

**INVESTIGATION OF ISCHEMIA-REPERFUSION EFFECTS ON ATF3
AND GADD45G GENE EXPRESSION IN RAT KIDNEY TISSUES USING
RT-PCR AND REAL-TIME PCR**

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**Programme : Molecular Biology - Genetics and
Biotechnology**

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JUNE 2009

İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

**İSKEMİ-REPERFÜZYONUN ATF3 VE GADD45G GEN
EKSPRESYONLARI ÜZERİNDEKİ ETKİLERİNİN RT-PCR VE REAL-
TIME PCR TEKNİKLERİ KULLANILARAK SIÇAN BÖBREK
DOKULARINDA İNCELENMESİ**

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ABBREVIATIONS

ADP	: Adenosine diphosphate
ARF	: Acute renal failure
ATF	: Activating transcription factor
ATP	: Adenosine triphosphate
bax	: Bcl-2-associated X protein
bp	: Base pair
bZip	: Basic leucine zipper
C3a, C5a	: Anaphylatoxins, soluble fragments of C3 and C5
C5b-9	: Terminal complement complex, membrane attack complex
cAMP	: Cyclic adenosine monophosphate
CD11/18	: Cluster of differentiation 11/18
Cdc2	: Cell division cycle 2
cDNA	: Complementary DNA
CDS	: Coding DNA sequences
c-Fos	: Fos oncogene
cIAP2	: Cellular inhibitor of apoptosis 2
c-Jun	: Jun oncogene
CR6	: Cytokine response 6
CREB	: cAMP responsive element binding protein
C_t	: Cycle threshold
dd	: Distilled
DEPC	: Diethyl pyrocarbonate
DNA	: Deoxyribonucleic acid
DTT	: 1,4-Dithiothreitol
E	: Amplification efficiency
EDTA	: Ethylenediaminetetraacetic acid
EGR-1	: Early growth response protein-1
EtBr	: Ethidium bromide
F	: Forward (left) primer
GADD45a,	
b, g	: Growth arrest- and DNA damage-inducible alfa, beta, gamma
GAPDH	: Glyceraldehyde-3-phosphate dehydrogenase
GOI	: Gene of interest
HP	: Heat preconditioning
HSP	: Heat-shock protein
ICAM-1, -2	: Intercellular adhesion molecule-1, -2
IFN	: Interferon
IκB	: Inhibitory subunit of NF κ B
IL-1β, -4,	
-6, -8	: Interleukin-1 β , -4, -6, -8
IP	: Ischemic preconditioning
IRF-1	: Interferon regulatory factor-1
IRI	: Ischemia-reperfusion injury

JNK	: c-Jun N-terminal kinase
JunB	: Jun-B oncogene
LTB4	: Leukotriene B4
MAPK	: Mitogen-activated protein kinase
MCP-1	: Monocyte chemotactic protein-1
MEKK4	: Mitogen-activated protein kinase kinase kinase 4
min	: Minute
MMP-9	: Matrix metalloproteinase-9
mRNA	: Messenger ribonucleic acid
MyD	: Myeloid differentiation
NC	: Negative control
NFκB	: Nuclear factor κB
NHE	: Sodium-hydrogen exchanger
NO	: Nitric oxide
NOS	: Nitric oxide synthase
nt	: Nucleotide
p53	: Tumor protein 53
PARP	: Poly-(ADP-ribose)-polymerase
PCNA	: Proliferating cell nuclear antigen
PCR	: Polymerase chain reaction
pI	: Isoelectric point
PLA₂	: Phospholipase A ₂
PMN	: Polymorphonuclear leukocytes
R	: Reverse (right) primer
ROS	: Reactive oxygen species
RT	: Reverse transcription
s	: Second
SAPK	: Stress-activated protein kinase
TBE	: Tris-boric acid-ethylenediaminetetraacetic acid
TNF-α	: Tumor necrosis factor-α
TXA₂	: Thromboxane A ₂
U	: Enzyme unit
UPL	: Universal probe library
UV	: Ultraviolet
V	: Voltage
XIAP	: X-linked inhibitor of apoptosis

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INVESTIGATION OF ISCHEMIA-REPERFUSION EFFECTS ON ATF3 AND GADD45G GENE EXPRESSION IN RAT KIDNEY TISSUES USING RT-PCR AND REAL-TIME PCR

SUMMARY

Ischemia is a state of tissue oxygen deprivation because of inadequate blood supply owing to constriction of the blood vessels or to obstruction. Reperfusion is the restoration of blood flow to the ischemic tissue. Whereas ischemic injury is mainly due to oxygen-deprived cellular necrosis, reperfusion is highly responsible from the injury because it produces an inflammatory response that both heightens local damage and leads to systemic insult. Thus, ischemia-reperfusion injury (IRI) causes damage in several organs. Pathophysiology of the IRI is complex and inflammatory mechanisms play a major role.

Ischemic preconditioning (IP) and heat preconditioning (HP) are two methods of minimizing IRI that can be applied to an organ before an ischemia-reperfusion condition to make the organ tolerant to IRI. *Activating transcription factor 3 (Atf3)* and *growth arrest- and DNA damage-inducible gamma (Gadd45g)* are two genes that have been shown to be induced by several stress conditions such as ischemia-reperfusion. In a previous study with microarray analysis, it was observed that *Atf3* and *Gadd45g* genes were upregulated when rat kidney tissues were studied for gene expression upon ischemia-reperfusion, ischemia-reperfusion with heat preconditioning and with ischemic preconditioning. In this study, expression levels of these genes upon the same conditions were investigated in total RNA samples from rat kidney tissues by using RT-PCR and Real-Time RT-PCR techniques.

In both techniques, reaction mixtures were prepared as multiplex samples by using the internal control gene (β -Actin) primers in the same reaction tube. In RT-PCR experiments, the annealing condition of 56°C for 30 s and 32 cycles of PCR amplification were determined as optimum conditions for the multiplex reactions of both the *Atf3* and *Gadd45g* genes. cDNA products of the multiplex RT-PCR were loaded to agarose gel, and relative quantification of gene expression was done using the band volumes obtained by the BioCapt software. In multiplex Real-Time PCR experiments, C_T values of the obtained cDNAs were determined, and relative quantification of gene expression was done by using the $2^{-\Delta\Delta C_t}$ method. Relative quantification of the treated samples were determined according to Sham control samples.

The resulting values of both techniques were compared with the previously obtained microarray results. Results obtained have shown significant expression changes under different conditions and were compatible with the microarray data. For both *Atf3* and *Gadd45g* genes, the highest increase in gene expression level was observed in the IP-conditioned samples and especially in the sample with 60 min reperfusion time. For these samples, *Atf3* showed higher expression values than *Gadd45g*.

İSKEMİ-REPERFÜZYONUN ATF3 VE GADD45G GEN EKSPRESYONLARI ÜZERİNDEKİ ETKİLERİNİN RT-PCR VE REAL-TIME PCR TEKNİKLERİ KULLANILARAK SIÇAN BÖBREK DOKULARINDA İNCELENMESİ

ÖZET

İskemi, kan damarlarının büzülmesine ya da tıkanmasına bağlı yetersiz kan akışından kaynaklanan doku oksijen yoksunluğu durumudur. Reperfüzyon, kan akışının iskemik dokuya yeniden gelmesidir. İskemik hasar, oksijenden yoksun kalan hücrelerin nekrozundan kaynaklanırken hasardan yüksek oranda reperfüzyon sorumludur, çünkü reperfüzyonun yol açtığı enflamatuvar cevap hem bölgesel hasarı artırır hem de sistemik kötüleşmeye neden olur. Böylece, iskemi-reperfüzyon hasarı (IRI) birçok organa zarar verir. IRI'nin patofizyolojisi karmaşıktır ve enflamatuvar mekanizmalar büyük bir rol oynar.

İskemik önkoşullama (IP) ve ısı önkoşullaması (HP) IRI'yi azaltmanın iki yöntemidir ve bir organı IRI'ye dayanıklı hale getirmek için iskemi-reperfüzyon durumundan önce uygulanabilir. Aktive edici transkripsiyon faktörü 3 (*Atf3*) ile büyümenin tutuklanması ve DNA hasarı ile indüklenen gama (*Gadd45g*), iskemi-reperfüzyon gibi çeşitli stres koşulları tarafından indüklendiği gösterilmiş olan iki gendir. Daha önce yapılmış olan bir mikroarray analiz çalışmasında, sıçan böbrek dokularında, iskemi-reperfüzyon, ısı önkoşullamasını takiben iskemi-reperfüzyon ve iskemik önkoşullamayı takiben iskemi-reperfüzyon koşullarında gen ekspresyonu çalışıldığında *Atf3* ve *Gadd45g* genlerinin anlatımının arttığı gözlenmiştir. Bu çalışmada, bu genlerin aynı koşullar altındaki anlatım seviyeleri, sıçan böbrek dokularından elde edilmiş olan total RNA örnekleri üzerinde RT-PCR ve Real-Time RT-PCR teknikleri kullanılarak çalışılmıştır.

Her iki teknikte de, reaksiyon karışımları, aynı reaksiyon tüpünde internal kontrol genin (β -Aktin) primerleri kullanılarak multipleks örnekler olarak hazırlandı. RT-PCR çalışmalarında, 56°C'de 30 saniye primer bağlanma koşulu ve 32 döngülük PCR amplifikasyonu, hem *Atf3* hem de *Gadd45g*'nin multipleks reaksiyonları için en uygun koşullar olarak belirlendi. Multiplex RT-PCR'ın cDNA ürünleri agaroz jele yüklendi ve rölatif gen ekspresyonu miktarı, BioCapt yazılımı ile elde edilen bant hacimleri kullanılarak hesaplandı. Multiplex Real-Time PCR çalışmalarında, elde edilen cDNA'ların C_T değerleri belirlendi ve $2^{-\Delta\Delta C_t}$ yöntemi kullanılarak rölatif gen ekspresyonu miktarı hesaplandı. Farklı koşullar uygulanan örneklerin rölatif gen ekspresyonu miktarı tayini, Sham kontrol örneklerine göre belirlendi.

Her iki tekniğin sonuçları, daha önce elde edilmiş olan mikroarray sonuçlarıyla karşılaştırılmıştır. Elde edilen sonuçlar, mikroarray verileriyle uyumlu şekilde, farklı koşullar altında anlamlı ekspresyon değişimleri olduğunu göstermiştir. Hem *Atf3* hem de *Gadd45g* genleri için en fazla gen ekspresyonu artışı IP uygulanan örneklerde, özellikle de 60 dakikalık reperfüzyon örneğinde görülmüştür. Bu örnekler için *Atf3*, *Gadd45g*'den daha yüksek ekspresyon değerleri göstermiştir.

1. INTRODUCTION

1.1 Ischemia-Reperfusion Injury (IRI)

Ischemia is a state of tissue oxygen deprivation resulting from inadequate blood flow. Reperfusion is the restoration of blood flow to the ischemic tissue. Introduction of molecular oxygen into cells upon reperfusion elicits a cascade of adverse reactions that injure tissue [1, 2].

Ischemia-reperfusion injury (IRI) causes organ damage in the brain, heart, lungs, liver, kidneys and skeletal muscle [1]. Conditions that trigger IRI include the different forms of acute vascular occlusions (stroke, myocardial infarction, limb ischemia) with the respective reperfusion strategies (thrombolytic therapy, angioplasty, and operative revascularization), also routine surgical procedures (organ transplantation, free-tissue-transfer, cardiopulmonary bypass, vascular surgery) and major trauma/shock [3].

In kidney transplantation, IRI occurs in situations such as graft harvesting, cold storage and surgery. Clinical consequences of IRI have been considered to be delayed graft function and acute rejection in the short term and chronic rejection late after transplantation [4].

1.1.1 Pathophysiology of IRI

The pathophysiology of the IRI is complex (Table 1.1) and inflammatory mechanisms play a major role (Figure 1.1). The pathogenetic events mainly involve injured endothelium and activated leukocytes and interaction of the two.

The initial metabolic change during tissue ischemia is energy depletion as a result of defective resynthesis of adenosinetriphosphate (ATP) and degradation of it finally to hypoxanthine. Under physiological conditions, hypoxanthine is converted to xanthine by the enzyme xanthine dehydrogenase. However, under ischemic conditions, xanthine dehydrogenase is converted by Ca^{2+} dependent proteases to xanthine oxidase that is capable of generating reactive oxygen species (ROS) [3, 5].

Table 1.1 : Effects of ischemia-reperfusion injury [3].

Molecular effects	Cellular effects
ATP depletion	Endothelial cell dysfunction/ swelling
Defective ATP-resynthesis	Leukocyte (PMN) recruitment
Increase in hypoxanthine	Oxidative burst (PMN)
Activation of xanthine oxidase	Impaired vasodilatation (NO-mediated)
Generation of ROS (O_2^- , H_2O_2 , $OH^\cdot$, $ONOO^-$)	Enhanced vasoconstriction (endothelin-mediated)
Antioxidant (glutathione) depletion	Endothelial barrier disruption
Intracellular Na^+ and Ca^{++} -overload	Expression of adhesion molecule: (P-/L-selectin, ICAM-1/2)
Activation of NHE	Liberation of matrix-degrading proteases (Elastase, MMP-9)
Activation of PLA_2	
Activation of PARP	
Activation of $NF\kappa B$	
Activation of Toll-like- receptor-signaling	
Subcellular effects	Mediators
Mitochondrial dysfunction/swelling	Arachidonic acid metabolites (LTB_4 , TXA_2)
Translocation of <i>bax</i>	Cytokines (IL-1 β , IL-6, TNF- α)
Efflux of cytochrome c	Chemokines (IL-8, MCP-1)
Lipid peroxidation	Activated complement (C3a, C5a, C5b-9)
DNA strand breaks	
Cell membrane damage	
Increased cell membrane permeability	
Cytoskeletal derangements	

In IRI, homeostasis of oxygen metabolism is altered and highly reactive oxygen species such as H_2O_2 , $OH^\cdot$ and O_2^- accumulate. Upon ischemia, antioxidant defenses are depleted and therefore highly destructive hydroxyl radical ($OH^\cdot$) elevates causing direct damage to cellular membranes as well as proteins and inducing lipid peroxidation. Following restoration of oxygen supply with reperfusion, the production of ROS is increased intensely by dysfunctional mitochondria, xanthine oxidase and NO synthase (NOS) systems. In addition to cellular lipids and proteins, ROS react directly with DNA, leading to cell injury and cell death [2, 3].

Depletion of the ATP stores inhibits the active ion transport systems (the sodium [Na^+]-potassium [K^+]-ATPase) of ischemic cells. This results in intracellular accumulation of sodium and calcium (Ca^{2+}) ions [6, 4]. Consequently, cellular influx of water and swelling occur. Intracellular calcium increase exerts detrimental effects

by activating the intracellular enzymes proteases and endonucleases which are important in proapoptotic signaling, and it activates plasma-membrane phospholipase A₂ which is an enzyme that activates the arachidonic acid cascade. This cascade leads to the production of leukotrienes, which are potent chemotactic agents important for the interaction between leukocytes and endothelial cells [3, 4].

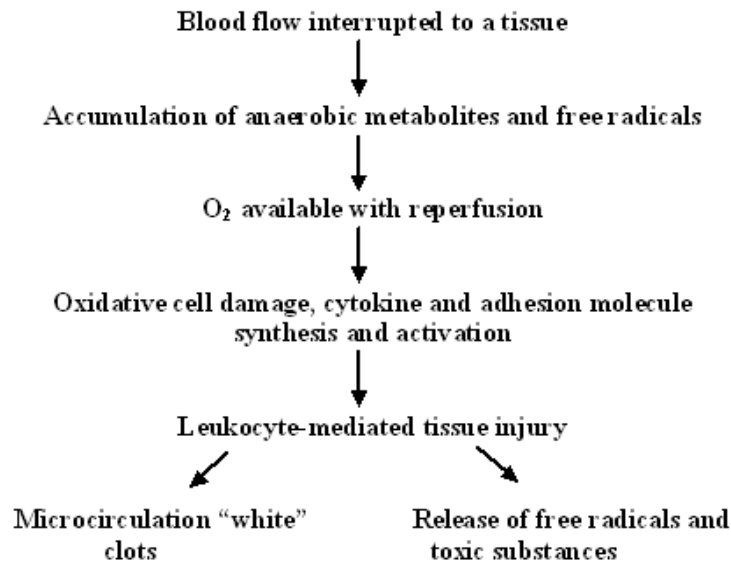


Figure 1.1 : Proposed inflammatory cascade in ischemia-reperfusion injury [1].

The influx of sodium and calcium induces a consecutive flow of water from the vessels into the cells. Swelling of endothelial cells reduces the vascular lumen and leads to an obstruction of the capillary flow. Consequently, the local hemodynamic is disturbed, platelets begin to aggregate and leukocytes come in a physically closer distance to endothelial cells [7]. In addition, following reperfusion, NO which has vasodilation properties is decreased whereas endothelin-1, the most powerful vasoconstrictor, is dramatically increased. Additionally, disruption of the endothelial barrier due to disorganization of junctional adhesion proteins leads to increased fluid filtration at the capillary level and macromolecular leakage. All these events lead to reduction of capillary perfusion despite adequate restoration of blood flow, and constitute the “no-reflow” phenomenon, although early reports have attributed it to blockage of capillaries by neutrophils preventing reperfusion [3, 8].

The degree of tissue damage strongly correlates with the number of recruited leukocytes. Various pro-inflammatory factors such as leukotriene B₄ and the complement activation product C5a activate circulating neutrophils. The process of

leukocyte adhesion is initiated by polymorphonuclear leukocytes (PMN) which are attracted to the vascular intima and stimulate activities of other leukocyte populations [4]. To emigrate into inflamed tissue, leukocytes initially tether to and roll on the vascular endothelium (Figure 1.2). This relatively loose adhesion is mediated by the P- and E-selectins on the endothelium and L-selectin on leukocytes, while the integrin molecules CD11/CD18 on leukocytes and their receptors ICAM-1 and ICAM-2 on the endothelium are responsible for sticking and firmer adhesion. Leukocyte transmigration is the final stage and occurs between endothelial cells. Leukocytes then travel through the extracellular matrix to the site of tissue injury that is guided by a concentration gradient of cytokines and chemokines produced at the site of injury [1, 4].

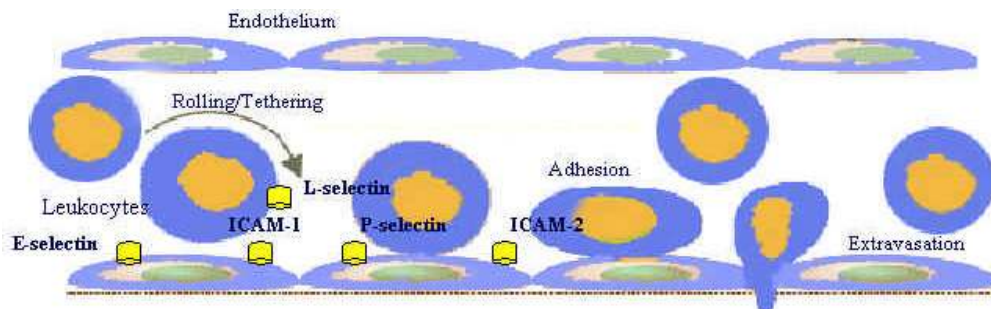


Figure 1.2 : Current model of leukocyte migration to inflamed tissue [1].

In conclusion, ischemia-reperfusion initiates a complex interplay between the endothelium, different types of blood cells and leukocytes, leading to microvascular injury and cell necrosis or apoptosis.

1.2 Preconditioning Before IRI

While ischemic injury is mainly due to oxygen-deprived cellular necrosis, reperfusion produces an inflammatory response that both heightens local damage and leads to systemic insult. Various methods of limiting reperfusion injury have been described such as oxygen radical scavenging, leucodepletion, induced hypothermia and controlled reperfusion [9].

1.2.1 Ischemic preconditioning (IP)

In 1986, Murry and colleagues described the phenomenon of ischemic preconditioning (IP) which proved to be one of the most powerful methods of

minimising ischemia-reperfusion injury (IRI) [9]. Murry et al. demonstrated in the canine heart that four cumulative 5 min ischemia-5 min reperfusion periods conferred protection from a subsequent 40 min sustained ischaemic insult and myocardial infarct size was reduced to 25% of that seen in the control group. On the other hand, when an identical preconditioning protocol was followed on the animals which then received a sustained 3 hours occlusion, there was no difference between infarct size in the preconditioned and control groups. (Note that all animals were allowed 4 days of reperfusion after sustained ischemia). The protective effect of preconditioning in the 40 min study may have been due to reduced ATP depletion and/or to reduced catabolite accumulation during the sustained ischaemic occlusion. As a result of the study, the multiple anginal episodes that often precede myocardial infarction in man may delay cell death after coronary occlusion, and thereby allow for greater salvage of myocardium through reperfusion therapy [10]. IP is effective within minutes, suggesting that preformed mediators are responsible for its effects. Locally released agonists such as adenosine, bradykinin, catecholamines and opioids trigger the protective response through various cell surface G-protein coupled receptors. Kinases such as protein kinase C, tyrosine kinase and p38MAPKinase participate in the signalling pathway. The mitochondrial ATP-sensitive potassium (K_{ATP}) channels may serve as the end-effector of preconditioning [9].

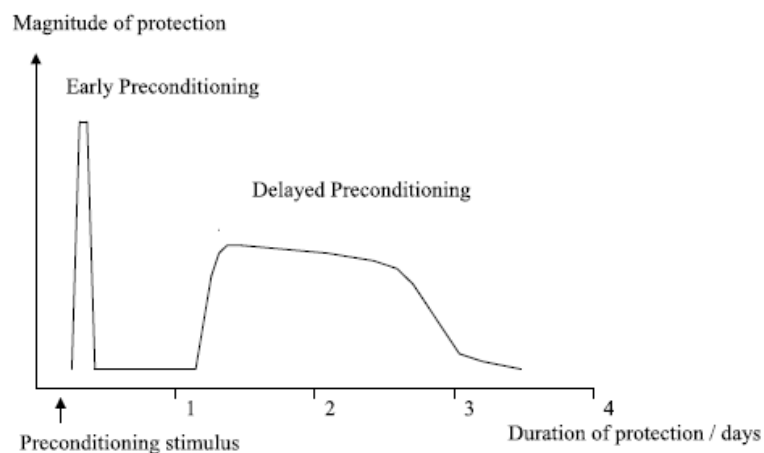


Figure 1.3 : Biphasic protection induced by a single preconditioning episode [9].

IP induces a biphasic protection from IRI: an initial strong protective stimulus that is brief and a later, less powerful but longer lasting protection (Figure 1.3). Repeated preconditioning episodes are neither additive nor cumulative (Figure 1.4) [9].

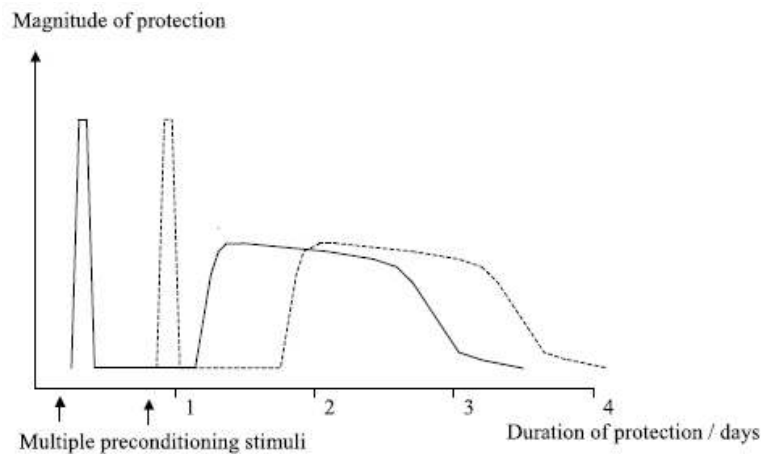


Figure 1.4 : Multiple preconditioning episodes convert biphasic protection into a monophasic pattern. Note that the magnitude of protection is not increased and the duration of protection is dictated by the last preconditioning stimulus [9].

According to a comprehensive review by Pasupathy and Homer-Vanniasinkam (2005), IP utilises endogenous mechanisms in skeletal muscle, liver, lung, kidney, intestine and brain in animal models to convey varying degrees of protection from IRI [9]. Recent reports also suggest that human heart, liver, lung and skeletal muscle acquire similar protection after IP. Preconditioned tissues exhibit reduced energy requirements (conservation of energy substrates), diminished metabolism, better electrolyte homeostasis and genetic reorganisation, giving rise to the concept of ‘ischemia tolerance’. IP also induces ‘reperfusion tolerance’ with less reactive oxygen species and activated neutrophils released, reduced cytokine production and apoptosis, and enhanced microcirculatory perfusion compared to nonpreconditioned tissue. Systemic IRI is also diminished by preconditioning. IP is ubiquitous but more research is required to fully translate these findings to the clinical arena [9].

In a study, rats were randomized to sham operation, 45 min renal ischemia, ischemic preconditioning with four cycles of 8 min renal ischemia and 5 min reperfusion followed by 45 min renal ischemia and systemic adenosine pretreatment before 45 min renal ischemia. 45 min of renal ischemia followed by 24 hours of reperfusion resulted in marked rises in blood urea nitrogen and creatinine. Ischemic

preconditioning and adenosine pretreatment protected renal function and improved renal morphology. It was demonstrated for the first time that rat kidneys can be preconditioned to attenuate IRI, and adenosine infusion before ischemic insult protects renal function via A₁ adenosine receptor activation [11].

In another study, it was found in rats that 15 min of warm ischemia and 10 min of reperfusion in the kidney is the most suitable one-cycle schedule for preconditioning since it protects from both warm and cold ischemia. Prolongation of the reperfusion window (20 or 40 min) abolished the preconditioning protection. The beneficial effect of preconditioning is related to the local production of NO, and IP has promising therapeutic value in clinical renal transplantation [12].

1.2.2 Heat preconditioning (HP)

The heat-shock response consists of the expression of a family of highly conserved proteins that are known as heat-shock proteins (HSPs) [13, 14, 15] and for their cytoprotective function in a variety of injury models [16]. Recent experimental studies have elucidated that the induction of HSPs is capable of reducing ischemic necrosis in myocardial, neuronal, and renal tissue [17]. The protective role of heat preconditioning (HP) or HSPs is thought to be due to their ability to facilitate refolding, assembly, and stabilization of denatured proteins. However, precise molecular mechanisms are not well known. The protective effect of HSP-70 is partially mediated through inhibition of NF- κ B pathway-related inflammation, as well as modulation of cell necrosis or apoptosis. HP stabilizes I κ B proteins and suppresses their activation in liver IRI or myocardial inflammation. In addition, HSP-70 has been demonstrated to be protective in renal tubular cell apoptosis [16].

Jo et al (2006) have investigated the *in vivo* effect of HP on inflammation and tubular cell necrosis and apoptosis in ischemic ARF in rats. They found that HP attenuated the renal injury, and it was accompanied by inhibition of NF- κ B activation with subsequent decrease in inflammation and also decrease in tubular cell necrosis and apoptosis. HSP-70 is thought to be responsible for this beneficial effect [16].

Another study in rats showed that HP induces renal HSP72 and, for the first time, HSP32. HP increases survival following transplantation after 40 hours cold ischemia (cold storage) and acts by improving several parameters of kidney function including proteinuria, volume output and creatinine clearance [18].

According to a recent study on mice, local heat preconditioning of myocutaneous tissue markedly increases flap survival by maintaining adequate nutritive perfusion rather than inducing ischemic tolerance. The protection is caused by the increased arteriolar blood flow due to significant arteriolar dilation, which is mediated through the carbon monoxide-associated vasoactive properties of HSP-32 [17].

1.3 *ATF3* Gene, its Products and Role in Stress

1.3.1 *ATF3* gene and its products

Activating transcription factor 3 (ATF3) is a member of the mammalian activating transcription factor/cAMP responsive element binding protein (ATF/CREB) family of basic leucine zipper (bZip)-type transcription factors [19, 20]. Human *ATF3* gene is localized on the q32.3 region of the chromosome 1 [21]. ATF3 protein is composed of 181 amino acids, and the basic region and leucine zipper domain from 88 to 147 amino acids are required for dimer formation and specific DNA binding [19, 20].

The homodimer of ATF3 represses transcription from various promoters with ATF sites, probably by stabilizing inhibitory co-factors at the promoter, whereas heterodimers with c-Jun or JunB activate transcription [19, 20]. In addition to the heteromeric forms, various spliced isoforms of ATF3 may further generate functional diversity in different cellular context [20]. The splice variant ATF3 Δ Zip was isolated in serum-stimulated HeLa cells. It lacks the leucine zipper domain and does not bind to DNA. It stimulates transcription, presumably by sequestering inhibitory co-factors away from the promoter [19]. ATF3 Δ Zip2a and -b were isolated from cells treated with various stimuli such as A23187, TNF- α , endoplasmic reticulum stress, or oxidative stress. These two isoforms encode the C-terminally truncated protein of 135 amino acids. ATF3 Δ Zip2 lacks the leucine zipper domain and thus is incapable of DNA binding [22]. It sensitizes cells to apoptotic cell death, partially through suppressing nuclear factor (NF)- κ B-dependent transcription of antiapoptotic genes such as *cIAP2* and *XIAP* [20]. ATF3 Δ Zip2c and ATF3 Δ Zip3 were identified in amino acid- and glucose-deprived cells [23]. Another isoform, ATF3b, is implicated in mediating cAMP signaling of proglucagon transcription in pancreatic α -cells [24].

1.3.2 Induction of Atf3 expression by extracellular signals

It has been hypothesized that *ATF3* is an immediate-early gene that responds to extracellular signals. The evidences are that first, the mRNA level of ATF3 is relatively low in most cell types examined, but greatly increases upon serum induction or during liver regeneration. Second, the 3' untranslated region of ATF3 mRNA contains several AUUUA sequences, a characteristic of the mRNAs of many immediate-early genes. Third, ATF3 is similar to c-Fos (a well characterized immediate-early gene) in the bZip region [25].

When the expression of ATF3 was examined by *in situ* hybridization in different tissues after a variety of treatments, the following correlation was noticed: Signals that induce tissue injury increase the levels of ATF3 mRNA, but signals do not induce tissue injury fail to do so. Considering the ischemia-reperfusion injury-related results, it was found that myocardial ischemia and myocardial ischemia coupled with reperfusion induce ATF3 in the heart, and renal ischemia-reperfusion injury induces ATF3 in the kidney (Table 1.2). It can be hypothesized from all results that induction of ATF3 is a part of the cellular stress responses [25].

Table 1.2 : Examples of treatments that induce the expression of ATF3 in tissues of whole organisms [25].

Tissues	Treatments
Liver	Partial hepatectomy Alcohol Carbon tetrachloride Acetaminophen Cyclohexamide Hepatic ischemia
Heart	Ischemia Ischemia-reperfusion
Kidney	Ischemia-reperfusion
Brain	Seizure
Peripheral nerves	Axotomy
Skin	Wounding
Thymus	Anti-CD3 ϵ

ATF3 has also been demonstrated to be induced in cultured macrophage cells by treatment with cytokines such as interferons (IFNs) and interleukin-4 (IL-4), in various cell types by genotoxic agents such as ionizing radiation, methyl methanesulfonate and ultraviolet (UV) light, and by agents known to induce cell

death or the JNK/SAPK signaling pathway such as doxorubicin, Fas antibody, anisomycin and cyclohexamide [25].

1.3.3 Signaling pathways involved in the induction of Atf3 by extracellular signals

JNK/SAPK pathway plays a role in the induction of ATF3 by stress signals [25]. Also, IL-6 and p53 are involved in the induction of ATF3 by extracellular signals. In IL-6 deficient mice, the induction is attenuated, but can be rescued by IL-6 injection [26]. The induction of ATF3 by genotoxic agents is p53-dependent in some cells [27].

1.3.4 Atf3 in cellular stress responses

Induction of ATF3 is a part of the cellular stress responses. However, it is not clear whether it is a beneficial stress response (such as heat shock response) or a detrimental stress response (such as inflammatory response). ATF3 may play a role in regulating cell cycle machinery [25].

1.4 GADD45G Gene, its Product and Role in Stress

1.4.1 GADD45G gene and its product

Myeloid differentiation (*MyD*) primary response and growth arrest- and DNA damage-inducible (*Gadd*) genes comprise a set of overlapping genes, including known (*IRF-1*, *EGR-1*, *Jun*) and novel (*MyD88*, *Gadd45a*, *MyD118/Gadd45b*, *CR6/Gadd45g*, *MyD116/Gadd34*) genes which are coordinately induced upon the onset of terminal myeloid differentiation and exposure of cells to stress stimuli. *MyD/Gadd* genes play a role in blood cell development, and are also involved in inflammatory responses to invading micro-organisms, and response to environmental stress and physiological stress, such as hypoxia, which results in ischemic tissue damage [28, 29].

The *Gadd45* gene family (*Gadd45a*, *Gadd45b*, *Gadd45g*) encodes for small (18 kDa), evolutionary conserved proteins that are highly homologous to each other (55-57% overall identity at the amino acid level), are highly acidic (pI = 4.0-4.2), and are primarily localized within the cell nucleus. These are a family of growth suppressive and apoptotic proteins which serve similar but not identical functions and interact

with cell cycle proteins [28, 29] and play a role in the G₂/M checkpoint in response to DNA damage [30].

CR6 (cytokine response gene 6) gene is the third member of the *Gadd45* gene family [31] and currently called as *Gadd45g* (*Gadd45γ*, *Gadd45gamma*) [21]. It was originally identified as an immediate early response gene in T cells stimulated by interleukin-2 [32]. The full-length sequences of murine and human *Gadd45g* cDNAs were determined [31, 33]. The human *Gadd45g* gene is localized on the q22.1-q22.2 region of the chromosome 9 [21]. It encodes a 159 amino acid length protein, which is named growth arrest- and DNA damage-inducible protein, gamma [34].

1.4.2 Induction of Gadd45g by extracellular signals

Gadd45 family members are rapidly induced by genotoxic stress agents, as well as by terminal differentiation and apoptotic cytokines [35].

1.4.3 Gadd45g in stress signaling

Gadd45 genes have been implicated in stress signaling in response to physiological or environmental stressors, which results in cell cycle arrest, DNA repair, cell survival and senescence, or apoptosis. Functioning as stress sensors, *Gadd45* proteins physically interact with other cellular proteins which are implicated in cell cycle regulation and the response of cells to stress. These include PCNA, p21, cdc2/cyclinB1, and the p38 and JNK stress response kinases. It remains to be determined what deterministic factors dictate whether *Gadd45* and partner proteins function in either cell survival or apoptosis (Figure 1.5) [35].

Evidence was obtained that *Gadd45b* and *Gadd45g* activate p38/JNK signaling by interacting with MEKK4, and cytokine production in effector T cells. MEKK4 is an upstream activator of the stress induced p38/JNK kinases, and the stress-responsive p38 and JNK MAPK pathways regulate cell cycle and apoptosis [33, 35].

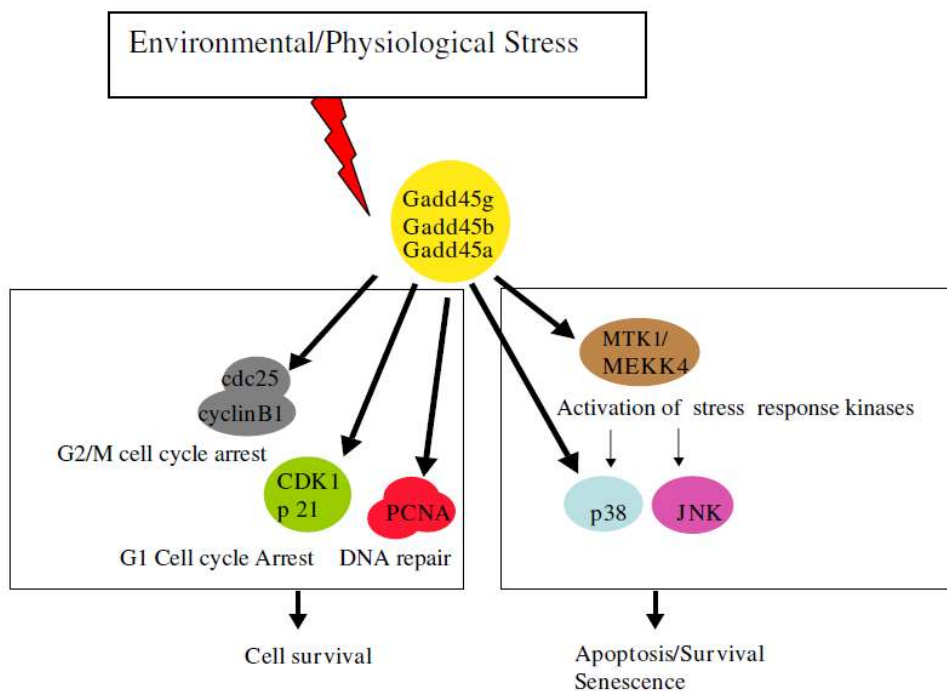


Figure 1.5 : Gadd45g in stress signaling : Coordination of cellular stress responses via interaction with partner proteins [35].

1.4.4 Gadd45g in cellular stress responses

Gadd45g was shown to have a role in cell cycle arrest by blocking G₁/S and G₂/M transitions. It mediates G₂/M cell cycle arrest due to interacting with and inhibiting the kinase activity of the cdc2/cyclinB1 complex. Its interaction with p21 may play a role in G₁ cell cycle arrest [35].

Gadd45g has a pro-apoptotic function, and was shown to have a role in neuronal cell death. However, interaction of Gadd45g protein with PCNA may promote cell survival by enhancing DNA repair [35].

1.5 Multiplex Polymerase Chain Reaction (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. Multiplex PCR considerably saves time and effort in the laboratory. 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-transcription (RT)-PCR.

An efficient multiplex PCR usually requires strategic planning and optimization of reaction conditions. For a successful multiplex PCR assay, adjusting of primer concentration for each target sequence, concentration of the PCR buffer, balance between the magnesium chloride and deoxynucleotide concentrations, cycling temperatures, and amount of template DNA and Taq DNA polymerase are important [36, 37].

1.6 Quantitative Reverse Transcription PCR (QRT-PCR)

Reverse transcription PCR (RT-PCR) represents a sensitive and powerful tool for analyzing RNA. It has tremendous potential for quantitative applications, however, a comprehensive knowledge of its technical aspects is required. This technique is highly sensitive that it permits analysis of gene expression from very small amounts of RNA (even at the level of the content of a single cell). Moreover, this method can be applied on a large number of samples and/or many different genes in the same experiment. This method provides quantification of specific RNA transcripts and the detection of any variation in their expression levels under different experimental conditions [38, 39]. Figure 1.6 presents the steps in a qRT-PCR approach.

In this thesis work, an RNA standard as described in Figure 1.6 was not used. The RNA standard shown in Figure 1.6 is a homologous, external RNA standard. In this thesis work, an internal (endogenous) standard RNA (β -Actin) was co-amplified with the target RNA in the same tube to be used for relative quantification.

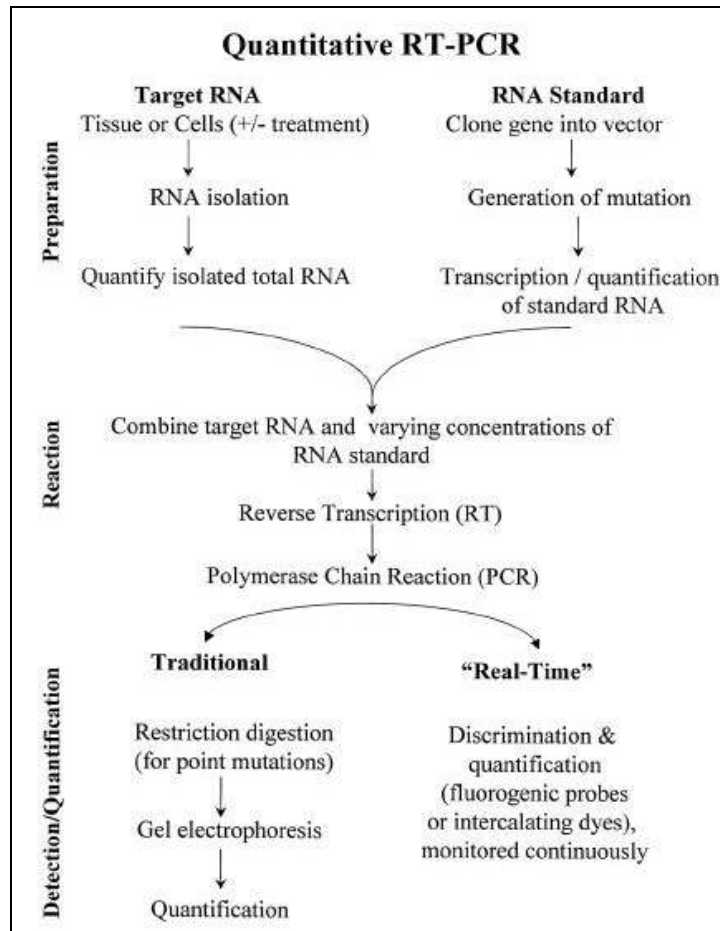


Figure 1.6 : Flowchart of the technical steps in qRT-PCR [38].

1.7 Quantitative Real-Time Reverse Transcription PCR (QReal-Time RT-PCR)

The real-time PCR is the technique that uses fluorescent reporter molecules to monitor the production of amplification products during each cycle of the PCR reaction, thus combines nucleic acid amplification and detection steps into a single step. The time (or PCR cycle) where the target amplification is first detected is usually referred to as cycle threshold (C_T) at which fluorescence intensity is greater than background fluorescence. The greater the quantity of target DNA in the starting material, the faster a significant increase in fluorescent signal will appear, yielding a lower C_T [40, 41].

There are many benefits of using real-time PCR method over other methods to quantify gene expression [40, 41]:

- Quantitative data can be produced with 7 to 8 log orders of magnitude.

- Gel electrophoresis to detect amplification products is not required.
- Real-time PCR assays are 10,000- to 100,000-fold more sensitive than RNase protection assays, 1000-fold more sensitive than dot blot hybridization, and can even detect a single copy of a specific transcript.
- Real-Time PCR can reliably detect gene expression differences as small as 23% between samples and have lower coefficients of variation (cv; SYBR Green at 14.2%; TaqMan at 24%) than end point assays such as band densitometry (44.9%) and probe hybridization (45.1%).
- Real-time PCR can also discriminate between messenger RNAs (mRNAs) with almost identical sequences, and requires much less RNA template than other methods of gene expression analysis.

The major disadvantage of real-time PCR is that it requires expensive equipment and reagents. In addition, a powerful experimental design and a deep understanding of normalization techniques are necessary for accurate conclusions [40].

Real-time PCR can be performed as either a one-step reaction, where the entire reaction from cDNA synthesis to PCR amplification is performed in a single tube, or as a two-step reaction, where reverse transcription and PCR amplification occur in separate tubes [40]. Figure 1.7 shows the general steps in a real-time PCR experiment, from RNA isolation to data analysis.

There are two methods of quantification of real-time PCR data: absolute quantification and relative quantification. Absolute quantification uses a standard curve that is generated by serially diluted DNA or RNA standards (such as 1:10 diluted, 1:100 diluted and so on) of known concentrations. The standard curve produces a linear relationship between C_T and initial amounts of standard sample, thus allows the determination of the concentration of unknowns based on their C_T values. This method assumes all standards and samples have approximately equal amplification efficiencies. In addition, the concentration of serial dilutions should comprise the levels in the experimental samples, therefore a more concentrated RNA or cDNA sample than the unknown samples should be used to generate a standard curve [40].

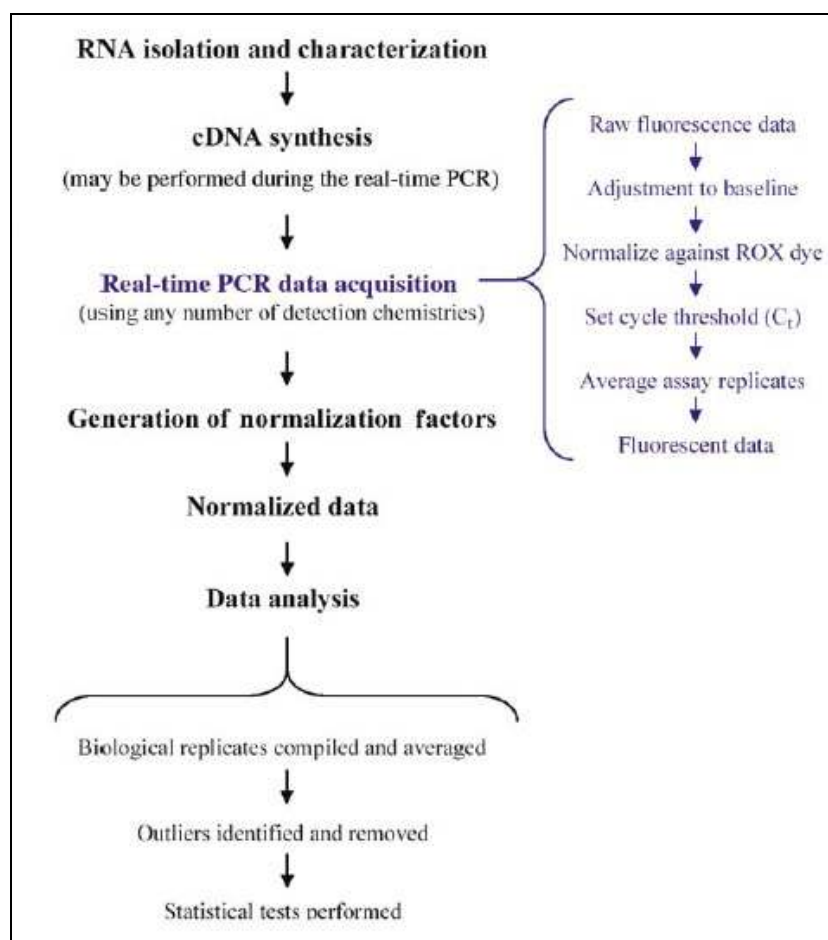


Figure 1.7 : Steps performed during quantitative real-time PCR [40].

1.7.1 Relative quantification

Relative quantitation of gene expression allows quantifying differences in the expression level of a specific target gene between different samples. The data output is expressed as a fold-change or a fold-difference of expression levels. For example, one can look at the change in expression of a particular gene over a given time period in treated vs. untreated samples. For this, a calibrator sample (i.e. untreated at day 0) and an endogenous control gene (i.e. Beta-Actin) to normalize input amounts are chosen. For all samples, at first, both target and endogenous control gene levels are assessed by real-time PCR. Secondly, target level is normalized by endogenous control level for all samples. Finally, the normalized results are then expressed in a format such as “At day 30, sample A had a 10-fold greater expression level of the target gene than at day 0” [42].

Endogenous control is a gene sequence contained in a sample that is used to normalize target quantities. In addition to the target sequence, an endogenous control is quantified as a means of correcting results that can be skewed by input nucleic acid loading differences. Endogenous control has its own set of primers and probe in the reaction tube. Endogenous control genes are thought to have stable expression such as housekeeping genes like *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase), *ACTB* (Beta-Actin) and rRNAs. These genes are present in all nucleated cell types, and mRNA synthesis of these genes is considered to be stable in various tissues, even under experimental treatments. However, these genes have been shown to be affected by different treatments, biological processes, and even different tissues or cell types [40, 42, 43]. Therefore, when using a housekeeping gene for normalization, one must validate its stability with her/his own samples rather than relying on previously published materials [40].

Amplification efficiency (E) of the reaction is an important consideration when performing relative quantitation. The ideal E value is “1” which means that the PCR product concentration doubles during every cycle within the exponential phase of the reaction. However, many PCRs do not have ideal E values. Amplification efficiency is calculated using data collected from a standard curve with the following formula [40]:

$$\text{Exponential amplification} = 10^{(-1/\text{slope})} \quad (1.1)$$

$$\text{Efficiency} = [10^{(-1/\text{slope})}] - 1 \quad (1.2)$$

There are several methods of relative quantitation. Here the most widely used two methods will be described: standard curve method and comparative C_T method.

1.7.2 Standard curve method for relative quantification

This method uses a standard curve which is generated by using serially diluted standards of known concentrations as mentioned also in the description of absolute quantification above. Concentrations of target gene and endogenous control gene are determined by interpolating from the standard curve for all experimental samples. Normalization and then relative expression to calibrator sample for each treated sample are then calculated using the concentration values. Because sample quantity is divided by calibrator quantity, standard curve units are eliminated, requiring only the relative dilution factors of the standards for quantification [40, 42].

This method requires the least amount of validation because the PCR efficiencies of the target and endogenous control do not have to be equivalent. It gives highly accurate quantitative results [42]. It is also the simplest quantification method [40]. However, this method is useful when testing low numbers of targets and small numbers of samples and when looking for very discrete expression changes, because it requires that each reaction plate contain standard curves, and requires more reagents and more space on a reaction plate [42].

1.7.3 Comparative C_T (ΔΔC_T) method for relative quantification

This method is similar to the standard curve method, except it is a mathematical model and uses arithmetic formulas to obtain the relative quantitation of gene expression. The use of this method is only possible when the PCR efficiencies of the target and endogenous control are relatively equivalent. Because the comparative C_T method does not require a standard curve, it is useful when assaying a large number of targets and/or samples since all reaction wells are filled with sample reactions rather than standards. Thus, fewer reagents are used [40, 42].

In this method, the amount of target, normalized to an endogenous reference and relative to a calibrator, is given by the following formula [44]:

$$2^{-\Delta\Delta C_t} \quad (1.3)$$

1.8 Aim of the Study

In a previous study with microarray analysis, it was observed that *Atf3* and *Gadd45g* genes were upregulated when rat kidney tissues were studied for gene expression upon ischemia-reperfusion, ischemia-reperfusion with heat preconditioning and with ischemic preconditioning [45, 46]. In this study, the aim was to investigate the expression levels of these genes under the same conditions in total RNA samples from rat kidney tissues by using quantitative (q) RT-PCR and qReal-Time RT-PCR techniques, and to compare the results with the previously obtained microarray results.

2. MATERIALS AND METHODS

2.1 Materials and Laboratory Equipment

2.1.1 Equipments

The laboratory equipment used in this study is listed in Appendix A.

2.1.2 Chemicals, Enzymes, Markers, and Buffers

For agarose gel electrophoresis, agarose from AppliChem was used. 10X TBE was prepared with 121.14 g Tris-hydroxymethyl-aminomethan (BDH Laboratory Supplies, England), 61.83 g Boric acid (MERCK, Germany), 7.44 g EDTA (Carlo Erba Reagents), and adding distilled water to complete the volume to 1 liter. 1X TBE was then prepared by 1:10 diluting the 10X TBE. Three μ l and 7.5 μ l EtBr (10 mg/ml; Sigma) was used respectively in the mini and midi gels. #SM0323 marker (GeneRuler 100bp Plus DNA Ladder) from Fermentas was used. For the DNA contamination testing experiments, recombinant *Taq* DNA Polymerase (#EP0402) from Fermentas was used. For making the PCR tubes sterile and free of RNase, 1:1000 diluted diethyl pyrocarbonate (DEPC; MP Biomedicals) was prepared with deionized water. Ethanol (70% v/v; Sigma-Aldrich) was used for common sterilization prior to setting of PCR.

2.1.3 Molecular biological kits

For RT-PCR, SuperScript III One-Step RT-PCR System with Platinum *Taq* DNA Polymerase kit (for 100 reactions) of Invitrogen was used to synthesize cDNA from total RNA in one step. For Real-Time PCR, Transcriptor High Fidelity cDNA Synthesis Kit (for 50 reactions) of Roche was used for two-step cDNA synthesis prior to PCR, and LightCycler Taqman Master kit (for 96 reactions) of Roche was used for PCR on the LightCycler Carousel-Based System using Hydrolysis (Taqman) Probes.

2.2 RNA Samples

Total RNAs were used in the experiments. RNAs had been previously isolated from kidney tissues of rats (*Rattus norvegicus*) by using peqGOLD TriFast (peqLab) kit [45]. The rats were 30 weeks-old, 220-358 g, male Wistar rats and were exposed to ischemia by clamping the left kidneys. A group of the rats had been exposed to “heat preconditioning (HP)” and another group to “ischemia preconditioning (IP)” before the application of ischemia-reperfusion, whereas the third group of rats had been exposed to no preconditioning before. The HP group was exposed to 42 °C for 5 min, 24 h before ischemia. IP was applied as 3 cycles of ischemia for 5 min and the following reperfusion for 10 min. Following the preconditioning, the rats were exposed to ischemia for 45 min (I45) and immediately afterwards, to no reperfusion (for 0 min, R0), to reperfusion for 15 min (R15), or for 60 min (R60).

Each RNA sample had two copies (A and B) which were obtained separately from two groups of rats. Thus, the RNA samples were composed of three main groups:

1. Heat Preconditioning (HP) group: The rats in this group were exposed to heat preconditioning before ischemia-reperfusion. These samples had sample numbers between 1 and 6.
2. No preconditioning group: The rats in this group were not exposed to any preconditioning before ischemia-reperfusion. The sample numbers were between 13 and 18.
3. Ischemia Preconditioning (IP) group: The rats in this group were exposed to ischemia preconditioning before ischemia-reperfusion. The sample numbers were between 25 and 30.

Additionally, samples called “Sham-A” and “Sham-B” were obtained from rats which were exposed only to the same surgical manipulation with other rats, but neither to preconditioning nor ischemia-reperfusion. They served as the control samples of the study.

For this thesis work, one copy (A or B) of each RNA sample was chosen. Thus, experiments were conducted with the RNA sample numbers 1, 3, 5, 14, 16, 18, 26, 28, 29 and also Sham-A, Sham-B (Table 2.1). The RNAs were stored at – 80 °C.

Table 2.1 : Total RNA samples used in this study [45].

N0	Abbreviations of applied conditions	Explanation	Probe1	Probe2	Mixture (μg)	Concentration ($\mu\text{g}/\mu\text{l}$)	A 260/280	Amount (μl)
1	HP_I45_R0_A	Heat preconditioning - Ischemia for 45 min - No reperfusion - Copy A	A 53 a	A 78	ca. 30	1.61	2.30	3
3	HP_I45_R15_A	Heat preconditioning - Ischemia for 45 min - Reperfusion for 15 min - Copy A	A 73 b	A 74 b	ca. 30	1.57	2.32	21
5	HP_I45_R60_A	Heat preconditioning - Ischemia for 45 min - Reperfusion for 60 min - Copy A	A 44 a	A 43	ca. 30	1.97	2.27	7
14	I45_R0_B	Ischemia for 45 min - No reperfusion - Copy B	A 14	A 18	ca. 30	1.55	1.42	4.5
16	I45_R15_B	Ischemia for 45 min - Reperfusion for 15 min - Copy B	A 35	A 36 a	ca. 30	1.85	1.47	3.5
18	I45_R60_B	Ischemia for 45 min - Reperfusion for 60 min - Copy B	A 19	A 67 a	ca. 30	2.32	1.62	5.5
26	IP_I45_R0_B	Ischemia preconditioning - Ischemia for 45 min - No reperfusion - Copy B	A 65	A 96	30	1.87	1.96	12
28	IP_I45_R15_B	Ischemia preconditioning - Ischemia for 45 min - Reperfusion for 15 min - Copy B	A 60 a	A 62 a	30	2.52	1.81	7
29	IP_I45_R60_A	Ischemia preconditioning - Ischemia for 45 min - Reperfusion for 60 min - Copy A	B 55	B 56	30	1.80	1.64	7.5
31	Sham_A	Control with neither preconditioning nor ischemia-reperfusion - Copy A	A 39 a	A 30	30	2.07	1.96	9
32	Sham_B	Control with neither preconditioning nor ischemia-reperfusion - Copy B	A 29 a	A 42	30	2.25	2.27	10

2.3 Multiplex Reverse Transcription-Polymerase Chain Reaction (Multiplex RT-PCR)

RT-PCR was used to amplify the cDNA sequences of the *Atf3* and *Gadd45g* genes, and studied as multiplex. For this purpose, a pair of primers (forward, F and reverse, R) for the internal control gene Beta-Actin was used as the second primer pair in the reaction.

2.3.1 Primer design

For the design of primers, coding DNA sequences (CDS) of *Atf3*, *Gadd45g* and *Actb* genes of the *Rattus norvegicus* were used from the UCSC Genome Browser website [47] (Appendix B). The primer pairs for the genes were designed by using the Primer3Plus website [48] and are listed in the Table 2.2 below. The primers were obtained from GenOva (Turkey).

The primers were confirmed by Amplify 3X software [49] by checking whether they actually bind to the related sequences on the genes. The efficiency of binding, the amplicon sizes and the primer dimer constructions were also determined by using this software. Hairpin structure analysis was performed with the aid of SciTools on the IDT website [50].

Table 2.2 : Primer pairs used in the RT-PCR study.

Gene	Primer (F: Forward, R: Reverse)	Primer Sequence	T _m (°C)	GC%	Product Size
ATF3	Primer_2_F	5' CTCCTGGGTCACCTGGTGTTT 3'	60.0 °C	55.0 %	203 bp
ATF3	Primer_2_R	5' CCGCCTCCTTTTCTCTCAT 3'	60.7 °C	50.0 %	
GADD45G	Primer_1_F	5' TTGCACGAACCTCTGCTGTC 3'	60.2 °C	50.0 %	174 bp
GADD45G	Primer_1_R	5' CGCCTGGATCAACGTAAAAT 3'	60.0 °C	45.0 %	
GADD45G	Primer_3_F	5' GGAAAGCATTGCACGAACCTT 3'	60.3 °C	45.0 %	182 bp
GADD45G	Primer_3_R	5' CGCCTGGATCAACGTAAAAT 3'	60.0 °C	45.0 %	
Beta-Actin	Primer F	5' TTGCTGACAGGATGCAGAAG 3'	60.1 °C	50.0 %	122 bp
Beta-Actin	Primer R	5' CAGTGAGGCCAGGATAGAGC 3'	59.9 °C	60.0 %	

2.3.2 Primer dilution

All primers were diluted initially to a final concentration of 100 μ M (Table 2.3). Subsequently, 1:10 dilutions were made from those stock primer solutions to a final concentration of 10 μ M. Thus, the primers were ready to be used directly in the RT-PCR reactions.

Table 2.3 : Dilution procedure for primer stock solutions.

Primers	Initial dry amount (Picomoles)	Added water (μ l)	Stock concentration (μ M)
Atf3-F	155077	1550.77	100
Atf3-R	117561	1175.61	100
Gadd45g-1F	92930	929.30	100
Gadd45g-1R	82390	823.90	100
Gadd45g-3F	101505	1015.05	100
Gadd45g-3R	60952	609.52	100
Beta-Actin-F	135100.02	1351	100
Beta-Actin-R	141349.68	1413.5	100

2.3.3 RNA templates

All RNA templates shown in Table 2.1 were diluted 1:10 initially. The volume of each RNA template to be used in the RT-PCR mixture was then calculated to have 100 ng of template amount in the reaction mixture (Table 2.4) . When preparing RT-PCR mixtures, the calculated volumes were taken from the 1:10 diluted stocks.

Table 2.4 : RT-PCR reaction amounts of the RNA templates.

RNA Template No	1	3	5	14	16	18	26	28	29	Sham-A	Sham-B
1:10 diluted concentration (ng/ μ l)	161	157	197	155	185	232	187	252	180	207	225
Reaction volume (μ l)	0.62	0.63	0.51	0.65	0.54	0.43	0.53	0.40	0.56	0.48	0.45
Starting amount (ng)	100	100	100	100	100	100	100	100	100	100	100

2.3.4 RT-PCR mixture

SuperScript III One-Step RT-PCR System with Platinum *Taq* DNA Polymerase kit (Invitrogen) was used for the preparation of RT-PCR mixture. A standard mixture of RT-PCR (except RNA templates and water) with a final volume of 50 μ l was prepared according to the supplier's instructions.

The composition of the standard RT-PCR mixture is shown in Table 2.5. This mixture was prepared individually in 200 μ l-sterile tubes, not as a mastermix for multiple reactions. Sterile and nonpyrogenic distilled water (Eau Bi-Distillée, Galen İlaç Sanayi, Turkey) was used in the reaction mixtures.

Table 2.5 : Composition of the standard RT-PCR mixture.

Components	Stock Concentration	Volume	Final Concentration
2X Reaction Mix	0.4 mM of each dNTP, 3.2 mM MgSO ₄	25 μ l	200 μ M of each dNTP, 1.6 mM MgSO ₄
Template RNA	155-252 ng/ μ l (Table 2.4)	0.40-0.65 μ l (Table 2.4)	0.8-1.3 ng/ μ l
Primer Mix (F + R) of Target Gene	10 μ M F primer, 10 μ M R primer	1 μ l	0.2 μ M F primer, 0.2 μ M R primer
Primer Mix (F + R) of Beta-Actin gene	10 μ M F primer, 10 μ M R primer	1 μ l	0.2 μ M F primer, 0.2 μ M R primer
SuperScript III RT/ Platinum <i>Taq</i> Mix	—	2 μ l	—
Autoclaved distilled water	—	20.35-20.60 μ l	—
FINAL VOLUME		50 μl	

The RT-PCR mixture (Table 2.5) was only used during the main (finally optimized) multiplex reactions. Beta-Actin primers were not used in the optimization experiments. Additionally, in some optimization experiments, half volumes were used. A negative control (NC) with no target RNA, in which no amplification should be observed, was also used in each experiment.

2.3.5 RT-PCR cycle conditions

Initially used RT-PCR cycle conditions are shown in Table 2.6. Annealing temperature and duration, duration of the extension phase, and the cycle repeat number of the PCR were further optimized.

Table 2.6 : General RT-PCR cycle conditions.

Phase	Temperature (°C)	Time	Cycle Repeat Number
cDNA Synthesis	55	30 min	1
Initial Denaturation	94	2 min	1
PCR Amplification			
Denaturation	94	15 s	40
Annealing	53	90 s	
Extension	68	50 s	
Final Extension	68	5 min	1

2.3.6 Testing of primers and cycle number determination

The Atf3 and Gadd45g primers were tested by using the RNA sample 3 as target whether the primers are binding to their target sequences and amplification is observed or not. The RT-PCR program used is shown in Table 2.6.

For the determination of optimum cycle number (the number after which the band brightness is constant, i.e. saturation is observed), two tubes of the same reaction mixture (50 µl + 50 µl = 100 µl) were prepared for each gene and 10 µl of the reaction mixture was taken from the reaction tubes at the cycle numbers 23, 26, 29, 32, 35, 38 and 40.

2.3.7 Testing of genomic DNA contamination of RNA samples

RNA samples 3, 18 and 26 were tested initially using the both Atf3 and Gadd45g primers and in a second experiment, samples 1, 5, 14, 16, 28, 29, Sham-A and Sham-B were tested using the Gadd45g primers to see whether the samples have any genomic DNA contamination or not. Instead of the SuperScript III RT/ Platinum *Taq* Mix, only *Taq* DNA Polymerase (Fermentas) was used at 0.4 µl volume in the 50 µl

total PCR mixture. The initial cDNA synthesis stage was omitted. PCR conditions are shown in Table 2.7.

Table 2.7 : PCR conditions of genomic DNA contamination testing.

PCR Amplification Stages	Temperature ($^{\circ}\text{C}$)	Time	Cycle Repeat Number
Denaturation	94	15 s	40
Annealing	53	90 s	
Extension	68	50 s	
Final Extension	68	5 min	1

2.3.8 Testing of primer product/dimer formations

To test whether the PCR products of the genomic DNA contamination testing experiment actually indicate genomic DNAs or primer products/dimers, PCR reaction mixtures containing only F or R primer of a primer pair or the primer pair itself (F+R) were prepared with the Atf3 and Gadd45g-1 primers. Each forward and reverse primer was diluted 1:10 before the reaction, and the previously 1:10 diluted forward + reverse primer pairs were used. Thus, all the primer solutions had a starting concentration of 10 μM . Any template RNA was not used. Compositions of reaction mixtures are indicated in Table 2.8. For the PCR reaction, the PCR program indicated in Table 2.7 was used.

Table 2.8 : Compositions of PCR reaction mixtures prepared to test any primer product/dimer formation.

Components	Forward (F) primer	Reverse (R) primer	F + R mixture	Negative Control
1. dd H₂O	23.6 μl	23.6 μl	23.6 μl	24.6 μl
2. R_{mix}	25 μl	25 μl	25 μl	25 μl
3. Primers	1 μl	1 μl	1 μl	-
4. Taq DNA polymerase	0.4 μl	0.4 μl	0.4 μl	0.4 μl
Total reaction mixture	50 μl	50 μl	50 μl	50 μl

2.3.9 Optimization of PCR amplification for ATF3

Annealing conditions of 56 °C for 30 seconds and 60 °C for 30 seconds were tested for the Atf3 primer pair using the RNA sample number 26. Three tubes of the reaction mixture were prepared for each condition: two tubes with the Atf3 primers and one tube of negative control. During reactions, 10 µl sample was taken from the tubes at the 23., 26., 29., 32., 35., 38. and 40. cycles.

2.3.10 Optimization of PCR amplification for GADD45G

Optimization experiments were conducted using two couples of primers (Primer-1F, -1R and Primer-3F, -3R) for the Gadd45g gene and the RNA sample number 3 as target. Because the first used primer pair (Primer-1) amplified a non-specific region, the second primer pair (Primer-3) was then tested.

2.3.10.1 Gradient-PCR

In Gradient-PCR approach, to determine the optimum annealing temperature, different annealing temperatures can be tested at the same time for the same PCR reaction mixture in a thermal cycler. For the first primer pair (Primer-1), 11 different temperatures (55 °C-65 °C temperature gap) were tested at the same time. For this purpose, 11 tubes of the reaction mixture and an NC tube were prepared. The reaction mixture was scaled down to a total volume of 25 µl (Table 2.9). Gradient-PCR program used is shown in Table 2.10.

Table 2.9 : Reaction mixture for Gradient-PCR with Primer-1.

Components	Sampe-3 (x11)	NC (x1)
dd H ₂ O	10.685 µl	10.685 µl
Rmix	12.5 µl	12.5 µl
Primer mix (F+R)	0.5 µl	0.5 µl
Template	0.315 µl	0.315 µL (dd H ₂ O)
Enzyme	1 µl	1 µl
TOTAL	25 µl	25 µl

Table 2.10 : Gradient-PCR conditions for the first primer pair of Gadd45g.

Phase	Temperature (°C)	Time	Cycle Repeat Number
cDNA Synthesis	55	30 min	1
Initial Denaturation	94	2 min	1
PCR Amplification			
Denaturation	94	15 s	
Annealing	55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65	30 s	40
Extension	68	50 s	
Final Extension	68	5 min	1

For the second primer pair (Primer-3), 59 °C-64 °C temperature gap was tested in Gradient-PCR, and 30 s extension time was used. The full-scale reaction volume (50 µl total) was used for reaction mixtures.

2.3.10.2 Touchdown-PCR

In Touchdown-PCR approach, a high-low temperature gap is used for specific primer binding. The highest temperature is above the melting temperature of the primers and decreased gradually. Non-specific binding of the primers to non-target sequences of DNA is avoided at the higher temperatures [51].

Thus, firstly, a Touchdown RT-PCR experiment was conducted using the Primer-3 pair. It had 4 PCR amplification stages: the first two had 3 cycle repeat, the third one 4 cycle repeat and the last amplification stage had 30 cycle repeat number (totally 40 cycles). Annealing temperature was decreased by 1 °C at each amplification stage as 63-62-61 and finally 60 °C. Annealing time was 30 s, whereas the extension time 15 s. The full-scale reaction volume (50 µl total) was used for reaction mixtures.

A second Touchdown RT-PCR experiment that had 16 PCR amplification stages was set for both of the primer pairs. The first 15 amplification stages were run for 2 cycles, whereas the last stage was run for 10 cycles. Annealing temperature of the first PCR stage was 63 °C (the highest) and decreased by 1 °C at each PCR stage up to 48 °C as the lowest temperature. Annealing and extension times were both 30 s. A total reaction volume of 25 µl was used as indicated in Table 2.9.

The third Touchdown RT-PCR experiment was set for both of the primer pairs. It had 6 PCR amplification stages which had 70-68-66-64-62 and finally 61 °C annealing temperatures decreasing gradually by 2 °C at each amplification stage. The first 5 stages had 3 cycle repeat, whereas the last one (with the 61 °C annealing temperature) had 25 cycle repeat number. Annealing time was 30 s, extension time was 50 s. The half measurements of the reaction mixture were used, and two tubes (25 µl + 25 µl = 50 µl) were prepared with each primer pair. In this case, 5 µl reaction mixture was taken from the tubes during the reaction at cycle no: 23, 26, 29, 32, 35, 38, and 40.

The last Touchdown RT-PCR experiment was set for both of the primer pairs. It had 8 PCR amplification stages which had 68-67-66-65-64-63-62 and finally 61 °C annealing temperatures decreasing gradually by 1 °C at each amplification stage. The first 7 stages had 2 cycle repeat, the last one had 26 cycle repeat number. Annealing time was 30 s, extension time was 50 s. Five µl reaction mixture was taken from the tubes during the reaction at cycle numbers: 23, 26, 29, 32, 35, 38, and 40.

2.3.10.3 Further optimization of annealing and extension times

During these experiments, only annealing and extension times were changed at certain annealing and extension temperatures. Other conditions of RT-PCR program were the same as indicated in Table 2.6.

Annealing times of 30, 20 and 10 s were tested at 61 °C with the Primer-1 pair. At 61 °C 30 s annealing condition, extension times of 50, 30, 13 and 11 s were tried. The reaction mixture that was used in the experiments is shown in Table 2.9.

With the Primer-3 pair, firstly, extension times of 20, 15 and 10 s were tried at the 60 °C 30 s annealing condition. Secondly, using the 10 s extension time, the annealing time was reduced to 20 s. The full-scale reaction volume (50 µl total) was used for reaction mixtures.

2.3.11 Agarose gel electrophoresis

Electrophoresis of RT-PCR products was done using 150 ml (2.25 g agarose in 150 ml 1X TBE) and 60 ml (0.9 g agarose in 60 ml 1X TBE) agarose gels of 1.5 % (w/v). TBE buffer (1X) diluted from 10X stock TBE solution was used both in the gels and the electrophoresis tank. EtBr was added 7.5 µl to 150 ml gels and 3 µl to 60 ml gels.

Ten μ l of cDNA samples were loaded into the wells mixing with 5 μ l diluted (in initial experiments) or with 2 μ l undiluted 6X DNA Loading Dye (#R0611, Fermentas) in the multiplex experiments, and also 5 and 7 μ l with 1 μ l of the undiluted loading dye in the test experiments. Five μ l GeneRuler™ 100bp Plus DNA Ladder (#SM0323, Fermentas) (Figure C.1) marker was loaded with multiplex RT-PCR products, whereas 3 μ l and 5 μ l of other markers (#SM1103 and #SM1203, Fermentas) (Figure C.2 and Figure C.3) were also loaded with the products of optimization experiments.

RT-PCR products were run at 100-120 V for at least 20 min. Bands of the products were observed under UV light with the BTX-26.M (Biolab) instrument, and photographs were taken using UV PhotoMW software.

2.3.12 Relative quantification

Volumes of the RT-PCR product bands were determined from gel images by using BioCapt software (Vilber Lourmat). After quantifying the volumes of the Beta-Actin bands and the target gene (Atf3, Gadd45g) bands of multiplex RT-PCR experiments, log₂ values of the volumes were calculated. For each cDNA sample to be normalized, the log₂ value of the target gene band was divided by the log₂ value of the Beta-Actin band, and then, average values were calculated for the duplicates. The results of the cDNA samples 1, 3, 5, 14, 16, 18, 26, 28, 29 were divided by the results of the cDNA samples 31 (Sham-A) and 32 (Sham-B) which are the control samples. Thus, the relative expression levels of the Atf3 and Gadd45g genes in the treated tissues to those in the non-treated tissues (Sham-A and Sham-B) were determined.

2.4 Multiplex Real-Time PCR

Multiplex Real-Time PCR was also used to quantify the expression levels of the Atf3 and Gadd45g genes. cDNA synthesis and real-time PCR kits and LightCycler 2.0 instrument of Roche were used.

2.4.1 cDNA synthesis

cDNA synthesis from the RNA samples was accomplished by using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche) according to the supplier's instructions.

cDNA synthesis had two stages: in the first stage, a template-primer mixture (11.4 μ l totally) was prepared for one 20 μ l reaction by adding the components in the order listed in Table 2.11.

Table 2.11 : Template-primer mixture (for one reaction) used in cDNA synthesis from RNA samples prior to real-time PCR experiments.

Component	Volume	Final concentration
Water, PCR-grade	8.4 μ l	–
Random Hexamer Primer (600 pmol/ μ l)	2 μ l	60 μ M
Total RNA	1 μ l	1 ng up to 4 μ g
Total volume	11.4 μl	

One μ l total RNA was used from the undiluted stock solution of the RNA sample to be used as standard sample (for standard curve) during real-time PCR experiments. For preparing other RNA samples, however, 1 μ l total RNA was used from the 1:10 diluted stock solution. After this step, the template-primer mixture was denatured by heating the tube at 65°C for 10 min in a thermal cycler (PCR device) with a heated lid (to minimize evaporation). This step ensures denaturation of RNA secondary structures. The denaturation was ended by immediately cooling the tube on ice.

The second stage of cDNA synthesis was the synthesis stage where the remaining components of the RT mix were added into the tube containing the template-primer mix in the order listed in Table 2.12. The components of the synthesis stage were prepared as a mastermix for multiple reactions.

Table 2.12 : Remaining components of RT mixture (for one reaction).

Component	Volume	Final Concentration
Transcriptor High Fidelity Reverse Transcriptase Reaction Buffer, 5X concentration	4 μ l	1X (8 mM MgCl ₂)
Deoxynucleotide Mix, 10 mM each	2 μ l	1 mM each
Protector RNase Inhibitor, 40 U/ μ l	0.5 μ l	20 U

Table 2.12 : (Continued) Remaining components of RT mixture (for one reaction).

Component	Volume	Final Concentration
DTT	1 μ l	5 mM
Transcriptor High Fidelity Reverse Transcriptase	1.1 μ l	10 U
Final volume	20 μl	

The tube was then placed in thermal cycler with a heated lid, which was programmed according to Table 2.13. Finally, the reaction was stopped by placing the tube on ice. The cDNA samples were stored at - 20 $^{\circ}$ C.

Table 2.13 : RT-PCR conditions used in cDNA synthesis prior to real-time PCR experiments.

Phase	Temperature ($^{\circ}$ C)	Time	Cycle Repeat Number
Incubation	50	30 min	1
Inactivation of the Reverse Transcriptase	85	5 min	

2.4.2 Primers and Probes

Primers and probes used in the real-time PCR experiments (Table 2.14 and 2.15) were designed specifically by using mRNA sequences of the genes on the ProbeFinder (version 2.44) software on Assay Design Center of the Universal ProbeLibrary website of Roche [52].

Table 2.14 : Data of the primers used in the real-time PCR experiments.

Primer	Length	Position	T _m ($^{\circ}$ C)	% GC	Sequence (5' to 3')	Amplicon size (nt)
ATF3 Left	22	383-404	59	50	tgtcagtcaccaagtctgaggt	88
ATF3 Right	19	452-470	60	47	cacttggcagcagcaattt	
GADD45G Left	19	107-125	59	58	cgtggccaggatacagttc	75
GADD45G Right	20	162-181	60	50	cagcagaagttcgtgcaatg	

Table 2.15 : Data of the probes used in the real-time PCR experiments.

Probe	Gene	Catalog number	Sequence
#70	ATF3	04688937001	ggcggcgg
#76	GADD45G	04688996001	cacagcca

Before starting the setting up the real-time PCR reaction, the dry lyophilized oligomer primers of the Atf3 and Gadd45g genes were diluted. The Beta-Actin primer pair was already ready-to-use in the kit and was not diluted further. All probes (Atf3, Gadd45g, Beta-Actin) were aliquoted as 10 μ l in 200 μ l sterile tubes, and the tubes were then covered with aluminium foil to prevent reaction with light.

Primer dilutions were made according to the IDT SciTools Resuspension Calculator software on the IDT website [53] by entering the “amount of oligo (OD₂₆₀)” and molecular weight data of the primers, and selecting the “Single-Stranded DNA” option. Dilution procedure is shown in Table 2.16.

Table 2.16 : Dilution procedure for the primers used in real-time PCR experiments.

Primer	Amount of sterile water to be added to the primer (μ l)	Stock concentration (μ M)
ATF3-F (Left)	733.3	100
ATF3-R (Right)	860.9	100
GADD45G-F	685	100
GADD45G-R	845.6	100

After preparing the main primer stocks of 100 μ M concentration, intermediate stocks were also prepared by diluting 20 μ l of the main stock at 1:5 (20 μ l primer and 80 μ l sterile water) to reach the final primer concentration of 20 μ M.

2.4.3 Real-Time PCR procedure

LightCycler TaqMan Master kit of Roche was used for the preparation of real-time PCR mixture. It is a ready-to-use hot start reaction mix for PCR on the LightCycler carousel-based system (LightCycler 2.0 instrument) using hydrolysis (TaqMan) probes [54]. PCR parameters are shown in Table 2.17.

Table 2.17 : PCR parameters that must be programmed for a LightCycler carousel-based system PCR run with the LightCycler TaqMan Master [54].

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

Pre-incubation is required for activation of FastStart Taq DNA Polymerase and denaturation of the DNA.

Preparation of the PCR mix for a 20 µl standard reaction was done in the order listed in Table 2.18. Multiple reactions were set by preparing a mastermix without including the target cDNA.

Table 2.18 : Multiplex real-time PCR mix (for one reaction).

Component	Concentration	Volume	Final Concentration
Water (PCR-grade)	–	9 µl	–
Primer Mix (UPL ⁺ Reference Gene Assay)	20 µM	0.4 µl	400 nM
Primer (GOI [*]) forward	20 µM	0.4 µl	400 nM
Primer (GOI) reverse	20 µM	0.4 µl	400 nM
Probe (UPL Reference Gene Assay)	10 µM	0.4 µl	200 nM
UPL probe (GOI)	10 µM	0.4 µl	200 nM

Table 2.18 : (Continued) Multiplex real-time PCR mix (for one reaction).

Component	Concentration	Volume	Final Concentration
TaqMan Master Mix	5X	4.0 μ l	1X
Total Volume		15 μl	
Final Volume (with 5 μ l cDNA)		20 μl	

*GOI: Gene of interest, ⁺UPL: Universal Probe Library

After preparing the mastermix, 15 μ l PCR mix was carefully pipetted into each precooled LightCycler capillary tube, and 5 μ l of the cDNA template was then added.

In some experiments to obtain standard curve, also singleplex PCR mixtures (without Beta-Actin primers and probe) were prepared in the order listed in Table 2.19.

Table 2.19 : Singleplex real-time PCR mix (for one reaction).

Component	Concentration	Volume	Final Concentration
Water (PCR-grade)	–	10.4 μ l	–
Primer (GOI [*]) forward	20 μ M	0.2 μ l	200 nM
Primer (GOI) reverse	20 μ M	0.2 μ l	200 nM
UPL ⁺ Probe (GOI)	10 μ M	0.2 μ l	100 nM
TaqMan Master Mix	5X	4.0 μ l	1X
Total Volume		15 μl	
Final Volume (with 5 μ l cDNA)		20 μl	

*GOI: Gene of interest, ⁺UPL: Universal Probe Library

2.4.4 Standard curve

For setting of the standard curve, cDNA sample numbers 3 and 32 (Sham-B) were used in the reactions in undiluted, 1:10 diluted, 1:100 diluted and 1:1000 diluted form. Thus, totally 5 tubes of the reaction mixture were prepared including also a negative control tube. The undiluted cDNAs were synthesized from the undiluted main RNA stocks. Thereafter, 1:10 diluted cDNA was prepared from the undiluted cDNA, the 1:100 diluted was prepared from the 1:10 diluted one and so on. Standard curve reactions were set as both multiplex and singleplex to compare the reaction efficiencies of the two reactions.

2.4.5 Multiplex reaction

Beta-Actin primer pair and probe were used as the second gene components with the components of either the target gene Atf3 or Gadd45g in the multiplex reactions. Multiplex reactions were set as duplicate, thus with an NC tube, totally 23 tubes of the reaction mixture were prepared.

2.4.6 Relative quantification

The $\Delta\Delta C_T$ method [42, 44] was used to calculate the relative gene expressions resulting from Real-Time PCR.

2.5 Statistical Analysis

The results of RT-PCR, Real-Time PCR and microarray methods which were used for quantification of gene expression were analyzed statistically to determine whether the three results were significantly different or not. Friedman test was applied for this purpose by using SPSS software (version 16.0). The Friedman test is a non-parametric test for testing the difference between several related samples. The null hypothesis (H_0) for the Friedman test is that there are no differences between the variables. If the calculated probability is low ($P \leq 0.01$, or $P \leq 0.05$) the null-hypothesis is rejected and it can be concluded that at least 2 of the variables are significantly different from each other (H_a) [55, 56]. Kendall's W [55] value was also determined for each condition (IR, HP-IR, and IP-IR). Thus, the Friedman test was used for the determination of the difference between the three columns (RT-PCR,

Real-Time PCR and microarray results), whereas the Kendall's W was used for the determination of the difference between the three lines (R0, R15 and R60 samples).

3. RESULTS

3.1 RT-PCR Primer Tests

Both Atf3 and Gadd45g primers worked appropriately, i.e. they were able to amplify the target sequences. However, the initially used primer pair (Primer-1) of Gadd45g also amplified an unwanted non-specific region (~ 300 bp) in addition to the 174 bp target sequence (Figure 3.1).

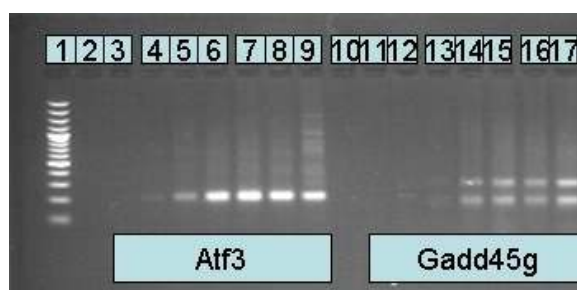


Figure 3.1 : Atf3 and Gadd45g primer products, using the RNA sample-3 as target. Explanations of the products indicated on the gel image are given in Table 3.1.

Table 3.1 : Explanations for the agarose gel lanes in Figure 3.1.

Lane number	Explanation	Lane number	Explanation	Lane number	Explanation
1	Marker #SM0323	2	Empty	10	Empty
		3	Atf3 at cycle number 23	11	Gadd45g at cycle number 23
		4	Atf3 at cycle number 26	12	Gadd45g at cycle number 26
		5	Atf3 at cycle number 29	13	Gadd45g at cycle number 29
		6	Atf3 at cycle number 32	14	Gadd45g at cycle number 32
		7	Atf3 at cycle number 35	15	Gadd45g at cycle number 35
		8	Atf3 at cycle number 38	16	Gadd45g at cycle number 38
		9	Atf3 at cycle number 40	17	Gadd45g at cycle number 40

Therefore, a second Gadd45g primer pair (Primer-3) was used. However, this primer pair also amplified the non-specific region (~ 300 bp).

3.2 Testing of Genomic DNA Contamination and Primer Products/Dimers

All RNA samples used in the study were tested for DNA contamination and it was confirmed that they had not been contaminated (Figure 3.2). The observed bands in Figure 3.2 indicate only the primer product/dimer formations which were verified later (Figure 3.3).

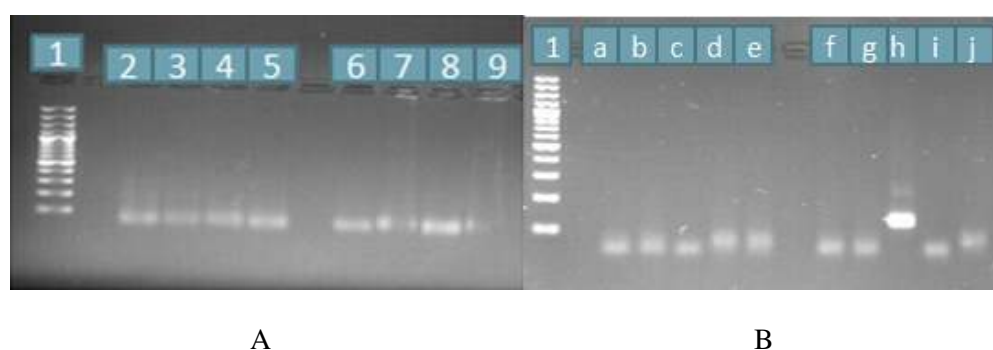


Figure 3.2 : DNA contamination test results of RNA samples no 3, 18, 26 (A), and 1, 5, 14, 16, 28, 29, Sham-A and Sham-B (B). Explanations for the products indicated on the gel image are given in Table 3.2.

Table 3.2 : Explanations for the agarose gel lanes in Figure 3.2.

Lane number	Explanation	Lane number	Explanation
1	Marker (#SM0323, Fermentas)	a	Sample-1 with Gadd45g primers
2	Sample-3 with Atf3 primers	b	Sample-5 with Gadd45g primers
3	Sample-18 with Atf3 primers	c	Sample-14 with Gadd45g primers
4	Sample-26 with Atf3 primers	d	Sample-16 with Gadd45g primers
5	NC with Atf3 primers	e	Sample-28 with Gadd45g primers
6	Sample-3 with Gadd45g primers	f	Sample-29 with Gadd45g primers
7	Sample-18 with Gadd45g primers	g	Sample Sham-A with Gadd45g primers
8	Sample-26 with Gadd45g primers	h	β -Actin primer control using Sample-3
9	NC with Gadd45g primers	i	Sample Sham-B with Gadd45g primers
		j	NC with Gadd45g primers

Figure 3.3 shows the agarose gel products of the verification experiment of primer product/dimer formations. These bands verified the product bands shown in Figure 3.2 and indicate the primer product/dimer formations, because, the bands (Figure 3.3) were only observed for the products of the primer pairs (F + R), and no product band was observed for the cases of single primers (F or R). Explanations for the products indicated in Figure 3.3 are given in Table 3.3.



Figure 3.3 : Agarose gel bands of primer product/dimer formations.

Table 3.3 : Explanations for the agarose gel lanes in Figure 3.3.

Lane number	Explanation	Lane number	Explanation	Lane number	Explanation
1	Marker #SM0323	2	Atf3 Forward (F) primer	7	Empty
		3	Atf3 Reverse (R) primer	8	Gadd45g Forward primer
		4	Atf3 F + R primers	9	Gadd45g Reverse primer
		5	Atf3 F + R primers	10	Gadd45g F + R primers
		6	Atf3 control with no primer	11	Gadd45g control with no primer

3.3 RT-PCR Results of ATF3

3.3.1 Optimum PCR conditions

When the RT-PCR products of the optimization experiments for Atf3 were analyzed by using the BioCapt software, the annealing condition of 56 °C for 30 s and 32 cycles of PCR amplification were determined as optimal conditions for the multiplex RT-PCR of Atf3 gene. The agarose gel bands of Atf3 at 56 °C were brighter than those at 60 °C (Figure 3.4) and image analysis results of 56 °C gave a more accurate graph (Table 3.5 and Figure 3.5).

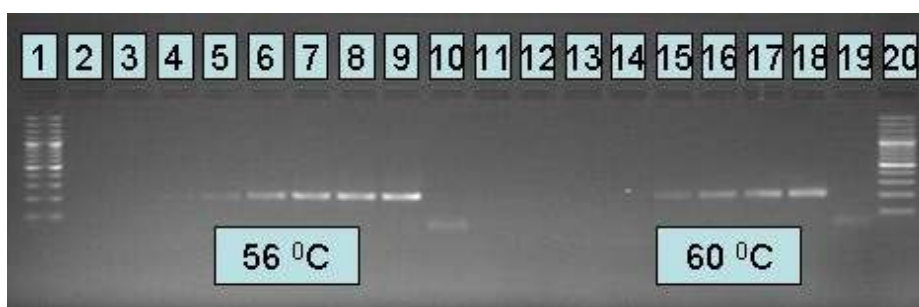


Figure 3.4 : RT-PCR products of the annealing optimization experiment at 56 °C and 60 °C for the Atf3 primers by using sample-26. Explanations for the lane numbers are given in Table 3.4.

Table 3.4 : Explanations for the agarose gel lanes in Figure 3.4.

Lane number	Explanation	Lane number	Explanation
1	Marker (#SM0323, Fermentas)	11	Empty
2	Empty	12	Cycle no 23 at 60 °C
3	Cycle no 23 at 56 °C	13	Cycle no 26 at 60 °C
4	Cycle no 26 at 56 °C	14	Cycle no 29 at 60 °C
5	Cycle no 29 at 56 °C	15	Cycle no 32 at 60 °C
6	Cycle no 32 at 56 °C	16	Cycle no 35 at 60 °C
7	Cycle no 35 at 56 °C	17	Cycle no 38 at 60 °C
8	Cycle no 38 at 56 °C	18	Cycle no 40 at 60 °C
9	Cycle no 40 at 56 °C	19	NC at cycle no 40 and 60 °C
10	NC at cycle no 40 and 56 °C	20	Marker (#SM0323, Fermentas)

Table 3.5 : Image analysis results (volume values) of the Atf3 gene bands shown in Figure 3.4.

Cycle repeat number		23	26	29	32	35	38	40
Volume	Threshold=120, 56 °C	0	0	345	3363	6356	7338	9372
	Threshold=120, 60 °C	0	0	289	14	1130	2170	4122

As shown in Table 3.5, the band volumes of Atf3 products at cycle number 29, 32, 35, 38 and 40 showed a more straight increase at 56 °C than those at 60 °C, also as shown in Figure 3.5. At the annealing temperature of 56 °C, there was less increase of the volume fold after the cycle number 32 with respect to cycles 29 and 32. It

seems that the band volume reached the saturation after cycle no 32 and thus, optimum cycle was the cycle no 32.

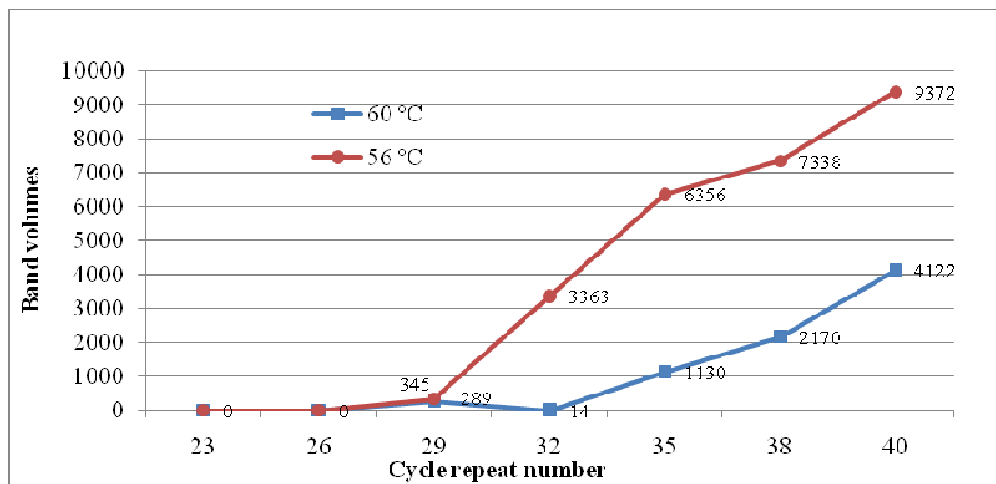


Figure 3.5 : Line graph showing the increase in the band volumes (y axis) given in Table 3.5 upon increase in the cycle repeat number (x axis).

3.3.2 Multiplex RT-PCR of Atf3

All RNA samples were run in duplicate. Resulting agarose gel (1.5 %, 150 ml) bands of cDNAs are shown in Figure 3.6 and explained in Table 3.6.

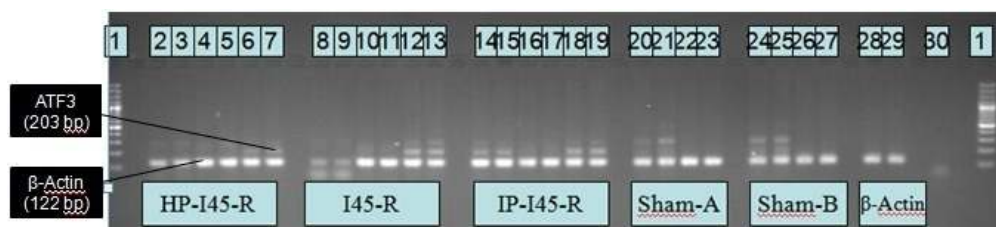


Figure 3.6 : Multiplex RT-PCR products of Atf3 and β -Actin genes. Explanations for the 30 lanes indicated on the gel image are given in Table 3.6.

Table 3.6 : Explanations for the agarose gel lanes in Figure 3.6.

Lane number	Explanation	Lane number	Explanation
1	Marker #SM0323 (Fermentas)	16-17	Sample-28 (IP-I45-R15-B)
2-3	Sample-1 (HP-I45-R0-A)	18-19	Sample-29 (IP-I45-R60-A)
4-5	Sample-3 (HP-I45-R15-A)	20-21	Sample Sham-A with Atf3 and β -Actin primers

Table 3.6 : (Continued) Explanations for the agarose gel lanes in Figure 3.6.

Lane number	Explanation	Lane number	Explanation
6-7	Sample-5 (HP-I45-R60-A)	22-23	Sample Sham-A with only β -Actin primers
8-9	Sample-14 (I45-R0-B)	24-25	Sample Sham-B with Atf3 and β -Actin primers
10-11	Sample-16 (I45-R15-B)	26-27	Sample Sham-B with only β -Actin primers
12-13	Sample-18 (I45-R60-B)	28-29	Controls with only β -Actin primers using sample-26
14-15	Sample-26 (IP-I45-R0-B)	30	NC with Atf3 and β -Actin primers

3.3.3 Relative quantification

Relative quantification of the RT-PCR products of Atf3 gene shown in Figure 3.6 was done as described in the “Materials and Methods” section of this Thesis (2.3.12. Relative quantification). Resulting volume values and calculations are given in Appendix D (Table D.1).

3.4 Multiplex Real-Time PCR Results of ATF3

Standard curve setting failed for Atf3 with the Sample 3 dilutions; as the undiluted cDNA had a C_T value higher than 27.00, and the 10-fold diluted cDNA had a C_T value higher than 40.00. As a result, the efficiency value of Atf3 was higher than 2.5 and not in the acceptable range (1.8-2.1) [57]. However, error and efficiency values of Atf3 were acceptable for the singleplex reaction, and also for the multiplex reaction of Beta-Actin gene (Table 3.7). Acceptable value of error is: ≤ 0.1 [57].

Table 3.7 : Efficiency and error values of Atf3 and β -Actin genes using cDNA of Sample 3 in real-time PCR experiments.

	β -Actin (in multiplex reaction)	ATF3 (in singleplex reaction)
Efficiency	2.09	2.01
Error	0.026	0.011

In the second set of experiments, Sham-B RNA was chosen as the sample for standard curve preparation, because it was one of the highly concentrated samples (2.25 µg/µl) with a high volume to produce enough cDNA stock. Again, 10-fold dilutions (from undiluted to 1:1000 diluted) were prepared. However, the same problem existed in C_T values: as the undiluted cDNA had a C_T value of 26.00 (a high C_T), the 10-fold diluted cDNAs had higher C_T values than 30.00. As a result, efficiency and error values of Sham-B were not acceptable for the multiplex reaction, either (Table 3.8). However, Sham-B had acceptable values in singleplex reaction, and also Beta-Actin had acceptable values both in multiplex and singleplex reactions (Table 3.8).

Table 3.8 : Efficiency and error values of Atf3 and β -Actin genes using Sham-B cDNA in real-time PCR experiments.

	ATF3 (in multiplex reaction)	ATF3 (in singleplex reaction)	β-Actin (in multiplex reaction)	β-Actin (in singleplex reaction)
Efficiency	1.53	2.07	1.92	1.94
Error	0.275	0.008	0.017	0.0003

As the standard curve failed for Atf3 multiplex reaction with both cDNA samples 3 and Sham-B, $\Delta\Delta C_T$ method was used to calculate the relative Atf3 gene expression in each treated sample. The calculations and the resulting fold-changes in gene expression are shown in Table 3.9 : if the Atf3 expression in Sham-A was considered as basis, i.e. as one-fold, in other words, when the calibrator is Sham-A, Sham-B had then two-fold expression of Atf3 relative to Sham-A. Also, when Sham-A was the calibrator, the expression in one sample was nearly two-fold of the expression in the same sample when the calibrator was Sham-B. The highest increase in Atf3 gene expression was observed in the ischemia preconditioning (IP) case, at reperfusion time of 60 min. The increase in the Atf3 gene expression of I45_R60_B, HP_I45_R60_A and IP_I45_R60_A samples were 19.43, 23.75, and 44.32-fold respectively, when Sham-A was used as the calibrator. When Sham-B was used as the calibrator, however, these fold-increase values changed as 9.68, 11.84 and 22.09, respectively.

Table 3.9 : Fold-changes in gene expression of Atf3 after HP-IR, only IR and IP-IR treatments, calculated by $\Delta\Delta C_T$ method.

Sample Number	Sample Name	Atf3 Average C_T	β -Actin Average C_T	$\Delta C_T = C_{T \text{ ATf3}} - C_{T \text{ BETA-ACTIN}}$	$\Delta\Delta C_T = \Delta C_T \text{ treated} - \Delta C_T \text{ Sham-A}$	Fold difference in Atf3 relative to Sham-A ($2^{-\Delta\Delta C_T}$)	$\Delta\Delta C_T = \Delta C_T \text{ treated} - \Delta C_T \text{ Sham-B}$	Fold difference in Atf3 relative to Sham-B ($2^{-\Delta\Delta C_T}$)
1	HP_I45_R0_A	29.95	20.90	9.06	1.26	0.42	2.26	0.21
3	HP_I45_R15_A	29.68	21.64	8.04	0.23	0.85	1.24	0.42
5	HP_I45_R60_A	24.73	21.50	3.24	-4.57	23.75	-3.57	11.84
14	I45_R0_B	33.08	28.45	4.63	-3.18	9.06	-2.18	4.52
16	I45_R15_B	30.64	23.42	7.22	-0.59	1.50	0.42	0.75
18	I45_R60_B	25.60	22.08	3.53	-4.28	19.43	-3.28	9.68
26	IP_I45_R0_B	26.15	21.47	4.69	-3.12	8.70	-2.12	4.33
28	IP_I45_R15_B	27.68	22.05	5.63	-2.18	4.53	-1.18	2.26
29	IP_I45_R60_A	23.32	20.99	2.34	-5.47	44.32	-4.47	22.09
31	Sham-A	30.03	22.23	7.81	0.00	1.00	1.01	0.50
32	Sham-B	28.62	21.82	6.80	-1.01	2.00	0.00	1.00

3.5 Comparison of Relative Gene Expression Results of ATF3 Obtained by RT-PCR, Real-Time PCR and Previous Microarray Analysis

RT-PCR and Real-Time PCR results of ATF3 gene expression were compared with the previously obtained microarray gene expression data [46] (Table 3.10).

Table 3.10 : Comparison of the relative quantification results of ATF3 gene expression obtained by RT-PCR, Real-Time PCR and previous microarray analysis.

Sample number	Sample name	Relative Quantification to Sham-A			Relative Quantification to Sham-B		
		RT-PCR Results	Real-Time PCR Results	Microarray Results	RT-PCR Results	Real-Time PCR Results	Microarray Results
1	HP-I45-R0-A	—	0.42	-1.18	—	0.21	-1.98
3	HP-I45-R15-A	0.78	0.85	0.08	0.67	0.42	-0.78
5	HP-I45-R60-A	1.16	23.75	4.08	1.00	11.84	3.29
14	I45-R0-B	0.63	9.06	-0.75	0.55	4.52	-1.22
16	I45-R15-B	1.02	1.50	0.37	0.88	0.75	-0.50
18	I45-R60-B	1.27	19.43	3.96	1.10	9.68	3.24
26	IP-I45-R0-B	1.18	8.70	3.07	1.02	4.33	2.31
28	IP-I45-R15-B	0.96	4.53	2.40	0.83	2.26	1.63
29	IP-I45-R60-A	1.24	44.32	4.54	1.07	22.09	3.65
31	Sham-A	1.00	1.00	—	0.86	0.50	—
32	Sham-B	1.16	2.00	—	1.00	1.00	—

3.6 RT-PCR Results of GADD45G

3.6.1 Primer-1 and Primer-3 results

In the Gadd45g optimization experiments, because the first used primer pair (Primer-1) gave an undesirable non-specific product (Figure 3.7 B), the second primer pair (Primer-3) was tried. However, the second primer pair also resulted in the same non-specific product (Figure 3.7 A).

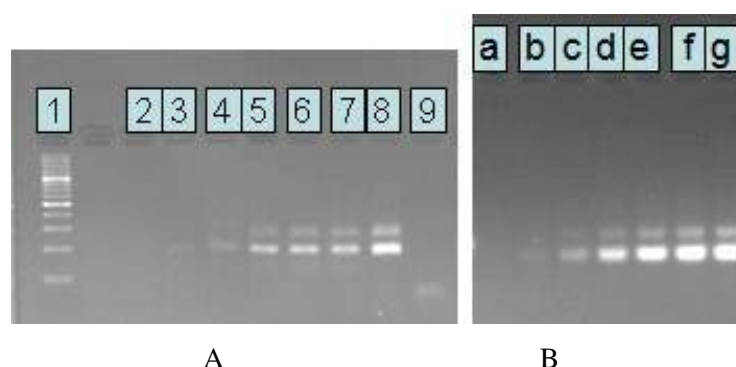


Figure 3.7 : Agarose gel bands of products of first (B) and second (A) Gadd45g primer pairs. The full-scale reaction volume (50 μ l of total) was used for reaction mixtures of both primer pairs. RNA Samples 3 and 26 were used respectively for the second and first primers. For both primer pairs, annealing condition was 56 $^{\circ}$ C for 30 s, and the other conditions of the RT-PCR experiments were the same as shown in Table 2.6.

Explanations for the gel lanes indicated in Figure 3.7 are given in Table 3.11.

Table 3.11 : Explanations for the agarose gel lanes in Figure 3.7.

Lane number	Explanation	Lane number	Explanation
1	Marker #SM0323 (Fermentas)	9	NC with Primer-3
2	Cycle no 23 with Primer-3	a	Cycle no 23 with Primer-1
3	Cycle no 26 with Primer-3	b	Cycle no 26 with Primer-1
4	Cycle no 29 with Primer-3	c	Cycle no 29 with Primer-1
5	Cycle no 32 with Primer-3	d	Cycle no 32 with Primer-1
6	Cycle no 35 with Primer-3	e	Cycle no 35 with Primer-1
7	Cycle no 38 with Primer-3	f	Cycle no 38 with Primer-1
8	Cycle no 40 with Primer-3	g	Cycle no 40 with Primer-1

Volumes of the agarose gel bands shown in Figure 3.7 are given in Table 3.12. Threshold volume was 146 for the bands of the products of both primers.

Table 3.12 : Volumes of Primer-1 and Primer-3 products shown in Figure 3.7.

Cycle number	Primer-3 products			Primer-1 products		
	Non-specific band volumes	Gadd45g band volumes	Gadd45g/Non-specific volume ratio	Non-specific band volumes	Gadd45g band volumes	Gadd45g/Non-specific volume ratio
23	-	-	-	-	-	-
26	-	131	-	-	18	-
29	-	669	-	0	3268	-
32	389	4700	12,08	33	16645	504,39
35	1229	7488	6,09	1679	25490	15,18
38	1432	7651	5,34	3309	28424	8,59
40	4397	13657	3,11	4576	30011	6,56

3.6.2 Gradient-PCR results

None of the annealing temperatures tested in Gradient-PCR experiments for both the primer pairs were successful to prevent the amplification of the non-specific product (Figure 3.8).

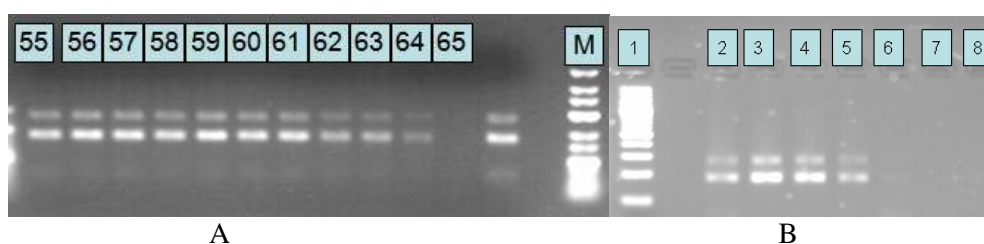


Figure 3.8 : Agarose gel images of the products of Gradient-PCR experiments by using Primer-1 (A) and Primer-3 (B) primers of Gadd45g and RNA Sample 3 as template. Explanations for the lanes indicated on the gel image are given in Table 3.13.

Table 3.13 : Explanations for the agarose gel lanes in Figure 3.8.

Lane number	Explanation	Lane number	Explanation
55-65	55-65 °C Annealing temperatures for Primer-1	4	Annealing at 61 °C for Primer-3

Table 3.13 : (Continued) Explanations for the agarose gel lanes in Figure 3.8.

Lane number	Explanation	Lane number	Explanation
M	Marker #SM1203 (Fermentas)	5	Annealing at 62 °C for Primer-3
1	Marker #SM0323 (Fermentas)	6	Annealing at 63 °C for Primer-3
2	Annealing at 59 °C for Primer-3	7	Annealing at 64 °C for Primer-3
3	Annealing at 60 °C for Primer-3	8	NC, Annealing at 60 °C for Primer-3

Volumes of the agarose gel bands shown in Figure 3.8 are given in Table 3.14. Threshold volume was 120 for the Primer-1 products, and 200 for the Primer-3 products.

Table 3.14 : Volumes of Primer-1 and Primer-3 products shown in Figure 3.8.

Annealing temperature	Primer-1 products			Primer-3 products		
	Non-specific band volumes	Gadd45g band volumes	Gadd45g/Non-specific volume ratio	Non-specific band volumes	Gadd45g band volumes	Gadd45g/Non-specific volume ratio
55	2250	9080	4.04	-	-	-
56	2996	10727	3.58	-	-	-
57	2667	10488	3.93	-	-	-
58	2139	9237	4.32	-	-	-
59	3027	10878	3.59	174	9891	56.84
60	2426	10071	4.15	6278	24636	3.92
61	2277	10171	4.47	1992	20643	10.36
62	580	5392	9.30	0	4617	-
63	448	4911	10.96	0	0	-
64	0	1485	-	0	0	-
65	0	0	-	-	-	-

3.6.3 Optimum PCR conditions

Any of the optimization experiments could not eliminate the non-specific band (~300 bp). Increasing the annealing temperature and decreasing the annealing and extension times, decreased the volume and brightness of the agarose gel band for both the non-specific product and Gadd45g. However, the first primer pair was determined to bind to the target sequence more efficiently compared with the second primer (Figure 3.7 and 3.8, and Table 3.12 and 3.14), as the first primer was already specified as the best primer by the Primer3Plus program. The first primer pair could amplify the target sequence at 56 °C and higher temperatures (Figure 3.8 A). However, these results were obtained using only the RNA Sample 3 as template, and amplification may not be observed at higher temperatures than 56 °C when using other samples as template. At the 56 °C of annealing temperature, brightness and volume of agarose gel band of Sample 3 product by using Primer-1 reached the saturation at the cycle number 35 (Figure 3.7 B and Table 3.12). Thus, beginning from cycle number 35, the band brightness and volume became constant in comparison to the cycle numbers before 35, and cycle number 32 seemed as the optimum cycle repeat number for Sample 3. As a result of these and the other optimization experiments, the annealing condition of 56 °C for 30 s, extension time of 50 s, and 32 cycles of PCR amplification were determined as optimum conditions for multiplex RT-PCR of Gadd45g using Primer-1.

3.6.4 Multiplex RT-PCR of Gadd45g

All RNA samples were run in duplicate. Resulting agarose gel (1.5 %, 150 ml) bands are shown in Figure 3.9 and explained in Table 3.15.

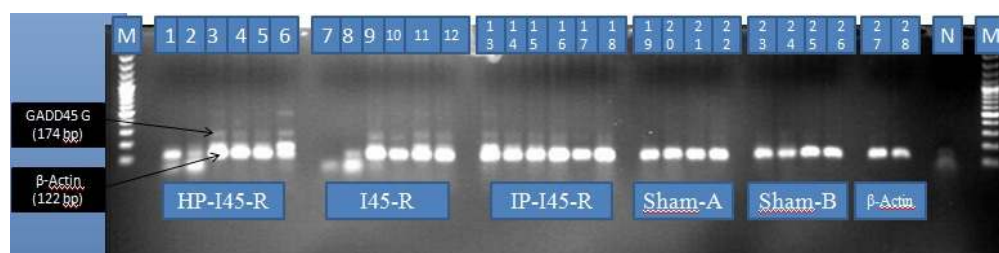


Figure 3.9 : Multiplex RT-PCR products of Gadd45g and β -Actin genes. Explanations for the lanes indicated on the gel image are given in Table 3.15.

Table 3.15 : Explanations for the agarose gel lanes in Figure 3.9.

Lane number	Explanation	Lane number	Explanation
M	Marker #SM0323	15-16	Sample-28 (IP-I45-R15-B)
1-2	Sample-1 (HP-I45-R0-A)	17-18	Sample-29 (IP-I45-R60-A)
3-4	Sample-3 (HP-I45-R15-A)	19-20	Sample Sham-A with Gadd45g and β -Actin primers
5-6	Sample-5 (HP-I45-R60-A)	21-22	Sample Sham-A with only β -Actin primers
7-8	Sample-14 (I45-R0-B)	23-24	Sample Sham-B with Gadd45g and β -Actin primers
9-10	Sample-16 (I45-R15-B)	25-26	Sample Sham-B with only β -Actin primers
11-12	Sample-18 (I45-R60-B)	27-28	Controls with only β -Actin primers using sample-26
13-14	Sample-26 (IP-I45-R0-B)	N	NC with Gadd45g and β -Actin primers

3.6.5 Relative quantification

Relative quantification of the RT-PCR products of Gadd45g gene observed in Figure 3.9 was done as described in the “Materials and Methods” section of this Thesis (2.3.12. Relative quantification). Resulting volume values and calculations are shown in Appendix D (Table D.2). Because no band volume was detected in Sham samples, resulting value for each sample could not be divided by a Sham value. Therefore, Sham values were considered as 1-fold, and the normalized value for each sample was considered as the fold-change expression to Sham samples.

3.7 Multiplex Real-Time PCR Results of GADD45G

A standard curve was obtained successfully for Gadd45g gene using the 10-fold dilutions of cDNA Sample 3. The efficiency and error values of multiplex reaction are shown in Table 3.16, which were both in acceptable ranges. Thus, Sham-B was not used as a new sample for a second set of experiments to obtain a new standard curve.

Table 3.16 : Efficiency and error values of Gadd45g and β -Actin genes using Sample 3 cDNA in real-time PCR experiments.

	Gadd45g (in multiplex reaction)	β-Actin (in multiplex reaction)
Efficiency	2.14	1.94
Error	0.002	0.026

However, it is generally recommended to set the standard curves of both Atf3 and Gadd45g genes using the same standard sample. As the cDNA Sample 3 did not work as a standard with Atf3, and thus the relative Atf3 gene expression was calculated using the $\Delta\Delta C_T$ method, the relative gene expression of Gadd45g was also calculated using the same method. The calculations and the resulting fold-changes in Gadd45g gene expression are shown in Table 3.17.

Table 3.17 : Fold-changes in gene expression of Gadd45g after HP-IR, only IR and IP-IR treatments, calculated by $\Delta\Delta C_T$ method.

Sample Number	Sample Name	Gadd45g Average C_T	β -Actin Average C_T	$\Delta C_T = C_{T \text{ GADD45G}} - C_{T \text{ BETA-ACTIN}}$	$\Delta\Delta C_T = \Delta C_T \text{ treated} - \Delta C_T \text{ Sham-A}$	Fold difference in Gadd45g relative to Sham-A ($2^{-\Delta\Delta C_T}$)	$\Delta\Delta C_T = \Delta C_T \text{ treated} - \Delta C_T \text{ Sham-B}$	Fold difference in Gadd45g relative to Sham-B ($2^{-\Delta\Delta C_T}$)
1	HP_I45_R0_A	27.58	21.03	6.55	1.22	0.43	1.53	0.35
3	HP_I45_R15_A	27.57	21.84	5.73	0.40	0.76	0.71	0.61
5	HP_I45_R60_A	26.34	22.11	4.23	-1.11	2.15	-0.80	1.74
14	I45_R0_B	34.48	29.25	5.23	-0.11	1.08	0.20	0.87
16	I45_R15_B	28.63	23.58	5.05	-0.29	1.22	0.03	0.98
18	I45_R60_B	26.85	22.69	4.16	-1.18	2.26	-0.87	1.82
26	IP_I45_R0_B	26.22	21.94	4.28	-1.06	2.08	-0.75	1.68
28	IP_I45_R15_B	27.12	22.53	4.59	-0.75	1.68	-0.44	1.35
29	IP_I45_R60_A	25.60	21.59	4.01	-1.33	2.50	-1.02	2.02

Table 3.17 : (Continued) Fold-changes in gene expression of Gadd45g after HP-IR, only IR and IP-IR treatments, calculated by $\Delta\Delta C_T$ method.

Sample Number	Sample Name	Gadd45g Average C_T	β -Actin Average C_T	$\Delta C_T = C_{T \text{ GADD45G}} - C_{T \text{ BETA-ACTIN}}$	$\Delta\Delta C_T = \Delta C_T \text{ treated} - \Delta C_T \text{ Sham-A}$	Fold difference in Gadd45g relative to Sham-A ($2^{-\Delta\Delta C_T}$)	$\Delta\Delta C_T = \Delta C_T \text{ treated} - \Delta C_T \text{ Sham-B}$	Fold difference in Gadd45g relative to Sham-B ($2^{-\Delta\Delta C_T}$)
31	Sham-A	27.62	22.28	5.34	0.00	1.00	0.31	0.81
32	Sham-B	26.81	21.78	5.03	-0.31	1.24	0.00	1.00

As in Atf3 gene expression, the highest increase in Gadd45g gene expression was observed in the ischemia preconditioning (IP) case with 60 min of reperfusion time. Generally, expression levels in HP-treated samples were lower than those in the only ischemia-reperfusion-treated samples (Table 3.17), whereas expression in IP-treated samples was higher than in the only ischemia-reperfusion-treated samples (Table 3.17).

3.8 Comparison of Relative Gene Expression Results of GADD45G Obtained by RT-PCR, Real-Time PCR and Previous Microarray Analysis

RT-PCR and Real-Time PCR results of GADD45G gene expression were compared with the previously obtained microarray gene expression data [46] (Table 3.18).

Table 3.18 : Comparison of the relative quantification results of GADD45G gene expression obtained by RT-PCR, Real-Time PCR and previous microarray analysis.

Sample number	Sample name	Relative Quantification to Sham-A			Relative Quantification to Sham-B		
		RT-PCR Results	Real-Time PCR Results	Microarray Results	RT-PCR Results	Real-Time PCR Results	Microarray Results
1	HP-I45-R0-A	—	0.43	-0.57	—	0.35	-0.43
3	HP-I45-R15-A	0.66	0.76	0.41	0.66	0.61	0.60

Table 3.18 : (Continued) Comparison of the relative quantification results of GADD45G gene expression obtained by RT-PCR, Real-Time PCR and previous microarray analysis.

Sample number	Sample name	Relative Quantification to Sham-A			Relative Quantification to Sham-B		
		RT-PCR Results	Real-Time PCR Results	Microarray Results	RT-PCR Results	Real-Time PCR Results	Microarray Results
5	HP-I45-R60-A	0.61	2.15	1.02	0.61	1.74	1.28
14	I45-R0-B	—	1.08	-0.01	—	0.87	0.31
16	I45-R15-B	0.76	1.22	-0.09	0.76	0.98	0.17
18	I45-R60-B	0.75	2.26	0.88	0.75	1.82	1.13
26	IP-I45-R0-B	0.42	2.08	1.01	0.42	1.68	1.22
28	IP-I45-R15-B	0.38	1.68	0.94	0.38	1.35	1.19
29	IP-I45-R60-A	—	2.50	1.52	—	2.02	1.69
31	Sham-A	—	1.00	—	—	0.81	—
32	Sham-B	—	1.24	—	—	1.00	—

3.9 Results of Statistical Analysis

The Friedman non-parametric test was applied to both the Atf3 and Gadd45g results of RT-PCR, Real-Time PCR and previous microarray analysis. For both genes, the relative gene expressions with respect to Sham-A and Sham-B were determined to be significantly different (≤ 0.01) or not (> 0.01) between the three results. The statistical analysis was conducted separately for each case (HP, IR, IP). Thus, for each case, the number of subjects was three ($N = 3$, for R0, R15 and R60) and the number of conditions was also three ($k = 3$, for RT-PCR, Real-Time PCR and microarray analysis). Zero “0” was used as the relative expression value for the samples with no expression value (-). In addition to the Friedman test that was used to determine the difference between the conditions (k), Kendall’s W value was also

used to determine the difference between the subjects (N). Kendall's W ranges from 0 (complete difference) to 1 (no difference) [55].

For the expression of Gadd45g gene, the level of significance was 0.097 ($W = 0.778$) for the HP samples calibrated to Sham-A; 0.264 ($W = 0.444$) for the HP samples calibrated to Sham-B; 0.097 ($W = 0.778$) for both the IR samples calibrated to Sham-A and Sham-B, and 0.05 ($W = 1.00$) for both the IP samples calibrated to Sham-A and Sham-B. As all of these values were higher than the significance level of 0.01, there was not a significant difference between the results of the three methods for all the three (HP, IR, IP) conditions of Gadd45g.

For the expression of Atf3 gene, the level of significance was 0.097 ($W = 0.778$) for HP samples calibrated to Sham-A; 0.264 ($W = 0.444$) for the HP samples calibrated to Sham-B; 0.097 ($W = 0.778$) for the IR samples calibrated to Sham-A; 0.264 ($W = 0.444$) for the IR samples calibrated to Sham-B, and 0.05 ($W = 1.00$) for both the IP samples calibrated to Sham-A and Sham-B. As all of these values were higher than the significance level of 0.01, there was not a significant difference between the results of the three methods for all the three (HP, IR, IP) conditions of Atf3.

4. DISCUSSION AND CONCLUSIONS

In this study, expression levels of Atf3 and Gadd45g genes under ischemia followed by reperfusion (IR), HP before ischemia-reperfusion and IP before ischemia-reperfusion conditions were investigated in total RNA samples from rat kidney tissues by using RT-PCR and qReal-Time RT-PCR techniques. Both techniques were successfully applied with necessary optimizations and it was observed that there were significant differences in the expression levels of the above-mentioned genes under different conditions. The results obtained were compared with the previously obtained DNA microarray results and were found to be compatible with the microarray data.

The overall comparison of the relative gene expression levels for Atf3 and Gadd45g indicated that, although there were differences in the absolute values, relative quantification based on the controls Sham-A or Sham-B generally yielded similar results (Table 3.10 and Table 3.18).

For Atf3 gene expression, it can be concluded that under HP and IP conditions prior to ischemia-reperfusion, the Atf3 gene expression increases when the reperfusion time increases. The highest and most significant level of Atf3 expression under HP and IP conditions was observed at 60 min reperfusion time (Table 3.10). For these conditions, the RT-PCR, Real-Time PCR and microarray results were all parallel. In the case of IR samples, there is also a general positive effect of prolonged reperfusion time on Atf3 gene expression, the 60 min reperfusion time sample having the highest relative gene expression level in this group (Table 3.10).

In the case of Gadd45g gene expression, Real-Time PCR and microarray results were also generally compatible. Also for Gadd45g, it was generally observed that the gene expression level increased with increasing reperfusion time, with the highest Gadd45g expression observed with 60 min reperfusion time samples of each treatment group. In IP group, there was a slight decrease in Gadd45g expression at the reperfusion time of 15 min, as compared to zero reperfusion time sample. This result was obtained both by Real-Time PCR and microarray data (Table 3.18).

Interestingly, similar observation was also made for the IP group samples of Atf3 gene expression (Table 3.10). It seems like a typical early response of IP-tissues upon IR treatment. RT-PCR results of Gadd45g were generally compatible with Real-Time PCR and microarray results at the 0- and 15 min-reperfusion times of the all three cases (HP, IR and IP), however, there was a decrease in the gene expression at the reperfusion time of 60 min in all the cases for RT-PCR results (Table 3.18). This can be explained by the great differences in the band volumes of duplicate samples (Table D.2). Because all experimental steps and calculations are done manually in RT-PCR method, this study shows one more time the low sensitivity of the RT-PCR method (Table D.1 and Table D.2) compared to Real-Time PCR method (Table D.3).

Interestingly, for both Atf3 and Gadd45g genes, the highest expression levels were observed in the IP group, at 60 min of reperfusion time. This is seemingly an important result implying the significant genetic effect of the beneficial IP treatment. For the 60 min reperfusion time and the IP samples, relative gene expression values of Atf3 were higher than the values of Gadd45g. For both genes, the highest values were observed with the Real-Time PCR results which again implies the high sensitivity of the technique. In addition to these findings, the long-term effects of reperfusion should also be studied with a similar experimental setup, to investigate the effects of reperfusion times longer than 60 min.

A previous study by Yin *et al.* (1997) [58] supports the result obtained in this study. In the study, Atf3 expression levels in rat kidney were examined by Northern analysis. No detectable levels of Atf3 mRNA was observed in control kidneys or kidneys subjected to ischemia for 1 h. However, within 20 min following reperfusion, Atf3 mRNA level increased significantly, reached maximal level by 1.5 h, was maintained at a high level for at least 3 h, and returned to the basal level by 24 h. It was concluded that ischemia followed by reperfusion leads to a rapid and transient expression of this stress-responsive gene in the kidney as a consequence of stress kinase activation.

In a similar study, Atf3 expression was shown to be increased also in both ischemic and ischemic-reperfused rat hearts by using *in situ* hybridization using antisense RNA probes [59]. Ischemia was applied for two hours and reperfusion for one hour. Atf3 expression in ischemic-reperfused hearts was higher than in ischemic hearts,

and Atf3 was expressed in a larger area in myocardium in the ischemia-reperfusion case. In that study, it was also shown and hypothesized that in non-stressed liver, gadd153/Chop10 (gadd stands for growth arrest and DNA-damaging; Chop stands for C/EBP-homologous protein) inhibits the limited amount of Atf3 by forming an inactive heterodimer with it, whereas in carbon tetrachloride (CCl₄)-injured liver, the synthesis of gadd153/Chop10 was repressed, allowing the induced Atf3 to function.

A recent *in vivo* study examined whether Atf3 plays a role in IRI [60]. At first, the changes in Atf3 levels were characterized in the kidney of a mouse model of IRI. In the kidney, at 12 and 24 h after ischemia there was a significant increase in Atf3 mRNA levels and corresponding increases in Atf3 protein levels. Immunofluorescent staining of kidney showed that, with IRI, Atf3 protein was localized principally in the nuclei of the distal tubules. Secondly, protective effect of overexpression of Atf3 was examined by using adenovirus gene transfer in the model of IRI in the mouse kidney. Overexpression of Atf3 mRNA and protein in the kidney was confirmed by RT-PCR and Western blot analysis. It was observed that the injury observed in the kidneys of mice injected with AdAtf3 (the adenovirus vector encoding the human *ATF3* gene) was attenuated compared with control mice. An improvement in renal function and in the acute tubular necrosis (ATN) score was also observed in the kidneys of IRI mice with Atf3 overexpression compared with control mice. As a result of the study, Atf3 was found to protect the kidney from IRI.

In an ischemic preconditioning study on rat spinal cord, it was observed that Atf3 was robustly induced (8-fold by qRT-PCR and 2-fold by microarray analysis) at 30 min of reperfusion after 6 min of ischemia and remained elevated until returning to baseline by 24 h, whereas it was not induced upon 3 min of ischemia [61].

In another study of cardiac ischemic preconditioning on rats by using microarray analysis, IP (two repeats of 5 min-ischemia and 10 min-reperfusion) down-regulated the expression of several transcripts including Atf3 which were up-regulated by IR (40 min-ischemia and 30 min-reperfusion) [62].

A recent study of brain ischemia-reperfusion model on rats by using microarray analysis showed 12.5-fold increase in Atf3 gene expression after 6 h-reperfusion; and 8.5-fold increase after 6 h-reperfusion and 15.4-fold increase after 24 h-reperfusion in Gadd45g gene expression following 15 min of ischemia [63].

A recent study also examined gene expression profiles in response to myocardial infarction after 24 h of ischemia by using microarray analysis in murine model. In Atf3 and Gadd45g gene expression, 15-fold and 12-fold increase was observed respectively, compared with Sham controls [64].

In parallel with the findings mentioned above, fold-change increases in Atf3 and Gadd45g gene expression were observed in this study as reperfusion time was increased under different conditions (HP, IR, IP) with constant ischemia duration (45 min) (Table 3.10 and Table 3.18). The down-regulation of Atf3 was observed in the cardiac IP study [62], however, the response of Atf3 gene expression may be variable in different organs as in the cases of kidney [58] and heart [59] mentioned above.

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APPENDICES

APPENDIX A : Laboratory equipment

APPENDIX B : Coding DNA sequences (CDS) used for the design of the primers that were used in RT-PCR experiments

APPENDIX C : Markers used during loading the cDNA products to agarose gel

APPENDIX D : Volume (from Multiplex RT-PCR experiments) and C_T values (from Multiplex Real-Time PCR experiments) of duplicate RNA samples

APPENDIX A

Balances	Precisa 125A SCS Precisa XB 620C
Centrifuges	Eppendorf Centrifuge 5424
Electrophoresis Equipment	E - C MINI CELL PRIMO EC320 E - C MIDI CELL PRIMO EC330 BIO-RAD PowerPac Basic
Gel Documentation System	Biolab BTX-26.M UVI PhotoMW Version 99.05 for Windows
Pipettes	Eppendorf 2.5 µl, 10 µl, 100 µl, 200 µl, 1000 µl
RT-PCR	Techne TC-412 Techne TC-312
Gradient PCR	Corbett PalmCycler
Real-Time PCR	Roche LightCycler 2.0
Vortex	Heidolph REAX top
Magnetic Stirrer	Labword-online
Deep Freezers & Refrigerators	- 80 °C Sanyo - 20 °C, + 4 °C Arçelik
PCR tubes	Deltalab 0.2 ml PCR tubes Axygen 0.5 ml PCR tubes

APPENDIX B

ATF3 (NM_012912, Strand -) :

ATGATGCTTCAACATCCAGGCCAGGTCTCTGCCTCAGAAGTCAGCGCGACCGCC
ATCGTCCCCTGCCTCTCACCTCCTGGGTCACTGGTGTTTGAGGATTTTGCTAACCT
GACACCTTTTGTCAAGGAAGAGCTGAGATTCGCCATCCAGAACAAGCACCTTTG
CCATCGGATGTCTCTGCGCTGGAGTCAGTCACCATCAACAACAGACCTCTGGA
GATGTCAGTCACCAAGTCTGAGGTGGCCCCTGAAGAAGATGAGAGAAAAAGGA
GGCGGCGGGAAAGAAACAAAATTGCTGCTGCCAAGTGTCGAAACAAGAAAAAA
GAGAAGACAGAGTGCCTGCAGAAGGAGTCAGAGAAACTGGAGAGTGTGAATGC
CGAACTGAAGGCCCAGATCGAGGAGCTGAAGAATGAGAAGCAGCATCTGATTT
ACATGCTCAACCTGCACCGGCCACGTGTATCGTCCGGGCTCAGAACGGGCGGA
CGCCGGAAGACGAGAGGAACCTTTTTATCCAACAGATAAAAGAAGGAACATTG
CAGAGCTAA

GADD45G (AB020978, Strand -) :

ATGACTCTGGAAGAAGTCCGTGGCCAGGATACAGTTCCGGAAAGCACAGCCAG
GATGCAGGGCGCCGGGAAAGCATTGCACGAACTTCTGCTGTCGGCGCAGCGCCA
GGGCTGTCTGACCGCTGGCGTCTACGAGTCCGCCAAAGTCCTGAATGTGGACCC
TGACAATGTGACCTTTTTCGTGCTGGCTGCCGACGAAGAAGATGAGGGCGACAT
AGCCCTGCAGATCCATTTTACGTTGATCCAGGCGTTCTGCTGCGAGAACGACATT
GACATCGTGCGCGTGGGAGACGTGCAGAGGCTGGCGGCGATCGTGGGCGCCGA
CGACGAGGGGGGCGCGCCGGGAGACTTGCATTGCATCCTCATTTCGAACCCCTAA
TGAAGACACATGGAAGGACCCTGCCTTGGAGAAGCTCAGTTTGTCTGCGAGGA
GAGCCGCAGCTTCAACGACTGGGTGCCAGCATCACCTTCCCGAGTGA

BETA-ACTIN (NM_031144, Strand +) :

ATGGATGACGATATCGCTGCGCTCGTCGTCGACAACGGCTCCGGCATGTGCAAG
GCCGGCTTCGCGGGCGACGATGCTCCCCGGGCCGTCTTCCCCTCCATCGTGGGCC
GCCCTAGGCACCAGGGTGTGATGGTGGGTATGGGTCAGAAGGACTCCTACGTGG
GCGACGAGGCCAGAGCAAGAGAGGCATCCTGACCCTGAAGTACCCCATTTGAA
CACGGCATTGTACCAACTGGGACGATATGGAGAAGATTTGGCACCACACTTTC
TACAATGAGCTGCGTGTGGCCCTGAGGAGCACCCCTGTGCTGCTCACCAGGCC
CCTCTGAACCCTAAGGCCAACCCTGAAAAGATGACCCAGATCATGTTTGAGACC
TTCAACACCCCAGCCATGTACGTAGCCATCCAGGCTGTGTTGTCCCTGTATGCCT
CTGGTCGTACCACTGGCATTGTGATGGACTCCGGAGACGGGGTCACCCACACTG
TGCCCATCTATGAGGGTTACGCGCTCCCTCATGCCATCCTGCGTCTGGACCTGGC
TGGCCGGGACCTGACAGACTACCTCATGAAGATCCTGACCGAGCGTGGCTACAG
CTTACCACCACAGCTGAGAGGGAAATCGTGCGTGACATTAAAGAGAAGCTGTG
CTATGTTGCCCTAGACTTCGAGCAAGAGATGGCCACTGCCGCATCCTTTCCTCC
CTGGAGAAGAGCTATGAGCTGCCTGACGGTCAGGTCATCACTATCGGCAATGAG
CGGTTCCGATGCCCCGAGGCTCTCTCCAGCCTTCCTTCCTGGGTATGGAATCCT
GTGGCATCCATGAAACTACATTCAATTCCATCATGAAGTGTGACGTTGACATCCG
TAAAGACCTCTATGCCAACACAGTGCTGTCTGGTGGCACCACCATGTACCCAGG
CATTGCTGACAGGATGCAGAAGGAGATTACTGCCCTGGCTCCTAGCACCATGAA
GATCAAGATCATTGCTCCTCCTGAGCGCAAGTACTCTGTGTGGATTGGTGGCTCT
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APPENDIX C

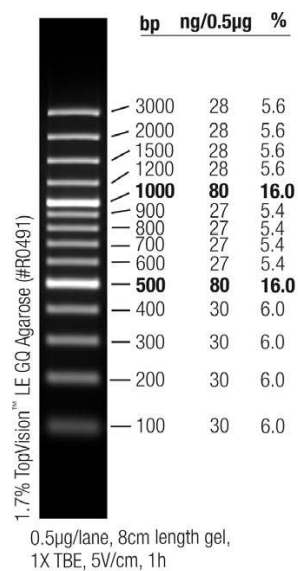


Figure C.1 : GeneRuler™ 100bp Plus DNA Ladder, ready-to-use (#SM0323, Fermentas)

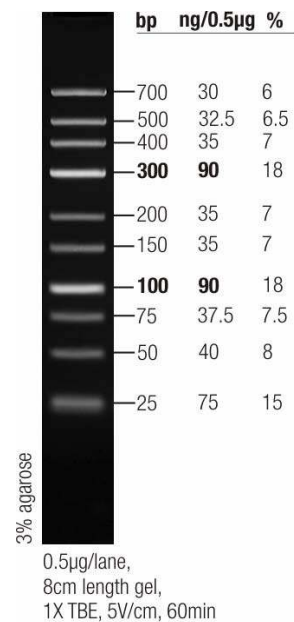


Figure C.2 : O'GeneRuler™ DNA Ladder, Low Range, ready-to-use (#SM1203, Fermentas)

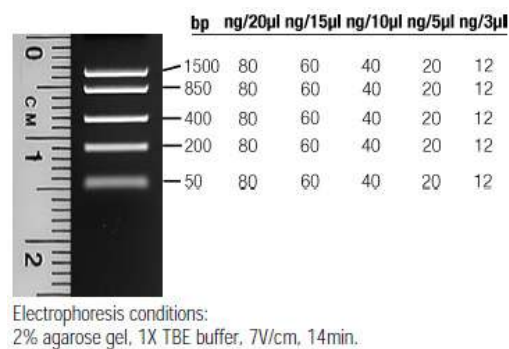


Figure C.3 : FastRuler™ DNA Ladder, Low Range, ready-to-use (#SM1103, Fermentas)

APPENDIX D

Table D.1 : Calculations of multiplex RT-PCR quantification of Atf3 gene expression for duplicate samples (Threshold = 120).

Sample Name	Band volume of Atf3	Band volume of β -Actin	Log2 value of Atf3 volume	Log2 value of β -Actin volume	Normalized value	Average of duplicate values
1_1	0	4679	—	12.1919845	—	
1_2	0	7140	—	12.8017084	—	---
3_1	37	11363	5.2094534	13.4720562	0.38668584	
3_2	928	12779	9.857981	13.6414873	0.72264708	0.554666
5_1	1916	11865	10.903882	13.5344245	0.80564060	
5_2	2773	11949	11.437232	13.5446023	0.84441253	0.825027
14_1	32	9183	5	13.1647498	0.37980213	
14_2	115	9286	6.8454901	13.1808416	0.51935152	0.449577
16_1	1165	16197	10.186114	13.983439	0.72844128	
16_2	1052	15025	10.038919	13.8750774	0.72352166	0.725981
18_1	5275	14249	12.364955	13.7985731	0.89610392	
18_2	5214	11990	12.348175	13.5495440	0.91133509	0.90372
26_1	3185	13831	11.637078	13.7556179	0.84598728	
26_2	3067	14459	11.582613	13.8196802	0.83812450	0.842056
28_1	681	12024	9.4115110	13.5536293	0.69439047	
28_2	609	12923	9.2502984	13.6576534	0.67729779	0.685844
29_1	3762	11241	11.877284	13.4564828	0.88264403	
29_2	3640	10017	11.829723	13.2901629	0.89011119	0.886378
ShamA2_1	232	9973	7.857981	13.2838118	0.59154564	
ShamA2_2	2235	10500	11.126059	13.3581017	0.83290720	0.712226
ShamA1_1		11965		13.5465328		
ShamA1_2		10220		13.3191076		
ShamB2_1	1572	8654	10.618386	13.0791514	0.81185584	
ShamB2_2	1991	8634	10.959278	13.0758134	0.83813352	0.824995
ShamB1_1		7543		12.8809227		
ShamB1_2		8173		12.9966500		
β -Actin_1		6867		12.7454643		
β -Actin_2		5584		12.4470832		

ShamA2 and ShamB2 samples contain both Atf3 and β -Actin primers whereas ShamA1 and ShamB1 samples are control samples and contain only the β -Actin primers. β -Actin duplicate are also control samples and contain only β -Actin primers.

Table D.2 : Calculations of multiplex RT-PCR quantification of Gadd45g gene expression for duplicate samples (Threshold = 122).

Sample Name	Band volume of Gadd45g	Band volume of β -Actin	Log2 value of Gadd45g volume	Log2 value of β -Actin volume	Normalized value	Average of duplicate values
1_1	0	12108	—	13.563673	—	
1_2	0	17478	—	14.093253	—	—
3_1	819	17804	9.6777196	14.119914	0.68539509	
3_2	490	17112	8.9366379	14.062721	0.63548428	0.6604397
5_1	101	16904	6.6582115	14.045077	0.47406016	
5_2	1775	19686	10.793603	14.264882	0.75665561	0.6153579
14_1	0	0	—	—	—	
14_2	5	21161	2.3219281	14.369120	0.16159153	—
16_1	3149	24281	11.620678	14.567540	0.79771038	
16_2	1078	17930	10.074142	14.130088	0.71295675	0.7553336
18_1	2741	21871	11.420487	14.416732	0.79216892	
18_2	1031	19053	10.009829	14.217731	0.70403842	0.7481037
26_1	634	22325	9.3083390	14.446373	0.64433746	
26_2	7	16904	2.8073549	14.045077	0.19988177	0.4221096
28_1	39	17801	5.2854022	14.119671	0.37432900	
28_2	43	19310	5.4262648	14.237061	0.38113659	0.3777328
29_1	0	12631	—	13.624681	—	
29_2	4	18444	2	14.170864	0.14113466	—
ShamA2_1	0	12000	—	13.550747	—	
ShamA2_2	0	12780	—	13.641600	—	—
ShamA1_1		12187				
ShamA1_2		12916				
ShamB2_1	0	9722	—	13.247037	—	
ShamB2_2	0	7260	—	12.825754	—	—
ShamB1_1		10463				
ShamB1_2		10022				
β -Actin_1		7970				
β -Actin_2		4984				

ShamA2 and ShamB2 samples contain both Gadd45g and β -Actin primers whereas ShamA1 and ShamB1 samples are control samples and contain only the β -Actin primers. β -Actin duplicate are also control samples and contain only β -Actin primers.

Table D.3 : C_T values of duplicate samples from multiplex real-time PCR experiments for Atf3 and Gadd45g genes.

Sample name	ATF3 C_T values	GADD45G C_T values
1_1	29.97	27.48
1_2	29.93	27.67
3_1	29.65	27.56
3_2	29.70	27.57
5_1	24.75	26.33
5_2	24.71	26.34
14_1	32.86	33.99
14_2	33.29	34.96
16_1	30.82	28.59
16_2	30.46	28.66
18_1	25.64	26.83
18_2	25.56	26.86
26_1	26.20	26.13
26_2	26.10	26.30
28_1	27.74	27.22
28_2	27.61	27.01
29_1	23.33	25.56
29_2	23.31	25.63
ShamA_1	30.04	27.68
ShamA_2	30.02	27.55
ShamB_1	28.53	26.95
ShamB_2	28.70	26.66

CURRICULUM VITAE



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