mode
Pre-Incubation	1	95	10 min	None
Amplification	45	95	10 sec	None
		60	30 sec	None
		72	1 sec	single
Cooling	1	40	30 sec	None

The protocol for a Universal Probe Library assay using the LightCycler TaqMan® Master consists of the following parts: pre-Incubation for activation of FastStart Taq DNA Polymerase and denaturation of the DNA, amplification of the target cDNA and cooling the rotor and thermal chamber.

In a multicolor reaction, the wavelengths of light emitted by the dyes overlap, causing one channel to pick up signals from a dye measured by another channel. This bleed-over of fluorescence signal can result in uninformative data. Color

compensation experiment is applied to correct the bleed-over (Roche Applied Science). Channel 530 was used for CXCL2 and DUSP1 gene, channel 560 for β -actin. Both channels were selected during analyses.

2.4.4 Properties of the probes

A good example for selection of probes is the use of “ Universal Probe Library” of Roche . Universal ProbeLibrary is based on only 165 short hydrolysis probes, labeled at the 5' end with fluorescein (FAM) and at the 3' end with a dark quencher dye. Locked Nucleic Acids (LNA) are incorporated into the sequence of each UPL probe in order to maintain the specificity and melting temperature that hybridizing qPCR probes require (Roche Diagnostics Corporation, 26 march 2009).

LNA's are bicyclic DNA nucleotide analogues with increased binding strengths compared to standard DNA nucleotides. For this reason, LNA- based probes need to be only 8–9 bases long to achieve a T_m of 68 °C - 69°C under standard conditions (Dorak, 2005).

2.4.5 Real time PCR optimization

Table 2.19: The experiments conducted for real time PCR optimization

Gene	The sample that is used, (sample number)	Type of assay	Dilution series
DUSP1	HP_I45_R15_A (3)	multiplex	undiluted, 1/10, 1/100 , 1/1000
CXCL2	HP_I45_R15_A(3)	multiplex&singleplex	undiluted, 1/10, 1/100 , 1/1000
CXCL2	HP_I45_R15_A(3)	multiplex&singleplex	undiluted, 1/10, 1/100 , 1/1000
CXCL2	HP_I45_R15_A (3)	multiplex&singleplex	undiluted, 1/10, 1/100 , 1/1000
CXCL2	Sham B	multiplex	undiluted, 1/10, 1/100 , 1/1000
CXCL2	Sham B	singleplex	undiluted, 1/10, 1/100 , 1/1000
CXCL2	HP_I45_R15_A (3)	multiplex&singleplex	Undiluted, 1/2 , 1/4 , 1/8

The first real time PCR optimization for DUSP1 gene optimization was done with DUSP1 gene as multiplex assay with dilution series of undiluted, 1/10, 1/100, 1/1000 to calculate the efficiency and the error of DUSP1 and β -actin. The standard curve experiment was set with sample number 3 (HP_I45_R15_A) (Table 2.19).

Real time PCR optimization experiments for CXCL2 gene were done as singleplex and multiplex assays (Table 2.19). The composition of multiplex assay mixtures were shown in Table 2.15. Singleplex assay components are given in Table 2.20.

Table 2.20: Components of Singleplex assay

Components	Volume (μl)
Water, PCR-grade	10.4
Primer (GOI) forward	0.2
Primer (GOI) reverse	0.2
UPL probe (GOI)	0.2
LightCycler® TaqMan® Master,	4
TOTAL VOLUME	15

Four of the PCR optimization experiments of CXCL2 gene were done using sample 3 (HP_I45_R15_A). Two of them were done using Sham B which has higher RNA concentration than sample 3. Dilution series of undiluted, 1/10, 1/100, and 1/1000 dilution samples were prepared with 2 μl cDNA. Dilution series of undiluted, 1/2, 1/4, and 1/8 diluted samples were done using 10 μl cDNA (Table 2.19).

Primer and probe volumes of CXCL2 gene were increased from 0.4 μl to 0.6 μl using sample 16 (I45_R15_B).

2.5 Expression Analysis

2.5.1 Relative Quantification of the Samples of RT-PCR

The volumes of PCR products of all samples were calculated by using “Biocapt Software” (Vilber-Lourmat, Marne LaValle, France). After the volume of DUSP1 and β-actin bands were calculated for each sample, log2 values of the results were calculated. The logarithmic results of DUSP1 gene expression were normalized with logarithmic results of β-actin gene expression by dividing DUSP1 results by β-actin results. These results were then divided by the logarithmic results of Sham A and Sham B (controls). The resulting ratios indicate the relative expression levels of each sample. These calculations were applied also for CXCL2 gene.

2.5.2 Relative quantification of the samples of Real-Time PCR

The relative quantification of the samples was done by $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak, 2001). Sham A and Sham B were chosen as calibrators. The gene expression changes were calculated according to calibrators. ΔC_T value of DUSP1

and CXCL2 were calculated by normalizing the average Ct values of the genes via subtracting the average Ct value of β -actin. ΔC_T value of calibrators were subtracted from ΔC_T value of genes and $\Delta\Delta C_T$ value was obtained for Sham A and Sham B, separately. The minus value of $\Delta\Delta C_T$ value was placed as the power of 2. $2^{-\Delta\Delta C_T}$ gave the relative quantification of the genes.

2.6 Statistical Analysis

The Friedman two-way analysis of variance by ranks (Friedman (1937)) is employed with rank-order data in a hypothesis testing situation involving a design with two or more dependent samples (Sheskin, 2004). Friedman test can also be defined is a distribution-free nonparametric test ANOVA for a randomized block design when comparing k treatments for significant difference among the means (Deep, 2006). It is used to detect the differences between multiple groups. The procedure involves ranking for each row, then considering the values of ranks by columns.

Assumptions of Friedman test: i) The samples from the k populations must be dependent (Glenberg and Andrzejewski, 2007), ii) The data to be analyzed are continuous and at least ordinal level of measurement, iii) The data from a randomly selected sample are either a) multiple observations from a single sample across more than two time periods or conditions or b) blocks of matched subjects in which the subjects from a given block are each randomly assigned to one of the three or more conditions (Pett, 1997).

The formula of Friedman test is:

$$F_r = \frac{12}{bk(k+1)} \sum_{j=1}^k R_j^2 - 3b(k+1) \quad (2.1)$$

where b= the number of the blocks, k= the number of rows, R_j = sum of the ranks in the jth column, and F_r = Friedman test statistic.

Friedman Test is used to test the relationship between the results of RT-PCR, Real-time PCR and microarray with the program SPSS (version 13). Hypothesis testing is the process of inferring from a sample whether or not to accept a certain statement about the population. The statement itself is called the hypothesis. The hypothesis to be tested is called the null hypothesis and is denoted by H_0 . The alternative hypothesis, denoted by H_1 , is the negation of the null hypothesis, and usually consists of a statement equivalent to saying H_0 is not true (Conover, 1998). The p value of a

test is the critical boundary between acceptance and rejection of null hypothesis (Deep, 2006). α (level of significance) is the maximum probability of rejecting a true null hypothesis (Conover, 1998). α is decided to be chosen as 0.01.

The hypothesis used in this thesis are as follows:

H_0 : There is no significant difference between the results of three methods.

H_1 : There is significant difference between the results of three methods.

Reject H_0 (the null hypothesis) if $p \text{ value} < \alpha$; $p < 0.01$

Accept H_0 (the null hypothesis) if $p \text{ value} > \alpha$; $p > 0.01$

Kendall's coefficient of concordance, developed independently by Kendall, and Babington-Smith (1939) and Wallis (1939), is a measure of correlation/association that is employed for three or more sets of ranks and allows researcher to evaluate the degree of agreement between m sets of ranks for n subjects/objects. The parameter estimated by the correlation coefficient will be represented by the notation W . The range of the possible values within which Kendall's coefficient of concordance may fall is $0 < W < 1$. When there is a complete agreement among all m sets of ranks, the value of W will be 1. When there is no pattern of agreement among m sets of ranks, W will be 0. Friedman test and Kendall's coefficient of concordance are based on the same statistical model (Sheskin, 2004). Kendall's coefficient of concordance can be used in the same situation where Friedman test statistic is applicable although it was probably intended primarily as a measure of "agreement in rankings" in the rows, rather than as a test statistic (Conover, 1998).

If $W < 0.70$ the results of the methods are unrelated.

$W > 0.70$ the results of the methods are related.

3. RESULTS

3.1 RT-PCR

3.1.1 Optimization of PCR cycle number for DUSP1 and CXCL2

PCR products' amplicon size of CXCL2 is 179 bp with the first primer set, 180 bp with the second primer set and DUSP1 is 222 bp.

Oligonucleotide primer set of DUSP1 gene resulted in successful PCR amplifications. However, the first primer set of CXCL2 gene did not give good PCR results. Although the band of CXCL2 gene (179 bp) was observed, there was a nonspecific band between 200 and 300 bp. The second best primer set suggested by Primer 3 and Amplify 3 softwares was tested for the next experiments. The nonspecific band was still observed in agarose gels.

Before starting with the rt-PCR experiment, optimization was done for cycle numbers for each of the two genes, DUSP1 and CXCL2. The results for DUSP1 gene are shown in Figure 3.1. The optimum cycle number was observed as 32 for DUSP1. Thus the following PCR program was used in the rt-PCR experiments: 55 °C (30 min) for cDNA synthesis, 94 °C (2 min) for initial denaturation and then 32 cycles at 94 °C (15 sec) / 53 °C (90 sec) / 68 °C (50 sec) and a final extension at 68 °C (5 min).

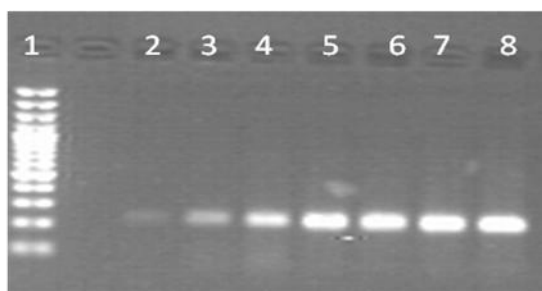
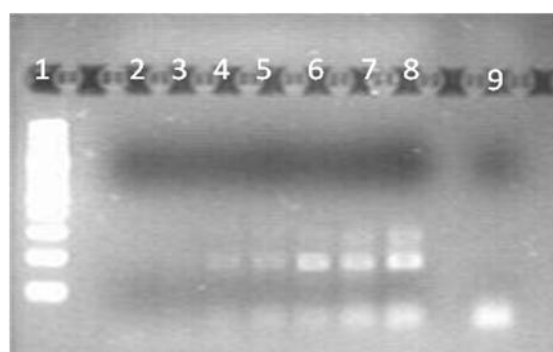


Figure 3.1: Optimization of PCR cycle number for DUSP1

Table 3.1: Explanation of bands in Figure 3.1

Lane	Explanation	Lane	Explanation
1	Marker	5	32 nd cycle
2	23 rd cycle	6	35 th cycle
3	26 th cycle	7	38 th cycle
4	29 th cycle	8	40 th cycle

The results for CXCL2 gene are shown in Figure 3.2. The optimum cycle number was observed as 35 for CXCL2. Thus the following PCR program was used in the rt-PCR experiments: 55 °C (30 min) for cDNA synthesis, 94 °C (2 min) for initial denaturation and then 35 cycles at 94 °C (15 sec) / 58 °C (30 sec) / 68 °C (30 sec) and a final extension at 68 °C (5 min).

**Figure 3.2:** Optimization of PCR cycle number for CXCL2**Table 3.2:** Explanation of bands in Figure 3.2

Lane	Explanation	Lane	Explanation
1	Marker	5	32 nd cycle
2	23 rd cycle	6	35 th cycle
3	26 th cycle	7	38 th cycle
4	29 th cycle	8	40 th cycle
		9	Control

3.1.2 PCR optimization

DNA contamination test experiment was conducted to check whether the nonspecific band was resulting from DNA contamination during RNA isolation. For this purpose, the same PCR reaction was performed using Taq polymerase instead of RT/Platinum Taq Mix. The results are shown in Figure 3.3. The results revealed that the mRNAs did not contain any DNA contamination.

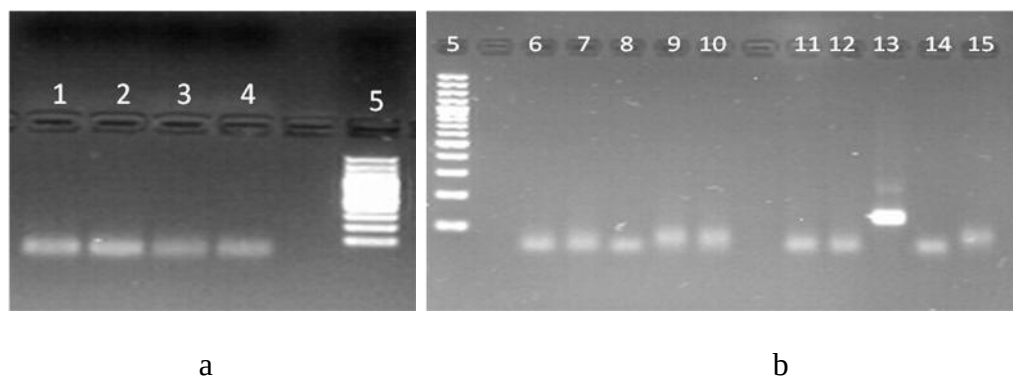


Figure 3.3: DNA contamination experiment gel images a) sample 3, 18, 26 and control b) sample 1, 5, 14, 16, 28, 29, Sham-A and Sham-B.

Table 3.3: Explanation of bands in Figure 3.3

Lane	Explanation	Lane	Explanation
1	Sample 3	9	Sample 16
2	Sample 18	10	Sample 28
3	Sample 26	11	Sample 29
4	Control	12	Sham A
5	Marker	13	β -actin
6	Sample 1	14	Sham B
7	Sample 5	15	Control
8	Sample 14		

Primer dimers experiment was also conducted to test if the bands were primer dimers. For this purpose, PCR reaction mixtures containing only one primer of each primer pairs and both primers of the primer pairs for CXCL2 by using 0.4 μ l Taq DNA polymerase (2 units) were prepared. This procedure was performed for all of the samples.

The results are shown in Figure 3.4 which indicate that the bands were primer dimers. Only the primer pairs showed the same band, but the single primers did not show any band because of the fact that EtBr only binds to double stranded DNA.

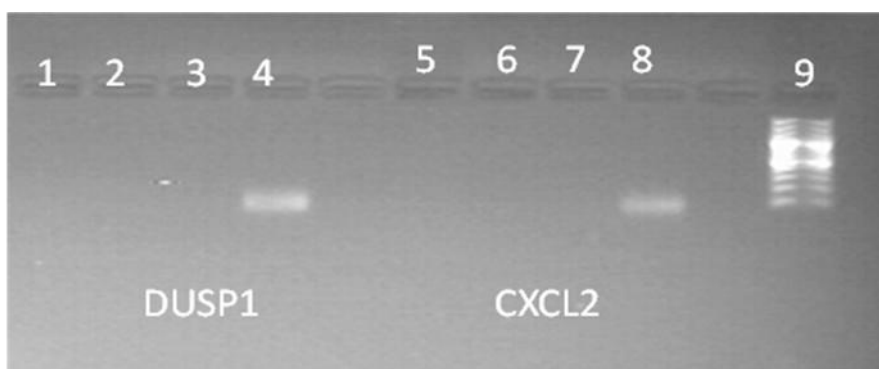


Figure 3.4: Gel images of Primer dimers control using CXCL2 and DUSP1 primers

Table 3.4: Explanation of bands in Figure 3.4

Lane	Explanation	Lane	Explanation
1	DUSP1 R	5	CXCL2 R
2	DUSP1 F	6	CXCL2 F
3	DUSP1 F+R	7	CXCL2 F+R
4	DUSP1 K	8	CXCL2 K
		9	Marker

Annealing temperature and time were optimized for DUSP1 by varying these parameters between 53 °C (90 sec); 56 °C (30 sec) and 60 °C (30 sec). The band of 53 °C (90 sec) conditions was brighter than the other conditions for annealing time and temperature (Figure 3.5 and 3.6).

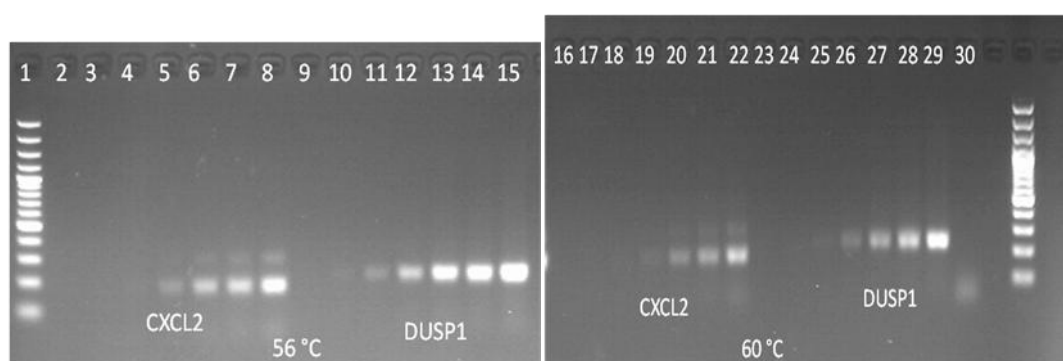


Figure 3.5: Gel images of CXCL2 and DUSP1 gene optimization using 56 °C (30 sec) and 60 °C (30 sec)

Table 3.5: Explanation of bands in Figure 3.5

Lane	Explanation	Lane	Explanation	Lane	Explanation
1	Marker	11	29. cycle, 56 °C, DUSP1	21	38. cycle, 60 °C, CXCL2
2	23. cycle, 56 °C, CXCL2	12	32. cycle, 56 °C, DUSP1	22	40. cycle, 60 °C, CXCL2
3	26. cycle, 56 °C, CXCL2	13	35. cycle, 56 °C, DUSP1	23	23. cycle, 60 °C, DUSP1
4	29. cycle, 56 °C, CXCL2	14	38. cycle, 56 °C, DUSP1	24	26. cycle, 60 °C, DUSP1
5	32. cycle, 56 °C, CXCL2	15	40. cycle, 56 °C, DUSP1	25	29. cycle, 60 °C, DUSP1
6	35. cycle, 56 °C, CXCL2	16	23. cycle, 60 °C, CXCL2	26	32. cycle, 60 °C, DUSP1
7	38. cycle, 56 °C, CXCL2	17	26. cycle, 60 °C, CXCL2	27	35. cycle, 60 °C, DUSP1
8	40. cycle, 56 °C, CXCL2	18	29. cycle, 60 °C, CXCL2	28	38. cycle, 60 °C, DUSP1
9	23. cycle, 56 °C, DUSP1	19	32. cycle, 60 °C, CXCL2	29	40. cycle, 60 °C, DUSP1
10	26. cycle, 56 °C, DUSP1	20	35. cycle, 60 °C, CXCL2	30	Control

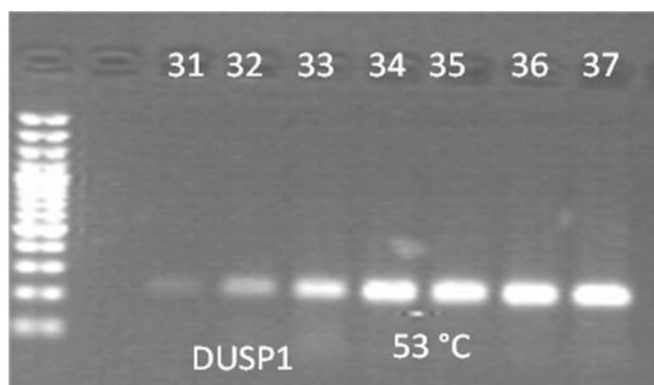


Figure 3.6: Gel image of DUSP1 gene optimization using 53 °C (90 sec)

Table 3.6: Explanation of bands in Figure 3.6

Lane	Explanation	Lane	Explanation
31	23.cycle, 53 °C, DUSP1	35	35. cycle, 53 °C, DUSP1
32	26. cycle, 53 °C, DUSP1	36	38. cycle, 53 °C, DUSP1
33	29. cycle, 53 °C, DUSP1	37	40. cycle, 53 °C, DUSP1
34	32. cycle, 53 °C, DUSP1		

CXCL2 gene optimization using the first primer set was continued with 56 °C (30 sec) and 60 °C (30 sec). When the nonspecific band was observed again, gradient PCR was decided to be performed 55 °C- 65 °C and the extension time was reduced from 50 sec to 30 sec. At all temperatures, however, the band between 200 kb and 300 kb was clearly visible (Figure 3.7).

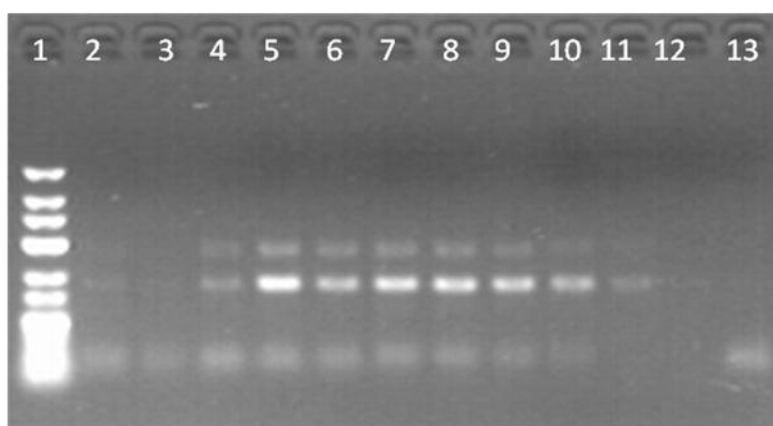


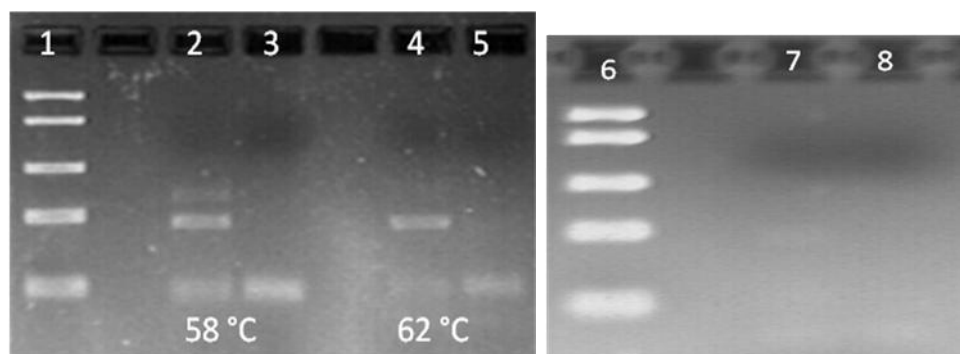
Figure 3.7: Gel images of Gradient PCR with first primer set (CXCL2 gene)

Table 3.7: Explanation of bands in Figure 3.7

Lane	Explanation	Lane	Explanation
1	Marker	8	61 °C
2	55 °C	9	62.1 °C
3	56 °C	10	63.1 °C
4	56.9 °C	11	64 °C
5	57.9 °C	12	65 °C
6	58.8 °C	13	60.4°C(Control)
7	60.4 °C		

The brighter band of CXCL2 gene during Gradient PCR was 58 °C (Figure 3.7). The annealing time at this temperature was reduced from 30 sec to 20 sec.

The nonspecific band at 62 °C was the least visible one when the nonspecific band was compared with those at the other temperatures (Figure 3.7). Afterwards the optimizations were kept going with this temperature by reducing the annealing time to 20 sec and 10 sec (Figure 3.8). The extension time was then reduced from 50 sec to 30 sec and even to 11 sec and 10 sec (Figure 3.9).

**Figure 3.8:** Gel images of CXCL2 gene using a) 58 °C (20 sec), 62 °C (20 sec) b) 62 °C (10 sec) with first primer set**Table 3.8:** Explanation of bands in Figure 3.8

Lane	Explanation	Lane	Explanation
1	Marker	5	Control, 62 °C (20 sec)
2	Sample 3, 58 °C (20 sec)	6	Marker
3	Control, 58 °C (20 sec)	7	Sample 3, 62 °C (10 sec)
4	Sample 3, 62 °C (20 sec)	8	Control, 62 °C (10 sec)

Further optimization was performed by adding MgSO_4 to the concentration mixtures to a final concentration of 1.8 mM and 2mM MgSO_4 to reduce nonspecific band (Figure 3.9).

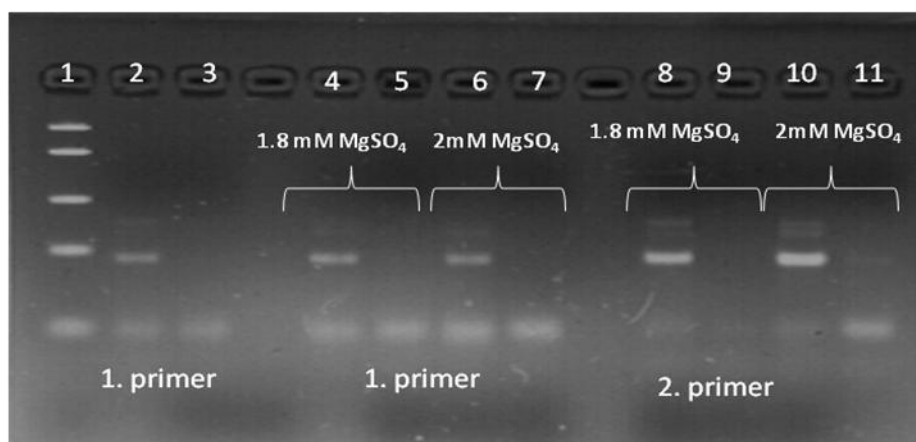


Figure 3.9: Gel images of CXCL2 gene using first and second primer set and different MgSO_4 concentration

Table 3.9: Explanation of bands in Figure 3.9

Lane	Explanation	Lane	Explanation
1	Marker	7	Control (annealing 62°C,30 sec;extension 68°C 50sec)
2	Sample3 (annealing 62°C, 30 sec;extension 68°C 11sec)	8	Sample3 (annealing 64°C,30 sec;extension 68°C 50sec)
3	Control (annealing 62°C,30 sec;extension 68°C 11sec)	9	Control (annealing 64°C,30 sec;extension 68°C 50sec)
4	Sample3 (annealing 62°C,30 sec;extension 68°C 50sec)	10	Sample3 (annealing 64°C,30 sec;extension 68°C 50sec)
5	Control (annealing 62°C,30 sec;extension 68°C 50sec)	11	Control (annealing 64°C,30 sec;extension 68°C 50sec)
6	Sample3 (annealing 62°C,30 sec;extension 68°C 50sec)		

However, none of these optimization procedures using the first primer set of CXCL2 were successful and the nonspecific band could not be eliminated.

The optimizations that were done using the second primer set began with annealing temperature and time as 53°C (90 sec) and continued with 56°C (30 sec). Gradient PCR was then done as with the first primer set by using the second primer set (Figure 3.10). The nonspecific band at 64°C was the least visible nonspecific band, among all

other samples tested at different temperatures. Additionally, 1.8 mM and 2mM MgSO₄ was also added to the PCR mixture for further optimizaitaion.

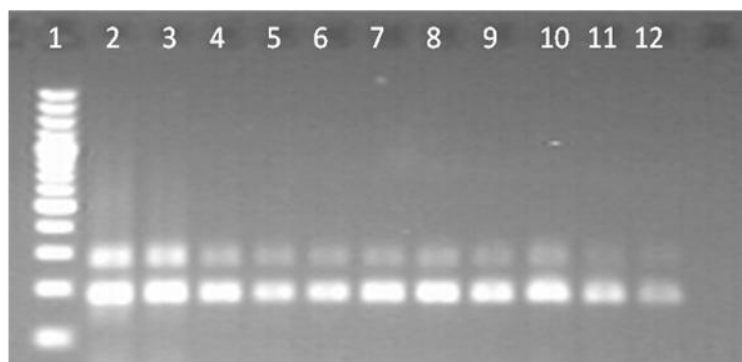


Figure 3.10: Gel images of Gradient PCR with second primer set (CXCL2 gene)

Table 3.10: Explanation of bands in Figure 3.10

Lane	Explanation	Lane	Explanation
1	Marker	7	60 °C
2	55 °C	8	61 °C
3	56 °C	9	61.9 °C
4	57.1 °C	10	62.9 °C
5	58.1 °C	11	63.9 °C
6	59.1 °C	12	65 °C

However, none of these experiments could eliminate the nonspecific band.

Touchdown PCR was carried out using different temperatures and cycle numbers. The optimum Touchdown PCR experiment was the one applied according to the following program: 55 °C (30 min) for cDNA synthesis, 94 °C (2 min) for initial denaturation and then 40 cycles at 94⁰ C (15 sec), the annealing temperature by 1 °C every cycle from 70 °C to a touchdown of 64 °C (30 sec) at which 35 cycles were carried out; 68 °C (30 sec) and a final extension at 68 °C (5 min) (Figure 3.11). The nonspecific band was visible at the other touchdown PCR experiments.

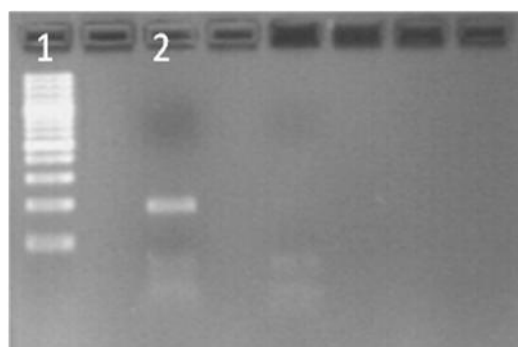


Figure 3.11: Gel images of optimum Touchdown PCR

Table 3.11: Explanation of bands in Figure 3.11

Lane	Explanation
1	Marker
2	Sample 3

The nonspecific band disappeared only in sample 3 (HP_I45_R15_A) and the band of CXCL2 gene was observed only at the 40th cycle. Before 40th cycle, no amplification product was observed. When the experiment was done using sample number 18, there was no amplification product even at the 40th cycle (Figure 3.12).

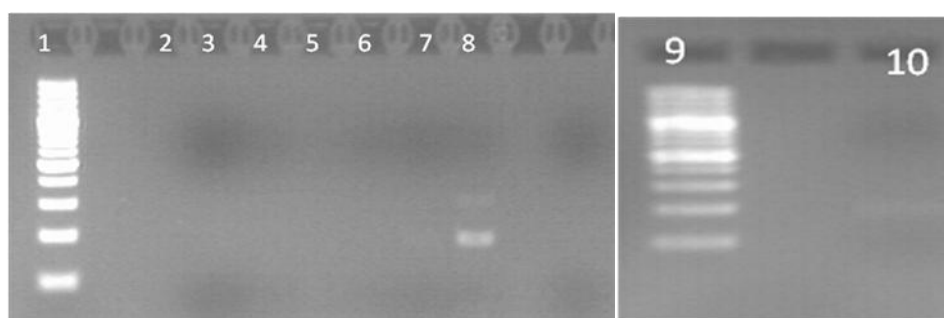


Figure 3.12: Gel images of optimum touchdown PCR at the 40th cycle using
a) sample 3 b) sample 18

Table 3.12: Explanation of bands in Figure 3.12

Lane	Explanation	Lane	Explanation
1	Marker	6	Sample 3, 35.cycle
2	Sample 3, 23. cycle	7	Sample 3, 38.cycle
3	Sample 3, 26. cycle	8	Sample 3, 40.cycle
4	Sample 3, 29. cycle	9	Marker
5	Sample 3, 32. cycle	10	Sample 18, 40. cycle

Finally, as a result of all optimizations, annealing temperature and time were chosen as 58 °C and 30 sec; and extension temperature and time were chosen as 68 °C and 30

sec, respectively. While the volume of the nonspecific band was decreasing, the specific band volume was also decreasing. Thus, 58°C was chosen as the optimal annealing temperature, in order not to lose the specific band (Figure 3.7).

3.1.3 Multiplex rt-PCR

All samples were run in duplicate.



Figure 3.13: DUSP1 Multiplex RT-PCR results

Table 3.13: Explanation of bands in Figure 3.13

Lane	Explanation	Lane	Explanation	Lane	Explanation
1	Marker (#SM0323, Fermentas)	12-13	Sample-18 (I45-R60-B)	22-23	Sample-31 (Sham-A) with only beta-actin primers
2-3	Sample-1 (HP-I45-R0-A)	14-15	Sample-26 (IP-I45-R0-B)	24-25	Sample-32 (Sham-B) with DUSP1 and beta-actin primers
4-5	Sample-3 (HP-I45-R15-A)	16-17	Sample-28 (IP-I45-R15-B)	26-27	Sample-32 (Sham-B) with only beta-actin primers
6-7	Sample-5 (HP-I45-R60-A)	18-19	Sample-29 (IP-I45-R60-A)	28-29	Controls with only beta-actin primers (sample 26)
8-9	Sample-14 (I45-R0-B)	20-21	Sample-31 (Sham-A) with DUSP1 and beta-actin primers	30	Control with DUSP1 and beta-actin primers and with no template
10-11	Sample-16 (I45-R15-B)				

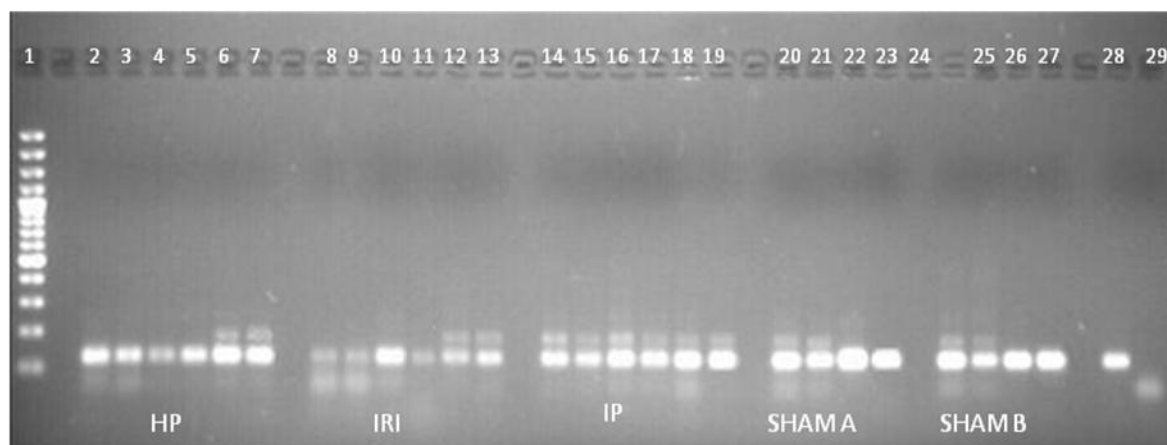


Figure 3.14: CXCL2 Multiplex RT-PCR results

Table 3.14: Explanation of bands in Figure 3.14

Lane	Explanation	Lane	Explanation	Lane	Explanation
1	Marker (#SM0323, Fermentas)	12-13	Sample-18 (I45-R60- B)	22-23	Sample-31 (Sham-A) with only beta-actin primers
2-3	Sample-1 (HP- I45-R0-A)	14-15	Sample-26 (IP-I45- R0-B)	24-25	Sample-32 (Sham-B) with CXCL2 and beta- actin primers
4-5	Sample-3 (HP- I45-R15-A)	16-17	Sample-28 (IP-I45- R15-B)	26-27	Sample-32 (Sham-B) with only beta-actin primers
6-7	Sample-5 (HP- I45-R60-A)	18-19	Sample-29 (IP-I45- R60-A)	28-29	Controls with only beta-actin primers (sample 26)
8-9	Sample-14 (I45-R0-B)	20-21	Sample-31 (Sham-A) with CXCL2 and beta-actin primers	30	Control with CXCL2 and beta-actin primers and with no template
10-11	Sample-16 (I45-R15-B)				

3.2 Real-time PCR Experiments

DUSP1 and CXCL2 gene expression analysis was also performed using real-time PCR, a more sensitive method than rt-PCR.

The total RNA population was reverse transcribed with random hexamers so that different PCR analyses could be performed on the same cDNA sample. Reaction mix are given as given in the Table 2.13 and 2.14.

The error rates of the Real-Time PCR reactions of DUSP1 and β -actin were about 0.3% and the efficiencies of these reactions were approximately 2 (Table 3.15).

Table 3.15: Error rates and efficiencies of DUSP1 and β -actin real-time PCRs.

	DUSP1	β-actin
Error Rate	0.0317	0.0252
Efficiency	2.062	2

The error rates and efficiencies of DUSP1 and beta actin were in an acceptable range (Table 3.1). However, the efficiency of CXCL2 gene was not between 1.8 and 2.2 and error rate was higher than 2%. Firstly, sample number 3 was used to set the standard curve optimization because of its high volume. The Ct of undiluted sample was over 30 cycles and Ct's of the dilutions of 1/10 and 1/100 near 40 cycles, and of 1/1000 was over 40 cycles. The higher Ct values reduce the efficiency. When unsatisfactory efficiency values were obtained, the sample changed with Sham B (control) because of higher concentration of Sham B. However, the results did not change.

The standards were prepared in undiluted, 1/2 diluted, 1/4 diluted and 1/8 diluted forms to prevent the Ct values from reaching 40 cycles. However, neither the efficiency nor the error values were still in an acceptable range, but β -actin efficiency and error values were in an acceptable range during CXCL2& β -actin multiplex assay (Table 3.16).

Primer and probe volumes of CXCL2 gene were increased from 0.4 μ l to 0.6 μ l using sample 16 (I45_R15_B) to test if the high Ct value could be reduced. However, Ct value of sample 16 remained unchanged.

Table 3.16: Error rates and efficiencies of CXCL2 and β -actin Real-Time PCRs.

	CXCL2	β-actin
Error Rate	>>>0.2	0.00776
Efficiency	2.347	2.105

3.3 Relative Quantification of RT-PCR Results

3.3.1 DUSP1 gene expression levels as quantified by RT-PCR

When the mRNA levels were normalized and calibrated according to the endogenous control and the standard dilutions, relative expression levels were obtained. Shams were considered to be 1 as relative expression units.

When the calibrator was chosen as Sham A for DUSP1 gene, the lowest gene expression level was observed within the ischemia reperfusion injury group (N=1; 0.075 relative expression) and the highest gene expression was observed within ischemic preconditioning group (N=1; 1.028 relative expression) (Figure 3.15) .

In the heat preconditioning group and ischemia reperfusion injury group, as it was observed that the reperfusion time increased, DUSP1 gene expression also increased.

In the HP-I45-R0-A sample (N=1; 0.180 relative expression), DUSP1 gene was downregulated as compared to Sham A. As the reperfusion time increased, DUSP1 gene was upregulated in both samples HP-I45-R15-A (N=1; 1.023 relative expression) and HP-I45-R60-A (N=1; 1.03 relative expression) but there was no significant difference when these two samples were compared with Sham A.

Upregulation was observed in I45-R15-B (N=1; 0.99 relative expression) and I45-R60-B (N=1; 1.019 relative expression) compared to I45-R0-B (N=1; 0.075 relative expression) (Figure 3.15).

In the ischemic preconditioning group, at 15 min reperfusion, the expression of DUSP1 gene slightly decreased. It was upregulated in 60 min reperfusion samples. However, downregulation and upregulation were not significant when the samples were compared with Sham A (Figure 3.15).

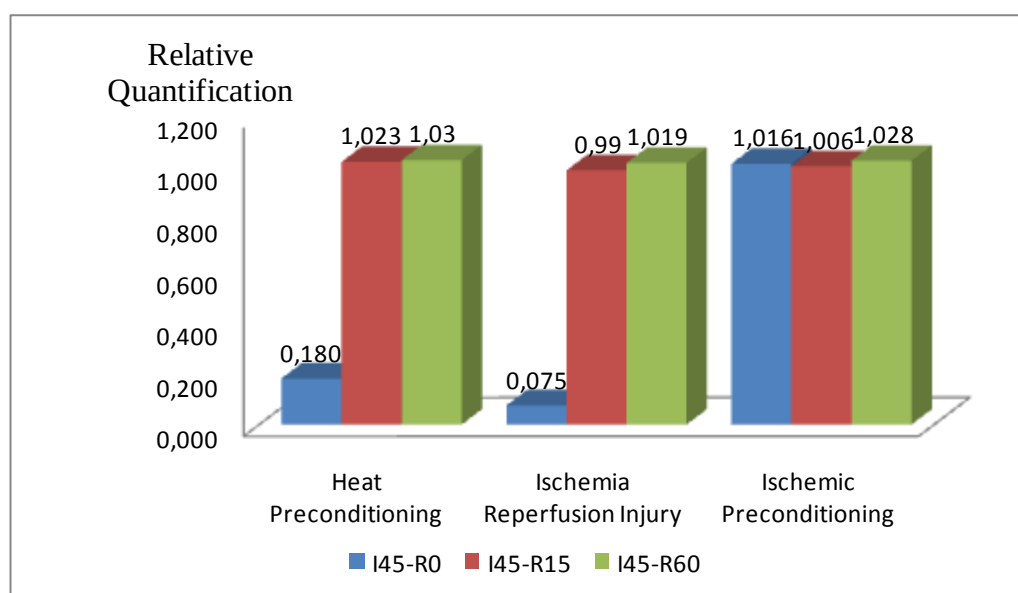


Figure 3.15: Relative quantification of DUSP1 gene expression normalized to Sham A according to rt-PCR

There was no significant difference in the results of DUSP1 gene expression in each sample whether the calibrator was Sham A or Sham B (Figure 3.15 and 3.16).

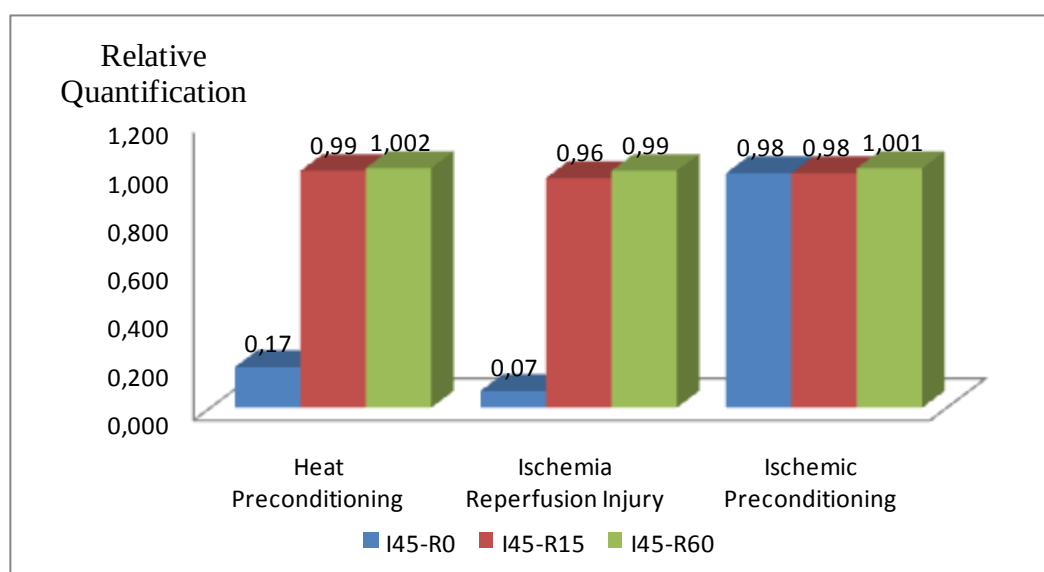


Figure 3.16: Relative quantification of DUSP1 gene expression normalized to sham B according to RT-PCR results.

3.3.2 CXCL2 gene expression levels as quantified by rt-PCR

CXCL2 gene was not expressed in samples HP-I45-R0-A, HP-I45-R15-A, I45-R0-B and I45-R15-B. However, it was upregulated in samples HP-I45-R60-A (N=1; 1.09 relative expression) and I45-R60-B (N=1; 1.12 relative expression) (Figure 3.17).

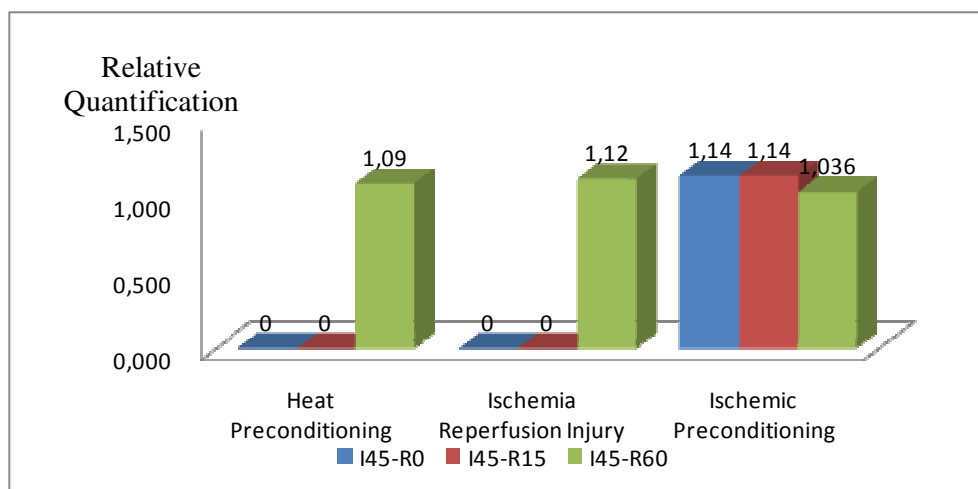


Figure 3.17: Relative quantification of CXCL2 gene expression normalized to sham A according to RT-PCR results

The relative expression levels of those two samples were higher when the results were normalized to Sham B (Figure 3.18). In ischemic preconditioning group, all three samples were upregulated in comparison to both Sham A and Sham B. Samples IP-I45-R0-B and IP-I45-R15-B had the same fold increase values.

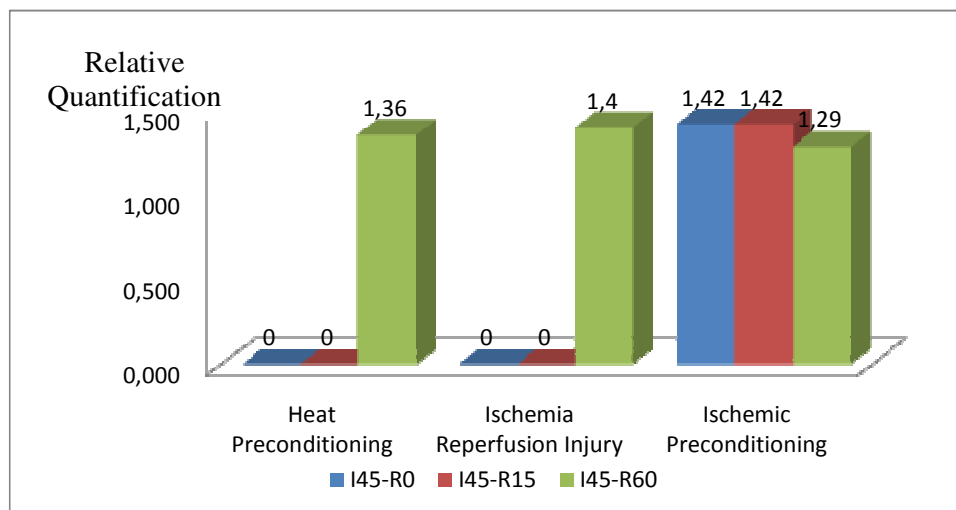


Figure 3.18: Relative quantification of CXCL2 gene expression normalized to sham B according to RT-PCR results

3.4 Relative Quantification of Real-Time PCR Results

3.4.1 DUSP1 gene expression levels as quantified by real-time PCR

When the calibrator was chosen as Sham A, DUSP1 was observed to be downregulated in Sample HP-I45-R0-A. Increase in gene expression was observed when the reperfusion time increased to 15 min for the sample HP-I45-R15-A. However, the DUSP1 expression of both of these samples were the lower compared to that of Sham A (Figure 3.19).

A reperfusion time of 60 min in the sample HP-I45-R60-A resulted in more than two fold increased expression levels compared to Sham A.

Sample I45-R0-B had 0.43 Relative Expression Units but as the reperfusion time increased to 15 min (Sample I45-R15-B) Relative Expression Units decreased to 0.36. The highest relative expression in the ischemia-reperfusion group was observed with the sample I45-R60-B corresponding to 60 min reperfusion time (Figure 3.19).

All samples in the ischemic preconditioning group had higher relative expression levels for DUSP1, compared to Sham A. Among all 9 samples from 3 different conditions, sample IP-I45-R60-A had the highest relative expression (N=1; 2.84 relative expression) (Figure 3.19).

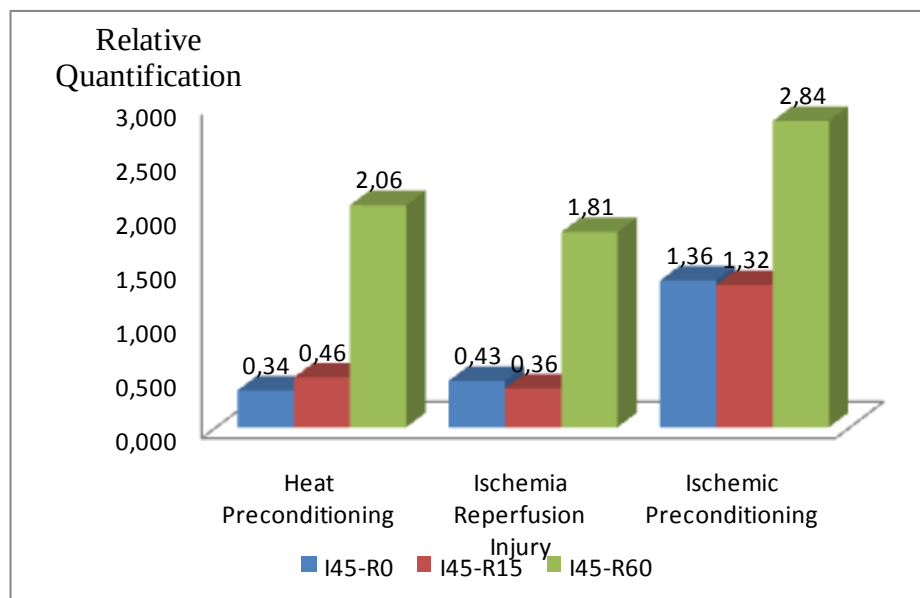


Figure 3.19: Relative quantification of DUSP1 gene expression normalized to sham A according to Real-time PCR results

DUSP1 gene expression levels were higher when normalized to Sham B, compared to normalisation to Sham A for all samples (Figure 3.19 and 3.20). This difference

was particularly significant for samples IP-I45-R60-A and I45-R60-B. More than 3 fold increase was observed in gene expression of the sample IP-I45-R60-A when the calibrator was Sham B. The relative expression unit was 2.1 for sample I45-R60-B, but only 1.81 for the same sample, when Sham A was the calibrator.

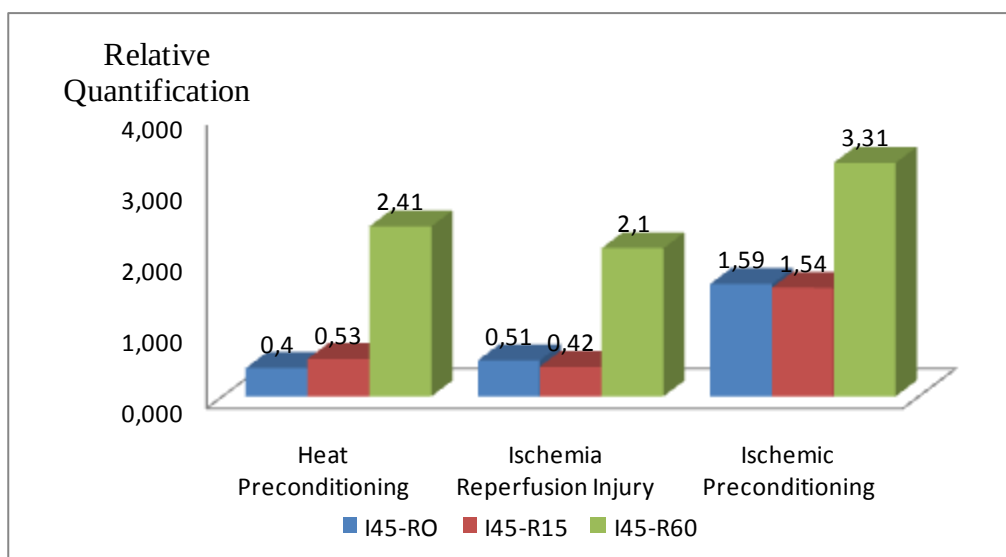


Figure 3.20: Relative quantification of DUSP1 gene expression normalized to sham B according to Real-time PCR results

3.4.2 CXCL2 gene expression levels as quantified by real-time PCR

When the calibrator was chosen as Sham A, CXCL2 gene expression level for samples HP-I45-R0-A and HP-I45-R15-A were more than one fold. CXCL2 gene was not expressed in sample I45-R0-B. The highest relative expression was observed at sample I45-R60-B. The relative expression levels of all samples of ischemic preconditioning group was more than 5-fold. All samples that were reperused for 60 min had the highest relative expression in their particular group (Figure 3.21).

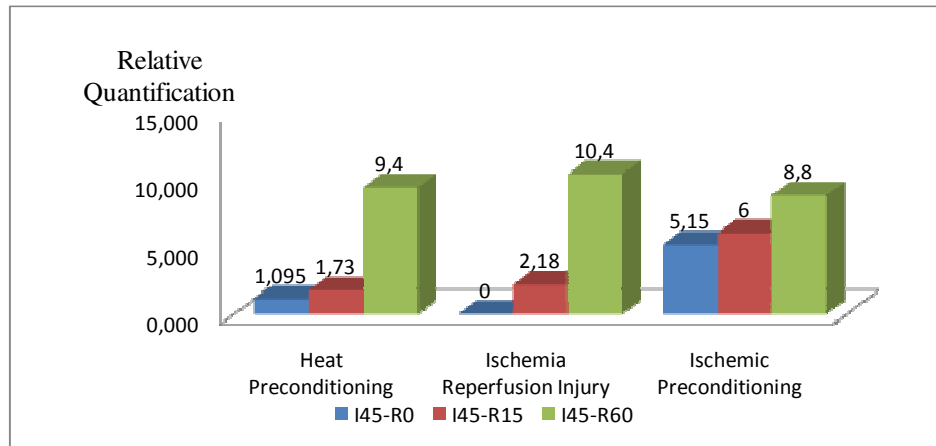


Figure 3.21: Relative quantification of CXCL2 gene expression normalized to sham A according to real-time PCR results

For CXCL2 gene, the relative expression level that were calculated according to Sham A were two-fold than the relative expression levels calculated according to Sham B (Figure 3.22).

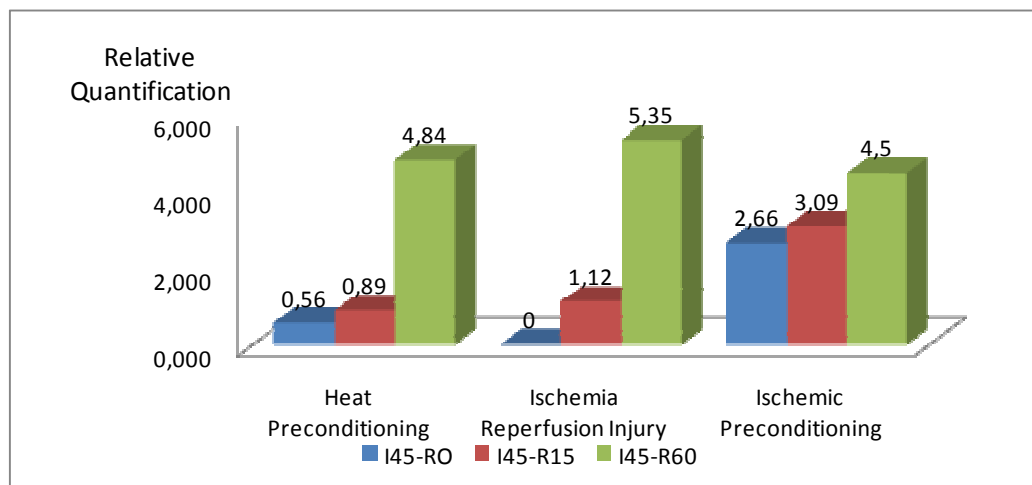


Figure 3.22: Relative quantification of CXCL2 gene expression normalized to sham B according to real-time PCR results

3.5 Comparison of RT-PCR&Real-time PCR results with previously obtained microarray results and Statistical Analysis

Table 3.17: Comparison of DUSP1 gene RT-PCR&Real-time PCR results with previously obtained microarray results

SAMPLES	DUSP1/shamA ratio			DUSP1/shamB ratio		
	RT-PCR	Real-time pcr	M.array	RT-PCR	Real-time pcr	M.array
HP-I45-R0-A (1)	0.180	0.34	-1.27	0.17	0.4	-1.51
HP-I45-R15-A (3)	1.023	0.46	-1.15	0.99	0.53	-1.2
HP-I45-R60-A (5)	1.03	2.06	1.6	1.002	2.41	1.47
I45-R0-B (14)	0.075	0.43	-0.97	0.07	0.51	-1.24
I45-R15-B (16)	0.99	0.36	-0.79	0.96	0.42	-1.01
I45-R60-B (18)	1.019	1.81	1.14	0.99	2.1	1.18
IP-I45-R0-B (26)	1.016	1.36	1.06	0.98	1.59	1.19
IP-I45-R15-B (28)	1.006	1.32	1.2	0.98	1.54	1.1
IP-I45-R60-A (29)	1.028	2.84	1.05	1.001	3.31	1

Table 3.18: Comparison of CXCL2 gene RT-PCR&Real-time PCR results with previously obtained microarray results

SAMPLES	CXCL2/shamA ratio			CXCL2/shamB ratio		
	RT-PCR	Real-time pcr	M.array	RT-PCR	Real-time pcr	M.array
HP-I45-R0-A (1)	0	1.095	-0.18	0	0.56	0.14
HP-I45-R15-A (3)	0	1.73	-0.31	0	0.89	0.24
HP-I45-R60-A (5)	1.09	9.4	1.66	1.36	4.84	1.52
I45-R0-B (14)	0	0	0.03	0	0	-0.04
I45-R15-B (16)	0	2.18	-0.35	0	1.12	-0.16
I45-R60-B (18)	1.12	10.4	1.92	1.4	5.35	1.94
IP-I45-R0-B (26)	1.14	5.15	1.49	1.42	2.66	1.53
IP-I45-R15-B (28)	1.14	6	0.91	1.42	3.09	1.01
IP-I45-R60-A (29)	1.036	8.8	1.44	1.29	4.5	1.52

Friedman test was used to test the relationship between RT-PCR, real-time PCR and microarray results. The results of DUSP1 and CXCL2 gene expression were tested when normalized to Sham A and Sham B. For a good correlation, W value should be between 0.70 and 1, and the P value should be more than 0.01, in order not to have any significant differences between RT-PCR, real-time PCR and microarray results.

Table 3.19: p values and Kendall coefficient of concordance for RT-PCR, real-time PCR and microarray of DUSP1 and CXCL2 gene expression

	DUSP1&ShamA		Dusp1&ShamB		CXCL2&ShamA		CXCL2&ShamB	
	P value	W	P value	W	P Value	W	P Value	W
HP	0.264	0.444	0.264	0.444	0.097	0.778	0.050	1
IRI	0.050	1	0.264	0.444	0.441	0.273	0.097	0.778
IP	0.264	0.444	0.097	0.778	0.097	0.778	0.148	0.636

p values showed that the results of the three experimental methods were not significantly different from each other, thus indicating a good correlation. Kendall's coefficient of concordance values of the results were generally in an acceptable range (Table 3.19).

4. DISCUSSION & CONCLUSIONS

Acute renal failure (ARF) is an inflammatory consequence of a sudden and temporary interruption of blood flow in the kidneys and it remains as a common issue in the clinical practice which lack satisfactory therapeutic options (de Paiva et al., 2008).

Important cause of morbidity and mortality in hospitalized intensive care unit patients is acute renal failure from ischemic damage (Hamar et al., 2004).

ARF pathogenesis leads to the sudden interruption of blood flow to the kidneys starting inflammation and innate immune responses such as production of reactive oxygen species, complement and neutrophils activation, up-regulation of adhesion molecules and tubular cell death (de Paiva et al., 2008).

Upregulation of adhesion molecules, chemokines and HSPs in experimental brain dead donor kidneys are known and upregulation of these genes are also confirmed in cardiac death donor kidneys (Kusaka et al., 2006).

The involvement of renal IRI in upregulation of proinflammatory cytokines and the actual role of cytokines has become apparent (Bertocchi et al., 2008).

Real time PCR is the most sensitive method for the detection and quantification of gene expression levels, in particular for low abundant transcripts in tissues with low RNA concentrations, from limited tissue sample and for the elucidation of small changes in mRNA expression levels (Pfaffl, 2005). Thus, results from real time PCR and RT-PCR experiments were compared with each other, and also to previous microarray results with the same samples.

The results of RT-PCR and Real-time PCR experiments show that CXCL2 gene was not expressed when the sample is only subjected to ischemia (I45-R0-B). The results obtained from gene expression analysis using quantitative real-time PCR, indicated that expression levels increased with reperfusion time. Apparently, when the blood flow began, CXCL2 gene was expressed because of the ROS production upon blood flow and the beginning of inflammation. In the heat preconditioning (HP) and

ischemia reperfusion (IR) groups, 60 min reperfusion time samples had the highest CXCL2 gene expression levels, according to both RT-PCR and real-time PCR results. The results according to Sham A and Sham B controls differed from each other for CXCL2 gene in their absolute values, but not the general tendency in gene expression levels. The Real-time PCR and RT-PCR results were in agreement with microarray results for CXCL2. CXCL2 gene was induced in all samples except HP-I45-R0-A, HP-I45-R15-A. There seems to be a downregulation for CXCL2 gene expression normalized to Sham B in HP-I45-R0-A and HP-I45-R15-A samples according to Real-time PCR results, but no expression was observed for same samples in rt-PCR analysis. This may result from the differences in sensitivities of rt-PCR and real-time PCR, the latter being more sensitive (Figures 3.17, 3.18, 3.21 and 3.22). IPC generally increase CXCL2 gene expression. The increase in reperfusion time in IPC group did not cause a significant increase in CXCL2 gene expression. In HP and IRI, however, CXCL2 gene expression increased significantly as a function of reperfusion time (Figures 3.21 and 3.22).

Salt, alcohol, phenol and other inhibitors carried over from the RNA isolation process can affect the apparent RT efficiency (Pfaffl, 2005). RNAs had previously been isolated using peqGOLD TriFastTM (Tektaş, Ph.D Thesis), and this method involved the use of phenol. It is likely that reason for the low efficiency of CXCL2 results could be due to the use of phenol in RNA isolation.

Priming method that was used to initiate cDNA synthesis can be either gene specific or non-specific (Pfaffl, 2005). Nonspecific cDNA synthesis was chosen because the experiments for two genes were applied for the same samples. In nonspecific method, random hexamer was chosen as Pfaffl (2005) observed that it was the most efficient primer in nonspecific primers.

Two general methods for the quantitative detection of the amplicon have become established: gene specific fluorescent probes or specific double strand DNA binding agents as SYBR green (Pfaffl, 2005). In RT-PCR experiments, a nonspecific band was observed for CXCL2 samples, thus it was decided to use Taqman probe for real-time PCR experiments to obtain more specific results.

Regarding quantification, the first decision was the use of Roche LightCycler Relative quantification program. However, CXCL2 efficiency values was not within

the acceptable range. Thus, the method for relative quantification was changed to $\Delta\Delta C_t$ method.

The dynamic range of the performed calibration curve can be up to nine orders of magnitude from $<10^1$ to $>10^{10}$ start molecules, depending on the applied standard material (Pfaffl, 2005). As the samples that were used in this study were RNA samples of limited amount, it was not possible to use 10^2 , 10^3 start molecules. Instead, undiluted, 10^{-1} , 10^{-2} , and 10^{-3} start molecules were used. The use of more undiluted samples could bring the efficiency values between 1.8 and 2.2.

In all experiments in this study, the genes of interest and the housekeeping gene (beta-actin) serving as an internal control were co-amplified in the same reaction tube. Thus, they were amplified under the same conditions. Normalization with genes that were in the same tube should have minimized technical problems.

During PCR, formation of primer dimers competes with formation of specific PCR product, leading to reduced amplification efficiency and a less successful specific Real-Time PCR product (Pfaffl, 2005). Although probe based real time PCR was used, primer dimer formation was observed for CXCL2 gene.

Different tissues exhibit different PCR efficiencies, caused by RT inhibitors, PCR inhibitors, and by variations in the total RNA fraction pattern extracted (Pfaffl, 2005). The efficiency of CXCL2 gene may be affected by these inhibitors.

CXCL2 efficiency was reduced by late amplification cycles. All samples for CXCL2 gene have high threshold cycle values above the acceptable range of quantification [$C_t > 30$ cycles]. This high threshold cycle value reduces the efficiency of CXCL2 gene. Behrends et al. (2007) observed the same result for the same gene. CXCL2 gene was also expressed in the control samples Sham A and Sham B.

In response to extracellular stimulation, MKP-1 mRNA levels are usually increased by 10–100 fold within 15-60 min (Wang and Liu, 2007). In all groups, DUSP1 gene expression was highest when reperfusion time was 60 min. The Real-time PCR and RT-PCR results were generally in agreement with microarray results for DUSP1. According to real-time PCR results, downregulation of DUSP1 was observed in HP-I45-R0-A, HP-I45-R15-A, I45-R0-B, I45-R15-B samples (Figures 3.19 and 3.20). DUSP1 gene was induced in all of the samples of ischemic preconditioning

group. These results imply that DUSP1 gene is downregulated in response to hypoxia.

Microarray, RT-PCR and Real-time PCR results can be discordant with each other. The discordance of these results may be explained by the decreased sensitivity of the microarray per se or by the relatively low expression levels of these genes (Bruder et al., 2007).

Real-time PCR also offers a much wider dynamic range of up to 10^7 -fold (compared to 1000-fold in conventional RT-PCR) in comparison to conventional RT-PCR (Dorak, 2006). The ranges of relative quantification values were indeed higher in our real-time PCR results, compared with those of rt-PCR and microarray (Tables 3.17 and 3.18).

The lower values of Kendall's coefficient of concordance could possibly be explained by the zero expression levels and minus values of some microarray data.

To summarize, the early preconditioning effects on DUSP1 and CXCL2 gene expression levels were investigated in detail in this study by successfully applying rt-PCR and real-time PCR techniques and comparing them to previously obtained microarray results. Generally, it was observed that, as the reperfusion time increased, the gene expression levels also increased, up to 60 min reperfusion time. Thus, it remains to be investigated what changes would occur in gene expression for reperfusion times longer than 60 min. Such new experiments would be useful to investigate late preconditioning effects.

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CURRICULUM VITAE



Candidate's full name: Şeyma Hande TEKARSLAN

Place and date of birth: Istanbul, 07.06.1983

Permanent Address: 19 May Quarter İnönü Street No:11 Kozyatagi/ Istanbul

Universities and

Colleges attended: B.Sc. : Istanbul University Faculty of Business Administration
(2002-2006)

Istanbul University Faculty of Sciences-Department of
Biology (2004-2007) (Double Major Program)

M.Sc. : Istanbul Technical University Molecular Biology-
Genetics and Biotechnology (2007-2009)