Effect of Time Restricted Feeding and its Endocrine Regulation in Hepatectomized Fibrotic rats-A Histomorphological and Biochemical Study

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2016

CERTIFICATE

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DECLARATION

I hereby declare that the findings and conclusion are of my own investigation. No part of this work has been presented elsewhere for any other degree.

Aysha Ambreen

DEDICATED

TO

RESPECTABLE PARENTS

&

MY FAMILY

Table of Contents

List of Figures i
List of Tables v
List of Abbreviations/Acronyms vii
Acknowledgement xi
Abstract xii
CHAPTER # 1 1
INTRODUCTION 2
1.1 Liver 2
1.2 Liver Regeneration 2
1.3 Partial Hepatectomy as a Model of Liver Regeneration 3
1.4 Traditional Model of Liver Regeneration 4
1.5 Revised model of liver regeneration 4
1.6 Fibrotic and Cirrhotic Liver Regeneration 5
1.7 Hormonal Status 6
1.8 Oxidative Stress 7
1.9 Mineral Metabolism 7
1.10 Circadian Rhythms 8
1.11 Circadian Clock and Liver Regeneration 9
1.12 Circadian Clock and Hepatic Fibrosis 9
1.13 Circadian Clock Entrainment 10
1.14 Feeding Regimens 10
1.15 Objectives 12
CHAPTER # 2 13
MATERIALS AND METHODS 14
2.1 Animals 14
2.2 Induction of Fibrosis 14
2.3 Experimental Design 14
2.4 Partial Hepatectomy 15
2.5 Dissection 16
2.6 Relative Liver Weight 17

2.7 Liver Regeneration Rate 17
2.8 Histomorphological Examinations 17
2.9 Biochemical Analysis 21
2.10 Statistical Analysis 31
CHAPTER # 3 32
FIBROTIC LIVER REGENERATION UNDER TEMPORAL FEED RESTRICTION 32
3.1 Introduction 33
3.2 Results 35
3.3 Conclusion 71
CHAPTER # 4 73
ADRENAL AND THYROID HORMONE ALTERATIONS DURING FIBROTIC LIVER REGENERATION UNDER TIME RESTRICTED FEEDING 73
4.1 Introduction 74
4.2 Results 75
4.3 Conclusion 81
CHAPTER # 5 82
OXIDATIVE STRESS 82
5.1 Introduction 83
5.2 Results 84
5.3 Conclusion 91
CHAPTER # 6 92
MINERAL METABOLISM 92
6.1 Introduction 93
6.2 Results 94
6.3 Conclusion 104
CHAPTER # 7 105
DISCUSSION 106
REFERENCES 126

List of Figures

Figure #	Title	Page #
2.1	Schematic presentation of experimental design.	15
3.1	Percentage Increase in Body weights of rats following eleven weeks of olive oil/CCl_4 treatment.	36
3.2	Percent body weight change of rats exposed to two weeks of time restricted feeding (tRF) regimens.	36
3.3	Percent body weight change of rats 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens.	38
3.4	Liver regeneration rate (%) of rats 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens.	38
3.5	Effect of time restricted feeding (tRF) on Proliferating cell nuclear antigen count (PCNA) after partial hepatectomy.	41
3.6a	Histomorphometric alterations in vehicle rats before and 48 hours post partial hepatectomy (PHx) under temporal feed restriction.	44
3.6b	Histomorphometric alterations in fibrotic rats before and 48 hours post partial hepatectomy (PHx) under temporal feed restriction.	45
3.7a	Mean AgNOR count per cell of rats 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens.	47
3.7b	AgNOR Proliferative index (pAgNOR) of rat hepatocytes 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens.	47
3.7c	AgNOR size variation in rat hepatocytes 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens.	48
3.7d	AgNOR distribution of rat hepatocytes 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimen.	48

3.8	AgNOR staining in regenerating rat liver.	49
3.9	Glycogen deposition in rat liver after partial hepatectomy under the effect of timed feed restriction.	58
3.10	ALT levels of rats 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens.	60
3.11	AST levels of rats 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens.	60
3.12	ALP levels of rats 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens.	61
3.13	LDH levels of rats 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens.	61
3.14	Bilirubin levels of rats 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens.	62
3.15	Total serum protein of rats 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens.	62
3.16	Serum albumin content of rats 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens.	64
3.17	Triglycerides (TG) content of rats 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens.	64
3.18	Serum glucose levels of rats 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens.	65
3.19	Changes in hepatic extracellular matrix (ECM) content under time restricted feeding (tRF) regimens following partial hepatectomy in rat.	67

3.20	Alpha-Smooth muscle actin (a-SMA) immunohistochemical staining in rat liver 48 hours after partial hepatectomy.	68
3.21	Hepatic hydroxyproline content of rats 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens.	69
3.22	Hepatic ultrastructural examination of rat liver following partial hepatectomy under time restricted feeding.	70
4.1	Serum corticosterone levels of rats 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens.	77
4.2a	Serum triiodothyronine (T3) of rats 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens.	78
4.2b	Serum thyroxine (T4) of rats 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens.	78
4.2c	Serum triiodothyronine/thyroxine (T3/T4) ratio of rats 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens.	79
4.3a	Liver triiodothyronine (T3) of rats 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens.	79
4.3b	Liver T4 of rats 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens.	80
4.3c	Liver triiodothyronine/thyroxine (T3/T4) ratio of rats 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens.	80
5.1	Hepatic CAT levels of rats 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens.	86
5.2	Hepatic POD levels of rats 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens.	86

5.3	Hepatic SOD levels of rats 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens.	87
5.4	Hepatic GSH levels of rats 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens.	87
5.5	Hepatic GPx levels of rats 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens.	88
5.6	Hepatic GSR levels of rats 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens.	89
5.7	Hepatic TBARS levels of rats 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens.	90

List of Tables

Table #	Title	Page #
3.1a	Relative liver weight (%) of rats 48 hours post partial hepatectomy after exposure to two weeks of time restricted feeding (tRF) regimens.	39
3.1b	Two way ANOVA for relative liver weight (P-value).	39
3.2a	Proliferation cell nuclear antigen (PCNA) and mitotic count in hepatocytes 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens.	40
3.2b	Two way ANOVA for hepatocyte PCNA index (P-value).	40
3.3a	Binuclear cell count (%) of rat hepatocytes, pre and 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens.	43
3.3b	Two way ANOVA for Pre-PHx binuclear hepatocyte count (P-value).	43
3.4a	Area (μm^2) of rat hepatocytes, pre and 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens.	51
3.4b	Two way ANOVA for pre and post PHx rat hepatocytes area (P-value).	51
3.5	Nuclear Area (μm^2) of rat hepatocytes, pre and 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens.	52
3.6	Nuclear diameter (μm) of rat hepatocytes, pre and post partial hepatectomy (PHx) 48 hours after exposure to two weeks of time restricted feeding (tRF) regimens.	53
3.7	Nuclear perimeter (μm) of rat hepatocytes, pre and post partial hepatectomy (PHx) 48 hours after exposure to two weeks of time restricted feeding (tRF) regimens.	54
3.8a	Hepatic tissue composition of rats 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens	56
3.8b	Two way ANOVA for hepatictissue composition of rats 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens. (P-value)	57
6.1a	Hepatic sodium content (mg/g dry weight), pre and 48 hours post partial hepatectomy (PHx) after exposure to two weeks	97

	of time restricted feeding (tRF) regimens.	
6.1b	Two way ANOVA table for pre and post PHx hepatic sodium content.	97
6.2a	Hepatic potassium content (mg/g dry weight), pre and 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens.	98
6.2b	Two way ANOVA table for pre and post PHx hepatic potassium content.	98
6.3a	Hepatic calcium content (ug/g dry weight), pre and 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens.	99
6.3b	Two way ANOVA table for pre and post PHx hepatic calcium content.	99
6.4a	Hepatic magnesium content (mg/g dry weight), pre and 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens.	100
6.4b	Two way ANOVA table for pre and post PHx hepatic magnesium content.	100
6.5a	Hepatic iron content (ug/g dry weight), pre and 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens.	101
6.5b	Two way ANOVA table for pre and post PHx hepatic iron content.	101
6.6a	Hepatic copper content (ug/g dry weight), pre and 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens.	102
6.6b	Two way ANOVA table for pre and post PHx hepatic copper content.	102
6.7a	Hepatic zinc content (ug/g dry weight), pre and 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens.	103
6.7b	Two way ANOVA table for pre and post PHx hepatic zinc content.	103

List of Abbreviations/Acronyms

°C	Celsius
4-AAP	4 - Chlorophenol and 4-Aminoantipyrine
ADF	Alternate Day Fasting
AgNOR	Argyrophilic Nucleolar Organizer Region
AL	Ad Libitum
ALP	Alkaline Phosphatase
ALP	Atherogenic Lipoprotein Phenotype
ALT	Alanine Aminotransferase
ANOVA	Analysis of Variance
AST	Aspartate Transaminase
BCG	Bromocresol Green
CAT	Catalase Activity
CCl_4	Carbon Tetrachloride
CR	Caloric Restriction
D1	Deiodinase Type 1 (liver selenoenzyme)
DAB	Diaminobenzidine
DNA	Deoxyribonucleic Acid
DPX	Distyrene Plasticizer Xylene
DR	Dietary Restriction

DTNB	Dithio-bis Nitro Benzoic Acid
ECM	Excessive Extracellular Matrix
EDTA	Ethylene-Diamine-Tetraacetic Acid
EGF	Epidermal Growth Factor
GK	Glycerol Kinase
GPx	Glutathione Peroxidase Assay
GSH	Reduced Glutathione Assay
GSR	Glutathione Reductase Assay
H&E	Hematoxyline-Eosin
H_2O_2	Hydrogen Peroxide.
HCl	Hydrochloric Acid
HGF	Hepatocyte Growth Factor
HIER	Heat Induced Epitope Retrieval
HNO3	Nitric Acid
HSC	Hepatic Stellate Cells
IF	Intermittent Fasting
IL-6	Interleukin-6
LDH	Lactate Dehydrogenase
LP	Lipid Peroxidation
LPL	Lipoprotein Lipase
LRR	Liver Regeneration Rate
mAgNOR	Mean Number of AgNORs in 100 Nuclei

MDA	Malondialdehyde
MDH	Maltate Dehydrogenase
NAD^+	Nicotinamide Adenine Dinucleotide
NADH	Nicotinamide Adenine Dinucleotide + Hydrogen
NADPH	Nicotinamide Adenine Dinucleotide Phosphate-Oxidase
NaOH	Sodium Hydroxide
NBT	Nitrobluetetrazolium
pAgNOR	Proliferative Count of Agnor
PAS	Periodic Acid-Schiff
PBS	Phosphate Buffer Saline
PCNA	Proliferating Cell Nuclear Antigen
PFA	Paraformaldehyde
PHx	Partial Hepatectomy
pNPP	p-Nitrophenyl Phosphate
POD	Peroxidase
RLW	Relative Liver Weight
ROS	Reactive Oxygen Species
SCN	SupraChiasmatic Nucleus
SOD	Superoxide Dismutase Activity
SPSS	Statistical Package for the Social Sciences
T3	Triiodothyronine
T4	Thyroxine

TBA	Thiobarbituric Acid
TBARS	Thiobarbituric Acid Reactive Substance Assay
TGF-α	Transforming Growth Factor α
TGF-β1	Transforming growth factor-beta 1
TIMP	Tissue inhibitors of metalloproteinases
TMB	Tetramethylbenzidine
TNF	Tumor Necrosis Factor
tRF	Time-Restricted Feeding.
tRF1	First Time Restricted Regimen
tRF2	Second Time Restricted Regimen
α-SMA	Alpha Smooth Muscle Actin

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Aysha Ambreen

Abstract

Owing to the numerous vital responsibilities granted to the liver, nature has imparted this organ the ability to regenerate in order to safeguard the functions performed by it. Hepatic regeneration is a remarkable physiological process with marvelous highly coordinated biological events that occur simultaneously and sequentially leading to the hypertrophy and hyperplasia of hepatocytes allowing the liver to regenerate to its original size following resection. In contrast to the normal liver fibrotic liver showed delayed and impaired regenerative response because of slow proliferation rate and increased extracellular matrix. Hepatocyte mitosis, trophic state and fibrosis are influenced by circadian clock oscillations. Circadian rhythms in the body are entrained by two important zeitgebers namely light and feed. Light is the dominant zeitgeber of the central master clock residing in the suprachiasmatic nucleus (SCN) while peripheral clocks are mainly influenced by feeding patterns viz. caloric restriction, intermittent fasting and time restricted feeding (tRF). The tRF is the only pattern that entrains peripheral clocks independent of SCN, especially the hepatic clock. Present study has been conducted to evaluate the effect of time-restricted feeding on the regeneration ability of liver following partial hepatectomy.

Animals were made fibrotic by eleven weeks intragastric carbon tetrachloride treatment while vehicle received olive oil. Each group was then either fed ad libitum or subjected to two weeks of time-restricted feeding i.e. time-restricted fed for 12 hours when the light was on (tRF1) and time-restricted fed for 4 hours only after 4hrs of light on and before 4hrs of light off (tRF2), before undergoing two-third partial hepatectomy (PHx). Liver regenerative capacity, functional capability along with hepatic antioxidant and mineral content was evaluated 48 hours post PHx. Finally adrenal and thyroid hormones concentrations, being the most influential hormones on liver have also been determined.

The tRF schedules markedly decreased the body weights of rats and recovered them significantly after PHx especially the full daytime feed restriction. Liver regeneration as assessed by estimating different somatometric, hypertrophic and hyperplasic estimates

indicated no effect of tRF on relative liver weight. However, liver regeneration rate was significantly increased by both feeding regimens specially the first schedule. The tRF regimen inhibited hyperplasia as percentage proliferation cell nuclear antigen and mitotic count were reduced by the treatments. The restricted feeding schedules did not affect AgNOR index in vehicle rats, however, fibrotic rats showed increased and heterogeneous AgNOR index when the feed was restricted to middle of the light phase. Both feed restriction schedules increased the binuclear cells that were decreased significantly during the regenerative process. A marked reduction in hepatocyte cellular area under temporal feed restriction was observed that remained restricted after PHx compared to ad libitum fed rats. Nuclear morphometry also followed the same trend in vehicle rats but fibrotic rats remained non-responsive to tRF regimens. Hepatic tissue analysis showed that tRF regimens increased the hepatic water, lipid and glycogen content with no effect on DNA and protein.

Hepatic functional capability evaluation indicated improvement in serum alanine transaminase and aspartate transaminase under tRF regimens with no significant effect on alkaline phosphatase, bilirubin, total protein and albumin levels. Lactate dehydrogenase activity was significantly restored in vehicle rats while glucose and triglyceride were differentially affected by temporal feeding restrictions. Decreased serum glucose under tRF1 while increased triglyceride levels under tRF2 in fibrotic rats was observed.

Temporal feed restriction decreased the extra cellular matrix in vehicle rats with little or no improvement in fibrotic rats as indicated by hydroxyproline quantification and histopathological evaluation by Gomori triple stain and α-SMA.

Serum corticosterone levels were increased by partial hepatectomy. Timed-feed restriction had a differential effect on corticosterone concentrations with no effect on vehicle rats while a considerable decrease and increase under the first and second regimen respectively was observed in fibrotic rats. Partial hepatectomy depressed the thyroid hormone levels which were raised by time-restricted feeding in vehicle and fibrotic rats under the first and second schedule respectively by significantly increasing serum thyroxine levels. Serum triiodothyronine/thyroxine ratio was considerably low in these

rats. Significantly reduced triiodothyronine and triiodothyronine/thyroxine ratio in fibrotic sham livers were remarkably increased by second schedule after partial hepatectomy.

The tRF treatments improved the hepatic catalase levels in both groups while peroxidase and superoxide dismutase levels did not show any significant effect under the treatments. No decrease in hepatic thiobarbituric acid reactive substance levels but significant increase in glutathione content under the tRF regimens was seen. Interestingly glutathione peroxidase levels were increased and decreased in vehicle and fibrotic rats respectively under the first feeding schedule. Glutathione reductase activity was significantly increased in vehicle rats with no effect on fibrotic rats under the second tRF regimen.

No significant change in hepatic sodium and potassium contents were observed except a considerable decrease under tRF2 in vehicle rats that tend to raise their levels during the regenerative process. Interestingly the first feeding regimen elevated while the second has depressed the hepatic magnesium levels during regeneration. Calcium and iron was non-significantly decreased in vehicle rats under the tRF treatments while copper was significantly reduced in control animals with a rise in fibrotic livers under the second regimen. Zinc was raised in fibrotic rats under the treatments.

Temporal feed restriction influenced liver regeneration by inhibiting hyperplasia, producing a shift from hepatocyte binuclearity to mononuclearity, reducing ploidy and hypertrophy, effecting hepatic tissue constituents, and lipid related metabolic events. The tRF regimens improved the hepatic functional and antioxidant capacity; and mineral metabolism during regeneration. Temporal feed restriction had produced different alterations in adrenal and thyroid hormones with respect to vehicle and fibrotic rats, which further influence liver regeneration being bound in a complex close relation with liver.

CHAPTER # 1

INTRODUCTION

1.1 Liver

Liver is the largest glandular body organ that performs numerous vital functions. This tissue is highly specialized for the regulation of nutrient metabolism. It acts as the major blood reservoir and the principal center of detoxification. Liver plays important role in the maintenance of numerous metabolites and hormones. Hepatic tissue also acts as a major component of the organism defensive response (Kietzmann *et al.*, 1997). It is a vital organ that supports nearly every organ in the body in some facet.

Liver injury can impair these and many other processes. Nature has imparted liver the ability to regenerate in order to safeguard the functions performed by it. This ability allows liver to recover the lost mass without endangering the viability of entire organism. This remarkable phenomenon of liver regeneration following injury or loss of liver mass is seen in all vertebrates, from humans to fish (Michalopoulos and DeFrances, 1997).

1.2 Liver Regeneration

The liver has an extraordinary capacity to regenerate from various types of injuries including massive destruction of hepatic tissue by toxins, viral agents, or by surgical desertion (Ai-Hadeedi *et al.*, 1990; Palmes and Spiegel, 2004; Alison *et al.*, 2009). It is a very complex phenomenon, associated with well-organized signaling events involving growth factors, cytokines, matrix remodeling, and several feedbacks of stimulation and inhibition of growth related signals (Michalopoulos and DeFrances, 1997).

In contrast with other regenerating tissues like bone marrow and skin, hepatic regeneration does not depend on stem progenitor cells instead all the mature liver cell populations i.e. hepatocytes, stellate cells, biliary epithelial cells, sinusoidal endothelial cells and Kupffer cells are involved in liver regeneration (Michalopoulus and De Frances, 1997). In fact this process is not a true regeneration as the liver does not recover the original lobular structure rather the remnant tissue increase in size. Only in severely

damaged liver with impaired hepatocyte proliferation facultative liver stem cells proliferate and assumed to contribute to regeneration (Zaret and Grompe, 2008; Alison *et al.*, 2009; Tanaka *et al.*, 2011). Among all the liver cells hepatocytes that account for about 80% of liver weight and about 70% of all liver cells are the most important one to take part in regenerative process. Most of the metabolic and synthetic functions of liver are also performed by the hepatocytes (Si-Tayeb *et al.*, 2010).

1.3 Partial Hepatectomy as a Model of Liver Regeneration

Liver regeneration after partial hepatectomy (PHx) in rodents is one of the most studied models of cell, organ, and tissue regeneration. In 1931, Higgins and Anderson devised an excellent model of 70% hepatectomy in adult rats (Higgins and Anderson, 1931), which has been extensively used in hepatic regeneration studies (Fausto, 2000).

The reproducibility of PHx in terms of mass removed and precision of timing of the sequence of events has made PHx the preferred experimental model for tissue regeneration. Rodents have multi-lobular liver and three of the five liver lobes representing 2/3 of the liver mass; can be easily removed without causing damage to the residual two lobes. The remnant lobes grow in size to restore the mass of the original five lobes. As the resection of lobes does not induce damage to the remaining liver tissue, thus the process of liver regeneration from the very beginning depend entirely on the processes relevant only to liver tissue and not to necrosis or inflammation. Further, because PHx can be performed within few minutes, the regenerative phenomena can be precisely timed. Moreover, it has clinical importance too because hepatectomy is a practical treatment for liver tumors, and liver transplantation is an important therapeutic option in patients with severe liver diseases. These attributes of the 70% PHx makes it the most popular and accepted model of liver regeneration through the years by many investigators (Michalopoulos and DeFrances, 1997; Michalopoulos, 2007; Mitchell and Willenbring, 2008).

1.4 Traditional Model of Liver Regeneration

Liver regeneration following PHx is a complex process involving multiple signaling pathways induced by a variety of hormones, growth factors and cytokines (Michalopoulos, 1990; Michalopoulos, 1997; Fausto, 2000). There stands an old traditional model of liver regeneration that postulates that following two-third PHx, all the remaining 1/3 hepatocytes undergo roughly one round of cell division leading to 60% of hepatocytes followed by a smaller percent of cells to enter cell cycle thus restoring the lost tissue mass by establishing the original hepatocyte number (Fausto *et al.*, 2006; Michalopoulos, 2007; Duncan *et al.*, 2009).

1.5 Revised model of liver regeneration

Most of the studies on liver regeneration are focused on the proliferation of hepatocytes as was emphasized in the long-standing traditional model. Although an elegant and simple model, evidences suggested that the traditional model of liver regeneration required revision. Miyaoka *et al.*, (2012) using imaging cytometric analysis and genetic tracing system, proposed a revised model of liver regeneration. They suggested cellular hypertrophy significantly contributes to liver regeneration alongwith hyperplasia as has been postulated by the traditional model. They showed that hepatocytes on average divide only about 0.7 times rather than one to two times during regeneration following 70% PHx. According to the revised model, the physiological process of hepatic regeneration following 70% PHx is a combination of hypertrophy (increase in cell size) and hyperplasia (increase in cell number) that led to the recovery of the original liver weight (Miyaoka *et al.*, 2012).

The model suggested that following 30% PHx, the liver restores its original mass by hypertrophy, neither the cell nor the nuclear number of hepatocytes changes. As only a small fraction of hepatocytes entered in cell cycle, their ploidy is not altered significantly. In contrast, when 70% of liver is removed, hypertrophy of hepatocytes occurs in the first response after PHx, followed by cell proliferation. Almost all hepatocytes enter into S phase, but approximately half replicate resulting in an increase in ploidy. Progression to mitotic phase is compromised in liver regeneration (Miyaoka *et al.*, 2012). During liver

regeneration, binuclear hepatocytes reduced and undergo unconventional cell division to produce two mononuclear daughter hepatocytes (Sulkin, 1943; Geschwind *et al.*, 1958, Gentric *et al.*, 2012).

The initiation, proliferation and termination of hepatic regenerative process involve several highly coordinated sequential biological events. PHx radically changes the blood flow into the liver. The increased blood flow generates stress that induces nitric oxide production, triggering regeneration (Macedo and Lautt, 1998; Wang and Lautt, 1998; Niiya *et al.*, 1999; Sato *et al.*, 1999; Schoen *et al.*, 2001). Hypertrophy facilitates the priming of hepatocytes by priming factors like tumor necrosis factor (TNF) and interleukin-6 (IL-6), to change their gene expression and move towards the proliferative phase. The cytotoxicity reducing agents also play an important role for the liver regenerative response. The levels of reactive oxygen species (ROS) and glutathione determine the proliferative or apoptotic effect of TNF on hepatocytes (Fausto, 2000; Miyoka *et al.*, 2012). Hepatocytes respond to the growth factors such as hepatocyte growth factor (HGF), transforming growth factor α (TGF-α) and epidermal growth factor (EGF), thus starting the progression phase. Hepatocyte proliferation is then followed by non-parenchymal cell proliferation. Striking increases in hepatocyte DNA synthesis occur within the initial 48 hours after PHx, followed by smaller but significant increases in hepatocyte mitosis that eventually lead to recovery of the lost liver mass (Fausto, 2004; Duncan *et al.*, 2009). Transforming growth factor-beta 1 (TGF-β1) is known to inhibit proliferation and terminate liver regeneration after the required mass has been gained (Michalopoulos and DeFrances, 2005).

1.6 Fibrotic and Cirrhotic Liver Regeneration

Although normal liver regenerates remarkably, fibrotic or cirrhotic livers though maintaining the ability to regenerate after PHx showed impaired and slow regeneration (Hashimoto and Watanabe, 1999; Andiran *et al.*, 2000; Kato *et al.*, 2005). Liver resection is the one of main therapeutic approaches for certain liver diseases such as hepatocellular carcinoma, in such cases the remnant liver is often cirrhotic and thus is less able to regeneration with significant risk of hepatic failure and postoperative deaths (Redaelli *et*

al., 2002). Improvement of fibrotic liver regeneration is an important task in the field of gastroenterology.

Hepatic fibrosis, the scarring of tissue, is a wound-healing process for chronic injury. It is characterized by excessive extracellular matrix (ECM) deposition, altered liver architecture and defective hepatocyte proliferation. The main source of the scar tissue during liver fibrosis and cirrhosis is the hepatic stellate cells (HSC). These are heterogeneous group of cells residing in the sub-endothelial space of Disse, and have been recognized as the principal fibrogenic hepatic cells. Following liver injury, these cells undergo activation and differentiation into fibrogenic myofibroblasts that produce extracellular matrix proteins (Bataller and Brenner, 2005; Friedman, 2008) leading to liver fibrosis that progresses to cirrhosis and finally hepatocellular carcinoma (Bissell *et al.*, 1990).

Several features are involved in the diminished regenerative capacity shown by the fibrotic and cirrhotic liver; however, two of them are the most important. The main reported factors responsible for the retarded growth of fibrotic liver following surgery are the reduced hepatocyte proliferative ability and increased and continuous deposition of ECM, with insufficient collagen breakdown (Andiran *et al.*, 2000). Decreased hepatocyte DNA synthesis and mitotic activity following PHx in carbon tetrachloride induced cirrhotic rats have been observed (Kanta and Chlumska, 1991; Bickel *et al.*, 1998). Similar results in dimethylnitrosamine induced liver cirrhosis after PHx was seen with depressed liver regenerative capacity (Ozawa *et al.*, 2006). Thioacetamide-induced liver cirrhotic rats showed that although PHx favors functional restoration of the liver but morphological alterations were not improved (Hashimoto and Watanabe, 1999). The therapeutic strategies for improved regeneration of fibrotic liver must focus on achieving better hepatocyte regenerative ability and increased ECM degradation (Hernández-Muñoz *et al.*, 2001). Further systemic and hepatic hormonal, oxidative, and mineral status also reported to affect the liver regenerative process.

1.7 Hormonal Status

The phenomenon of liver regeneration following partial hepatectomy is regulated by complex humoral mechanisms. Hormones like insulin, glucagon, glucocorticoids and

thyroid hormones, etc. reported to affect the hepatic regenerative process (Short et al., 1980; Leffert et al., 1988; Bucher 1991; Cervinkova and Simek 1992). Hepatocyte hyperplasia and hypertrophy; the main events of liver regeneration are both influenced by glucocorticoids (Tongiani et al., 1982; Barbason et al., 1989). Like glucocorticoids, thyroid status also affects liver regeneration. Thyroid hormones not only support (Alisi et al., 2005) liver regeneration but also aggravate hepatic fibrosis (Zvibel et al., 2010). Therefore, a perfect balance of thyroid hormones is very important during hepatic regeneration especially during fibrotic liver regeneration.

1.8 Oxidative Stress

Oxidative stress is an imbalance between the production of free radicals and the antioxidant defenses of cell. Oxidative stress results from the elevated production of reactive oxygen species (ROS) that cause damage to lipids, proteins and DNA (Schieber and Chandel, 2014). Liver regeneration following PHx requires a perfect balance between stimulating and inhibiting factors of hepatocyte proliferation. ROS and glutathione levels determine the proliferative or apoptotic effect of TNF on hepatocytes, the major cytokine that primes them to enter in the cell cycle (Fausto, 2000).Partial hepatectomy could cause oxidative stress (Miyake *et al.*, 2002). Free radicals may accumulate and cause oxygen toxicity (Kimball *et al.*, 1976).

Oxidative stress play important role in hepatic fibrosis development. It can initiate liver fibrosis by activating hepatic stellate cells (Lee *et al.*, 2001). Lipid peroxidation (LP), the cellular pathway involved in oxidative damage, can aggravate hepatic fibrosis by stimulating hepatic stellate cells to become fibrogenic (Vendemiale *et al.*, 2001). Increased lipid peroxidation during cirrhotic liver resection, results in impaired hepatic regeneration (Morimoto and Isselhard, 1992) as cirrhotic liver had reduced capacity to counteract oxidative stress (Cabre *et al.*, 2000; Balkan *et al.*, 2001).

1.9 Mineral Metabolism

The events of remarkable process of liver regeneration are strongly influenced by tissue minerals and metallothioneins (Mocchegiani *et al.*, 1997; Rink and Gabriel 2000).

PHx induces significant and interconnected changes in iron, zinc, calcium and magnesium levels not only in the regenerating liver but also in the lymphatic tissues and submandibular gland, indicating their importance during the regenerative processes. Tissue metals may affect the cell structural integrity, enzyme activities, transcription and replication factors, and transduction signals during liver regeneration. They may be involved in the cytokine production and in the development and maintenance of immune processes during the process (Milin*et al.*, 2005). Metalloproteinases and tissue inhibitors of metalloproteinases are involved in the ECM regulation during liver regeneration (Mohammed and Khokha, 2005a). Many growth factors are bind to hepatic ECM. Further, Metalloproteinases and tissue inhibitors of metalloproteinases (TIMP) levels are important in the regulation of HGF release during regeneration (Mohammed *et al.*, 2005). Several mineral metabolism disorders have been reported in hepatic diseases. Hepatic fibrosis resulted in the imbalance of mineral metabolism which play an important role in its pathogenesis (George, 2006), thus effecting the liver regeneration.

1.10 Circadian Rhythms

In mammals, most of the molecular, cellular, and organismal functions are subjected to well-controlled daily oscillations. Sleep-wake cycles, cardiovascular activity, blood pressure, body temperature, renal activity, hepatic and cellular metabolism and the secretion of many hormones and neurotransmitters are controlled by an endogenous time measuring system called the circadian clock (Schibler and Lavery, 1999; Panda *et al.*, 2002; Reppert and Weaver, 2002; Hastings *et al.*, 2008). Cell division (Scheving *et al.*, 1983, Bjarnason *et al.*, 1999; Bjarnason *et al.*, 2001), immune function (Haus and Smolensky, 1999) and behavior (Stephan and Zucker, 1972) are also regulated by circadian clocks that are synchronized to the environment by external cues.

The circadian system in mammals is organized hierarchically and works through the coordinated action of a light-entrained master pacemaker located in the suprachiasmatic nucleus (SCN) in the hypothalamus and a set of subordinated clocks in other oscillating tissues (Reppert and Weaver, 2002; Schibler and Sassone-Corsi, 2002; Hastings *et al.*, 2008). Clocks in non-SCN cells are generally referred to as peripheral clocks. Central

clocks are self-sustained and the master clock SCN have to re-entrain peripheral oscillators to prevent the dampening of circadian oscillations in peripheral organs such as liver, kidney, heart, and pancreas (Yamazaki *et al.*, 2000) through different neural connections and endocrine signals (Le Minh *et al.*, 2001; Takahashi *et al.*, 2008).

1.11 Circadian Clock and Liver Regeneration

Most of the studies focused on hepatocyte proliferation during liver regeneration which has been considered as the fundamental process during this phenomenon. An understanding of the regulation of hepatocyte replication is important in developing clinical approaches toward liver repair especially during fibrotic liver regeneration.

Cell division is reported to be regulated by the circadian clock (Scheving *et al.*, 1983). Mitosis is continuously taking place in the body as the life of each cell is limited and new cells are required to rebuild the organs. Evidence suggested that the circadian rhythms affect the timing of cell divisions in vivo. Dividing cells organize the processes required for their replication within circadian time (Hrushesky and Bjarnason, 1993). Key stages in the cell cycle correlate with rhythms in clock gene expression (Bjarnason *et al.*, 1999; Bjarnason *et al.*, 2001). Circadian regulation of cell division is found widely in organisms such as cyanobacteria (Mori and Johnson, 2000) and zebrafish (Dekens and Whitmore, 2008).

Hepatocytes, which do not divide normally, use circadian timing to organize mitotic activity during recovery from an injury (Barbason *et al.*, 1989; Matsuo *et al.*, 2003). Timing of hepatocyte entry into DNA synthesis after partial hepatectomy is cell autonomous. There present a molecular "mitotic clock" in the hepatocytes that regulates progression through cell cycle (Weglarz and Sandgren, 2000). In regenerating liver cells the cell cycle clock is under the control of SCN (Matsuo *et al.*, 2003). Studies indicated circadian variations in the mitotic index and DNA synthesis (DNAs) of not only hepatocytes but also in the sinusoid littoral cell populations of adult intact and hepatectomized male mouse (Badrán*et al.*, 1984; Surur *et al.*, 1985).

1.12 Circadian Clock and Hepatic Fibrosis

As the circadian clock system plays important role in regulating metabolism and energy homeostasis in the liver, circadian clock dysfunction lead to common chronic liver

diseases including hepatic fibrosis (Tong and Yin, 2013). Likewise, during carbon tetrachloride induced hepatic fibrosis in mice, impairment in the circadian rhythms of some hepatic clock genes has been observed (Chen *et al.*, 2010).

1.13 Circadian Clock Entrainment

Mammalian circadian rhythms are regulated by endogenous biological clocks, the master clock of which is located in the SCN. The period of this oscillation is not exact but approximately 24h and to keep synchrony with the environment, circadian rhythms need to be entrained daily by means of *zeitgebers*. *Zeitgebers* are signals that help synchronize the circadian clocks of body with the environment of which light, temperature, and food are the most important. Light or daily light-dark cycles are the most powerful *zeitgeber*, and functions by entraining the SCN, which receive photic information from the environment through retina through the retino-hypothalamic tract. Feeding regimens have been revealed to be the dominant *zeitgeber* for peripheral tissues (Damiola *et al.*, 2000; Stokkan *et al.*, 2001).

1.14 Feeding Regimens

There are three main types of feeding regimens that can entrain circadian clocks in both SCN and peripheral tissues thus affecting different physiological and metabolic processes.

1.14.1 Caloric Restriction

Caloric restriction (CR) also known as dietary restriction (DR) refers to a dietary regimen low in calories without malnutrition. CR restricts the amount of daily calories derived from carbohydrates, fats, or proteins to 60–75% of ad libitum feeding (Masoro *et al.*, 1995). CR increases the life span and delays the occurrence of age related pathophysiological changes, such as cancer, diabetes, cataracts and kidney diseases (Weindruch and Sohal, 1997; Roth *et al.*, 2002; Masoro, 2005) by increasing the resistance to oxidative stress (Sohal and Weindruch, 1996). CR synchronizes the peripheral clocks and thus influences clock-controlled physiological, metabolic and

behavioral systems (Froy and Miskin, 2010). The synchronization of peripheral oscillators by CR could be achieved either directly due to the temporal feeding as animals under CR consume most of their food within a short period of time or by synchronizing the SCN which by humoral or neuronal signals entrain the peripheral tissues (Froy and Miskin 2010).

1.14.2 Intermittent Fasting

Intermittent fasting (IF), also denoted as alternate day fasting (ADF) is the feeding regimen during which food is available ad libitum every other day. IF-treated mice eat on the day they have access to food approximately twice as much as those having continuous access to food (Anson *et al.*, 2003; Descamps *et al.*, 2005; Froy *et al.*, 2009). Intermittent fasting resulted in increased life span (Goodrick, 1990), improvement in glucose metabolism, cardioprotection, neuroprotection (Anson *et al.*, 2003; Ahmet *et al.*, 2005; Contestabile *et al.*, 2004; Mager *et al.*, 2006; Mattson, 2005; Sharma and Kaur, 2005), and increased resistance to cancer (Descamps *et al.*, 2005). IF entrain the peripheral rhythms with the involvement of SCN like CR (Froy and Miskin, 2010).

1.14.3 Time-Restricted Feeding

Time Restricted feeding (tRF) or restricted feeding RF corresponds to the dietary regimen during which feed is restricted to short periods of time during 24 hours and not available ad libitum, without any caloric reduction (Hirota and Fukada, 2004; Schibler *et al.*, 2003). Animals on tRF adjust their feeding schedule within a few days and consume all or most of their feed during the provided limited time (Froy *et al.*, 2006). Temporal feed restriction has profound effects on the behavior and physiology of animals. Many physiological activities that are normally controlled by the SCN, such as body temperature, locomotor activity, and heart rate, are altered by tRF (Mistlberger, 1994; Hara *et al.*, 2001; Boulamery-Velly *et al.*, 2005; Hirao *et al.*, 2006), suggesting that tRF is dominant over the SCN (Stephan, 2002; Oishi *et al.*, 2002) specially in peripheral tissues. It can reduce the levels of disease markers leading to better health (Sherman *et al.*, 2011). Even timed high-fat diet results in reduced body weight and improved metabolism compared to animals fed ad libitum (Sherman *et al.*, 2012). Further mice on daytime

restricted feeding exhibited slower tumor development than mice fed at night or ad-libitum (Filipski *et al.*, 2005; Wu *et al.*, 2004). Temporal feed restriction reported to improve metabolic and physiologic rhythms, it can improve glucose tolerance and motor function; and reduce adipose, liver steatosis and serum cholesterol (Hatori *et al.*, 2012).

Temporal feed restriction affects circadian oscillators in peripheral tissues, with no effect on the SCN (Damiola *et al.*, 2000; Stokkan *et al.*, 2001; Cassone and Stephan, 2002; Oishi *et al.*, 2002; Schibler *et al.*, 2003; Hirota and Fukada, 2004). Thus, tRF uncouples the SCN from the periphery, suggesting that peripheral tissue clocks and thus physiological processes can directly be regulated by nutritional synchronization (Lin *et al.*, 2008). When food availability returns to ad libitum, the SCN clock, whose phase remains unaffected by tRF, resets the peripheral oscillators (Damiola *et al.*, 2000).

Circadian clocks in the liver are reported to be strongly entrained by periodic meals (Hara *et al.*, 2001; Stephan, 2002). In fact, liver displays the fastest shift in the phase of clock-control genes under restricted feeding (Stokkan *et al.*, 2001). Entrainment of the liver by tRF to a specific phase without disrupting SCN-driven rhythms show the practicality and therapeutic efficacy of tRF in treating different liver disorders and complications including the disturbances during regeneration of fibrotic or cirrhotic liver.

1.15 Objectives

Chronic liver injuries result in liver fibrosis which leads to cirrhosis and finally hepatocellular carcinoma. Surgical resection is often required in certain liver pathologies. Extensive work has been done to reveal the remarkable phenomenon of liver regeneration but much work is required to improve the impaired regenerative capacity of fibrotic liver. As circadian rhythms have strong influence over mammalian liver processes including liver regeneration and fibrosis and feeding regimens specially temporal feeding can influence exclusively the hepatic physiological processes without disrupting other rhythms, present study has been conducted with the hypothesis that time-restricted feeding exposure to liver before hepatectomy affects the regenerative ability of fibrotic liver.

CHAPTER # 2

MATERIALS AND METHODS

2.1 Animals

Female Sprague-Dawley rats weighing 185-225g were used in the study. The animals were bred in the animal house facilities of Quaid-e-Azam University, Islamabad. They were housed in a windowless, air-conditioned room with temperature maintained at 25°C and strict 12h light-dark cycle with lights on at 7a.m. and off at 7p.m. The animals were allowed to consume standard rat pellet chow and water ad libitum.

2.2 Induction of Fibrosis

Fibrosis was induced by the modified method of Rivera *et al.*, (2001) and Proctor and Chatamra (1983). Briefly rats were given intragastric treatment of carbon tetrachloride (CCl_4, BDH, UK) at a dose of 412mg/kg dissolved in olive oil at ratio of 1:8 once in a week for eleven weeks while vehicle received olive oil at the same dose for the same duration. Body weights were weekly checked, and dosage of olive oil and CCl_4 was adjusted by weight change from the previous week.

2.3 Experimental Design

At the end of CCl_4/olive oil treatment each group was further sub-divided into three main groups: first group received food ad libitum (AL); second received food for twelve hours when the light was on i.e. 7a.m. to 7p.m. (tRF1) while the third group received food for four hours only after four hours of light on and before four hours of light off i.e. 11a.m. to 3p.m. (tRF2). Each group was maintained on the above mentioned restricted feeding regimens for two weeks. Weekly body weights were noted at the same time of the day after food presentation to the rats. After two weeks the animals in AL group were further subdivided into sham and partial hepatectomized group while restricted feeding groups were subjected to PHx only (Figure 2.1).

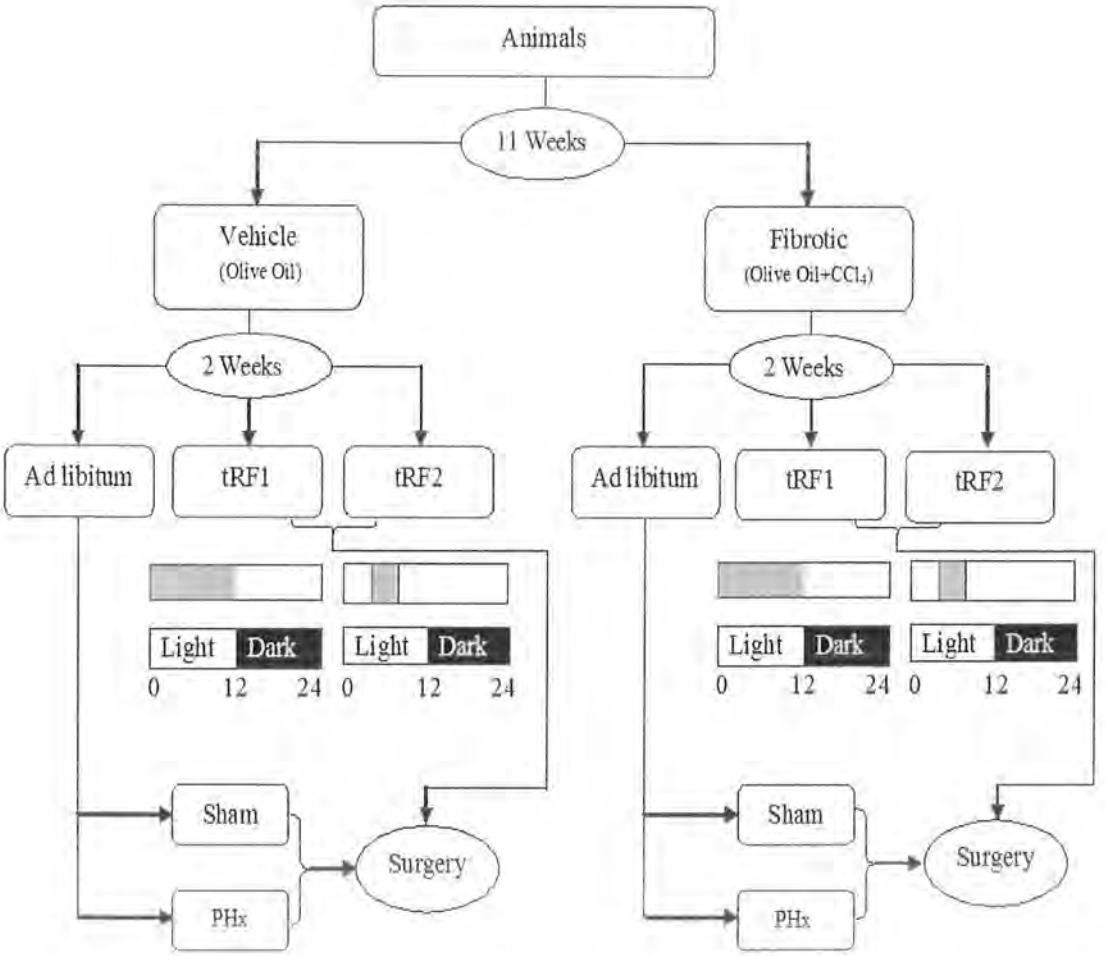

Figure 2.1: Schematic presentation of experimental design. PHx = Partial Hepatectomy, tRF = Time Restricted Feeding.

2.4 Partial Hepatectomy

Two-thirds hepatectomy (PHx) was performed under sterile conditions by the method of Higgins and Anderson (1931) between 9a.m. and 12p.m. to reduce the variability in the progression of liver regeneration associated with surgery time and the circadian clock. Briefly the peritoneum was opened by midline incision, the upper abdomen and lateral lower portions of both hemi-thoraces were compressed to exteriorize the liver without dividing the ligaments of the right and left lobes. The liver parenchyma was not touched because of the dangers of injuring the viscera and bleeding. A glysilk thread knot, surrounding the hilum and the hepatic vein, was tied and median and left lateral lobes of

the liver were resected. Because the rat liver is lobulated, the hilum of these lobes could be safely ligated without involving the vasculature of the remnant lobes. The abdomen was closed in two layers with absorbable suture chromic catgut in the musculature and glysilk in the skin. Rats were given approximately 0.5ml sterile saline intraperitoneally before closure of the abdomen to replace fluid loss from bleeding. As controls, sham-operated rats were subjected to midline laparotomy and gentle manipulation of the liver in the same manner as resected animals. The left and right liver lobes resected during hepatectomy were weighed to evaluate liver regeneration rate (LRR) and processed for histology as described in the following section.

2.5 Dissection

Forty eight hours after the surgery the rats were dissected out and the liver remnants were removed and weighed for LRR evaluation. Blood was collected from the dorsal aorta of rats and was centrifuged for 20 minutes at the rate of 3000rpm to separate serum. Serum was stored at -20°C in eppendorf tubes for hormonal and biochemical analysis.

For histopathological examination, parts of the excised livers were processed by fixing the specimens in 4% paraformaldehyde (PFA) followed by dehydration in ascending grades of ethyl alcohol and embedding in paraffin. While parts of liver samples were immediately frozen in liquid nitrogen and stored at -70°C until further biochemical analysis.

For ultrasturcual studies, small liver pieces were fixed in 4% formaldehyde and 1% glutaraldehyde in 0.1M phosphate buffer (pH 7.4) for 2 hours. Fixative was replaced with 8% (0.2M) sucrose in 0.1M phosphate buffer overnight. The tissues were then post-fixed with 1% OsO_4 in 0.1M phosphate buffer for 1 hour followed by rinsing in 0.1M phosphate buffer thrice for 10 minutes. After fixation, tissues were dehydrated with different ascending grades of acetone (30%-90%) for 15 minutes each and then twice with 100% acetone each for 30 minutes. Dehydration was followed by treatment with propylene oxide twice for 15 minutes each. For infiltration propylene oxide and resin were first mixed in 1:1 ratio and the specimens were placed in them for one hour and then 1:2 ratio for 24 hours tissue incubation.

For embedding, resin was poured in moulds and tissues were carefully placed in them. The moulds were then incubated at different temperatures for different time durations i.e. 38°C, 43°C and 63°C for 24, 24 and 48 hours respectively for polymerization. After polymerization the blocks were trimmed and 1μm semi-thin sections were cut with glass knives on an ultramicrotome. Sections were placed on water drops on a slide and stained with toluidine blue for light microscopy, on the basis of which ultrathin sections were cut. The sections were then transferred onto copper grids and stained with uranyl acetate. Ultrastructural observations were made under transmission electron microscope.

2.6 Relative Liver Weight

Relative liver weight (RLW), an indicator of liver regeneration was calculated by dividing the resected liver weight at the time of sacrifice by total body weight. The data was expressed as percentage:

RLW (%) = (Resected liver weight / Total body weight) × 100

2.7 Liver Regeneration Rate

The rate of liver regeneration rate was computed by the formula proposed by Fishback (1929). The formula gives the regeneration rate based on liver and body weight.

LRR (%) = 100× ((C-(A-B))/A

Where A denotes the estimated total weight of the liver at surgery; B denotes the excised liver weight at surgery; and C denotes the remnant liver weight at dissection. Estimated whole liver weight was obtained by the equation A = B/0.67 as two third hepatectomy was performed.

2.8 Histomorphological Examinations

After embedding in wax the tissues were mounted on blocks and 5μm thick tissue sections were cut on the rotary microtome.

2.8.1 Hematoxyline-Eosin Staining

Hematoxyline-Eosin (H&E) staining of liver samples was done for qualitative and quantitative histological examination. Briefly, deparaffinization was done in xylene and tissues were rehydrated in descending grades of ethanol. After washing in distill water the slides were stained with hematoxyline. Slides were then dipped in tap water for bright coloration, dehydrated and stained with eosin. The slides were moved to absolute alcohol for the completion of dehydration and mounted with distyrene plasticizer xylene (DPX). Lobular architecture of liver tissue was studied alongwith mitotic and binuclear cell counts on H&E stained slides. The mitotic count and binuclear cell frequency were estimated by dividing the number of mitotic and binuclear hepatocytes, respectively by the total number of hepatocytes per section. Hepatocyte area alongwith nuclear area, diameter and perimeter were also calculated by using Image-Pro software.

2.8.2 Histochemistry

2.8.2.1 AgNOR staining

AgNOR staining in liver sections was performed by modified procedure of Ploton *et al.*, (1986). Briefly 2% gelatin solution was made in 1% formic acid in which 50% silver nitrate solution was added in ratio 1:2 to obtain the working solution. After deparaffinizion with xylene and passing through descending alcohol concentrations, sections were rehydrated in several changes of ultrapure distilled water. After incubation with acid alcohol for 5 minutes, and rinsing in several times with distilled water, sections were soaked with working solution for 40 minutes at 37°C in a dark humidified chamber. Sections were then thoroughly washed with distilled water, dehydrated in alcohol and mounted with DPX.

For AgNOR enumeration, two types of counts were performed i.e. the mean number of AgNORs in 100 nuclei (mAgNOR) and the percentage of nuclei exhibiting five or more AgNOR granules/nucleus/100 cells called proliferative count (pAgNOR). AgNOR distribution and variation in size was also recorded by using the criteria of Khan *et al.*, (2006). All observations were made at 1000X magnification. Scores of size variation

were: 0 when the dots were more or less of uniform size; 1+ when of two different sizes; 2+ more than two different sizes but not those of 3+ and 3+ including all grades and sizes. The scores of dots distribution of AgNOR were considered as: 0, limited to nucleoli; 1+, occasional dispersion outside nucleoli; 2+, moderate dispersion outside nucleoli; 3+, widely dispersed through the nucleus.

2.8.2.2 Periodic acid-Schiff Staining

Periodic acid-Schiff (PAS) staining was done to detect glycogen in hepatocytes. For PAS staining fresh Schiff's reagent was prepared as followed:

One gram of basic fuchsin was dissolved in 200ml of boiling water on stirrer for five minutes. After cooling up to 50°C, 2g potassium metabisulfite was added in it. Solution was cooled to room temperature and 2ml HCl was added. This solution was kept in dark over night at room temperature. The following day 0.2g activated charcoal was added and mixed for 2 minutes followed by filtration. Schiff's reagent was stored in a dark bottle at 4°C.

For staining, the slides were dewaxed in xylene, transferred to alcohol, rinsed in distilled water, dipped in 1% periodic acid solution for five minutes, again rinsed in distilled water and washed in running tap water for 1 minute. Schiff's reagent was applied on slides for 15 minutes. Slides were rinsed in distilled water again and washed in running tap water for five minutes. For staining of nuclei the slides were dipped in Mayer's hematoxyline for one minute. Dehydration was done with ascending alcoholic grades. Clearing was done with xylene while for mounting DPX was used.

2.8.2.3 Gomori's trichrome stain

For collagenous connective tissue assessment in liver tissue, Gomori's trichrome staining was done. In that one-step trichrome procedure, chromotrope 2R (plasma stain) was combined with light green and aniline blue (connective tissue fiber stain) in a solution of phosphotungstic acid to which glacial acetic acid was added. Phosphotungstic acid favored the red staining of cytoplasm. The tungstate ion was specifically taken up by

collagen, and the connective tissue fiber stain subsequently bound to this complex, coloring the collagen greenish blue. Briefly, sections were deparaffinized in xylene and hydrated with descending alcohol grades to distilled water. Proper rinsing in distilled water was done before staining sections in Weigert's hematoxylin for 10 minutes. Slides were washed in running water for 10 minutes. Sections were stained for 15 to 20 minutes in Gomori's trichrome stain. Differentiation was done in 0.5% acetic acid by placing slides in it for 2 minutes. Dehydration was done with alcohol, clearing with xylene and mounting with DPX. After staining the nuclei looked black, cytoplasm red while collagen gave the greenish blue shade.

2.8.3 Immunohistochemistry

2.8.3.1 Proliferating cell nuclear antigen staining

Cell proliferation was also evaluated with immunohistochemical staining for proliferating cell nuclear antigen (PCNA) using Invitrogen PCNA staining kit. After deparaffinization in xylene and rehydration with alcohol, sections were treated with 3% hydrogen peroxide in methanol for 10 minutes to block the endogenous peroxidase activity. Heat induced epitope retrieval (HIER) was used to enhance the specific antigen staining by placing the slides in antigen retrieval solution at 89°C for 10 minutes. The solution was slowly cooled down to room temperature and the specimens were incubated with ready to use blocking solution at room temperature for 10 minutes. Tissues were then incubated with biotinylated monoclonal mouse anti-PCNA primary antibody in a moist chamber for an hour followed by rinsing with phosphate buffer saline (PBS). The specimens were incubated with streptavidin-peroxidase at room temperature for 10 minutes and with diaminobenzidine (DAB) chromogen for 5 minutes. Hematoxylin was used for counterstaining. Hepatocytes with PCNA-positive nuclei and the total number of hepatocytes were counted in five random microscopic fields at 400X magnification to compute the cell proliferation index.

2.8.3.2 Alpha-smooth muscle actin staining

Activated hepatic stellate cells were identified by alpha smooth muscle actin (α-SMA) immunostaining. Briefly following deparaffinization and rehydration, the sections were treated with 3% hydrogen peroxidase in methanol for 15 minutes to block endogenous peroxidase activity and were then washed with PBS. Antigen retrieval was performed by heating the slides for 10 minutes in a microwave oven in 0.1mol/L citrate buffer (pH 6.0). Tissue sections were treated with normal horse serum for 10 minutes to avoid non-specific immunoreactivity and were again washed twice with PBS. Sections were then incubated with primary mouse actin smooth muscle (BioGenex, USA clone 1A4) antibody for 30 minutes at room temperature. After washing with PBS, tissues were incubated at room temperature with biotinylated goat anti-mouse antibody for 10 minutes followed by incubation with streptavidin horseradish peroxidase conjugate at room temperature for 10 minutes. Slides were again washed with PBS and the bound peroxidase was detected using DAB chromogen. Hematoxylin was used for counterstaining.

2.9 Biochemical Analysis

Following biochemical analyses were performed in the study to evaluate the regenerative ability of fibrotic liver under tRF treatments following PHx.

2.9.1 Hepatic Tissue Composition

2.9.1.1 Liver water content

Liver water content was evaluated by placing the weighed tissue samples at 60°C for 48 hours in oven. The dried samples were again weighed and the difference in weight between the wet and dry samples was considered as the amount of water in the tissue. The results were expressed in g/100g.

2.9.1.2 Lipid

Lipid was extracted from rat liver tissue according to Folch method (Folch *et al.*,

1957). Briefly weighed amount of tissue was homogenized with chloroform: methanol (2:1) mixture. The reagent was added 20 times the volume of tissue sample. After dispersion, the mixture was agitated at room temperature in an orbital shaker for 20 minutes. After extraction the homogenate was centrifuged to recover the liquid phase which was then transferred to clean eppendorf tube and 0.2 volume of 0.9% saline was added into it. After vortexing some seconds, to separate the two phases, the mixture was again centrifuged at low speed (2000rpm). After centrifugation and siphoning of the upper phase, the lower chloroform phase containing lipids was taken into a pre-weighed vial and allowed to dry at 63°C in an oven to constant weight. Total lipid content was calculated from the difference in weight of the lipid containing and the empty vial.

2.9.1.3 Deoxyribonucleic Acid

Deoxyribonucleic Acid (DNA) was extracted using Gentra Puregene DNA kit by the following procedure. Frozen liver tissue was grounded with mortar and pestle. 300µl cell lysis solution was dispensed into1.5ml eppendorf tube and the grounded tissue was added into it. Cell lysis was completed by adding 1.5µl proteinase K, mixed by inverting 25 times and incubated at 55°C for 48 hours until the tissue had completely lysed. After cooling to room temperature, 100µl protein precipitation solution was added and vortexed vigorously for 20 seconds at high speed followed by centrifugation at 2000 × g for 10 minutes until tight protein pellet was formed. Isopropanol solution (300µl) was pipetted out in a clean 1.5ml microcentrifuge tube and the supernatant was carefully poured into it. The resulted solution is inverted gently 50 times to mix the solution properly and then centrifuged for 3 minutes at 2000 × g. After discarding the supernatant carefully the tube was drained by inverting it on a clean piece of absorbent paper, taking care that the pellet remained in the tube. To wash the DNA pellet 300µl of 70% ethanol was added in the tube and inverted several times and centrifuged for 1 min at 2000 × g. After removing the supernatant carefully the tube was drained on a clean piece of absorbent paper in such a way that the pellet remained in the tube and allowed to air dry for up to 15 minutes. Afterwards 50µl DNA hydration solution was added and vortexed for 5 seconds at medium speed to mix the solution which was incubated at 65°C for 1 hour to dissolve the DNA followed by overnight incubation at room temperature. Samples were centrifuged

briefly and transferred to storage tubes for DNA quantification. DNA was quantified spectrophotometrically by taking readings at wavelength of 260nm.

2.9.1.4 Protein

Hepatic protein content was determined using liver homogenates through Biuret method using Emapol total protein kit (Poland).

2.9.1.5 Glycogen

Hepatic Glycogen was extracted according to the procedure of Cook *et al.*, (1988). Briefly 0.1g tissue was rinsed in saline and heated in boiling water bath for 30 min in 0.4 ml 30% KOH. Solution was cooled to room temperature and after adding a drop of 10% $ZnSO_4$; 0.8 ml 95% ethanol was added. The solution was again boiled, allowed to cool to room temperature and was centrifuged at 3000rpm for 10 minutes. Supernatant was discarded; to the residue 0.4ml H_2O and 0.8ml 95% ethanol were added. The new solution was again heated to boil, cooled, centrifuged for 5 minutes and supernatant was discarded. 0.2-0.4ml 2.5N H_2SO_4 was added to the residue until the glycogen precipitated out and again boiled for 30 min. Sample was cooled and one drop of phenolphthalein was added. Few drops of 2.5N NaOH were added until pink color appeared. Few drops of 0.5N H_2SO_4 were added to discharge color. Aliquots were made for further analysis of glucose by the kit as described above.

2.9.2 Liver Function Assessment

To assess the liver functional capability, serum analysis of different biochemical parameters were performed in AMP Diagnostics chemistry analyzer. Enzyme activities were performed under the fed program in the machine while for metabolites absorbencies were noted at particular wavelengths for each test and their respective concentrations were found by the following formula/equation:

$$Cx = (\text{Absorbance of sample} / \text{Absorbance of standard}) \times Cs$$

where Cx and Cs are the concentrations of metabolites and standards respectively. Protein was determined at 25°C while all other reactions were performed at 37°C.

2.9.2.1 Alanine aminotransferase

Alanine aminotransferase (ALT), the most useful measure of liver cell injury, was determined in rat serum by using AMP Diagnostics kit through enzyme kinetic method. Basically, ALT transfers the amino group from alanine to α-oxoglutarate to form pyruvate and glutamate. The pyruvate enters in the reaction catalyzed by lactate dehydrogenase with NADH to produce lactate and NAD^+. The decrease in absorbance at 340 nm due to the consumption of NADH is proportional to the ALT activity in the sample that can be detected spectrophotometrically.

2.9.2.2 Aspartate transaminase

The AMP Diagnostics kit was used to determine aspartate transaminase (AST) through enzyme kinetic method. AST catalyzes the reaction between L-aspartateand oxoglutarate. In a chemical reaction, catalyzed by maltate dehydrogenase (MDH), the oxaloacetate formed is reduced by NADH to form maltate and NAD^+. The NADH oxidation rate is directly proportional to the catalytic AST activity and is determined by quantifying the decrease in absorbance at 340nm.

2.9.2.3 Alkaline phosphatase

By enzyme kinetic method alkaline phosphatase (ALP) was determined in rat serum using AMP Diagnostics kit. The enzyme ALP catalyzes the hydrolysis of phosphate esters in alkaline buffer and produces an organic radical and inorganic phosphate. The kit used p-nitrophenyl phosphate (pNPP) as a phosphatase substrate which turned yellow when dephosphorylated by ALP and could be detected at 405nm.

2.9.2.4 Lactate dehydrogenase

Lactate dehydrogenase (LDH) catalyzes the oxidation of lactate to pyruvate with simultaneous reduction of NAD^+ to NADH. The NAD^+ reduction rate can be measured at 340nm as an increase in absorbance.

2.9.2.5 Bilirubin

Total bilirubin could be converted to azobilirubin by diazotized sulfanilic acid that could be spectrophotometrically measured at 546nm. The AMP Diagnostics kit was used to determine total bilirubin in the serum.

2.9.2.6 Total protein

Cupric ions in alkaline solution react with protein to form purple complex which can be detected spectrophotometrically at an absorbance of 546nm (Biuret method). Total protein in serum was determined through Emapol total protein kit, Poland.

2.9.2.7 Albumin

Colorimetric determination of serum albumin was done using bromocresol green (BCG), a dye preferentially bound to albumin at an acidic pH of 3.8 to produce a shade of green color which could be measured spectrophotometerically at 620nm. The AMP diagnostics kit for serum albumin was used for this purpose.

2.9.2.8 Triglycerides

Triglycerides in rat serum were determined by using Gesan Triglycerides monoreagent kit. Triglycerides are hydrolyzed by lipoprotein lipase (LPL) to glycerol and fatty acids. Glycerol is then phosphorylated to glycerol-3-phosphate by ATP in a reaction catalyzed by glycerol kinase (GK) which is then further oxidized by glycerol phosphate oxidase producing dihydroxyacetone phosphate and hydrogen peroxide. The hydrogen per oxide, in the presence of peroxidase (POD), catalyzes the oxidative coupling of 4-chlorophenol and 4-aminoantipyrine (4-AAP) to form a red-colored quinoneimine dye

which can be spectrophotometrically measured at 500nm to determine triglycerides in the samples.

2.9.2.9 Glucose

Serum glucose was calorimetrically determined using AMP diagnostics kit. In the reaction glucose was oxidized under glucose oxidase to produce gluconic acid and hydrogen peroxide. The hydrogen peroxide is then oxidatively coupled with 4-aminophenazone and phenol in the presence of peroxidase to produce a red quinoneimine dye. The intensity of the pink color measured at 500 nm was proportional to the original glucose concentration.

2.9.3 Hormonal Analysis

2.9.3.1 Corticosterone

Serum corticosterone levels were determined by using Assay Max corticosterone ELISA Kit according to the manufacturer's instructions. All reagents including standard solutions and samples were prepared as instructed. Serum samples were diluted 1:200. The assay was performed at 25°C. Briefly 25µl of corticosterone standards and samples were added per well on which a polyclonal antibody specific for corticosterone has been pre-coated. Immediately after that 25µl of biotinylated corticosterone was added to each well on top of the standard and samples. Wells were tightly covered and incubated for two hours at room temperature. Corticosterone in standards and samples competed with the biotinylated corticosterone sandwiched by the immobilized antibody and streptavidin-peroxidase conjugate. Using automatic washer the plate was washed six times with 300µl of wash buffer so that all unbound material washed away. The plate was inverted to decant the contents on absorbent material and was hit 4-5 times to completely remove the liquid. Each well was then supplemented by 50µl of streptavidin-peroxidase conjugate and incubated for 30 minutes. After that 50µl of chromogen substrate tetramethylbenzidine (TMB) was added per well and further incubated for about 20 minutes till the optimal blue color develops. Plate was gently tapped to ensure thorough mixing. Bubbles in the well were broken with pipette tip. In the end, 50µl of stop solution

i.e. 0.5N hydrochloric acid was added to each well. The color changed from blue to yellow. The intensity of the color was measured by taking the absorbance on a microplate reader at a wavelength of 450nm immediately. Standard curve was formed labelling x-axis by the standard concentrations and y-axis by the corresponding mean 450nm absorbance. The concentrations of the unknown samples were determined from the standard curve and the values were multiplied by the dilution factor to get the final corticosterone concentrations in the samples in ng/ml.

2.9.3.2 Serum and liver triiodothyronine and thyroxine analysis

Levels of total triiodothyronine (T3) and thyroxine (T4) in serum and liver were determined by using enzyme immuno assay kits (Amgenix International Inc., Los Angeles, USA) according to the manufacturer instructions. In brief, 50µl of samples and standards were added to the microtiter wells coated with a certain amount of anti-T3 or anti-T4 antibody. A constant amount of T3 or T4 conjugated with horseradish peroxidase was added to the wells and incubated for 60 minutes at room temperature after thorough mixing. During incubation serum T3 or T4 and conjugated T3 or T4 competed for the limited binding sites on the anti-T3 or anti-T4 antibody. The wells were then washed by washing buffer 5 times to remove the unbound T3 or T4 conjugates. Blue color was developed when a solution of chromogen substrate TMB was added and incubated for 20 minutes. The color development was stopped by addition of 2N hydrochloric acid (HCl) and mixed thoroughly. Absorbance was measured at 450nm. The intensity of the color formed was directly proportional to the amount of enzyme present and was inversely related to the amount of unlabeled T3 or T4 in the sample. With the help of standards assayed in the same way, standard curve was plotted and the concentration of T3 or T4 in the unknown sample was determined. To prevent inter-assay variation, all samples of one experiment were measured within the same assay.

2.9.4 Assessment of Tissue Antioxidant and Lipid Peroxidation Status

To assess the hepatic antioxidant and lipid peroxidation status 10% homogenate in 100 mM KH_2PO_4 buffer containing 1 mM EDTA (pH 7.4) was prepared by grinding the tissue in electrical homogenizer. The homogenate was then centrifuged at 12000 × g for

30 minutes at 4°C for collection of the supernatant which was used for the following biochemical analyses:

2.9.4.1 Catalase activity

Hepatic catalase (CAT) activity was determined spectrophotometrically as described by Aebi (1984). Briefly 0.1ml liver homogenate was mixed with 2.5 ml 50mM phosphate buffer (pH 5.0). The reaction was initiated by the addition of 0.4 ml 5.9mM H_2O_2 to the reaction solution. Changes in absorbance were noted after every 30 seconds at 240 nm. Results were expressed as international units per gram tissue.

2.9.4.2 Peroxidase activity

The peroxidase activity (POD) activity in liver was measured according to the method of Chance and Maehly (1955) by guaiacol oxidation method. About 0.1ml of liver homogenate was mixed with 2.5ml phosphate buffer (50mM) of pH 5.0. After that 0.1ml of guaiacol (20mM) and 0.3ml H_2O_2 (40mM) were added to allow the reaction to take place. POD activity was measured by changes in absorbance of reaction solution at 470nm for every 30 seconds. Results were expressed as international units per gram tissue.

2.9.4.3 Superoxide dismutase activity

The superoxide dismutase (SOD) activity was determined according to the method of Giannopolitis and Ries (1977) by measuring its ability to inhibit the photochemical reduction of nitrobluetetrazolium (NBT). The reaction solution (3ml) contained 50μM NBT, 1.3μM riboflavin, 13mM methionine, 50mM phosphate buffer (pH 7.8) and 75nM EDTA. 50μl liver homogenate was used in the assay. Two sets of tubes were prepared. First set of tubes containing the reaction solution was irradiated under a light (15W fluorescent lamps) at 78μM m-2s-1 for 15 minutes while the other set of test tube was wrapped in aluminum foil and placed in dark without light for 15 minutes. The absorbance of samples was recorded at 650nm spectrophotometrically. One unit of SOD activity was defined as the amount of enzyme which caused 50% inhibition of photochemical reduction of NBT under the specified conditions.

2.9.4.4 Reduced glutathione assay

Hepatic reduced glutathione (GSH) levels were measured in the tissue samples according to the method of Jollow *et al.*, (1974) by using 1,2-dithio-bis nitro benzoic acid (DTNB). Briefly, 1ml of supernatant was precipitated with 1ml (4%) sulfosalicylic acid. The samples were then kept at 4°C for 1 hour and centrifuged at 1200 × g for 20 minutes at 4°C. Reaction mixture was prepared by mixing 0.1ml filtered aliquot, 2.7ml phosphate buffer (0.1M; pH 7.4) and 0.2ml of DTNB (100mM) to make the final assay volume of 3ml. The development of yellow color was taken as an indicator and read at an absorbance of 412nm spectrophotometrically. GSH concentration in liver tissue was calculated using the standard curve of GSH standard solutions. The concentration of GSH was expressed as micromole per gram of wet tissue.

2.9.4.5 Glutathione peroxidase assay

Glutathione peroxidase (GPx) activity was determined by previously repeated method of Mohandas *et al.*, (1984). About 0.1ml of liver homogenate was mixed with 1.49ml phosphate buffer (0.1M; pH 7.4), 0.1ml sodium azide (1mM), 0.1ml EDTA (1mM) and 0.05ml GSH (1mM). Further 0.05ml glutathione reductase (9U/ml) along with 0.01ml H_2O_2 (0.25mM) and 0.1ml NADPH (0.2mM) were added. Disappearance of NADPH at 340nm was recorded at 25°C. Enzyme activity was calculated using molar extinction coefficient of NADPH i.e. 6.22×10^3 M-1cm-1. The results were expressed as nM NADPH oxidized/min/g tissue.

2.9.4.6 Glutathione reductase assay

Hepatic Glutathione reductase (GSR) activity was determined by the method devised by Carlberg and Mannervik (1975) using NADPH as a substrate. Briefly, 0.1ml of liver homogenate was mixed with 1.65ml phosphate buffer (0.1M, pH 7.6) and 0.1ml EDTA (0.5mM). Afterwards 0.05ml oxidized glutathione (1mM) and 0.1ml NADPH (0.1mM) were added to make volume of solution up to 2ml. Disappearance of NADPH at 340nm at 25°C was taken as an indicator of enzyme activity. Enzyme activity was calculated as U/g liver weight using molar extinction coefficient of NADPH i.e. 6.22×10^3 M-1cm-1.

2.9.4.7 Thiobarbituric acid reactive substance assay

The quantitative determination of lipid peroxidation as evidenced by the formation of thiobarbituric acid reactive substances (TBARS) was determined according to the method of Iqbal *et al.*, (1996). The amount of malondialdehyde (MDA) formed was measured by the reaction with thiobarbituric acid (TBA) and used as an index of lipid peroxidation. Briefly 0.2ml liver homogenate was mixed with 0.2ml ascorbic acid (100mM), 0.58 ml phosphate buffer (0.1M; pH 7.4) and 0.02ml ferric chloride (100mM). The reaction mixture was incubated at 37°C for 1 hour in a shaking water bath. The reaction was terminated by the addition of 1.0ml of trichloroacetic acid (10%). Further 1.0ml 0.67% TBA was added in the reaction assay. All the tubes were then kept in boiling water bath for 20 minutes and then transferred to crushed ice-bath before centrifugation at 2500 × g for 10 minutes. Optical density of the supernatant was measured at 535nm through spectrophotometer against a reagent blank. The results were expressed as nM/g tissue using molar extinction coefficient of 1.56×10^{-5} /M/cm.

2.9.5 Mineral Analysis

Hepatic mineral content was estimated by atomic absorption spectrophotometry. Metal concentrations of sodium, calcium, magnesium, potassium, iron, copper and zinc had been determined in dry liver samples. Briefly samples were subjected to acid digestion by using 3 ml of HNO_3 in a volumetric flask. Digestion of tissues was carried out on a hotplate at 200°C to 250°C. The digests were then cooled to room temperature and volume of the digest was raised with deionized water up to 10 ml. Each digest was filtered through Whatman filter paper no. 42 and transferred to polyethylene bottles. Concentration of metals was determined through calibration curve of standard solutions using fast sequential atomic absorption spectrometer (Varian, Spectra AA-240, FS, USA).

2.9.6 Measurement of Hepatic Hydroxyproline Content

A modification of the method described by Reddy and Enwemeka (1996) and Woessner (1961) was used to determine hydroxyproline content in dry liver samples. Samples were hydrolyzed in 2N NaOH for 10 minutes at 65°C. They were first incubated

for 20 min at 120°C, then in 6N HCl for 20 min at 120°C. 10mg/ml in 4N NaOH activated charcoal solution was added to the samples and centrifuged. After centrifugation, the supernatants were analyzed for hydroxyproline. Both the standards and the processed tissue samples were incubated with 0.5ml of chloramine-T solution (50mM chloramine-T, 30% v/v Ethylene glycol monomethyl ether, 50% (v/v) hydroxyproline buffer (0.26M citric acid, 1.46M sodium acetate, 0.85M sodium hydroxide, 1.2% v/v glacial acetic acid), distilled H_2O for the remaining volume) for 20 minutes at room temperature, followed by 0.5ml of 3.15M perchloric acid for 5 minutes at room temperature. Care had been taken that after each addition the samples were mixed well. 0.5ml P-dimethylaminobenzaldehyde solution (1.34M p-dimethylaminobenzaldehyde dissolved in Ethylene glycol monomethyl ether) was added to each sample and incubated for 20 minutes at 60°C for color development. The absorbencies of standards and samples were read at 557nm on a spectrophotometer with results reported as mg hydroxyproline/g of dry tissue.

2.10 Statistical Analysis

Statistical Package for the Social Sciences (SPSS) version 17 was used to perform the statistical analyses. The results were expressed as mean ± standard error. Student's t-test was used for the statistical comparison of variables between two groups while comparisons among multiple groups were done by one-way and two way analysis of variance (ANOVA). For two-way ANOVA data was compared with a factor for group (2 levels) and a factor for "treatments" (8 levels). One-way ANOVA was used to determine significant differences among treatments (4 levels).Logarithmic transformation was applied on the data where required. In case of significant findings, one-way ANOVA was followed by Tukey's post hoc test whereas two-way ANOVA was followed by Bonferroni post hoc test. Non-parametric analysis was done by Kruskal Wallis test; when the Kruskal Wallis test showed significance; Mann Whitney U test was used to determine the groups among which the differences were statistically significant. For all analyses P-values less than 0.05 were considered significant.

CHAPTER # 3

FIBROTIC LIVER REGENERATION UNDER TEMPORAL FEED RESTRICTION

3.1 Introduction

Hepatic regeneration is a remarkable physiological process with marvelous sequential changes in gene expression, growth factors and morphologic structure (Michalopoulos and DeFrances, 1997). Liver regeneration following PHx involves the participation of all mature cell populations comprising the organ specially the hepatocytes, the main cellular components of liver. They are highly differentiated cells that do not divide under normal physiological conditions, however, hepatic resection lead to their hypertrophy and hyperplasia allowing the liver to regenerate to its original size (Fausto, 2000; Miyaoka *et al.*, 2012).

In contrast to normal liver fibrotic and cirrhotic liver has impaired and slow regeneration (Andiran *et al.*, 2000; Suárez-Cuenca *et al.*, 2008). Defective regeneration and dysfunction of cirrhotic liver result in postoperative deaths after surgery (Redaelli *et al.*, 2002). Reduced heptocellular proliferation and increased extracellular matrix are the main reasons for retarded regeneration of fibrotic and cirrhotic livers (Andiran *et al.*, 2000; Takeda *et al.*, 2001; Kato *et al.*, 2005).

Improvement of fibrotic liver regeneration is an important task in the field of gastroenterology. As hepatocyte proliferation is one of the fundamental processes required for liver regeneration (Fausto, 2000) therefore, an understanding of the regulation of hepatocyte division is very important for developing therapeutic approaches that can facilitate fibrotic liver regeneration.

Hepatocytes need to be primed by extrinsic factors including metabolic changes and altered cytokines expression following 70% PHx to enter into proliferative phase (Moolten and Bucher, 1967; Fisher *et al.*, 1971). TNF-α and IL-6 causes hepatocytes to move to cell cycle (Michalopoulos and DeFrances, 1997; Fausto, 2000; Taub 2004). Once hepatocytes reached to synthesis phase the sequence and timing of subsequent molecular events is directed by a program intrinsic to the hepatocyte itself. There present a mitotic clock in hepatocytes that regulate its division following priming by external factors (Weglarz and Sandgren, 2000).

Most of the mammalian physiological systems are under circadian rhythms (Panda *et al.*, 2002; Reppert and Weaver, 2002). Almost all cells and tissues have the molecular machinery capable of generating circadian rhythms (Davidson *et al.*, 2004; Lowrey and Takahashi, 2004). Cell division is reported to be regulated by the circadian clock in certain tissues (Scheving *et al.*, 1972; Scheving *et al.*, 1978). Diurnal variations in DNA synthesis and mitotic index in rodent liver have also been characterized. Not only in adult but in regenerating liver cells, the cell cycle is under the control of master circadian oscillator (Barbason *et al.*, 1989; Matsuo *et al.*, 2003). Hepatocytes as well as sinusoid littoral cells of mice liver showed circadian rhythm in their cell cycle during regeneration following PHx (Badrán*et al.*, 1984; Surur *et al.*, 1985). Furthermore it has been found that PHx-induced liver regeneration generates four continuous waves of hepatocyte proliferation that coupled with three waves of hepatic fat accumulation (Zou *et al.*, 2012). The dry mass of hepatocytes that represent the hepatocyte trophic status; another main event of liver regeneration following PHx has been reported to follow circadian oscillation (Tongiani *et al.*, 1982). Not only in regeneration, circadian clock also play an important role during hepatic fibrosis pathogenesis (Chen *et al.*, 2010; Tong and Yin, 2013).

In mammals, light is the main *zeitgeber* and the circadian system works through the coordinated action of a light-entrained master pacemaker located in the SCN and a set of subordinated clocks in the peripheral organs (Reppert and Weaver, 2002). Recently, feeding time has shown to be a dominant Zeitgeber for peripheral tissues (Damiola *et al.*, 2000; Stokkan *et al.*, 2001). There are three main types of feeding regimens, that affect circadian rhythms especially in peripheral tissues such as calorie restriction (CR), intermittent fasting (IF) and time restricted feeding (tRF). All three types of feeding regimens resulted in better health and reduced disease outcome (Froy and Miskin, 2010).

Both CR and IF entrain the peripheral clocks with the participation of SCN (Froy and Miskin, 2010) while tRF affects circadian oscillators in peripheral tissues, with no effect on the central pacemaker in the SCN (Damiola *et al.*, 2000; Stokkan *et al.*, 2001; Schibler *et al.*, 2003; Hirota and Fukada, 2004). Limiting the time and duration of food availability with no calorie reduction is termed as tRF (Hirota and Fukada, 2004; Schibler *et al.*,

2003). This feeding regimen uncouples the SCN from the periphery, suggesting that peripheral tissue clocks and thus physiological processes can be regulated by nutritional synchronization.

Circadian clock genes in liver are reported to be strongly entrained by feeding regimens (Hara *et al.*, 2001). In fact liver displays the fastest shift in the phase of clock-control genes under tRF (Stokkan *et al.*, 2001). Entrainment of the liver by tRF to a specific phase without disrupting SCN-driven rhythms, such as sleep and body temperature, might show its practicality and therapeutic efficacy in treating different liver disorders and complications including the disturbances during regeneration of fibrotic or cirrhotic liver.

Present study has been conducted with the hypothesis that time-restricted feeding exposure to liver before hepatectomy may affect the regeneration ability of fibrotic liver.

3.2 Results

3.2.1 Body Weights

In the present study, a non-significant 6% decrease in body weights of CCl_4 treated fibrotic rats as compared to vehicle had been observed (Figure 3.1). The tRF regimens produced interesting findings regarding body weights of animals. During two weeks of temporal restricted feeding, the weight of animals under tRF schedules decreased markedly compared to the animals fed ad libitum especially rats under tRF1strikingly lost their weight. The rats under tRF2 which received food for only four hours surprisingly did not lose weight during the feed restriction period; fibrotic rats had less weight as compared to their vehicle counterparts (Figure 3.2).

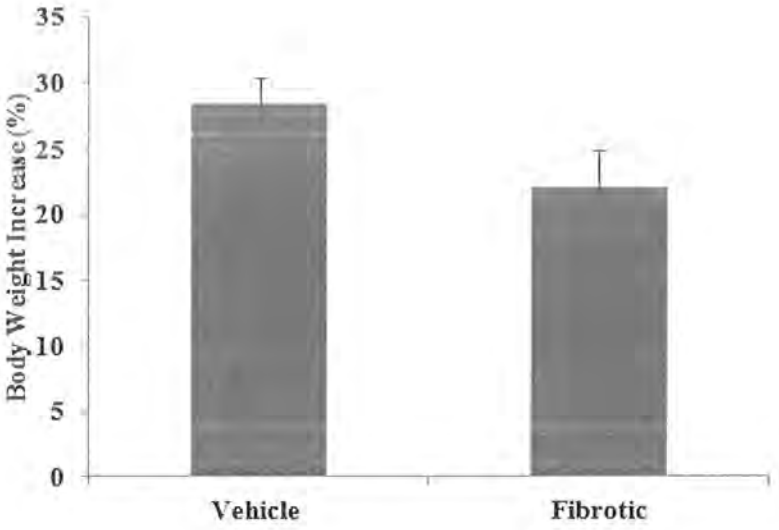

Figure 3.1: Percentage Increase in Body weights of rats following eleven weeks of olive oil/CCl_4 treatment. CCl_4 treatment resulted in non-significant body weight decrease, (n = 23-30/group).

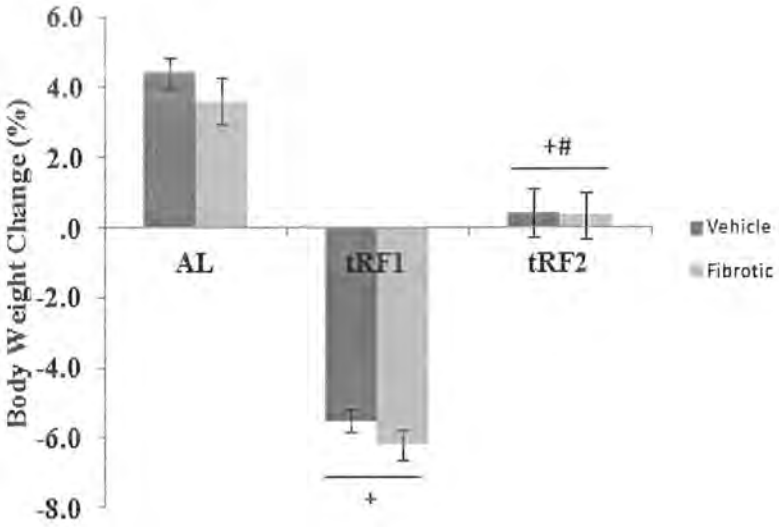

Figure 3.2: Percent body weight change of rats exposed to two weeks of time restricted feeding (tRF) regimens. The tRF schedules markedly decreased the body weights especially the regimen tRF1. Fibrosis Effect: F=1.264, P=0.272. $^{+}$(P<0.001) AL vs tRF regimens, $^{\#}$(P<0.001) tRF1 vs tRF2 (2-way ANOVA). (n=7-12/group).

Significant reduction in the body weights of the rats was observed post PHx. Both tRF regimens improved the gain in body weight after PHx especially tRF1 under which the highest rate of weight increase was observed 48 hours post PHx (Figure 3.3).

3.2.2 Liver Regeneration Assessment

Liver regenerative ability of rats 48 hours post PHx had been determined by different somatometric, hyperplasic and hypertrophic estimates.

3.2.2.1 Relative liver weight

As expected, sham animals in both groups had more RLW as compared to hepatectomized groups. Vehicle and fibrotic sham rats had almost same RLW. Non significant difference was observed among RLW of different treatment groups (Table 3.1a & 3.1b).

3.2.2.2 Liver regeneration rate

Feed restriction to day time had improved regenerative capacity of liver 48 hours following PHx in both vehicle and fibrotic rats specially the first schedule as detected by the outcomes of LRR. Fibrotic rats had slower regeneration rate than vehicle (Figure 3.4).

3.2.2.3 Proliferating cell nuclear antigen

As compared to sham a burst of proliferation had been seen in hepatectomized rats as measured by PCNA count. Both feed restriction regimes had decreasing effect on percentage PCNA count as studied at the given time of the day. However the number of PCNA count per field was more in time restricted feeding groups. In vehicle rats significant reduction was seen only in tRF2 regimen but under the first schedule PCNA count was also quite less. Fibrotic animals also showed non-significant decreasing trend especially under the first schedule. Fibrotic rats had always lower PCNA count as compared to their vehicle counterparts (Table 3.2a & 3.2b, Figure 3.5).

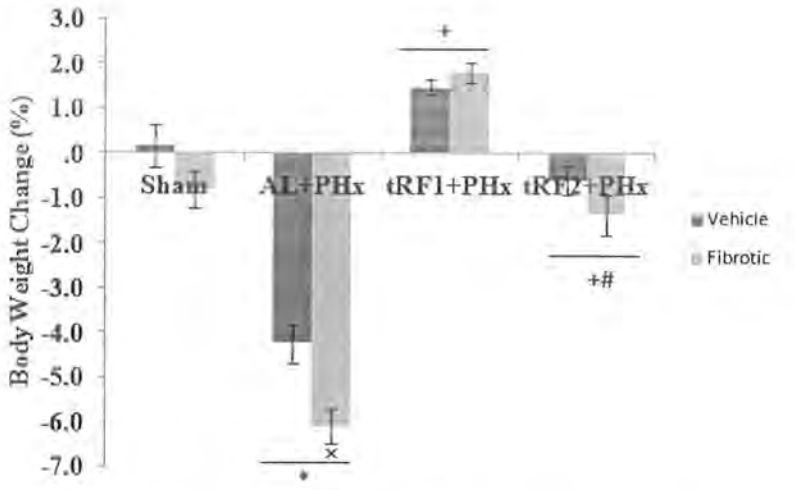

Figure 3.3: Percent body weight change of rats 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens. The tRF regimens improved the gain in body weight after PHx especially tRF1. Fibrosis Effect: F=8.269, P=0.007. *(P<0.001) Sham vs PHx; $^{+}$(P<0.001) AL vs tRF regimens; $^{\#}$(P<0.001) tRF1 vs tRF2 (2-way ANOVA). $^{\times}$(P<0.05) Vehicle vs Fibrotic (Student's t-test). (n=5-8/group).

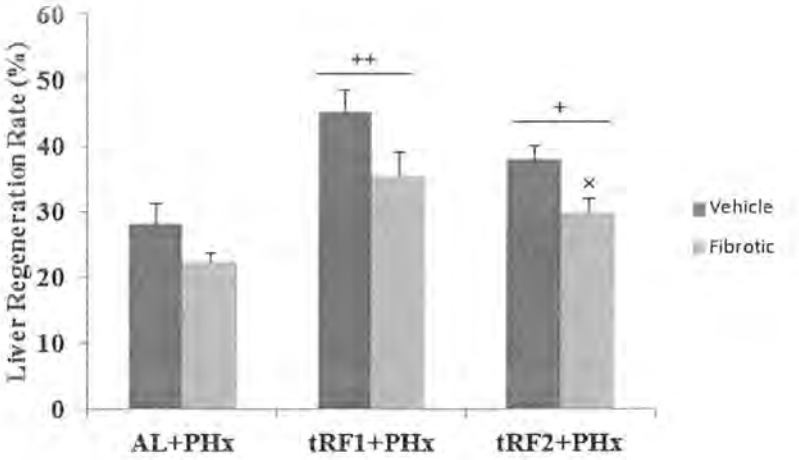

Figure 3.4: Liver regeneration rate (%) of rats 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens. The tRF regimens had significantly improved liver regenerative rate of rats. Fibrosis Effect: F=9.429, P=0.006. +(P<0.05) and ++(P<0.001) AL vs tRF regimens (2-way ANOVA). $^{\times}$(P< 0.05) Vehicle vs Fibrotic (Student's t-test). (n=5-8/group).

Table 3.1a: Relative liver weight (%) of rats 48 hours post partial hepatectomy after exposure to two weeks of time restricted feeding (tRF) regimens.

Groups	Sham	AL+PHx	tRF1+PHx	tRF2+PHx
Vehicle	3.43 ± 0.14	1.92 ± 0.15	2.0 ± 0.14	1.96 ± 0.14
Fibrotic	3.53 ± 0.15	1.93 ± 0.09	1.98 ± 0.17	1.96 ± 0.16

All findings are non-significant. (n = 5-8/group).

Table 3.1b: Two way ANOVA for relative liver weight (P-value).

Fibrosis Effect	F=0.237	P=0.629
Treatments Effect (Bonferroni post hoc test)		
Sham vs. PHx	0.000	*
AL vs. tRF1	1.000	Ns
AL vs. tRF2	1.000	Ns
tRF1 vs. tRF2	1.000	Ns

*: Statistically significant; ns: non-significant

Table 3.2a: Proliferation cell nuclear antigen (PCNA) and mitotic count in hepatocytes 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens.

	PCNA count (%)		Mitotic count (%)	
	Vehicle	Fibrotic	Vehicle	Fibrotic
AL+PHx	40.65 ± 3.08	30.91 ± 2.70*	4.58 ± 0.24	3.34 ± 0.30**
tRF1+PHx	33.51 ± 2.07	22.63 ± 2.24*	3.04 ± 0.21	2.04 ± 0.14**
tRF2+PHx	28.70 ± 2.88	26.30 ± 1.97	2.13 ± 0.15	1.75 ± 0.12

*(P<0.05) and **(P<0.005)Vehicle vs Fibrotic (Student's t-test). (n=5-8/group).

Table 3.2b: Two way ANOVA for hepatocyte PCNA index (P-value).

	PCNA count (P-value)	Mitotic count (P-value)
Fibrotic Effect	0.002 (12.53)	0.000 (34.126)
AL vs tRF1	0.016	0.000
AL vs tRF2	0.021	0.000
tRF1 vs tRF2	1.000	0.048

P-values for treatment effects are computed using Bonferroni post hoc test. and P<0.05 is considered significant. F statistic in brackets.

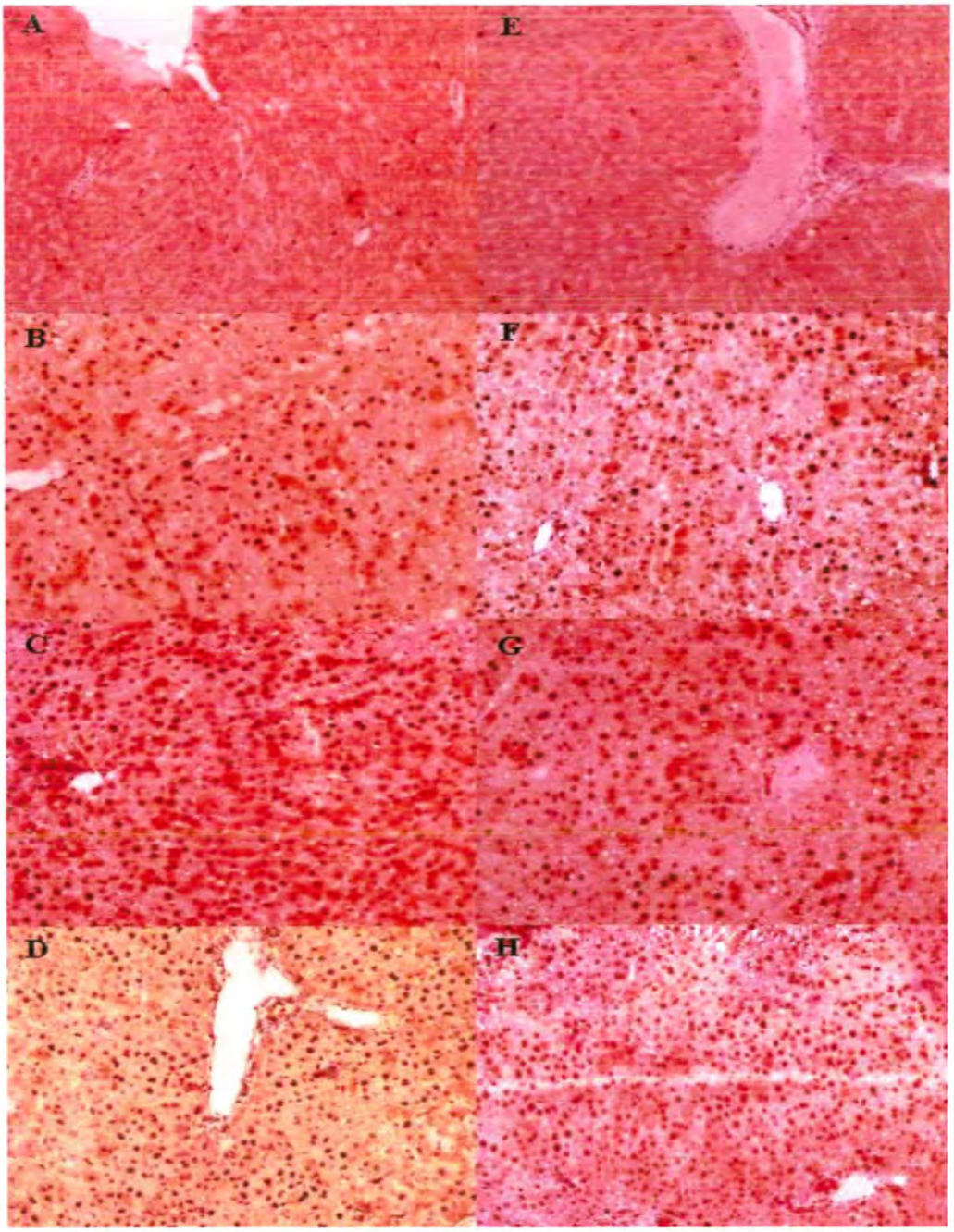

Figure 3.5: Effect of time restricted feeding (tRF) on Proliferating cell nuclear antigen count (PCNA) after partial hepatectomy. A burst of proliferation had been seen in partially hepatectomized rats compared to sham as is evident by increased PCNA activity (brown dots). tRF respectively decreased and increased the percentage and per field PCNA count 48 hours following PHx at the given day time. Vehicle group (A-D); Fibrotic (E-H). Sham (A& E), PHx (B & F), tRF1 (C & G) and tRF2 (D & H). (200X).

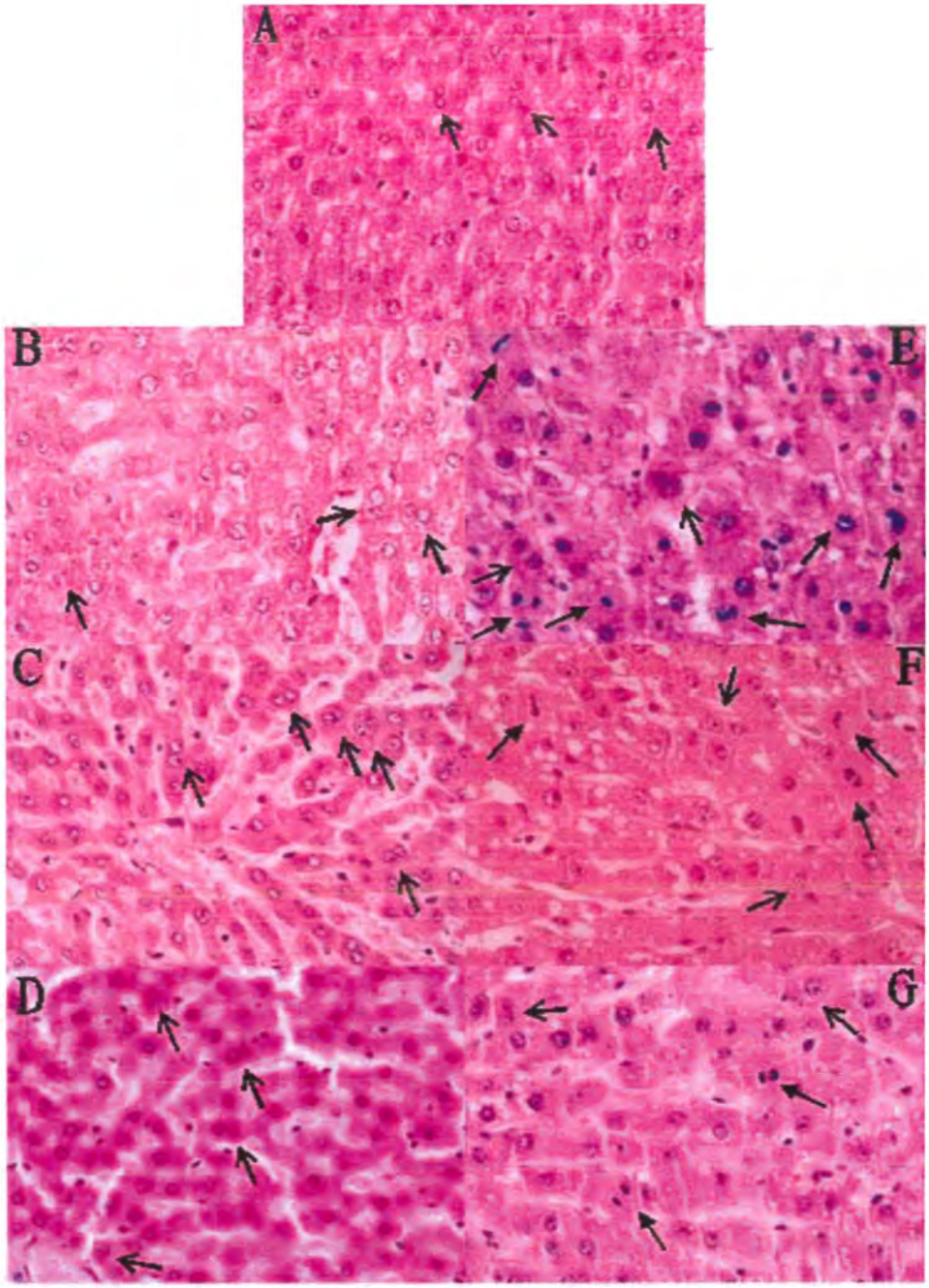

Figure 3.6a: Histomorphometric alterations in vehicle rats before and 48 hours post partial hepatectomy (PHx) under temporal feed restriction. Cells at different stages of cell cycle had been observed in regenerating rats (E-G). Feeding regimens had decreased percentage hepatocyte mitotic activity after PHx specially the second regimen (F&G). PHx increased the hepatocyte nuclear and cellular area while binuclear hepatocyte frequency was decreased (E). The tRF regimens doubled the binuclear cells (C&D) that were markedly reduced after PHx (F&G). The tRF regimens decreased the hepatocellular and nuclear area (C&D) that remained restricted after PHx (F&G). Bold arrow indicated mitotic cells while simple arrows represented binuclear cells (H&E; 400X).

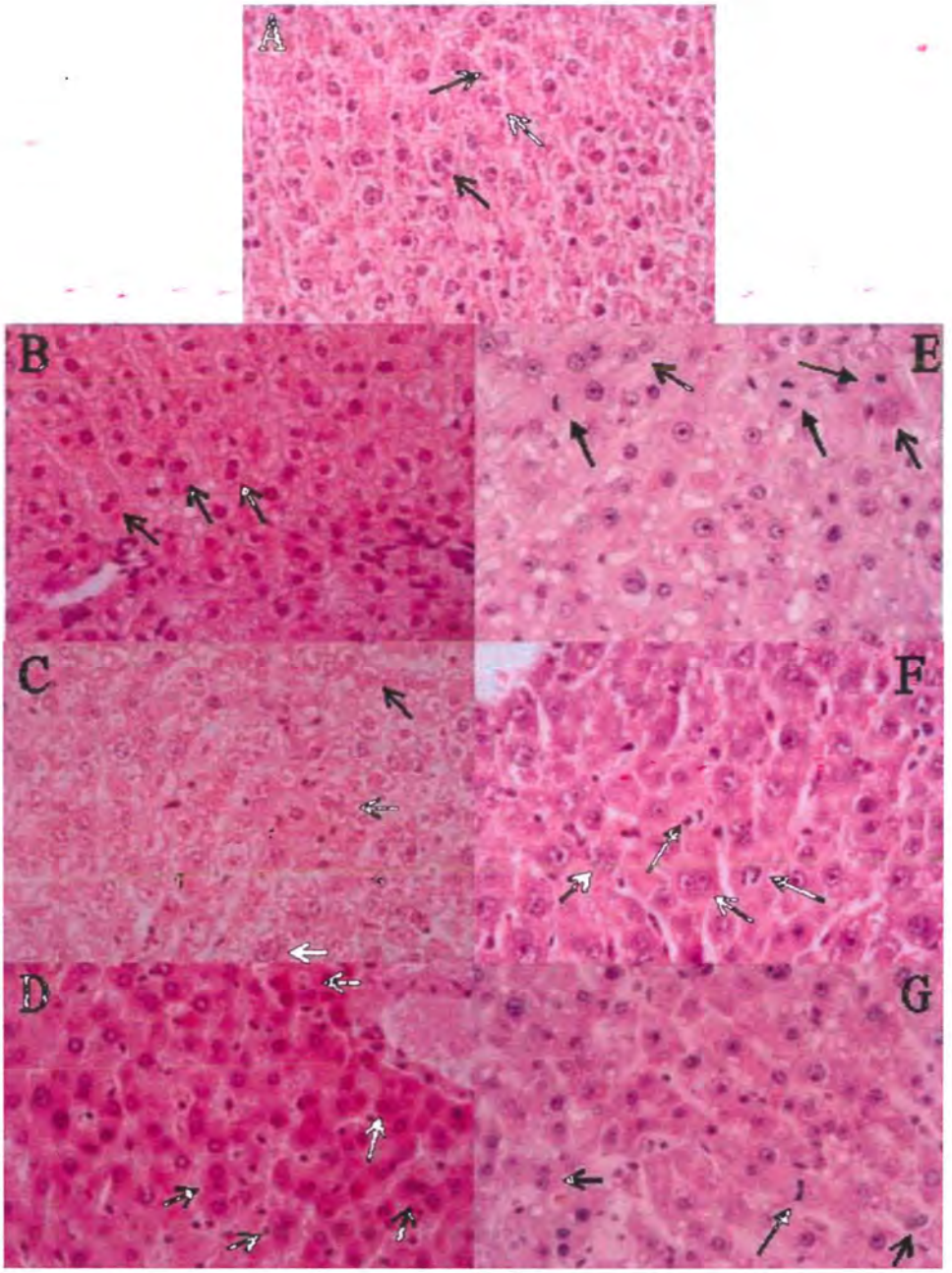

Figure 3.6b: Histomorphometric alterations in fibrotic rats before and 48 hours post partial hepatectomy (PHx) under temporal feed restriction. Cells at different stages of cell cycle had been observed in regenerating rats (E-G). Feeding regimens had decreased percentage hepatocyte mitotic activity after PHx specially the second regimen (F&G). PHx increased the hepatocyte nuclear and cellular area while binuclear hepatocyte frequency was decreased (E). The tRF regimens increased the binuclear cells (C&D) that were markedly reduced after PHx (F&G). The tRF regimens decreased the hepatocellular area (C&D) that was increased after PHx (F&G). The nuclear area remained uneffeced by temporal feed restriction before (C&D) and after PHx (F&G). Bold arrow indicated mitotic cells while simple arrows represented binuclear cells (H&E; 400X).

both tRF schedules resulted in an increased mAgNOR count especially tRF2 significantly affected the count (Figure 3.7a)

Proliferative AgNOR Index (pAgNOR)

In contrast to mAgNOR count, pAgNOR index showed a significant increasing trend after PHx. Temporal feed restriction could not affect pAgNOR in vehicle rats but both tRF schedules increased the pAgNOR index in fibrotic rats with significant increase under tRF2 (Figure 3.7b).

AgNOR Size Variation per Cell

Partial hepatectomy increased AgNOR size variation per nucleus. Again no effect of tRF after PHx on AgNOR size variation in vehicle rats was seen. The regimen tRF2 increased the AgNOR size variation in fibrotic rats but not to statistically different extent (Figure 3.7c).

AgNOR Distribution Variation per Cell

Non-significant increase in AgNOR distribution per nucleus after PHx had been observed especially in vehicle rats. Temporal feed restriction had no effect on vehicle rats while tRF2 significantly increased AgNOR distribution in fibrotic rats (Figure 3.7d).

3.2.2.7 Hepatocyte area

Before PHx significant decrease in hepatocyte area under temporal feed restriction in vehicle and fibrotic rats had been observed. A marked reduction in hepatocyte area under tRF1 and tRF2 in vehicle and fibrotic rats respectively was noticed.

PHx resulted in increased hepatocyte area as was evident by pre and post PHx comparisons in all groups irrespective of treatments or groups. The tRF1 regimen had reduced hepatocyte area in both vehicle and fibrotic groups. Surprisingly tRF2 regimen lessened the hepatocyte cellular area in vehicle rats only;

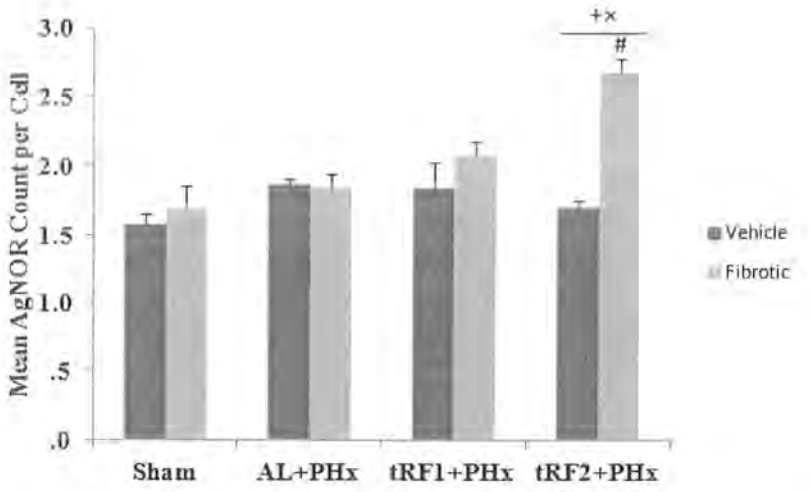

Figure 3.7a: Mean AgNOR count per cell of rats 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens. Differential effect of tRF on mAgNOR count with respect to vehicle and fibrotic rats was observed; with high mAgNOR levels under tRF2 in fibrotic rats and no effect on vehicle rats. Fibrosis Effect: F=15.123, P=0.000. $^{+}$(P<0.05) AL vs tRF regimens (2-way ANOVA). $^{\#}$(P<0.05) tRF1 vs tRF2 (1-way ANOVA). $^{\times}$(P<0.001) Vehicle vs Fibrotic (Student's t-test). (n=5-8/group).

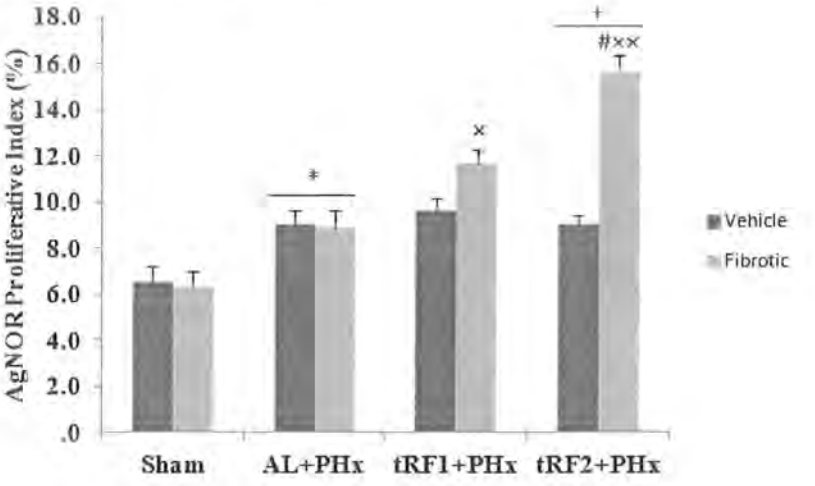

Figure 3.7b: AgNOR Proliferative index (pAgNOR) of rat hepatocytes 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens. Differential effect of tRF on pAgNOR count with respect to vehicle and fibrotic rats was observed. Fibrosis Effect: F=19.846, P=0.000. *(P<0.005) Sham vs PHx; $^{+}$(P<0.001) AL vs tRF regimens (2-way ANOVA). $^{\#}$(P<0.05) tRF1 vs tRF2 (1-way ANOVA); $^{\times}$(P<0.05), $^{\times\times}$(P<0.001) Vehicle vs Fibrotic (Student's t-test). (n=5-8/group).

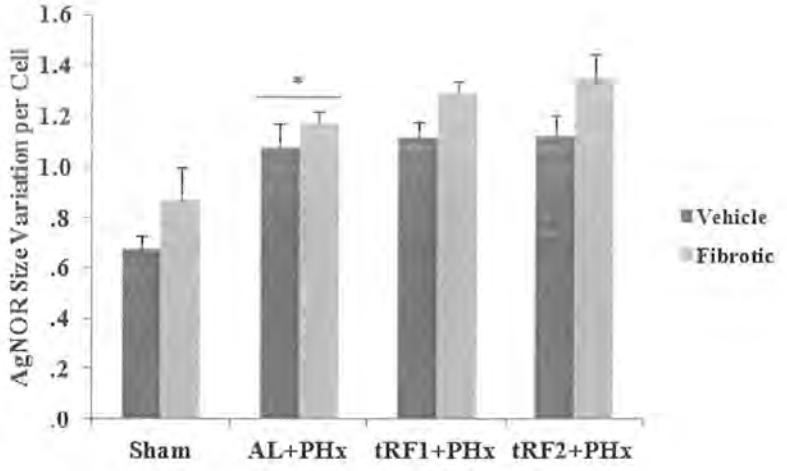

Figure 3.7c: AgNOR size variation in rat hepatocytes 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens. The tRF regimens had no effect on hepatocyte AgNOR size variation. Fibrosis Effect: F=9.021, P=0.006). *(P<0.005) Sham vs PHx (2-way ANOVA). (n=5-8/group).

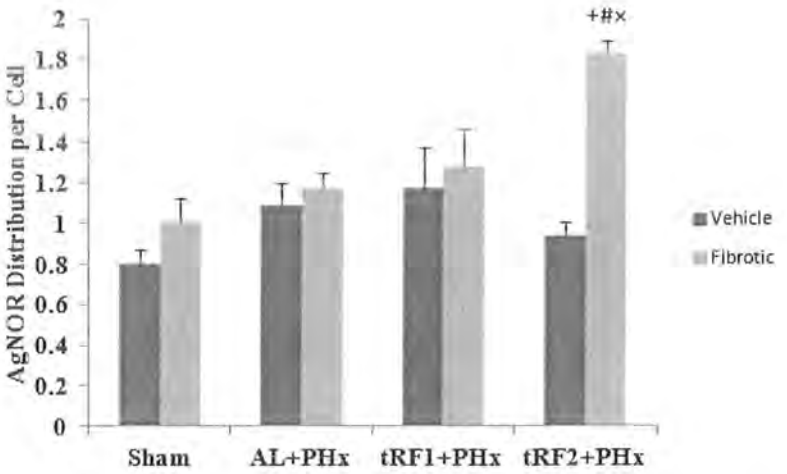

Figure 3.7d: AgNOR distribution of rat hepatocytes 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimen. Fibrosis Effect: F=12.723, P=0.001. +(P<0.005) AL vs tRF regimens; #(P<0.05) tRF1 vs tRF2 (1-way ANOVA). ×(P<0.001) Vehicle vs Fibrotic (Student's t-test). (n=5-8/group).

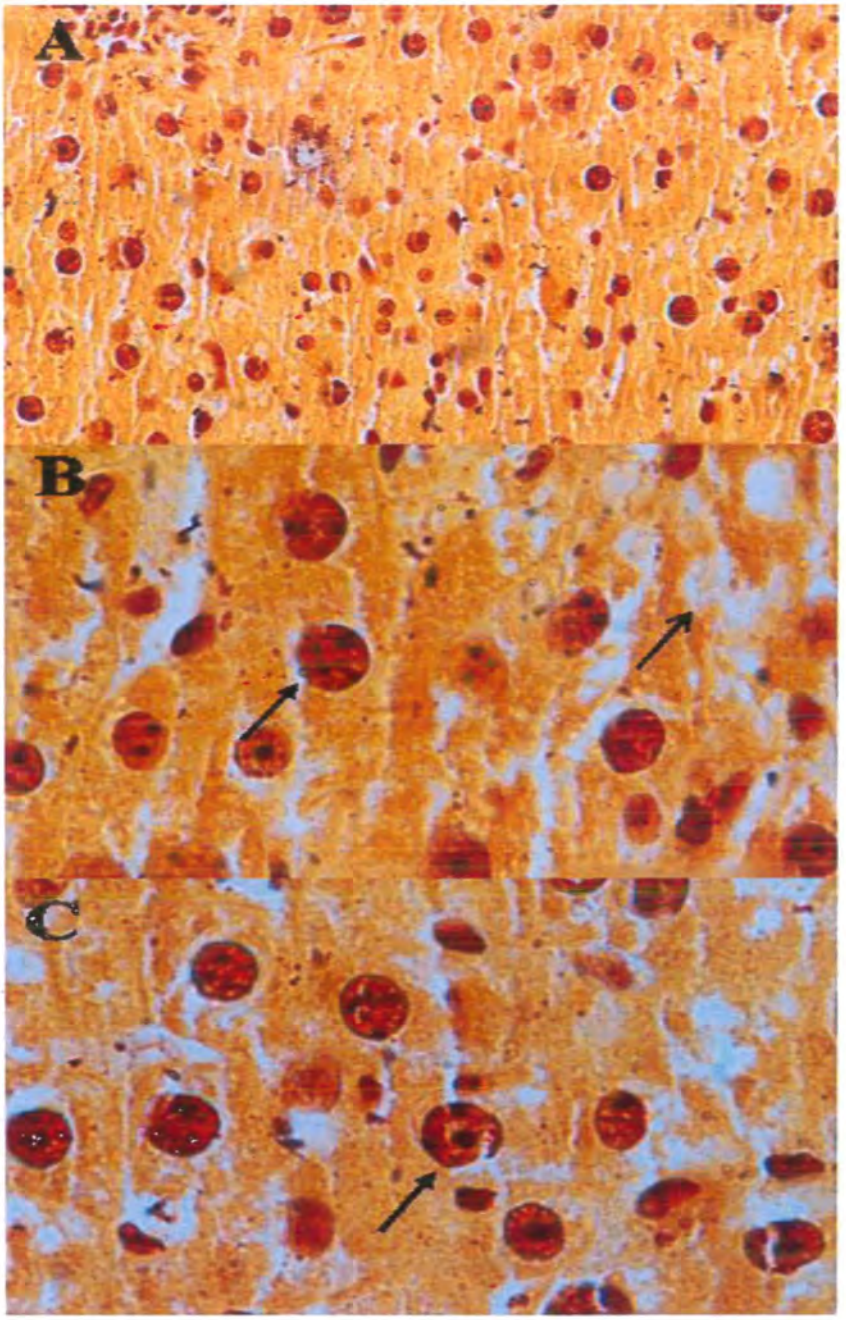

Figure 3.8: AgNOR staining in regenerating rat liver. Nucleus with five AgNOR dots could be seen (B) (Bold arrows). AgNOR dots with two degree size variation can also be viewed (C) (Bold arrows). Lipid (simple arrow) in the cytoplasm, a characteristic feature of liver regeneration after partial hepatectomy can be observed. (400X) & (1000X).

in fibrotic liver, however, hepatocyte area remained unchange under that schedule (Table 3.4a & 3.4b).

3.2.2.8 Nuclear area, diameter and perimeter

Fibrotic rats had less nuclear area than AL vehicle rats but this difference was not significant. Considering pre-PHx hepatocyte nuclear area, both vehicle and fibrotic rats responded differently to tRF regimens. Both tRF regimens significantly reduced nuclear area in vehicle. No change in fibrotic rats under the treatments before PHx had been seen.

Fibrotic rats had significantly less nuclear area than vehicle sham. PHx considerably increased the nuclear area in both vehicle and fibrotic rats as could be observed while comparing pre and post PHx values. Both schedules of temporal feed restriction had significantly reduced the nuclear area in vehicle rats. No difference regarding nuclear area had been observed in fibrotic rats under tRF regimens after PHx (Table 3.5). Nuclear diameter and perimeter also showed the same trend as of hepatocyte nuclear area in both groups (Table 3.6 & 3.7).

3.2.2.9 Hepatic Tissue Composition

Water

No significant effect of PHx on hepatic water content was observed. Both tRF regimens significantly increased water in liver tissue after PHx in both vehicle and fibrotic rats (Table 3.8a & 3.8b).

Lipid

PHx had increasing effect on hepatic lipid content as in both vehicle and fibrotic rats, elevated lipid levels were observed especially in fibrotic rats. Both tRF treatments increased the hepatic lipid content after PHx specially tRF1 in vehicle. In fibrotic rats hepatic lipid level under timed feed restriction was less than hepatectomized animals but more as compared to sham. Fibrotic rats had markedly

Table 3.4a: Area (μm^2) of rat hepatocytes, pre and 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens.

	Pre PHx		Post PHx	
	Vehicle	Fibrotic	Vehicle	Fibrotic
Sham	-	-	239.78 ± 6.47	$268.87 \pm 10.29^{\times}$
AL+PHx	260.28 ± 6.11	$280.65 \pm 7.42^{\times}$	$460.82 \pm 16.21^{¥¥}$	$324.36 \pm 9.72^{\times\times ¥}$
tRF1+PHx	218.74 ± 5.66	233.47 ± 5.94	$370.44 \pm 11.81^{¥¥}$	$268.93 \pm 10.80^{\times\times ¥}$
tRF2+PHx	231.02 ± 4.30	$193.77 \pm 5.6^{\#\#\times\times}$	$361.95 \pm 7.92^{+¥¥}$	$317.50 \pm 11.26^{\times\times \#¥¥}$

$^{+}$(P<0.001) AL vs tRF regimens; $^{\#}$(P<0.05), $^{\#\#}$(P<0.001) tRF1 vs tRF2 (1-way ANOVA). $^{\times}$(P<0.05), $^{\times\times}$(P<0.001) Vehicle vs Fibrotic (Student's t-test). $^{¥}$(P<0.01), $^{¥¥}$(P<0.001) pre vs post PHx (Student's t-test). (n=5-8/group).

Table 3.4b: Two way ANOVA for pre and post PHx rat hepatocytes area (P-value).

	Pre-PHx	Post-PHx
Fibrotic Effect	0.542 (0.407)	0.000 (62.277)
Sham vs PHx	———	0.000
AL vs tRF1	0.000	0.000
AL vs tRF2	0.000	0.129
tRF1 vs tRF2	0.304	0.299

P-values for treatment effects are computed using Bonferroni post hoc test. P<0.05 is considered significant. F statistic in brackets.

Table 3.5: Nuclear Area (μm^2) of rat hepatocytes, pre and 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens.

	Pre PHx		Post PHx	
	Vehicle	Fibrotic	Vehicle	Fibrotic
Sham	-	-	46.15 ± 0.65	$42.8 \pm 0.93^{\times\times}$
AL+PHx	40.71 ± 0.8	38.1 ± 1.22	$82.35 \pm 1.73^{*¥}$	$60.61 \pm 1.19^{*¥\times\times}$
tRF1+PHx	$35.39 \pm 0.6^{+}$	$38.59 \pm 0.82^{\times}$	$64.84 \pm 1.27^{+¥}$	$58.26 \pm 1.36^{¥\times}$
tRF2+PHx	$34.69 \pm 0.4^{+}$	$38.16 \pm 0.94^{\times\times}$	$59.7 \pm 0.77^{+\#¥}$	$60.42 \pm 1.82^{¥}$

*(P<0.001) Sham vs PHx; $^{+}$(P<0.001) AL vs tRF regimens; $^{\#}$(P<0.005) tRF1 vs tRF2; $^{\times}$(P<0.01), $^{\times\times}$(P<0.001) Vehicle vs Fibrotic; $^{¥}$(P<0.001) pre vs post PHx (Mann Whitney U test). (n = 5-8/group).

Table 3.6: Nuclear diameter (μm) of rat hepatocytes, pre and post partial hepatectomy (PHx) 48 hours after exposure to two weeks of time restricted feeding (tRF) regimens.

	Pre PHx		Post PHx	
	Vehicle	Fibrotic	Vehicle	Fibrotic
Sham	-	-	7.64 ± 0.05	$7.33 \pm 0.07^{\times\times}$
AL+PHx	7.16 ± 0.06	6.91 ± 0.11	$10.18 \pm 0.1^{*¥}$	$8.73 \pm 0.08^{*¥\times\times}$
tRF1+PHx	$6.69 \pm 0.05^{+}$	$6.97 \pm 0.07^{\times}$	$9.04 \pm 0.08^{*¥}$	$8.56 \pm 0.09^{¥\times}$
tRF2+PHx	$6.62 \pm 0.03^{+}$	$6.94 \pm 0.09^{\times\times}$	$8.68 \pm 0.05^{+\#¥}$	$8.72 \pm 0.13^{¥}$

*(P<0.001) Sham vs PHx; $^{+}$(P<0.001) AL vs tRF regimens; $^{\#}$(P<0.005) tRF1 vs tRF2; $^{\times}$(P<0.01), $^{\times\times}$(P<0.001) Vehicle vs Fibrotic; $^{¥}$(P<0.001) pre vspost PHx (Mann Whitney U test). (n = 5-8/group).

Table 3.7: Nuclear perimeter (μm) of rat hepatocytes, pre and post partial hepatectomy (PHx) 48 hours after exposure to two weeks of time restricted feeding (tRF) regimens.

	Pre PHx		Post PHx	
	Vehicle	Fibrotic	Vehicle	Fibrotic
Sham	-	-	24.01 ± 0.17	23.02 ± 0.24^{xx}
AL+PHx	22.51 ± 0.21	21.71 ± 0.35	31.98 ± 0.33$^{*¥}$	27.41 ± 0.24$^{*¥xx}$
tRF1+PHx	21.01 ± 0.18^{+}	21.89 ± 0.23^{x}	28.39 ± 0.28$^{*¥}$	26.88 ± 0.31$^{¥x}$
tRF2+PHx	20.81 ± 0.12^{+}	21.79 ± 0.28^{xx}	27.26 ± 0.17$^{+\#¥}$	27.41 ± 0.41$^{¥}$

*(P<0.001) Sham vs PHx; $^{+}$(P<0.001) AL vs tRF regimens; $^{\#}$(P<0.005) tRF1 vs tRF2; ^{x}(P<0.01), ^{xx}(P<0.001) Vehicle vs Fibrotic; $^{¥}$(P<0.001) pre vs post PHx (Mann Whitney U test). (n=5-8/group).

increased hepatic lipid than vehicle after PHx while elevated lipid level in vehicle rats compared to fibrotic counterparts under tRF1 was observed (Table 3.8a & 3.8b).

DNA

Post PHx, significant increase in hepatic DNA content was observed. No statistical significant difference among different treatment groups regarding hepatic DNA levels had been monitored in the study (Table 3.8a & 3.8b).

Protein

PHx had a non-significant increasing effect on hepatic protein content in vehicle rats while in fibrotic rats significantly raised liver protein levels were detected. No statistically significant difference among different groups in terms of protein content had been observed, however, in vehicle rats, tRF1 resulted in minor increase in hepatic protein content (Table 3.8a & 3.8b).

Glycogen

Hepatic glycogen content had been significantly decreased by PHx. Comparatively more decrease was seen in vehicle rats. Temporal feed restriction had recovered hepatic glycogen content. The tRF1 regimen had significantly while tRF2 non-significantly increased liver glycogen levels in both groups. Vehicle rats had always more hepatic glycogen levels than fibrotic rats (Table 3.8a & 3.8b).

PAS stain

Abundant and random glycogen expression as assessed by PAS histochemical staining had been observed in both vehicle and fibrotic sham rats. These rats were fed ad libitum. Partial hepatectomized vehicle and fibrotic rats had also shown random glycogen expression but considerably less than sham rats. The tRF1 regimen resulted in random while tRF2 regimen showed spatial glycogen pattern in vehicle rats. In fibrotic rats random but comparatively less glycogen content than controls had been viewed under tRF regimens (Figure 3.9).

Table 3.8a: Hepatic tissue composition of rats 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens.

		Sham	AL+ PHx	tRF1+ PHx	tRF2+ PHx
Water (g/g)	**Vehicle**	71.61 ± 0.73	70.03 ± 0.90	73.84 ± 0.53	72.71 ± 1.35
	Fibrotic	72.49 ± 0.71	71.73 ± 0.49	74.67 ± 0.56	74.96 ± 1.02
Lipid (mg/g)	**Vehicle**	35.86 ±1.49	50.74 ± 1.26	$81.35 \pm 4.34^{++}$	$63.63 \pm 2.2^{+\#}$
	Fibrotic	36.92 ± 1.64	$73.63 \pm 3.62^{*}$	$48.57 \pm 2.84^{*++}$	$59.43 \pm 3.71^{+}$
DNA (mg/g)	**Vehicle**	2.48 ± 0.19	3.81 ± 0.25	4.66 ± 0.46	4.41 ± 0.64
	Fibrotic	2.27 ± 0.22	3.48 ± 0.27	3.81 ± 0.29	3.69 ± 0.47
Protein (mg/g)	**Vehicle**	148.49 ± 20.77	197.5 ± 28.68	259.72 ± 25.64	209.88 ± 19.47
	Fibrotic	157.1 ± 14.68	$226.33 \pm 11.27^{*}$	206.43 ± 14.09	201.1 ± 16.42
Glycogen (mg/g)	**Vehicle**	43.35 ± 6.67	20.56 ± 3.23	36.12 ± 3.37	33.86 ± 2.76
	Fibrotic	32.84 ± 4.16	19.49 ± 2.56	27.79 ± 1.9	25.83 ± 4.71

*(P<0.05) Sham vs PHx; $^{+}$(P<0.05), $^{++}$(P<0.001) AL vs tRF regimens; $^{\#}$(P<0.005) tRF1 vs tRF2 (1-way ANOVA). *(P<0.001) Vehicle vs Fibrotic (Student's t-test). (n=5-8/group).

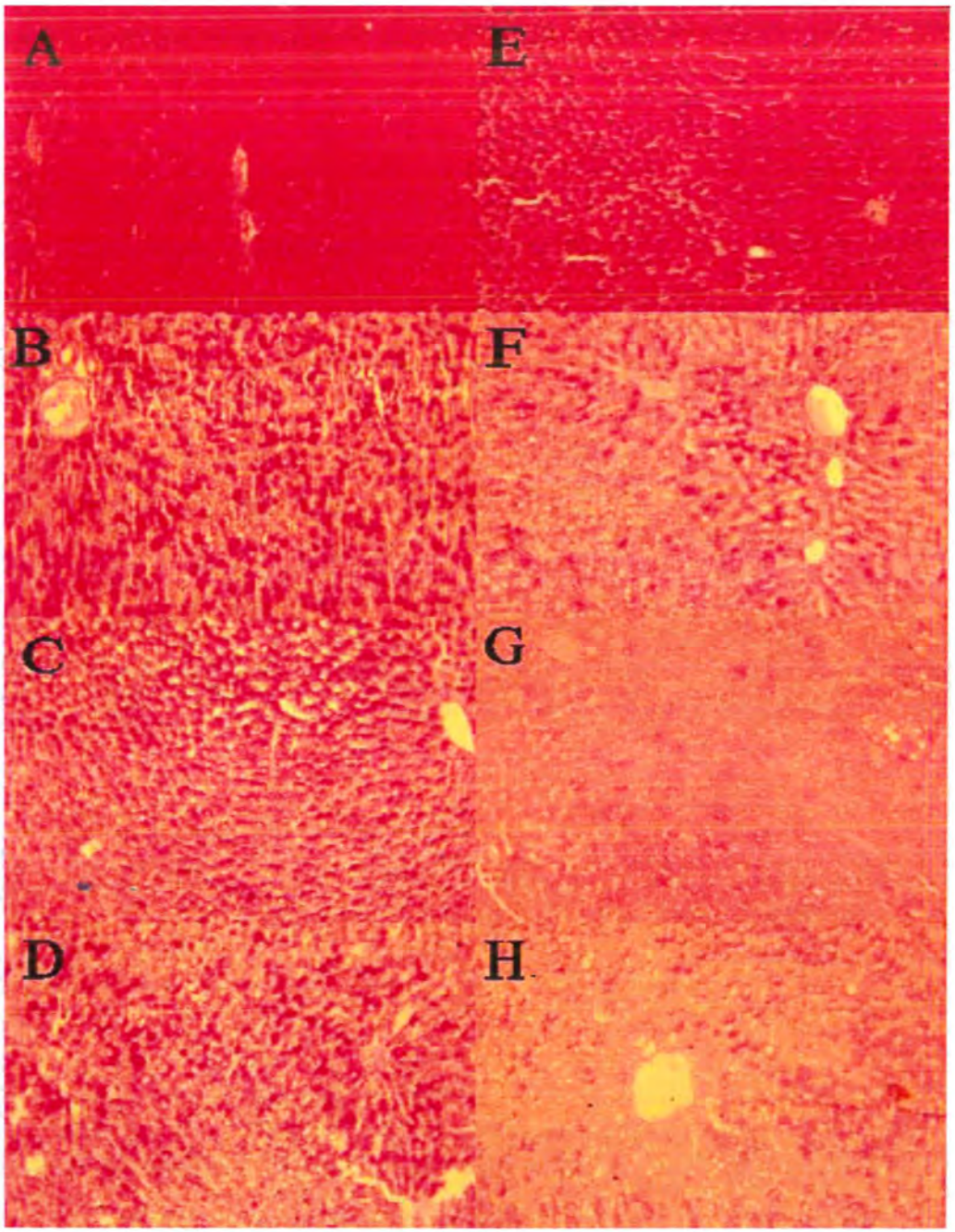

Figure 3.9: Glycogen deposition in rat liver after partial hepatectomy under the effect of timed feed restriction. Abundant and random glycogen expression could be observed in sham rats (A&E). Partially hepatectomized rats showed considerably less glycogen than sham (B&F). The tRF1 regimen resulted in random while tRF2 regimen showed spatial glycogen pattern in vehicle rats (C&D). In fibrotic rats random but comparatively less glycogen than controls under tRF regimens was observed (E&F). Vehicle group (A-D); Fibrotic (E-H). (PAS Stain), (200X).

3.2.3 Liver Functional Assessment

3.2.3.1 Alanine aminotransferase *(ALT)*

Hepatic fibrosis resulted in increased ALT levels as fibrotic rats had high enzymatic activity than vehicle rats. PHx significantly elevated the serum ALT levels in both vehicle and fibrotic rats. Both temporal feed restriction treatments had significant decreasing effect on serum ALT levels specially tRF1 (Figure 3.10).

3.2.3.2 Aspartate transaminase (AST)

Both fibrosis and hepatectomy significantly raised the serum AST levels. The tRF1 regimen decreased the AST levels while tRF2 had no effect on its activity in both vehicle and fibrotic rats (Figure 3.11).

3.2.3.3 Alkaline Phosphatase (ALP)

Hepatic fibrosis had a significant elevating effect on serum ALP levels. Both vehicle and fibrotic rats demonstrated significant increase in serum ALP activities 48 hours post PHx. No effect of timed feed restriction regimens had been monitored in both groups regarding serum ALP levels (Figure 3.12).

3.2.3.4 Lactate dehydrogenase (LDH)

PHx increased the serum LDH levels as their levels were significantly higher than sham animals in both groups. Timed feed restriction resulted in decrease in LDH levels specially tRF2 under which significant decrease in vehicle rats was seen. Moreover, fibrosis had an elevating effect on LDH levels (Figure 3.13).

3.2.3.5 Bilirubin

Fibrotic sham animals have considerably high bilirubin levels than vehicle. PHx had a significant raising effect on bilirubin concentrations but this effect was due to vehicle rats. No effect of temporal feed restriction had been detected in improving serum bilirubin levels (Table 3.14).

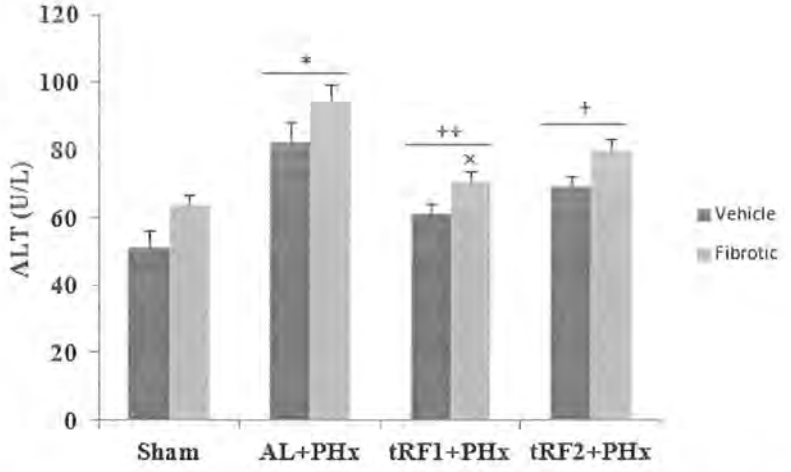

Figure 3.10: ALT levels of rats 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens. The tRF regimens improved the ALT content after PHx especially tRF1. Fibrosis Effect: F=15.102, P=0.001. *(P<0.001) Sham vs PHx, $^{+}$(P < 0.05) and $^{++}$(P < 0.001) AL vs tRF regimens (2-way ANOVA). $^{\times}$(P < 0.05) Vehicle vs Fibrotic(Student's t-test). (n=5-8/group).

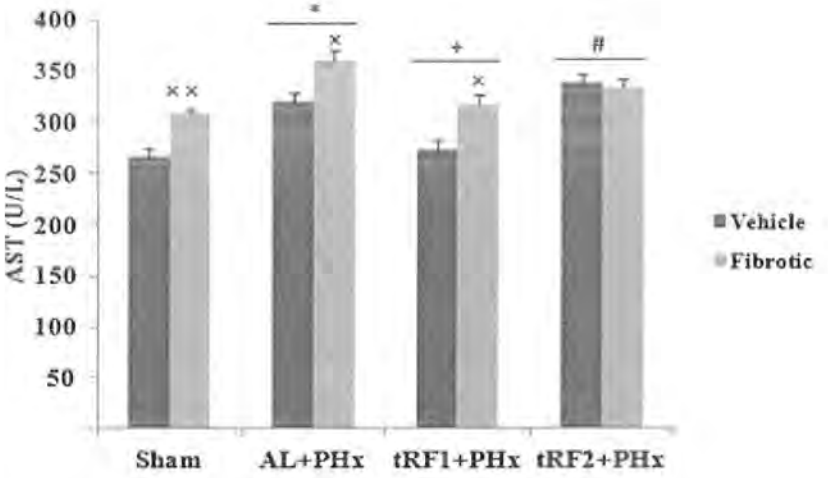

Figure 3.11: AST levels of rats 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens. The tRF regimens lowered the AST content after PHx. Fibrosis Effect: F=24.853, P=0.000. *(P<0.001) Sham vs PHx; $^{+}$(P<0.001) AL vs tRF regimens; $^{\#}$(P<0.001) tRF1 vs tRF2 (2-way ANOVA). $^{\times}$(P<0.05), $^{\times\times}$(P<0.*005)* Vehicle vs Fibrotic (Student's t-test). (n=5-8/group).

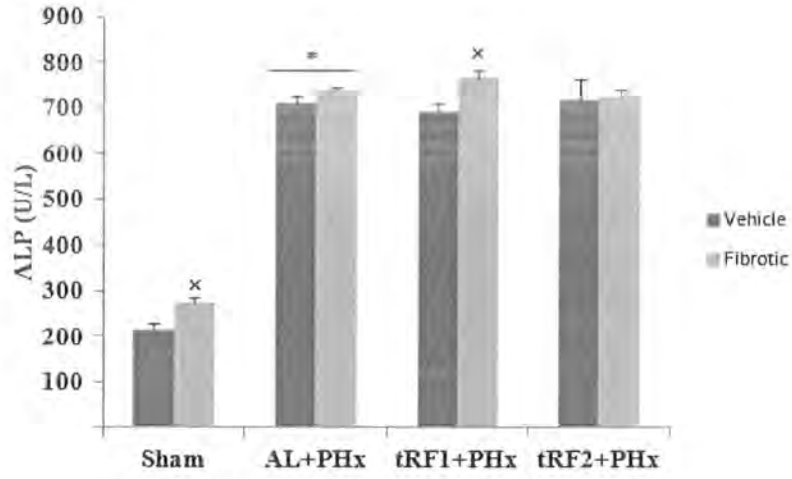

Figure 3.12: ALP levels of rats 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens. The tRF regimens produced no effect on ALP content after PHx. Fibrosis Effect: F=9.695, P=0.004. *(P<0.001) Sham vs PHx (2-way ANOVA). ×(P<0.05) Vehicle vs Fibrotic (Student's t-test). (n=5-8/group).

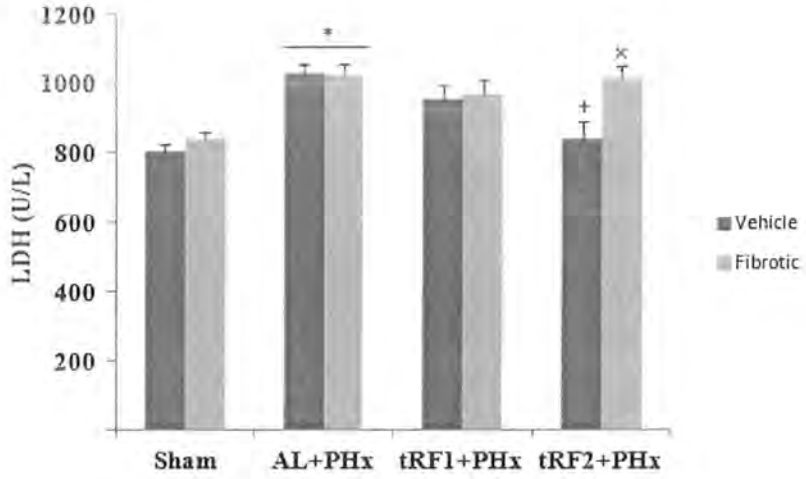

Figure 3.13: LDH levels of rats 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens. Improved LDH levels in vehicle rat under tRF2 after PHx had been detected. Fibrosis Effect: F=6.175, P=0.019. *(P<0.001) Sham vs PHx (2-way ANOVA). +(P<0.005) AL vs tRF regimen (1-way ANOVA). ×(P<0.05) Vehicle vs Fibrotic (Student's t-test). (n=5-8/group).

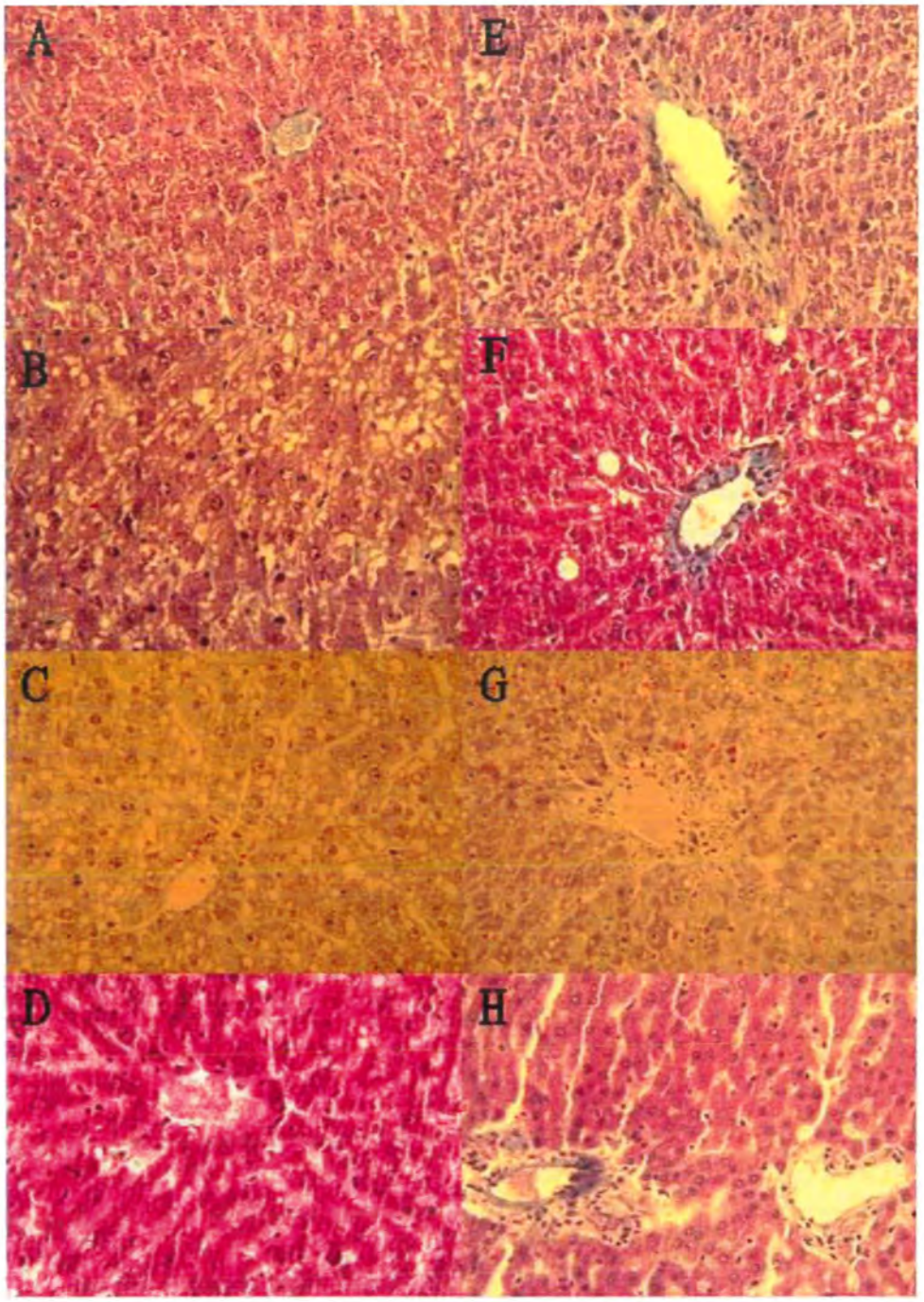

Figure 3.19: Changes in hepatic extracellular matrix (ECM) content under time restricted feeding (tRF) regimens following partial hepatectomy in rat. Gomori triple stain indicated increased extracellular matrix (highlighted in bluish green shade) in fibrotic rats compared to control. The tRF regimens did little or no reduction in hepatic collagen content in fibrotic rats following PHx. Vehicle group (A-D); Fibrotic (E-H). Sham (A& E), PHx (B & F), tRF1 (C & G) and tRF2 (D & H). (400X).

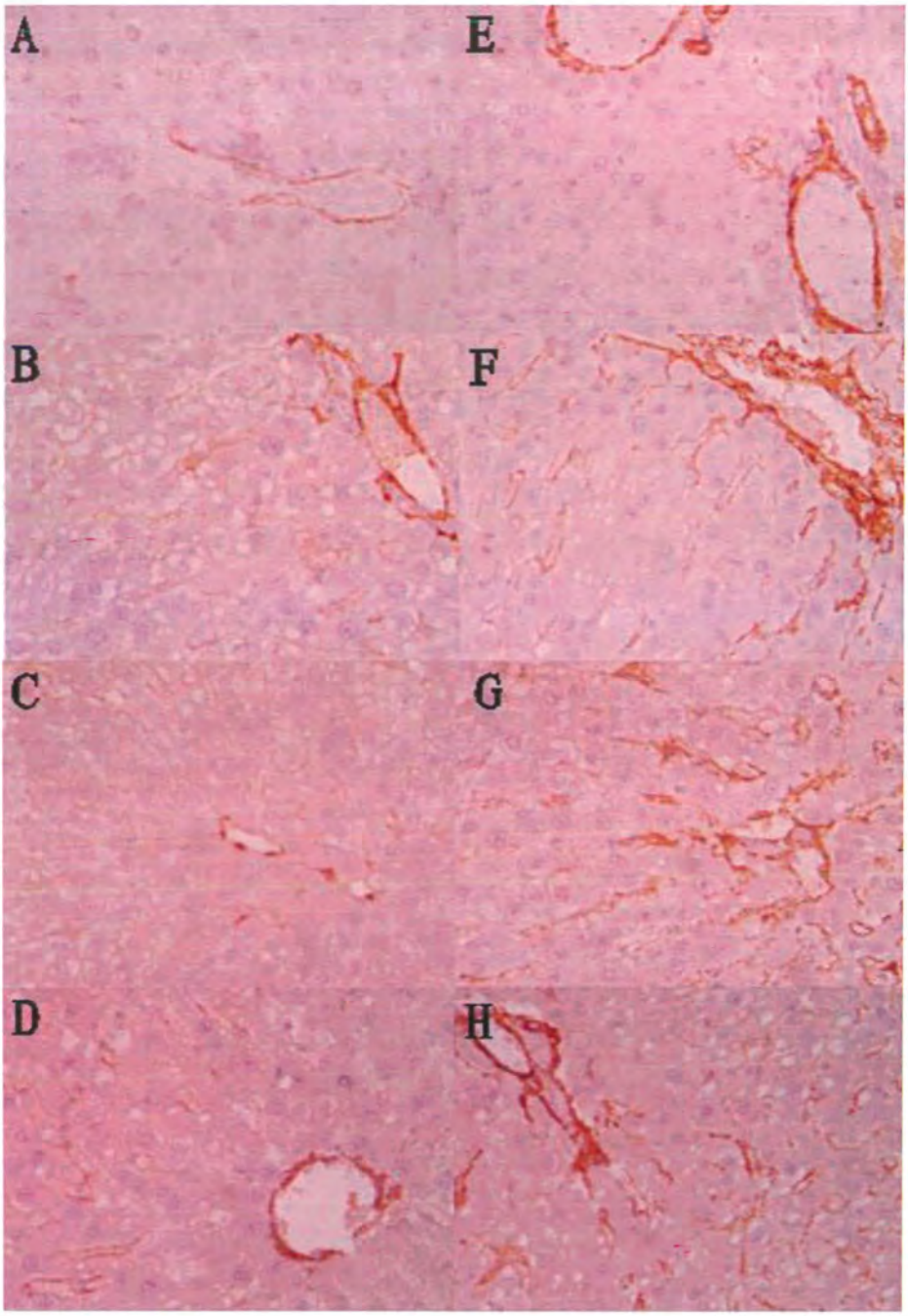

Figure 3.20: Alpha-Smooth muscle actin (a-SMA) immunohistochemical staining in rat liver 48 hours after partial hepatectomy. Activated HSCs as detected by α-SMA immunostaining were more numerous in partially hepatectomized rats than sham. α-SMA positive cells could be seen at hepatic vascular and sinusoidal spaces. The tRF schedules considerably decreased the activated HSC in vehicle rats, however, no improvement in fibrotic rats could be observed Vehicle group (A-D); Fibrotic (E-H). Sham (A& E), PHx (B & F), tRF1 (C & G) and tRF2 (D & H). (400X).

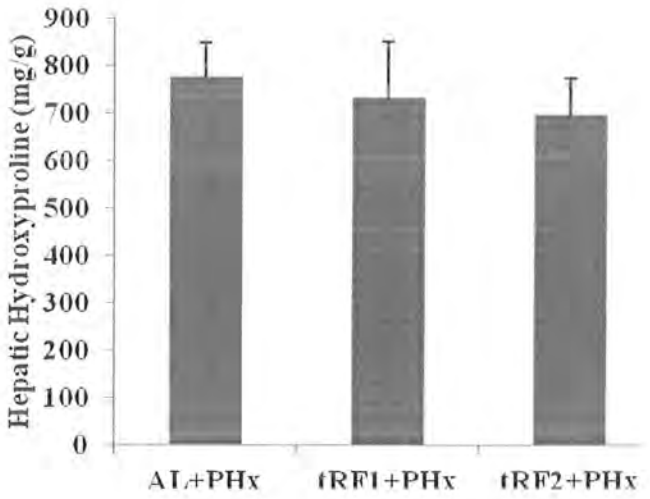

Figure 3.21: Hepatic hydroxyproline content of rats 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens. The tRF treatments did not reduce hepatic hydroxyproline content during fibrotic liver regeneration. (n=5-8/group).

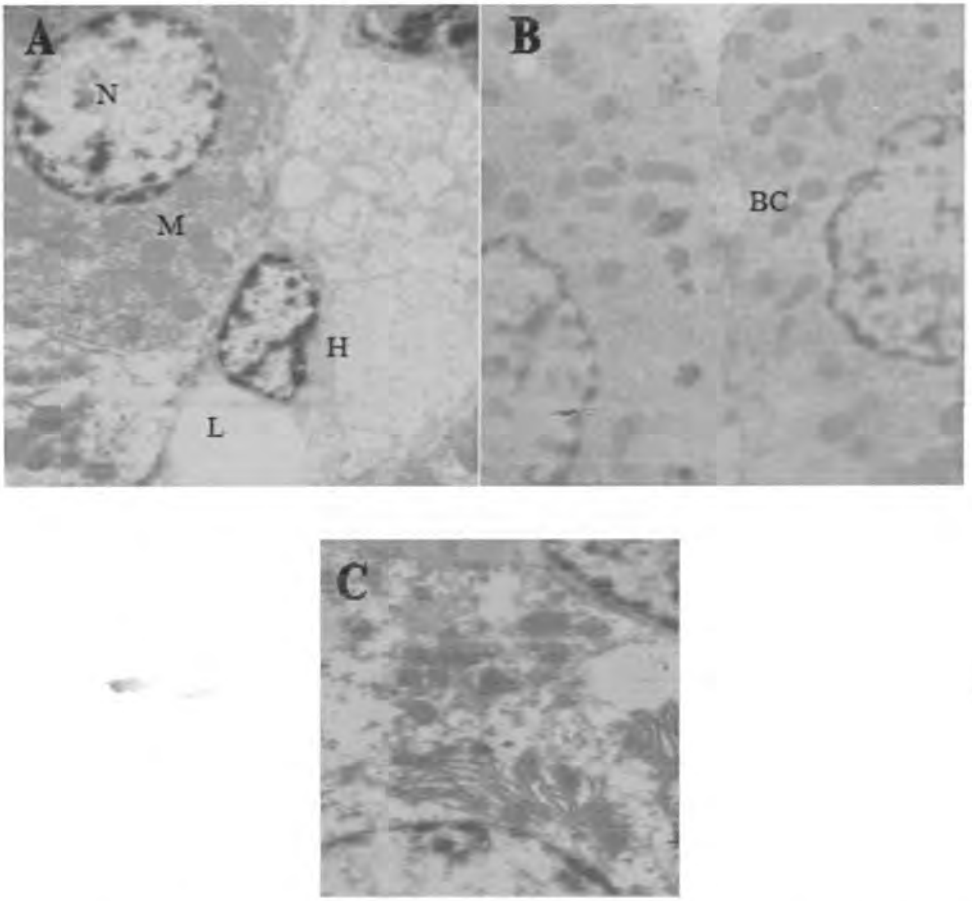

Figure 3.22: Hepatic ultrastructural examination of rat liver following partial hepatectomy under time restricted feeding. Normal hepatocyte with a well-defined nucleus, mitochondria with inactive hepatic stellate cell could be seen (A). Increased lipid accumulation in regenerating liver under tRF1 treatment was observed (C). M = Mitochondrion, N = Nucleus, HSC = Hepatic Stellate Cell, L = Lipid, BC = Bile canaliculi.

3.3 Conclusion

The tRF schedules markedly decreased the body weights of rats and recovered them significantly after PHx especially the full daytime feed restriction. Liver regeneration was assessed by estimating different somatometric hypertrophic and hyperplasic estimates. The results indicated no effect of tRF on relative liver weight however liver regeneration rate was significantly increased by both feeding regiemns specially the first schedule. The tRF regiemn has inhibed hyperplasia as percentage proliferation cell nuclear antigen and mitotic count were reduced by the treatments. The restricted feeding schedules did not affect AgNOR index in vehicle rats, however, fibrotic rats showed increased and heterogeneous AgNOR index when the feed was restricted to middle of the light phase. Both feed restriction schedules increased the binuclear cells that were decreased significantly during the regenerative process. A marked reduction in hepatocyte cellular area under temporal feed restriction was observed that remained restricted after PHx compared to ad libitum fed rats. Nuclear morphometry also followed the same trend in vehicle rats but fibrotic rats remained non-responsive to tRF regimens. Hepatic tissue analysis showed that tRF regimens increased the hepatic water, lipid and glycogen content with no effect on DNA and protein.

Hepatic functional capability evaluation indicated improvement in serum alanine transaminase and aspartate transaminase under tRF regimens with no significant effect on alkaline phosphatase, bilirubin, total protein and albumin levels. Lactate dehydrogenase activity was significantly restored in vehicle rats while glucose and triglyceride were differentially affected by temporal feeding restrictions. Decreased serum glucose under tRF1while increased triglyceride levels under tRF2 in fibrotic rats was observed.

Temporal feed restriction has decreased extra cellular matrix in vehicle rats with little or no improvement in fibrotic rats as indicated by hydroxyproline quantification and histopathological evaluation by Gomori triple stain and α-SMA.

The study concluded that tRF treatments markedly affected body weights. Temporal feed restriction influenced liver regeneration by inhibiting hyperplasia, producing a shift from binuclearity to mononuclearity, reducing ploidy and hypertrophy, and effecting

hepatic tissue constituents. The tRF regimens improved the functional capability of liver during regeneration and decreased extra cellular matrix in vehicle rats with little or no improvement in fibrotic rats.

CHAPTER # 4

ADRENAL AND THYROID HORMONE ALTERATIONS DURING FIBROTIC LIVER REGENERATION UNDER TIME RESTRICTED FEEDING

4.1 Introduction

Liver has a complex relation with both adrenal (Stalmans and Laloux, 1979) and thyroid gland (Malik and Hodgson, 2002). Multiple hepatic cellular pathways including hepatic metabolism and cell proliferation are affected by adrenal (Barbason *et al.*, 1987) and thyroid hormones (Feng *et al.*, 2000; Flores-Morales *et al.*, 2002). The phenomenon of liver regeneration is also influenced by these hormones. Thyroid hormones support (Alisi *et al.*, 2005) whereas adrenal hormones inhibit (Barbason *et al.*, 1989) hepatocyte proliferation, a key event of hepatic regeneration following PHx.

Adrenal hormones have a significant negative control over hepatic regeneration (Barbason *et al.*, 1989). Corticosterone circadian oscillations are not only responsible for the circadian synchronization of hepatocyte postnatal replication (Barbason *et al.*, 1987), but also involve in the circadian pattern of mitosis in the regenerating liver after PHx (Barbason *et al.*, 1989). Further the circadian rhythm of hepatocyte dry mass that represent the trophic state of hepatocyte; is reported to be regulated by corticosterone (Tongiani *et al.*, 1982).

Thyroid gland synthesizes and secretes two main hormones, thyroxine (T4), the major thyronine which functions as a pro-hormone and its deiodination product, triiodothyronine (T3), the active hormone. Tri-iodothyronine (T3) is considered a primary hepatic mitogen that induces hepatocyte proliferation in vivo and in vitro (Francavilla *et al.*, 1994). It is able to substitute PHx in stimulating re-growth of transplanted hepatocytes (Oren *et al.*, 1999; Cesarone *et al.*, 2000) and it can initiate the process of direct hyperplasia in intact liver (Ledda-Columbano*et al.*, 2000). DNA-synthesis and liver regenerative capacity after PHx can be enhanced by a single dose of T3 (Maliekal *et al.*, 1997; Malik *et al.*, 2003). Like other hepatic physiological processes, development of fibrosis in liver is also effected by thyroid hormones which accelerate the process through activation of HSCs (Zvibel *et al.*, 2010).

Time restricted feeding uncouples the peripheral clocks from the SCN especially in liver (Damiola *et al.*, 2000). Restricted feeding acts on peripheral oscillators through chemical cues like glucocorticoid hormones, as they cause phase shifts exclusively in peripheral tissues (Balsalobre *et al.*, 2000). Glucocorticoid signaling could be used by the master clock SCN to prevent the rapid uncoupling of peripheral oscillators. They can inhibit the uncoupling of peripheral and central circadian oscillators induced by temporal feed restriction. Glucocorticoid signaling exclusively slows down the phase changes that are generated by a feeding regimen that is in conflict with the normal activity phase, but has little effect when the phase is switched back from an abnormal to a normal feeding schedule (Le Minh *et al.*, 2001).

Different feeding regimens have been reported to markedly affect thyroid hormone levels, activity, and metabolism (Cokelaere *et al.*, 1996; Ortiz *et al.*, 2001). During temporal feed restriction an exclusively hepatic hypothyroidal-like condition is produced before food presentation that is recovered after food access due to variations in the activity of liver selenoenzyme deiodinase type 1 (D1), which transforms T4 into T3 (Aceves*et al.*, 2003).

Present study has been aimed to evaluate the effect of temporal restricted feeding schedules on adrenal and thyroid hormone alterations following PHx in normal and fibrotic rats.

4.2 Results

4.2.1 Corticosterone

Serum corticosterone levels of rat were increased 48 hours post 70% partial hepatectomy in both vehicle and fibrotic rats. The tRF treatments had a differential effect on serum corticosterone levels. In vehicle rats no effect of temporal feed restriction had been observed while in fibrotic rats, a considerable decrease and increase respectively under the first and second regimen was observed (Figure 4.1).

4.2.2 Serum T3 and T4 concentrations

Partial hepatectomy reduced serum T3 levels in both vehicle and fibrotic rats even after 48 hours post-surgery. No statistical significant effect of tRF regimens on serum T3 levels was observed in both vehicle and fibrotic rats (Figure 4.2a).

Reduced serum T4 levels had been observed 48 hours post PHx but the major reduction was demonstrated by vehicle rats. Both tRF treatments recovered serum T4 levels in vehicle rats especially significant increase was seen in animals under tRF1. This regimen had no effect on fibrotic rats. Surprisingly in fibrotic rats tRF2 regimen elevated the serum T4 level to a large extent (Figure 4.2b).

In both vehicle and fibrotic rats, serum T3/T4 ratio decreased to almost similar extent after PHx. Significantly less T3/T4 ratio in vehicle under tRF1 was observed due to less T3 and high T4. The increased serum T4 levels in fibrotic rats under tRF2 resulted in significantly decreased T3/T4 ratio in these animals (Figure 4.2c).

4.2.3 Liver T3 and T4 concentrations

In fibrotic rats, liver T3 concentration was considerably less than vehicle rats. In these rats except sham, liver T3 was more in all groups as compared to their vehicle counterparts. The tRF1 treatment had no effect on liver T3 both in vehicle and fibrotic rats. The tRF2 treatment, however, increased liver T3 in fibrotic rats with almost no effect on vehicle group (Figure 4.3a). Liver T4 concentration was almost same in all groups observed in the study (Figure 4.3b).

Fibrotic sham animals had the lowest liver T3/T4 ratio as liver T3 was less in these rats as compared to vehicle. PHx reduced liver T3/T4 ratio due to decreased liver T3 after PHx. Both tRF regimens recovered liver T3/T4 ratio to some extent in vehicle. The tRF2 treatment had significantly higher T3/T4 ratio in fibrotic rats due to high liver T3 (Figure 4.3c).

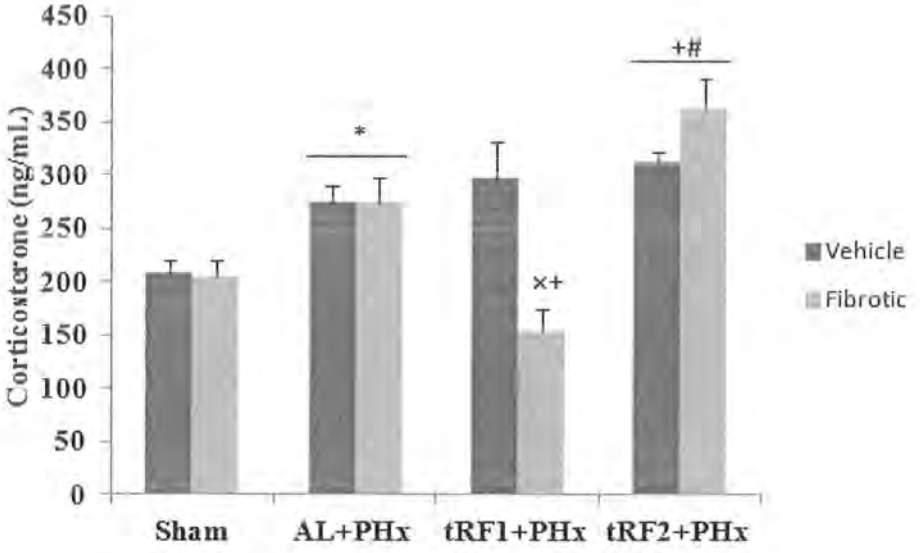

Figure 4.1: Serum corticosterone levels of rats 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens. Differential effect of timed-feed restriction on serum corticosterone levels with no effect on vehicle rats while a considerable decrease and increase in fibrotic rats respectively under the first and second regimen was observed. Fibrosis Effect: F=2.352, P=0.137. *(P<0.05) Sham vs PHx; $^{+}$(P<0.05) AL vs tRF regimens; $^{\#}$(P<0.001) tRF1 vs tRF2 (2-way ANOVA). $^{+}$(P<0.05) AL vs tRF regimens (1-way ANOVA). $^{\times}$(P< 0.05) Vehicle vs Fibrotic (Student's t-test). (n=5-8/group).

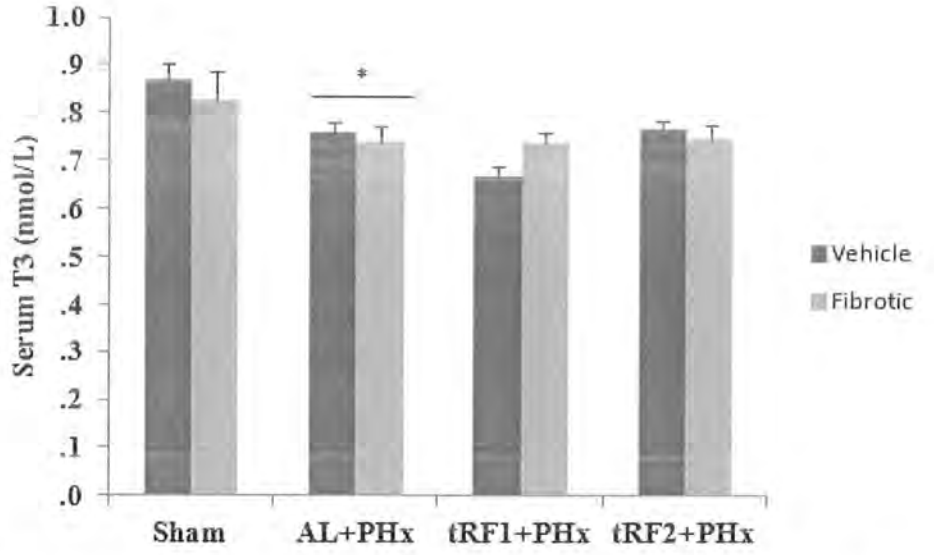

Figure 4.2a: Serum triiodothyronine (T3) of rats 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens. The tRF regimens could not produce statistical significant effect on serum T3 levels. Fibrosis Effect: F=0.000, P=0.988. *(P<0.05) Sham vs PHx (2-way ANOVA). (n=5-8/group).

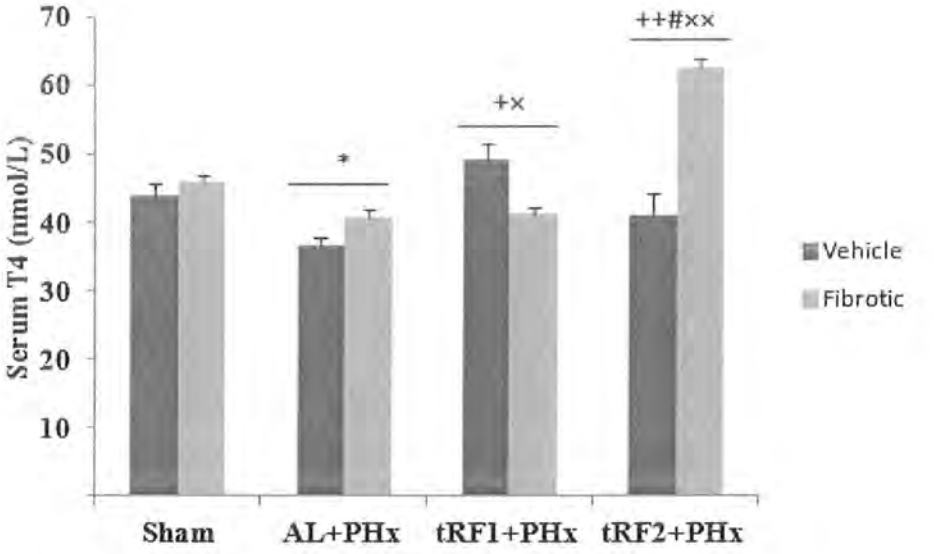

Figure 4.2b: Serum thyroxine (T4) of rats 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens. The tRF regimens had a differential increasing effect on serum T4 levels after PHx. Fibrosis Effect: F=10.976, P=0.003. *(P<0.05) Sham vs PH; $^{+}$(P<0.05); $^{++}$(P<0.001) AL vs tRF regimens; $^{\#}$(P<0.05) tRF1 vs tRF2 (2-way ANOVA). $^{\times}$(P<0.05), $^{\times\times}$(P<0.005) Vehicle vs Fibrotic (Student's t-test). (n=5-8/group).

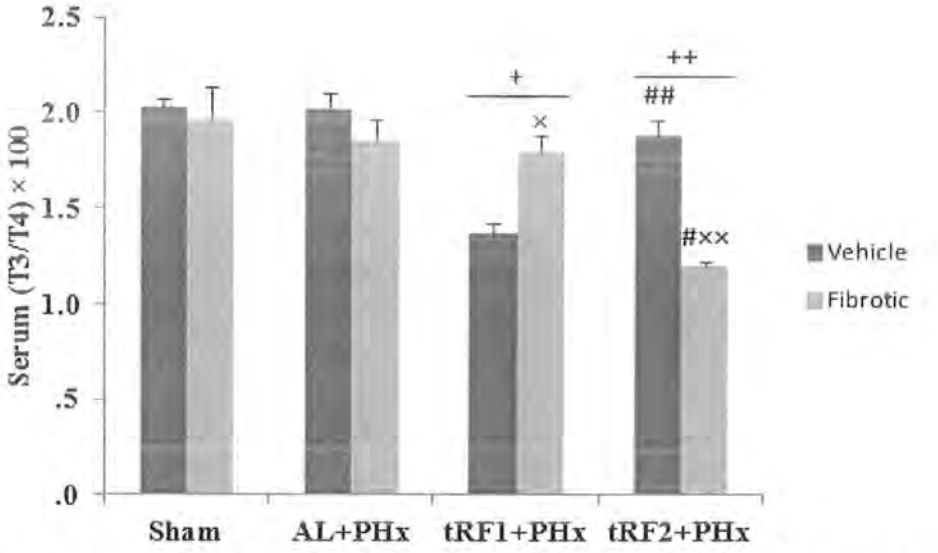

Figure 4.2c: Serum triiodothyronine/thyroxine (T3/T4) ratio of rats 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens. The tRF regimens differentially affected the serum T3/T4 ratios. Fibrosis Effect: F=3.455, P=0.075. $^{+}$(P<0.01), $^{++}$(P<0.001) AL vs tRF regimens (2-way ANOVA). $^{\#}$(P<0.01), $^{\#\#}$(P<0.005) tRF1 vs tRF2 (1-way ANOVA). $^{\times}$(P<0.01), $^{\times\times}$(P<0.001) Vehicle and Fibrotic (Students t-test). (n=5-8/group).

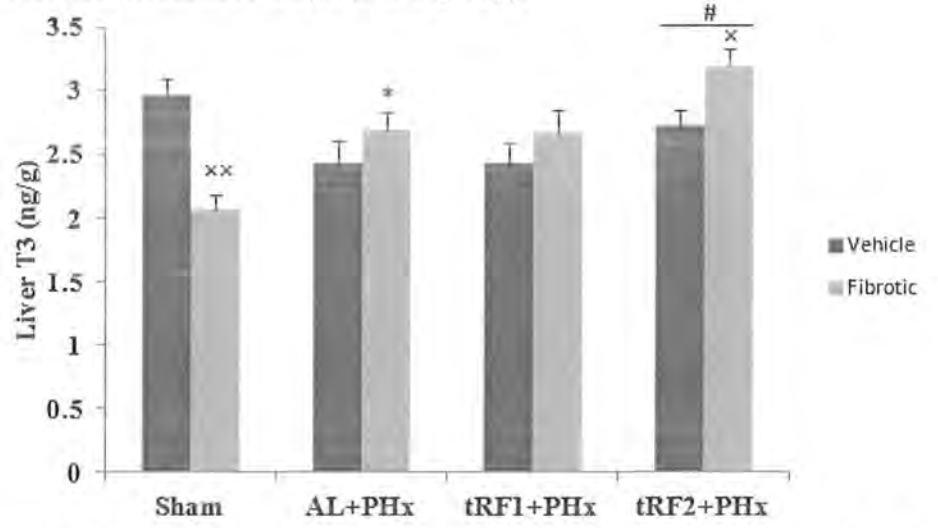

Figure 4.3a: Liver triiodothyronine (T3) of rats 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens. The tRF2 schedule has increasing effect on liver T3 concentrations in fibrotic rats. Fibrosis Effect: F=0.026, P=0.872 (This statistic insignificance is due to low T3 in fibrotic sham livers and high hepatic T3 concentrations in rest of the groups). *(P<0.05) Sham vs PHx (1-way ANOVA). $^{\#}$(P<0.05) tRF1 vs tRF2 (2-way ANOVA). $^{\times}$(P<0.05), $^{\times\times}$(P<0.005) Vehicle vs Fibrotic (Student's t-test). (n=5-8/group).

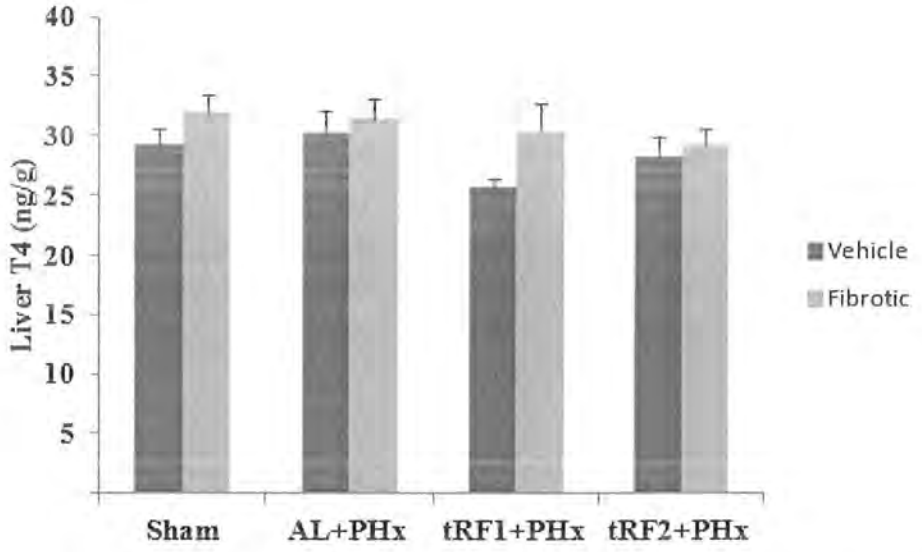

Figure 4.3b: Liver T4 of rats 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens. No significant difference among different treatment groups regarding liver T4 had been seen. Fibrosis Effect: F=4.372, P=0.046. (n=5-8/group).

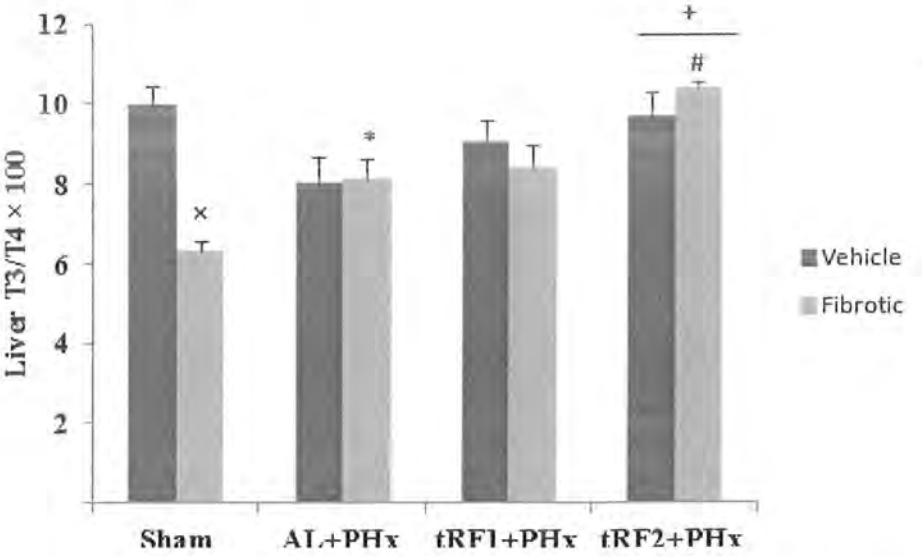

Figure 4.3c: Liver triiodothyronine/thyroxine (T3/T4) ratio of rats 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens. The tRF regimens increased the liver T3/T4 ratio especially tRF2. Fibrosis Effect: F=7.082, P=0.013. *(P<0.05) Sham vs PHx; $^{+}$(P<0.005) AL vs tRF regimen; $^{\#}$(P<0.01) tRF1 vs tRF2 (2-way ANOVA). $^{\times}$(P<0.001) Vehicle vs Fibrotic (Student's t-test). (n=5-8/group).

4.3 Conclusion

Serum corticosterone levels were increased by partial hepatectomy. Timed-feed restriction had a differential effect on corticosterone concentrations with no effect on vehicle rats while a considerable decrease and increase under the first and second regimen respectively was observed in fibrotic rats. Partial hepatectomy depressed the thyroid hormone levels which were raised by tRF in vehicle and fibrotic rats under the first and second schedule respectively by significantly increasing serum thyroxine levels. Serum T3/T4 ratio was considerably low in these rats. Significantly reduced T3 and T3/T4 ratio in fibrotic sham livers were remarkably increased by second schedule after PHx.

Time restricted feeding had produced different alterations in both adrenal and thyroid hormone levels with respect to vehicle and fibrotic rats during liver regeneration.

CHAPTER # 5

OXIDATIVE STRESS

5.1 Introduction

Partial hepatectomy induced hepatic regeneration model have shown that initiation of the liver regenerative response depends on many factors (Bedirli *et al.*, 2005). The process of regenerating liver requires a perfect balance between stimulating and inhibiting factors of hepatocyte proliferation as hepatocyte replication is one of the main events during liver regeneration following PHx. Reactive oxygen species (ROS), the byproduct produced during oxidative phosphorylation and glutathione levels determine the proliferative or apoptotic effect of TNF on hepatocytes, the major cytokine that prime the hepatocytes to move towards the synthesis phase of cell cycle (Fausto, 2000).

Partial hepatectomy could cause oxidative stress like other surgical procedures (Miyake *et al.*, 2002) as increased oxygen is demanded by the dividing cells to rebuild the lost hepatic tissue after PHx (Yoshioka *et al.*, 1998). As a result free radicals may accumulate and cause oxygen toxicity (Kimball *et al.*, 1976). Oxidative stress, the result of excessive production of ROS, can damage cells by activating lipid peroxidation (LP) and by altering protein and nucleic acid structures (Shen *et al.*, 1994; Pinkus, 1996).

Fibrotic and cirrhotic animals, though having the ability to regenerate, do not respond to surgical insults well, and show delayed and impaired regeneration following surgery (Kato *et al.*, 2005). Studies demonstrated that cirrhotic livers have reduced capacity to respond to oxidative stress (Cabre *et al.*, 2000; Balkan *et al.*, 2001). PHx-induced LP in massive resection of the cirrhotic liver may reported to be one of the several reasons involved in its defective regeneration (Morimoto and Isselhard, 1992). Oxidative stress plays important role in the etiopathogenesis of hepatic fibrosis. It aggravates the liver fibrosis through HSC activation (Lee *et al.*, 2001). Lipid peroxidation is reported to stimulate HSC activation (Vendemiale *et al.*, 2001), thus worsening the hepatic fibrosis.

Data suggested that caloric restriction reduces cellular injury and provides numerous health benefits by increasing the resistance to oxidative stress (Sohal and Weindruch, 1996; Hall *et al.*, 2000). Stimulation of cellular stress pathways by the intermittent fasting have also been reported in mediating its beneficial health effects (Anson *et al.*, 2003;

Mattson *et al.*, 2004; Mattson, 2008). Antioxidant potential of night-time feed restriction had also been reported (Jayakumar *et al.*, 2010). Temporal feed restriction has been shown to reduce the inflammation and related disease markers (Sherman *et al.*, 2012).

Present study is based on the postulation that like caloric restriction, intermittent fasting and night time feed restriction, daytime restricted feeding can reduce oxidative stress and hence can improve the regenerating ability of fibrotic liver following hepatectomy.

5.2 Results

5.2.1 Catalase

Hepatic fibrosis had a decreasing effect on hepatic catalase levels as liver CAT concentrations were always lower in fibrotic rats as compared to their vehicle counterparts. PHx significantly reduced the liver CAT levels in both groups post 48 hours. Temporal feed restriction had improved hepatic CAT levels in both vehicle and fibrotic rats specially the second schedule (Figure 5.1).

5.2.2 Peroxidase

Fibrotic sham animals had significantly higher POD levels than vehicle sham. In vehicle neither PHx nor treatments affected liver POD content. But in fibrotic rats significantly decreased POD levels 48 hours after PHx had been seen. Significant restoration in their levels in fibrotic animals had been observed specially under the first schedule (Figure 5.2).

5.2.3 Superoxide Dismutase

No statistical significant difference regarding hepatic SOD content in different study groups have been detected. However, fibrotic sham rats had considerably less SOD levels as compared to the vehicle and that levels were increased though non-significantly in these rats after PHx. Temporal feed restriction did not affect liver SOD content (Figure 5.3).

5.2.4 Glutathione

Hepatic fibrosis had a reducing effect on hepatic GSH levels. PHx resulted in increase in hepatic GSH content in fibrotic animals that were further increased by tRF1 regimen with little effect on vehicle rats. Interestingly very low GSH levels in vehicle livers compared to fibrotic under tRF2 had been monitored (Figure 5.4).

5.2.5 Glutathione Peroxidase

Fibrotic sham rats had significantly less hepatic GPx activity. PHx reduced hepatic GPx levels, though non-significant especially in vehicle rats. Differential effect of temporal feed restriction regarding hepatic GPx levels in vehicle and fibrotic rats had been observed. The tRF1 schedule showed non-significant improvement in vehicle rats while surprisingly much lowered hepatic GPx content of fibrotic rats under that schedule was recorded. The tRF2 schedule had no effect on hepatic GPx levels in both vehicle and fibrotic rats (Figure 5.5).

5.2.6 Glutathione Reductase

Reduced GSR levels were observed in fibrotic sham rats compared to vehicle. After PHx their levels reduced significantly in vehicle rats while remained statistically unchanged though increased, in fibrotic rats. The tRF2 schedule showed an increasing trend in liver GSR levels in vehicle rats while in fibrotic rats no alterations in their levels under tRF regimens following PHx had been observed (Figure 5.6).

5.2.7 Thiobarbituric acid substance

PHx significantly increased the hepatic TBARS levels 48 hours post-surgery. Interesting results regarding liver TBARS content in vehicle and fibrotic rats under timed feed restriction had been noticed. Vehicle rats had high TBARS values than fibrotic rats with tRF1 treatment. The tRF2 regimen, though non-significant, had decreasing trend in liver TBARS levels in both vehicle and fibrotic rats (Figure 5.7).

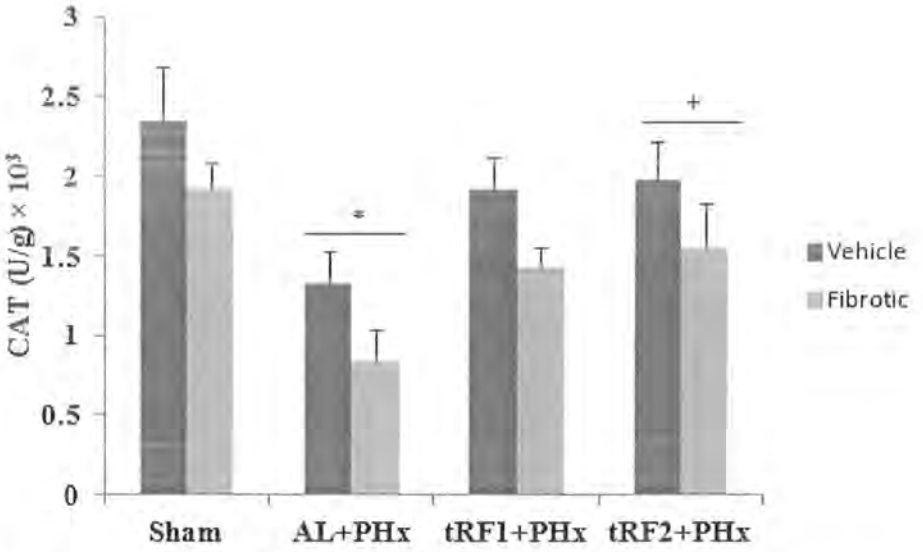

Figure 5.1: Hepatic CAT levels of rats 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens. The tRF regimens improved hepatic CAT levels after PHx especially tRF2. Fibrosis effect: F=8.174, P=0.008. *(P<0.005) Sham vs PHx; $^{+}$(P<0.05) AL vs tRF2 (2-way ANOVA). (n=5-8/group).

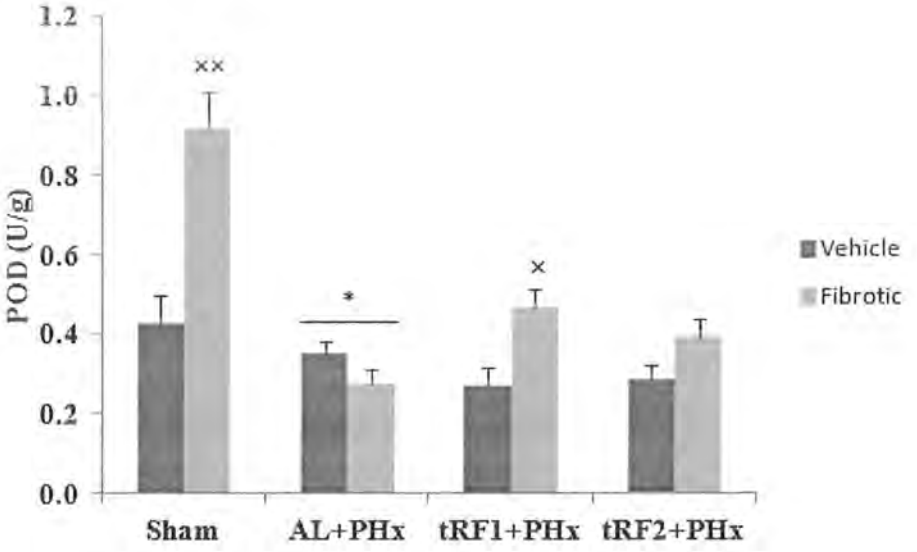

Figure 5.2: Hepatic POD levels of rats 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens. The tRF regimens increased the liver POD content in fibrotic rats after PHx especially tRF1. Fibrosis Effect: F=17.721, P=0.000. *(P<0.05) Sham vs PHx (2-way ANOVA). $^{\times}$(P<0.05), $^{\times\times}$(P<0.005) Vehicle vs Fibrotic (Student's t-test). (n=5-8/group).

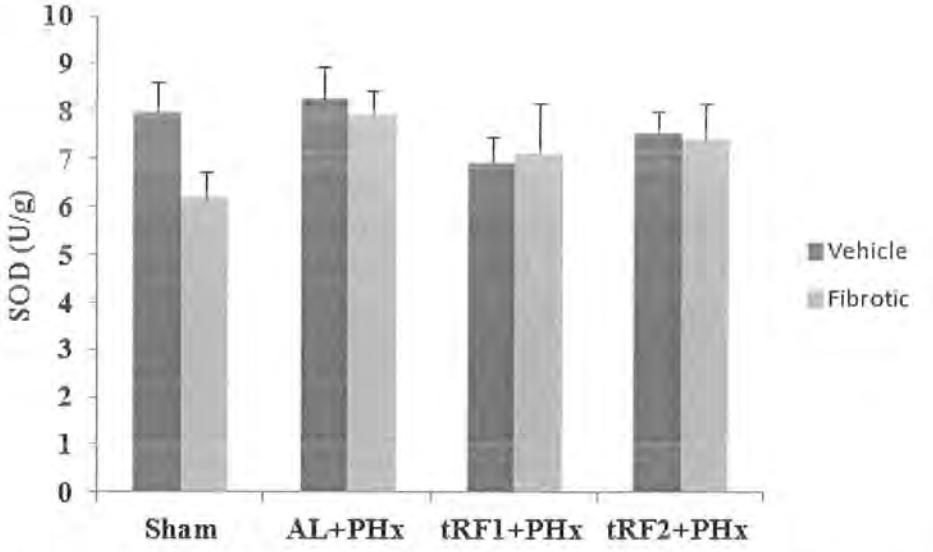

Figure 5.3: Hepatic SOD levels of rats 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens. Temporal feed restriction had not produced any statistical significant effect on hepatic SOD content. Fibrosis Effect: F=1.288, P=0.267. (n=5-8/group).

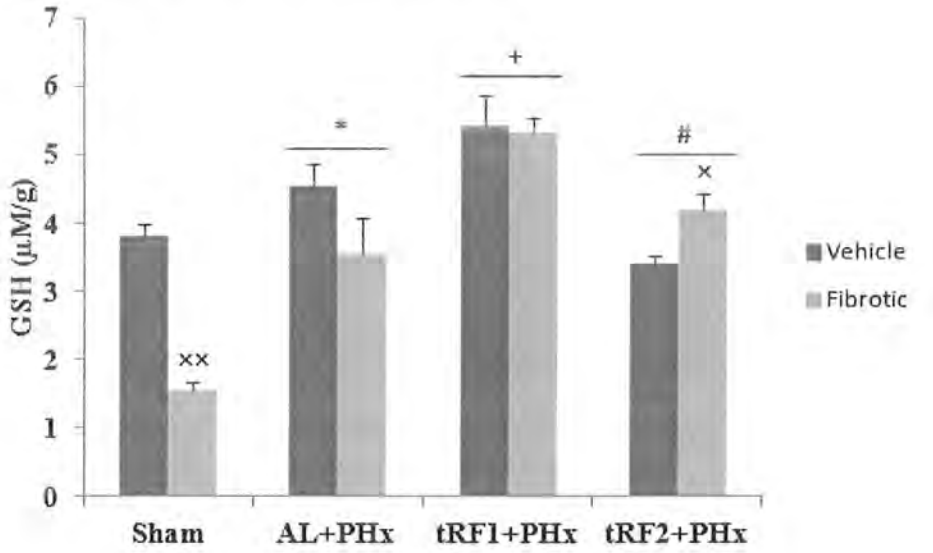

Figure 5.4: Hepatic GSH levels of rats 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens. The tRF1 regimen further rose the PHx induced increased hepatic GSH levels especially in fibrotic rats. Fibrosis Effect: F=20.086, P=0.000. *(P<0.001) Sham vs PHx; $^{+}$(P<0.005) AL vs tRF regimen; $^{\#}$(P<0.001) tRF1 vs tRF2 (2-way ANOVA). $^{\times}$(P<0.05), $^{\times\times}$(P<0.001) Vehicle vs Fibrotic (Student's t-test). (n=5-8/group).

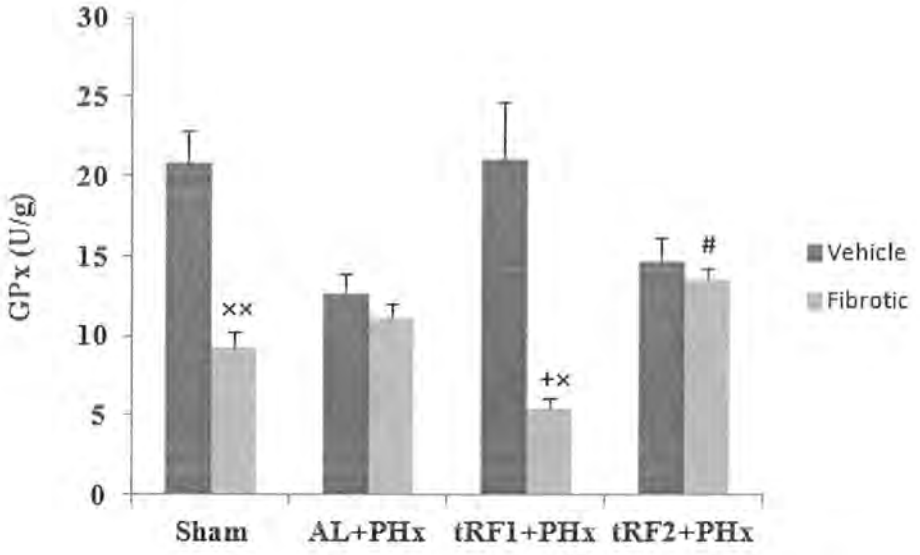

Figure 5.5: Hepatic GPx levels of rats 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens. Differential effect of temporal feed restriction regarding hepatic GPx levels in vehicle and fibrotic rats had been observed with increased and decreased liver GPx levels under tRF in vehicle and fibrotic rats respectively. Fibrosis Effect: F=34.565, P=0.000. $^{+}$(P<0.005) AL vs tRF regimen; $^{\#}$(P<0.001) tRF1 vs tRF2 (1-way ANOVA). $^{\times}$(P<0.005), $^{\times\times}$(P<0.001) Vehicle vs Fibrotic (Student's t-test). (n=5-8/group).

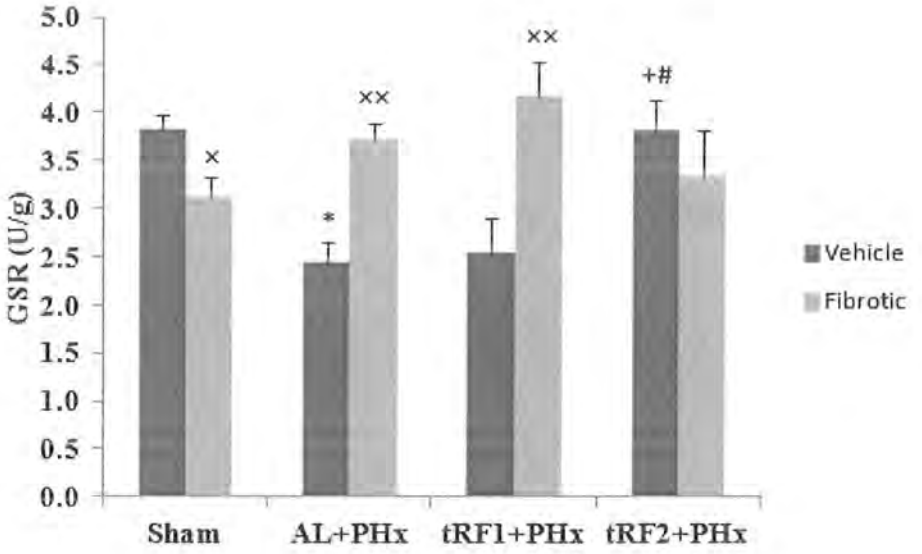

Figure 5.6: Hepatic GSR levels of rats 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens. The tRF2 regimen showed an increasing trend in liver GSR levels in vehicle rats while in fibrotic rats no alterations in their levels under the feeding treatments had been observed. Fibrosis Effect: F=3.280, P=0.079. *(P<0.05) Sham vs PHx; $^{+}$(P<0.05) AL vs tRF regimen; $^{\#}$(P<0.05) tRF1 vs tRF2 (1-way ANOVA). $^{\times}$(P<0.05), $^{\times\times}$(P<0.01) Vehicle vs Fibrotic (Student's t-test). (n=5-8/group).

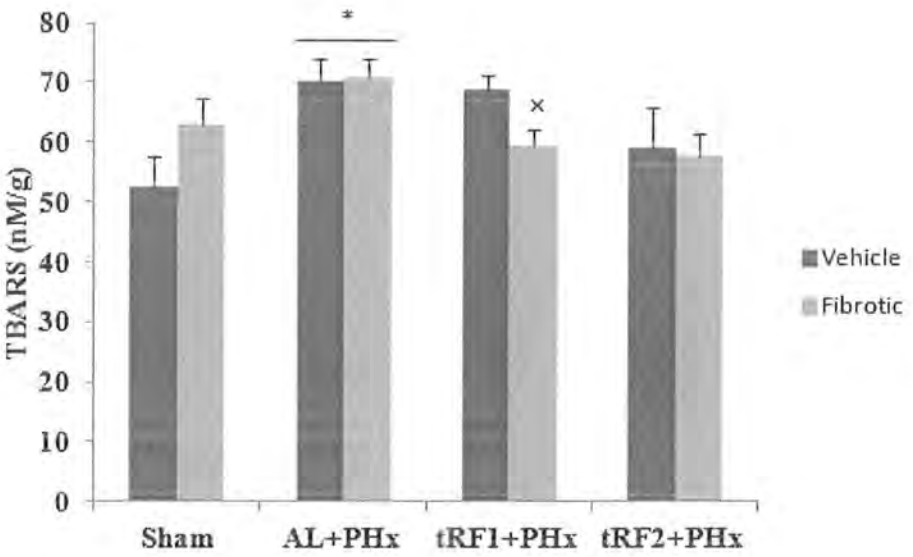

Figure 5.7: Hepatic TBARS levels of rats 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens. The tRF regimens lowered down the hepatic TBARS levels though non significantly in fibrotic rats. Fibrosis Effect: F=0.000, P=0.996. *(P<0.05) Sham vs PHx (2-way ANOVA). ×(P<0.05)Vehicle vs Fibrotic (Student's t-test). (n=5-8/group).

5.3 Conclusion

Partial hepatectomy decreased hepatic CAT and POD contents while SOD levels remain unchanged. Increased hepatic TBARS and GSH levels were observed after PHx while activities of GPx and GSR were reduced in vehicle but remain unchanged in fibrotic rats 48 hours post PHx.

The tRF treatments improved the hepatic CAT levels in both groups while POD and SOD levels did not show any significant effect by tRF regimens. Interesting results regarding hepatic lipid peroxidation system was seen. No decrease in hepatic TBARS levels but significant increase in GSH content under tRF1 was seen while non-significant decrease in TBARS levels with no improvement in hepatic GSH levels was observed under the second regimen. No effect of tRF on GSR activity in fibrotic rats was seen, however, a significant rise under tRF2 in vehicle rats was detected. GPx levels were increased under tRF1 in vehicle rats while decreased in fibrotic animals. The tRF2 regimen had no effect on hepatic GPx levels in both groups.

It could be concluded that temporal feed restriction affected the liver antioxidant defense system during liver regeneration following PHx especially the catalase activity and lipid peroxidation system.

CHAPTER # 6

MINERAL METABOLISM

calcium concentrations was seen. However in vehicle rats under tRF2 a considerable decreasing effect was seen. Fibrotic rats had significantly more hepatic calcium content under tRF2.

In the post PHx observations, statistically non-significant differences in hepatic calcium content among different study groups had been detected. However, fibrotic sham livers had considerably less calcium levels and an increasing trend in calcium levels 48 hours post PHx was observed in both vehicle and fibrotic rats. Vehicle and fibrotic rats behaved differently to the feed restriction regimens. In vehicle rats timed feed restriction resulted in reduced calcium levels while in fibrotic rats, treatments had no effect and calcium levels remain unchanged. Ad libitum and temporal feed restricted fibrotic rats had an increasing trend in liver calcium levels following PHx while levels of feed restricted vehicle rats remained unaffected (Table 6.3 &6.3b).

6.2.4 Magnesium

Fibrotic rats had less hepatic magnesium levels. Temporal feed restriction had a non-significant decreasing and increasing trend in vehicle and fibrotic rats respectively. After sham surgery magnesium liver content remained less in fibrotic rats compared to control. PHx led to increased hepatic magnesium concentrations and the levels were further enhanced by tRF1 in both vehicle and fibrotic rats. However tRF2 resulted in decreased hepatic magnesium levels. Comparisons between pre and post-surgery magnesium levels showed that their levels increased in AL and tRF1 treated rats while no significant change in its concentrations under tRF2 treatment was observed (Table 6.4a & 6.4b).

6.2.5 Iron

Temporal feed restriction had not produced any noticeable effect on hepatic iron content. Fibrotic rats had more iron levels than vehicle rats. Partial hepatectomy resulted in significant reduction in hepatic iron levels in fibrotic rats while in vehicle rats no effect was seen. Non-significant decrease and increase following PHx with timed feed restrictions had been detected in vehicle and fibrotic rats respectively. Both vehicle and fibrotic animals under tRF regimens showed decreasing trend in hepatic iron

concentrations after PHx as detected by pre and post-surgery comparisons. However, significant differences were observed only in fibrotic rats (Table 6.5a &6.5b).

6.2.6 Copper

Feed restricted vehicle showed no difference in hepatic copper levels before PHx however fibrotic rats showed a marked reduction in copper levels under tRF1. Interesting results regarding hepatic copper content had been observed in vehicle and fibrotic rats after PHx. Differential behavior under hepatectomy and temporal feed restriction had been seen in both groups. PHx resulted in significantly decreased hepatic copper levels in fibrotic animals while it had no effect in vehicle rats 48 hours post-surgery. In vehicle rats both regimens significantly lowered down copper levels in liver after hepatectomy while in fibrotic rats, tRF2 had significantly increased the copper levels. The tRF pretreatment tend to decrease the copper levels in vehicle rats while in fibrotic rats significant difference between pre and post copper levels had been seen in AL rats alongwith tRF1 treated rats. No statistical significant difference by tRF2 after PHx had been seen (Table 6.6a & 6.6b).

6.2.7 Zinc

Fibrotic rats had less hepatic zinc levels. These levels were significantly reduced under tRF2 in vehicle rats while in fibrotic rats an increasing trend though non-significant in hepatic zinc levels were detected under the same regimen. Statistically non-significant differences in hepatic zinc content among different study groups after PHx had been observed. However PHx had a reducing effect on hepatic zinc levels as zinc levels fell in both vehicle and fibrotic rats after surgery. In vehicle rats no effect of tRF has been seen while in fibrotic rats under feed restriction hepatic zinc levels returned to sham operated levels. Significant decrease in hepatic zinc levels in tRF1 treated vehicle rats between pre and post PHx levels had been seen while in fibrotic rats no difference was seen. (Table 6.7a & 6.7b).

Table 6.1a: Hepatic sodium content (mg/g dry weight), pre and 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens.

	Pre PHx		Post PHx	
	Vehicle	**Fibrotic**	**Vehicle**	**Fibrotic**
Sham	-	-	2.63±0.23	2.45±0.17
AL+PHx	2.83 ± 0.37	2.11 ± 0.12	2.97±0.30	2.52±0.50
tRF1+PHx	2.38 ± 0.28	2.09 ± 0.24	3.53±0.31$^{¥}$	3.41±0.41$^{¥}$
tRF2+PHx	1.98 ± 0.31	2.96 ± 0.20$^{+\#*}$	3.39±0.51	3.75±0.39

$^{+}$ (P<0.05) AL vs tRF regimens; $^{\#}$(P<0.05) tRF1 vs tRF2 (1-way ANOVA). *(P<0.05) Vehicle vs Fibrotic; $^{¥}$(P<0.05) pre vs post PHx (Student's t-test). (n=5-8/group).

Table 6.1b: Two way ANOVA table for pre and post PHx hepatic sodium content.

	Pre-PHx (P-value)	Post-PHx (P-value)
Fibrotic Effect	0.965 (0.002)	0.690 (0.161)
Sham vs PHx	———	1.000
AL vs tRF1	1.000	0.351
AL vs tRF2	1.000	0.134
tRF1 vs tRF2	0.902	1.000

P-values for treatment effects are computed using Bonferroni post hoc test. P<0.05 is considered significant. F statistic in brackets.

Table 6.2a: Hepatic potassium content (mg/g dry weight), pre and 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens.

	Pre PHx		Post PHx	
	Vehicle	Fibrotic	Vehicle	Fibrotic
Sham	-	-	4.90 ± 0.64	4.74 ± 0.43
AL+PHx	4.85 ± 0.55	3.61 ± 0.47	5.07±0.23	5.04 ± 0.57
tRF1+PHx	6.34 ± 0.48	3.99 ± 0.14$^{\times}$	6.81±0.71	5.22±0.51$^{¥}$
tRF2+PHx	2.60 ± 0.37	3.07 ± 0.38	5.37±0.55$^{¥¥}$	5.44±0.57$^{¥¥}$

$^{\times}$(P<0.005) Vehicle vs Fibrotic; $^{¥}$(P<0.05), $^{¥¥}$(P<0.01) pre vs post PHx (Student's t-test). (n=5-8/group).

Table 6.2b: Two way ANOVA table for pre and post PHx hepatic potasium content.

	Pre-PHx (P-value)	Post-PHx (P-value)
Fibrotic Effect	0.005 (10.115)	0.264 (1.300)
Sham vs PHx	———	1.000
AL vs tRF1	0.185	0.439
AL vs tRF2	0.008	1.000
tRF1 vs tRF2	0.000	1.000

P-values for treatment effects are computed using Bonferroni post hoc test. P<0.05 is considered significant. F statistic in brackets.

Table 6.3a: Hepatic calcium content (ug/g dry weight), pre and 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens.

	Pre PHx		Post PHx	
	Vehicle	Fibrotic	Vehicle	Fibrotic
Sham	-	-	307±39.42	232±46.13
AL+PHx	294.47 ± 23.65	245.58 ± 19.46	394±31.98[¥]	314±37.81
tRF1+PHx	283.38 ± 24.54	250.68 ± 15.33	256±44.89	346±50.97
tRF2+PHx	206.84 ± 20.10	268.70 ± 16.15[×]	255±25.38	342±46.40

[×](P<0.05) Vehicle vs Fibrotic; [¥](P<0.05) pre vs post PHx (Student's t-test). (n=5-8/group).

Table 6.3b: Two way ANOVA table for pre and post PHx hepatic calcium content.

	Pre-PHx (P-value)	Post-PHx (P-value)
Fibrotic Effect	0.684 (0.170)	0.861 (0.031)
Sham vs PHx	———	0.192
AL vs tRF1	1.000	0.934
AL vs tRF2	0.394	0.988
tRF1 vs tRF2	0.776	1.000

P-values for treatment effects are computed using Bonferroni post hoc test. P<0.05 is considered significant. F statistic in brackets.

Table 6.4a: Hepatic magnesium content (mg/g dry weight), pre and 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens.

	Pre PHx		Post PHx	
	Vehicle	Fibrotic	Vehicle	Fibrotic
Sham	-	-	731.09±66.84	483.06 ±39.8$^{\times}$
AL+PHx	735.64 ± 57.01	590.3± 79.81	1085.3±96.55$^{¥}$	858.94±42.18$^{¥}$
tRF1+PHx	654.81 ± 37.83	715.68 ± 36.26	1652.62±179.64$^{¥¥}$	1151.14±35.71$^{\times ¥¥}$
tRF2+PHx	584.32 ± 51.74	649.68± 18.54	689.65±97.79	596.43±48.67

$^{\times}$(P<0.05) Vehicle vs Fibrotic; $^{¥}$(P<0.05), $^{¥¥}$(P<0.001) pre vs post (Student's t-test). (n = 5-8/group).

Table 6.4b: Two way ANOVA table for pre and post PHx hepatic megnesium content.

	Pre-PHx (P-value)	Post-PHx (P-value)
Fibrotic Effect	0.9992 (0.000)	0.000 (19.574)
Sham vs PHx	———	0.000
AL vs tRF1	1.000	0.003
AL vs tRF2	1.000	0.000
tRF1 vs tRF2	0.677	0.000

P-values for treatment effects are computed using Bonferroni post hoc test. P<0.05 is considered significant. F statistic in brackets.

Table 6.5a: Hepatic iron content (ug/g dry weight), pre and 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens.

	Pre PHx		Post PHx	
	Vehicle	Fibrotic	Vehicle	Fibrotic
Sham	-	-	688.53 ± 71.84	784.84 ± 93.98
AL+PHx	646.29 ± 16.28	711.40 ± 58.46	663.06 ± 61.56	447.37 ± 14.34$^{*¥}$
tRF1+PHx	683.34 ± 30.38	763.39 ± 23.41	574.31 ± 59.06	584.38 ± 38.62$^{¥¥}$
tRF2+PHx	618.46 ± 43.94	744.78 ± 31.10*	490.78 ± 55.75	538.99 ± 54.01$^{¥}$

*(P<0.05) Sham and PHx; $^{+}$(P<0.001) AL vs tRF regimens (1-way ANOVA).*(P<0.05) Vehicle vs Fibrotic; $^{¥}$(P<0.05), $^{¥¥}$(P<0.01) pre vs post PHx (Student's t-test). (n = 5-8/group).

Table 6.5b: Two way ANOVA table for pre and post PHx hepatic iron content.

	Pre-PHx (P-value)	Post-PHx (P-value)
Fibrotic Effect	0.004 (10.251)	0.728 (0.123)
Sham vs PHx	———	0.076
AL vs tRF1	0.312	1.000
AL vs tRF2	1.000	1.000
tRF1 vs tRF2	0.528	1.000

P-values for treatment effects are computed using Bonferroni post hoc test. P<0.05 is considered significant. F statistic in brackets.

Table 6.6a: Hepatic copper content (ug/g dry weight), pre and 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens.

	Pre PHx		Post PHx	
	Vehicle	Fibrotic	Vehicle	Fibrotic
Sham	-	-	19.42±2.24	25.46±2.68
AL+PHx	18.76 ± 1.48	22.26 ± 1.38	22.83±1.73	$10.95\pm1.40^{\times ¥¥}$
tRF1+PHx	19.59 ± 1.49	$16.27 \pm 1.28^{+}$	$13.72\pm1.31^{+¥}$	$10.87\pm1.00^{¥¥}$
tRF2+PHx	16.58 ± 1.22	20.58 ± 1.51	$14.15\pm1.56^{+}$	$26.26\pm2.02^{++\times}$

$^{+}$(P<0.05), $^{++}$(P<0.001) AL vs tRF regimens (1-way ANOVA). $^{\times}$(P<0.005) Vehicle vs Fibrotic; $^{¥}$(P<0.05), $^{¥¥}$(P<0.01) pre vs post PHx (Student's t-test). (n=5-8/group).

Table 6.6b: Two way ANOVA table for pre and post PHx hepatic copper content.

	Pre-PHx (P-value)	Post-PHx (P-value)
Fibrotic Effect	0.252 (1.380)	0.512 (0.440)
Sham vs PHx	———	0.034
AL vs tRF1	0.310	0.038
AL vs tRF2	0.978	0.390
tRF1 vs tRF2	1.000	0.000

P-values for treatment effects are computed using Bonferroni post hoc test. P<0.05 is considered significant. F statistic in brackets.

Table 6.7a: Hepatic zinc content (ug/g dry weight), pre and 48 hours post partial hepatectomy (PHx) after exposure to two weeks of time restricted feeding (tRF) regimens.

	Pre PHx		Post PHx	
	Vehicle	Fibrotic	Vehicle	Fibrotic
Sham	-	-	63.82±5.64	63.31±1.14
AL+PHx	63.74 ± 2.95	57.66 ± 2.02	55.23±1.88$^{¥}$	50.80±4.29
tRF1+PHx	68.19 ± 1.53	60.59 ± 2.09$^{×}$	55.07±2.62$^{¥¥}$	62.64±2.72
tRF2+PHx	57.29 ± 2.30$^{\#}$	65.91 ± 3.09	51.20±2.56	61.39±4.20

$^{\#}$(P<0.05) AL vs tRF2 (1-way ANOVA). $^{×}$(P<0.05) Vehicle vs Fibrotic; $^{¥}$(P<0.05), $^{¥¥}$(P<0.005) pre vs post PHx (Student's t test). (n=5-8/group).

Table 6.7b: Two way ANOVA table for pre and post PHx hepatic zinc content.

	Pre-PHx (P-value)	Post-PHx (P-value)
Fibrotic Effect	0.407 (0.713)	0.310 (1.068)
Sham vs PHx	———	0.057
AL vs tRF1	0.667	0.522
AL vs tRF2	1.000	1.000
tRF1 vs tRF2	1.000	1.000

P-values for treatment effects are computed using Bonferroni post hoc test. P<0.05 is considered significant. F statistic in brackets.

6.3 Conclusion

Hepatic fibrosis led to reduced hepatic Na, K, Ca Mg and Zn content while Fe and Cu levels were raised. Partial hepatectomy tend to significantly or non-signicantly increase the hepatic Na, K, Ca and Mg levels while Fe and Zn contents were decreased in both vehicle and fibrotic rats however Cu levels were decreased in fibrotic rats only with little or no increae in vehicle rats.

Feed restrictions resulted in decreased and increased Na and Mg concentrations in vehicle and fibrotic rats respectively. After PHx both regiemns tend to raise Na levels while only the first schedule increased the hepatic Mg levels in both groups. The first feeding schedule increased while the second decreased the K levels in vehicle rats with no effect on fibrotic rats. Timed feed restriction resulted in reduced Ca levels before and after PHx while in fibrotic rats, treatments had no effect and Ca levels remain unchanged. Temporal feed restriction had not produced any noticeable effect on hepatic Fe content. Feed restriction did not affect hepatic Cu levels in vehicle rats before PHx however a marked reduction in their levels after surgery under the feeding regimens could be seen. In contrast, significantly lowered Cu levels under the first schedule were observed in fibrotic rats that remained lowered after hepatectomy. Timed feed restriction tends to raise the hepatic Zn levels in fibrotic rats during the liver regenerative process.

CHAPTER # 7

DISCUSSION

Comprehensible work has been done to reveal the marvelous phenomenon of liver regeneration (Satoh *et al.*, 1996; Ehrenfried *et al.*, 1997; Rininger *et al.*, 1997; Jaumot *et al.*, 1999; Hernández-Muñoz*et al.*, 2003; Miyaoka *et al.*, 2012), but little has been done on cirrhotic liver which show impaired regeneration following partial hepatectomy (Hashimoto and Watanabe, 1999; Andiran *et al.*, 2000) leading to postoperative mortalities. There requires a need for interventions that can improve postoperative regeneration and function of fibrotic liver because of the increased incidence of liver diseases and hepatocellular carcinoma that often require liver transplantation or surgical resection. As circadian rhythms have a strong influence over mammalian liver processes including liver regeneration (Atwood *et al.*, 2011) and hepatic fibrosis (Chen *et al.*, 2010); and feeding regimens specially temporal feeding can influence exclusively the hepatic physiological processes without disrupting other rhythms (Damiola *et al.*, 2000), present study has been conducted to evaluate the pre-operative effect of temporal feed restriction on different biochemical and morphological aspects of fibrotic liver regeneration.

Time restricted feeding has marked effects on body weights

Slower weight gain by CCl_4 induced fibrotic rats was observed as reported (Parsons *et al.*, 2004; Su *et al.*, 2013). Interesting results have been detected regarding body weights under tRF schedules both before and after PHx. Before PHx, tRF significantly decreased the body weights of animals confirming the previous studies (Díaz-Muñoz *et al.*, 2010). A more marked reduction in body weights was observed when food was restricted to full daytime period i.e. for 12 hours (tRF1) as compared to animals which received food for four hours in the middle of the light phase (tRF2). Feed restriction during the light phase results in reduction of body weights as compared to ad libitum (Martínez-Merlos *et al.*, 2004), while a possible explanation of body weight maintenance of animals under tRF2 during the feed restriction period might be the increased food anticipatory activity and hyperphagia as was observed in rats with food availability for just two hours in the light period (Martínez-Merlos *et al.*, 2004).

A significant reduction in the body weight of rats following PHx was seen as expected (Bülbüloğlu *et al.*, 2005), which was significantly maintained by tRF regimens especially the under the first schedule. That maintenance in the body weight might be due to hyperphagia as the animals were shifted from restricted feeding to ad libitum diet after PHx. It has already been reported that mice shifted from light phase restricted feeding to ad libitum diet consumed larger amount of food and showed greater weight gain (Hambly *et al.*, 2007). Moreover, light phase fed animals were shown to be resistant to stress-induced weight loss (Harris *et al.*, 2002) as was seen in the present study.

Liver Regeneration Assessment

The regenerative capacity of liver following 70% partial hepatectomy depends on both hyperplasia and hypertrophy (Miyaoka *et al.*, 2012) of the hepatocytes so the effect of temporal feed restriction on liver regeneration has been evaluated by measuring both hyperplasia and hypertrophy of cells in the study.

Hyperplasia was measured by mitosis, PCNA count and AgNOR counts in the study. Hepatic DNA content also provides information about the regenerative capacity of liver. Hepatocyte hypertrophy was assessed by measuring hepatic composition alongwith nuclear and hepatocytic area measurement. Somatometric parameters like RLW and LRR were also quantified in the study to better predict the liver regeneration capacity following tRF treatments.

Relative liver weight, an indicator of liver regenerative capacity had shown non-significant differencesamongdifferent treatment groups 48 hours post PHx in both vehicle and fibrotic rats in the study. tRF resulted in decrease in body and corresponding liver weight and sudden shift from timed feed restriction to ad libitum regiemn resulted in faster body and liver weight increase as was observed previously (Harris *et al.*, 2002; Díaz-Muñoz *et al.*, 2010; Bray *et al.*, 2012). So even the liver grows at faster rate but the ratio remained the same i.e. relative liver weight.

The regenerative capacity of liver as evaluated by LRR showed that liver regeneration of fibrotic rats was comparatively slower than vehicle rats as was reported before

(Andiran *et al.*, 2000). Significant increase in LRR specially when the feed was restricted to full light phase had indicated that temporal feed restriction had improved the regenerative capacity of liver 48 hours following PHx in both control and fibrotic rats. LRR showed the rate of liver growth and is based on weight changes. Before hepatectomy, rats were under temporal feed restriction and hence had decreased body and corresponding liver weights (Díaz-Muñoz *et al.*, 2010). Sudden shift from tRF to ad libitum resulted in faster increase in body and corresponding liver weights after stress (Bray *et al.*, 2012) raising the LRR.

Temporal feed restriction inhibited hyperplasia in regenerating liver following PHx

A burst of proliferation had been seen in partially hepatectomized rats as measured by PCNA count (Theocharis *et al.*, 1994; Assy *et al.*, 1998). Fibrotic and cirrhotic rats have reduced proliferation ability following PHx (Hashimoto and Watanabe, 1999; Andiran *et al.*, 2000; Kato *et al.*, 2005). Present study also revealed the negative effect of hepatic fibrosis on S phase hepatocytes as indicted by lower PCNA index in fibrotic rats during liver regeneration.

The circadian clock reported to regulate cell proliferation in those tissues where cell renewal and regeneration are necessary for normal physiological function. Different types of feed restrictions have influence over cell cycle and division. Dietary restriction inhibits cell proliferation in tissue-dependent manner in rats. In liver and glandular stomach cell division was inhibited by DR (Lu *et al.*, 2002). It also prevents tumorigenesis by increasing apoptosis and decreasing propagation in liver (James *et al.*, 1998). Calorie restriction also lowers cell proliferation and produce anti-cancer effects in some tissues (Bhattacharyya *et al.*, 2012). Food deprivation reduced cell proliferation and cell cycle gene expression in the zebrafish gut. Timed feeding can entrain the clock, and cell cycle components including PCNA in the zebrafish intestine (Peyric*et al.*, 2013). Both timed feed restriction regimens decreased the PCNA percentage in regenerating livers as studied at the given time of the day. Further, differential effect of timed feed restriction has been seen regarding PCNA count as in vehicle rats more reduction was seen under tRF2

regimen while in fibrotic animals increased reduction was observed under the first schedule.

After 70% PHx most of the hepatocytes entered S phase (Figure 3.5) (Grisham, 1962; Bucherand Swaffield1964) but less hepatocytes entered in M phase during regeneration, leading to the infrequent cell division and increase in ploidy (Miyaoka *et al.*, 2012). Same pattern had been observed in the present study. M phase of the cell cycle is reported to be compromised during liver regeneration (Miyaoka *et al.*, 2012). Hepatic fibrosis resulted in decrease in mitotic count as fibrotic rats had always less mitotic rate than their vehicle counterparts. Fibrotic and cirrhotic rats demonstrated to have decreased hepatocyte DNA synthesis and mitotic activity following PHx (Kanta and Chlumska, 1991; Suárez-Cuenca *et al.*, 2008; Bickel *et al.*, 1998).

Mammalian cells arrange the processes necessary for their duplication within circadian time (Hrushesky and Bjarnason, 1993).Cell division in the mammalian gut is under the control of circadian clock (Halberg *et al.*, 1954). Hepatocytes also use circadian timing to organize mitotic activity during recovery from an injury (Barbason *et al.*, 1989; Matsuo *et al.*, 2003). Tongiani *et al.*, (1982) found no effect of time restricted feeding on the circadian rhythms of hepatocyte mitosis. In the present study inhibitory effect of timed feed restriction on hepatocyte mitosis following PHx has been observed as mitotic count was significantly less under both regimens in both groups, especially under the second feeding regimen. In the study there were more cells in S phase of the cell cycle in fibrotic rats under tRF2 but less mitotic cells under that schedule was seen. This low mitotic response may be due to the effect of high corticosterone levels in these rats that has inhibitory effect on mitosis (Barbason *et al.*, 1987).

Nucleolar organizer regions are loops of nuclear DNA which contain ribosomal RNA genes in the form of clusters. With these regions are associated set of some proteins which show high affinity for silver hence named as argyrophilic nucleolar organizer region (AgNOR) proteins (Trere, 2000). AgNOR proteins accumulate in highly proliferating cells whereas their expression is very low in non-proliferating cells (Sirri *et al.*, 2000). Liver regeneration is also a highly proliferative phenomenon (Michalopoulos

and DeFrances, 1997) so AgNOR quantification has also been used to evaluate proliferative potential of regenerating liver following PHx (Amin *et al.*, 2010).

Interesting results regarding AgNOR index has been observed in the study. The mAgNOR count did not show significant increase 48 hours post PHx in consistent with the result of Ning *et al.* (2002), however, Amin *et al.*, (2010) has observed increased AgNOR count along with area hour after PHx. In contrast to mAgNOR count, pAgNOR values showed significant increase after PHx in the study making it to conclude that pAgNOR count better reflect the proliferative capacity of liver regeneration than mAgNOR count (Mourad *et al.*, 1992).

In the present study the proliferative phenomenon of liver regeneration has also shown an increasing effect on AgNOR size variation and distribution per nucleus. Time-restricted feeding did not affect any AgNOR parameter in vehicle rats, however, fibrotic rats showed increased and heterogeneous AgNOR values when the feed was restricted to the middle of light phase. AgNORs were reported to be heterogeneous in size and irregularly distributed in malignant tissues (Bukhari *et al.*, 2007).

Temporal feed restriction alters the phase of rhythms in both healthy and malignant tissue and hepatomas are differentially sensitive to circadian signals (Davidson *et al.*, 2004). In the present study temporal feed restriction has produced the same differential response after PHx as is evident by PCNA and AgNOR index. Both vehicle and fibrotic rats responded differently to the different temporal feeding regimens.

Temporal feed restriction produced a shift from binuclearity to mononuclearity, reduced ploidy and hypertrophy during the regenerative process

Hepatocyte binucleation is an interesting feature of adult liver. The number of binuclear hepatocytes decreases during the regeneration process as was observed in the present study (Beams and King, 1942; Sulkin, 1943; Wheatley, 1972) (Figures 3.6a & 3.6b and Table 3.3a). Fibrotic rats had significantly less binuclear cells in sham rats. Thioacetamide induced cirrhotic livers also showed significantly decreased hepatocyte binucleation (Gandillet *et al.*, 2003). Liver pathologies including hepatocellular

carcinoma and cirrhosis result in reduction in liver ploidy including binuclear hepatocytes (Schwarze *et al.*, 1984; Saeter *et al.*, 1988; Melchiorri *et al.*, 1994).

No difference in binuclear cell count had been seen when compared post PHx binuclear cell percentages but pre and post PHx comparisons showed that tRF has doubled the number of binuclear cells that were reduced to almost half after PHx showing that tRF treatments lead to mononuclear cell population after PHx. Tongiani *et al.*, (1982) found no effect of temporal feeding restriction on binuclear cell frequency but in the present study pre PHx results showed that feed restriction has raised the binuclear cells especially in vehicle rats. As tRF treatments increased the binuclear cell number it may be one of the reasons of reduced hepatocyte hyperplasia after PHx under the feeding regimens because binuclear hepatocytes do not participate in liver regeneration especially of injured livers (Jung-Rou *et al.*, 1989). Moreover, binucleation is considered as a sign of terminal differentiation (Liu *et al.*, 2010; Thornburg *et al.*, 2011).

PHx resulted in an increase in hepatocyte nuclear and cellular area as was evident by pre and post PHx comparisons in all groups irrespective of treatments or groups due to the increase in ploidy and cellular hypertrophy (Sulkin, 1943; Gentric *et al.*, 2012; Miyaoka *et al.*, 2012). Before PHx significant decrease in hepatocyte area under temporal feed restriction in both groups had been seen that remained reduced after PHx in vehicle rats. But in fibrotic rats interesting results regarding hepatocellular area has been observed. Under the first schedule comparatively less hepatocyte area was increased following PHx while an enlarged expansion in hepatocyte cellular area was observed under the second schedule. This may be due to the effect of corticosterone as hepatocyte dry mass is believed to be regulated by glucocorticoids i.e. corticosterone concentration, increase and decrease of which causes a decrease and increase of total solid content of hepatocytes (Tongiani *et al.*, 1982). tRF1 treated fibrotic rats had less corticosterone concentrations so their cellular area was also reduced while comparatively an elevated level of corticosterone had been observed under tRF2 treatment leading to enlarged hepatocyte size. Tongiani *et al.*, (1982), however, suggested that circadian rhythm of hepatocytes dry mass and weight classes is regulated by the light-dark regimen through the action of corticosterone and not by timed feeding. It could be seen that timed feeding

can affect corticosterone in partially hepatectomized rats which can then regulate hepatic dry mass and weight class distribution. Díaz-Muñoz *et al.*, (2010) also found increased hepatocyte size under timed feed restriction.

Vehicle and fibrotic rats responded differently to tRF regimens regarding hepatocyte nuclear area. Both tRF regimens significantly reduced nuclear area in vehicle rats that remained reduced compared to AL fed rats. No difference regarding nuclear area had been observed in fibrotic rats under tRF treatments. The decreased nuclear area may reflect the reduced ploidy state. It can be said that tRF has reduced ploidy in vehicle rats but did not affect the fibrotic rats. These results favor the hypothesis that the hepatocellular area changes under tRF was due to corticosterone because the comparatively decreased and increased hepatocellular area under tRF1 and tRF2 is not due to changes in cellular ploidy. Further we can say that tRF exposure to liver before PHx increases the cell pluripotency and replicative capacity of normal liver by decreasing binuclearity and ploidy as these are considered to be sign of terminal differentiation and senescence (Brodsky and Uryvaeva, 1977; Sigal *et al.*, 1995; Gupta, 2000; Thornburg *et al.*, 2011).

Timed feed restriction altered the hepatic tissue composition after partial hepatectomy

Hepatic tissue composition after hepatectomy showed that PHx increased hepatic lipid, DNA and protein content while glycogen levels were reduced with no effect on liver water level, 48 hours post-surgery. Harkness (1952) reported increased water and lipid content with decrease in glycogen and nitrogen levels 48 hours following PHx. tRF regimens affected the hepatic tissue composition by increasing the hepatic water, lipid and glycogen content with no effect on DNA and protein as was evident by hepatic tissue analysis.

Hepatocyte water content shows circadian rhythm that is suggested to be regulated by the light-dark cycle (Tongiani *et al.*, 1982). Tongiani *et al.* (1982) suggested that temporal feed restriction has no effect on circadian rhythms of water but Díaz-Muñoz *et al.*, (2010) detected significant variation in water content under the daytime feed restriction during the food anticipatory activity. Present study revealed that timed restricted feeding has

significant effect on hepatic water content as it was considerably raised during regeneration under the both tRF regimens compared to ad libitum fed rats.

Hepatic fat accumulation is a prominent phenomenon during liver regeneration (Koniaris *et al.*, 2003; Liao *et al.*, 2004; Michalopoulos, 2007; Dai *et al.*, 2008). Elevated lipid levels were observed in regenerating fibrotic rats in the study. Daytime feed restriction reported to lower the hepatic triacylglycerol levels that were returned to normal after feeding (Díaz-Muñoz *et al.*, 2010). Daytime feed restriction suggested to affect the rhythm of lipid metabolic contents (Escobar*et al.*, 1998). Increased hepatic lipid content was found after PHx under both tRF treatments especially in vehicle rats under the first regimen. In fibrotic rats hepatic lipid levels under timed feed restriction were more as compared to sham but less than hepatectomized animals. Zou *et al.*, (2012) observed four continuous waves of hepatocyte proliferation coupled with three waves of hepatic fat accumulation during PHx induced liver regeneration, and as increased lipid had been observed under tRF regimens so it might be the reason that at that time less hepatocyte proliferative activity was observed. Increased lipid accumulation in liver following PHx under tRF1 treatment in vehicle rats had been observed during ultrastructural studies too (Figure 3.22).

Increased hepatic DNA content because of increased DNA synthesis after PHx (Andiran *et al.*, 2000; Suárez-Cuenca *et al.*, 2008) had been observed. Feed restriction enhance hepatic DNA and protein, (Sachan and Su, 1986) but in the study temporal feed restriction showed no effect on hepatic DNA levels of regenerating livers, however, their levels had not been reduced ruling out the negative effect of tRF on hepatic DNA. Temporal feed restriction showed no effect on hepatic protein content after PHx. In non hepatectomized animals, higher protein synthesis rate by hepatocytes under DR than AL fed animals was reported. Dietary restriction prevents the age-related reduction in liver protein synthesis (Sachan and Su, 1986).

A moderate reduction in hepatic glycogen content that was reverted to normal levels after food ingestion under daytime feed restriction had been observed (Díaz-Muñoz *et al.*, 2010). The same group suggested that hepatic glycogen concentration is not under the

influence of dietary rhythms rather it is the fasting that change liver glycogen levels (Escobar*et al.*, 1998).In the present study, timed feed restriction had recovered hepatic glycogen content following PHx. tRF1 had significantly while tRF2 non-significantly increased liver glycogen levels. The results of PAS stain supported the biochemical findings. Timed feed restricted rats showed greater weight gain rate compared to AL rats after PHx suggesting that they consumed more food that led to increased glycogen content in them. The results of PAS stain and body weight gain under different feeding regimens are in agreement supporting the proposed suggestion.

Hepatic regenerative assessment following PHx under the effect of time restricted feeding by different somatometric, hyperplasic and hypertrophic factors revealed that though the tRF regimens inhibited hyperplasia and hypertrophy in regenerating liver but they improved the liver regeneration rate at the same time with no reduction in hepatic DNA and protein content alongwith other tissue constituents. Rather a shift from binuclearity to mononuclearity and decreased ploidy had been observed indicating that tRF potentiated the pluripotency and replicative capacity of regenerating liver. Moreover, in the study sampling was done on one time point only where increased hepatic lipid levels were observed that may masked the proliferative potential of regenerating liver as the phenomenon includes alternative waves of lipid and DNA synthesis (Zou *et al.*, 2012). Further studies with sampling at different circadian time points can better reveal the effect of temporal feed restriction on regenerative process especially in normal liver.

Hepatic functional capability was improved by temporal feed restriction

As liver function is impaired due to hepatic fibrosis and these livers are less able to regenerate, it is important to stimulate both the regeneration and function of the remnant fibrotic liver after hepatectomy. To evaluate liver function capability different serum biochemical tests were performed. Important liver enzymes like ALT, AST, ALP and LDH along with total bilirubin, protein and albumin, glucose and triglycerides were determined in different study groups 48 hours post partial hepatectomy.

Measurement of the hepatic enzyme activity in serum is of important value because it helps to assess the state of the liver and other organs (Venukumar and Latha, 2002;

Ulican *et al.*, 2003; Porchezhian and Ansari, 2005). Hepatic fibrosis resulted in elevated hepatic marker enzymes i.e. ALT, AST, ALP, LDH and total bilirubin in the study because of the damaged structural integrity of hepatocytes. When liver cell plasma membrane is damaged, a variety of enzymes and metabolites normally located in the cytoplasm are released into the blood circulation, a process known as cytolysis. The increased total bilirubin level in the serum could be attributed to the increased red blood cell destruction or damage of the liver tissue. In addition, hyperbilirubinaemia may result due to haemolysis and impaired secretion of bilirubin (Hall *et al.*, 2000). Hepatic fibrosis declined serum protein, albumin, glucose and triglycerides levels in the study. Several studies reported significantly lowered total protein and albumin levels during CCl_4 induced fibrosis (Huo *et al.*, 2011; Al-Attar, 2012). Declined serum glucose levels are in agreement with the reported studies (Khalaf *et al.*, 2009) but TG levels are usually raised during CCl_4 induced hepatic injury (Khan *et al.*, 2012).

In the present study partial hepatectomy led to a significant increase in liver function enzyme activities and total bilirubin levels, while significant decrease in total protein, albumin, glucose and triglyceride contents had been found in both vehicle and fibrotic rats. The obtained results regarding the effect of partial hepatectomy on the concentrations of hepatic enzymes and total bilirubin are in agreement with those reported studies of (Pelton *et al.*, 1998; Aly *et al.*, 2014; Cetinkunar *et al.*, 2015). The most likely explanation for hepatic markers elevation is their release and damage of liver parenchymal cells.

Feed restrictions results in decrease in ALT, AST, ALP, LDH, glucose and TG levels while total protein and albumin remain unaffected (Larson-Meyer *et al.*, 2008; Van Weyenberg *et al.*, 2008; Dehghan *et al.*, 2010).Both temporal feed restriction treatments had decreasing effect on serum ALT levels specially tRF1. That schedule had also restored the serum AST levels. ALT and AST are important and sensitive biomarkers of hepatic function, especially ALT. These are amino transferases enzymes, involved in the reactions of nitrogen removal from amino acids (transamination reaction). tRF treatments may improve the structural integrity of hepatocytes thus decreasing the hepatic enzyme circulation in bloodstream as ALT is the main and more specific hepatic enzyme than

AST. ALT is mainly found in liver as a cytoplasmic enzyme while AST is a cytoplasmic and mitochondrial enzyme and is found in many organs apart from liver including heart, skeletal muscle, kidney and brain tissues (Delva *et al.*, 1989).Timed feed restriction had no effect on ALP and total bilirubin levels in partial hepatectomized animals. Unchanged ALP and bilirubin levels under the feeding regimens may indicate that these treatments had no effect on the secretory mechanism of the hepatic cells. However LDH levels were reduced under the second regimen in vehicle rats indicating the improvement in liver function in these rats. As far as total protein and albumin levels are concerned, the current results demonstrated that tRF regimens had no significant effect on their levels following PHx. However, different feed restriction regimens tend to prevent the protein and albumin decline especially in aged animals (Fujita *et al.*, 1984; Hiragane and Ono 2003). In fibrotic rats increased TG content under tRF2 while decreased serum glucose levels under tRF1 were observed. The decreased glucose concentrations may be due to increased glycogenesis in these rats under the restricted feeding schedules.

Temporal feed restriction decreased the extra cellular matrix in vehicle rats with little or no improvement in fibrotic rats

As defective hepatocyte regenerative ability and increased ECM are the main causes of retarded liver regrowth after major hepatectomies in fibrotic and cirrhotic livers therefore the therapeutic strategies for improved regeneration of diseased liver must focus on ECM degradation as well as to enhance the liver regenerative capability (Hernández-Muñoz *et al.*, 2001). PHx resulted in increased ECM content in both vehicle as well as in fibrotic rats as was evident by increased expression of α-SMA (Figure 3.20) indicating that HSC got activated after PHx regardless of normal or diseased liver. Their activation in normal rats during the regenerative process shows that activated HSC play important role in this phenomenon (Mabuchi *et al.*, 2004). There are controversial reports regarding the nature of regenerated tissue in fibrotic or cirrhotic livers after PHx. Some reported diseased regenerated tissue after PHx (Andiran *et al.*, 2003) while others suggest that partial hepatectomy reduces the ECM content (Suárez-Cuenca *et al.*, 2008). Hashimoto and Watanabe (1999) also found that PHx favors functional restoration of the liver but not morphological alterations.

Temporal feed restriction has decreased extra cellular matrix in vehicle rats with little or no improvement in fibrotic rats as is evident by Gomori triple stain, α-SMA expression and hepatic hydroxyproline content (Figure 3.19, 3.20 & 3.21). That differential ECM reduction in vehicle rats is may be due to mineral alterations in the regenerating normal liver. In control liver tRF regimens tend to reduce Ca, Fe and Cu levels and as these metals are involved in collagen metabolism (Evans and Drouven, 1983; García-Fernández *et al.*, 2005) specially Fe and Cu results in fibrosis by promoting increased collagen synthesis (García-Fernández *et al.*, 2005) so reduced Fe and Cu may be responsible for decreased ECM in vehicle rats under tRF. Fibrotic rats did not show any considerable alteration in these metals under the feeding schedules so no difference in collagen content in these animals had been observed.

Time restricted feeding has produced different alterations in both adrenal and thyroid hormones with respect to vehicle and fibrotic rats

Anterior pituitary hormones display significant changes after partial hepatectomy which affect the liver regenerative process (Knopp *et al.*, 1991). Thyroid hormones support (Alisi *et al.*, 2005) whereas adrenal hormones inhibit liver regeneration (Barbason *et al.*, 1989). Partial hepatectomy has an increasing effect on serum corticosterone level 48 hour post PHx as was previously seen (Knopp *et al.*, 1991). It has been suggested that the increase in corticosterone levels after PHx is due to reduction in the hepatic capacity to inactivate corticosterone (Witek-Janusek and Marotta, 1981).

Glucocorticoids are the endocrine messengers that play important role in the entrainment of peripheral clocks (Dickmeis, 2009). Hepatic glucocorticoid signaling reported to be influenced by time feed restriction (Luna-Moreno *et al.*, 2012) though it does not raise the corticosterone levels in normal rats (Wilkinson *et al.*, 1978). In the study differential effect of timed feed restriction on serum corticosterone levels was observed. In vehicle rats no effect of temporal feed restriction had been seen while in fibrotic rats, significant decrease under tRF1 while non-significant but considerable increase under tRF2 was observed. Those corticosterone alterations in fibrotic rats influenced the mitotic and hypertrophic state of hepatocytes in these animals as

corticosterone influence mitosis by negatively regulating it (Barbason *et al.*, 1987) and also control the trophic state of hepatocytes (Tongiani *et al.*, 1982).

As both normal and malignant tissues are differentially sensitive to the temporal feed restriction (Davidson *et al.*, 2004), vehicle and fibrotic rats also behaved differently to the same feeding regimens. Temporal feed restriction uncouples the peripheral clocks from the master clock SCN especially in liver (Damiola *et al.*, 2000). SCN uses glucocorticoid signaling to prevent this uncoupling. It slows down the phase changes by the feeding regimens that are in conflict with the animals normal activity like those observed in the present study (Le Minh *et al.*, 2001). After PHx rats were moved to normal activity phase. When the animals were subjected back to normal feeding schedule glucocorticoid signaling has little effect (Le Minh *et al.*, 2001). In vehicle rats corticosterone levels remained unchanged compared to AL rats but fibrotic rats behaved differently and responded to both feeding regimens. That might be due to disturbed circadian clocks in these rats that were then differently responded by the tRF treatments. More detailed studies are required to reveal the facts as corticosterone show rhythmic behavior. Sampling at different circadian time points after PHx may better indicate the corticosterone role under tRF regimens in the hepatic regenerating animals.

There is controversial data regarding thyroid hormones alteration during liver regeneration after partial hepatectomy, some reported decrease in serum T4 and T3 levels (Knopp *et al.*, 1991; Kester *et al.*, 2009) while others observed decreased serum T4 and unchanged T3 levels during this phenomenon (Leffert and Alexander, 1976; Kester *et al.*, 2009). In the present study PHx depressed thyroid hormone levels that were recovered by temporal feed restriction in both groups under different tRF regimens as serum T4 was significantly increased 48 hours post PHx in both vehicle and fibrotic rats under the first and second schedule respectively due to which serum T3/T4 ratio was also changed and was significantly low in these animals under these treatments. Such markedly reduced serum T3/T4 ratio was reported to be beneficial for liver transplant patients (Brtko and Knopp, 1988). Moreover, neither feeding regimen has reduced serum T4 levels during liver regeneration after PHx which is thought to be detrimental for patients waiting for liver transplant (Van Thiel *et al.*, 1985) It can be said that time-restricted feeding alter

thyroid hormone levels after major hepatectomies in such a way as to improve survival rates in patients and that both vehicle and fibrotic rats behaved differently to the different tRF regimens.

Hypothyderal liver state was observed in sham fibrotic rats as liver T3 and thus T3/T4 ratio was significantly less in these animals. This hypothyderal state may be induced one, in order to protect the liver from deleterious effects of T3 on fibrosis (Zvibel *et al.*, 2010). Systemic T3 and T4 levels were affected by both PHx and time-restricted feeding but at tissue level no effect on T4 levels post 48 hour PHx was observed while T3 was decreased though not significantly in the present study. A decrease in both liver T3 and T4 after 24 hours of PHx have been reported but at 48 hours post PHx only reduction in T3 was observed (Kester *et al.*, 2009). Feed restriction to the middle of the light period effected liver T3 in both groups, especially very high levels of T3 was observed in fibrotic livers under this schedule, may be the high systematic T4 levels resulted in the high liver T3 levels in these animals.

High serum T4 was also seen in full daytime restricted fed vehicle rats but may be in fibrotic rats mid-light phase feed restriction affected thyroid hormone regulating enzymes in such a way so as to convert more serum T4 to liver T3 as it has been observed that temporal food restriction could produce a dramatic post translational change in liver D1 activity which decreases before food access and increases after food presentation (Aceves *et al.*, 2003). Moreover, D3 enzyme was found as an important modulator of thyroid hormone levels in the regenerating liver (Kester *et al.*, 2009). The increased local T3 may not be correlated with hepatic fibrosis as liver morphology was not worsened in the fibrotic rats. More detailed studies, however, are required to further reveal the facts.

Temporal feed restriction affected the liver antioxidant defense mechanism especially the* catalase activity and *lipid peroxidation system

Hepatic fibrosis leads to oxidative stress by increasing the production of free radicals and diminution in antioxidant defenses (Poli, 2000; De Minicis *et al.*, 2012). It plays important role in its development and aggravation (Lee *et al.*, 2001; Vendemiale *et al.*, 2001). PHx also results in oxidative stress (Miyake *et al.*, 2002) though there are

controversial reports regarding lipid peroxidation system (Carnovale *et al.*, 2000; Bilzerand Gerbes, 2000; Cheeseman *et al.*, 1986; Cheeseman *et al.*, 1988).Further increased oxidative stress during massive resection of cirrhotic liver may reported to be involved in impaired regeneration of cirrhotic livers (Morimoto and Isselhard, 1992).

In the study reduced hepatic CAT, POD and SOD, GSH, GSR, GSH and GPx content while increased liver TBARS levels were observed under the effect of hepatic fibrosis (Sahreen*et al.*, 2011; Chen, *et al* 2013). Hepatic POD values in sham rats were much higher than control animals however Sahreen *et al.*, (2011) observed less POD activity in CCl_4 injured rats.

PHx decreased liver CAT levels but hepatic POD and SOD content did not change 48 hours after following PHx in vehicle rats, however, in fibrotic rats POD levels were significantly decreased. That significant drop is due to increasingly high POD content in sham fibrotic rats. The decreasing effect of PHx on liver CAT was previously seen by Aly *et al.*, (2014). Increased hepatic TBARS levels alongwith reduced hepatic GPx and GSR contents were seen in hepatectomized vehicle rats. GSH levels were almost unchanged in these rats. In fibrotic animals liver GSH and TBARS content were raised with no effect on GPx and GSR activities. All these results indicate increased oxidative stress after PHx. During partial hepatectomy free radicals may accumulate as new cells are developing and increased oxygen is demanded by them during the regenerative process (Yoshioka *et al.*, 1998) thus resulting in oxidative stress (Miyake *et al.*, 2002). Controversial reports regarding the lipid peroxidative system induced by PHx have been observed. Some reported increased LP (Chen *et al.*, 1996; Bilzer and Gerbes, 2000; Carnovale *et al.*, 2000; Kaplowitz, 2000) while others observed reduced LP (Cheeseman *et al.*, 1986; Cheeseman *et al.*, 1988) following PHx. Reduced and increased hepatic GSH levels in vehicle and fibrotic livers respectively after PHx are in agreement with the reported study of Aly *et al.*, (2014).

ROS are kept at physiological levels by the processes regulated by circadian clocks in order to protect the organisms from oxidative stress (Stangherlin and Reddy, 2013). Different feeding regimens including temporal, caloric and energy feed restriction, and

intermittent fasting reported to improve the cellular stress mechanism (Eiam-ong *et al.*, 1996; Sohal and Weindruch, 1996; Ramsey *et al.*, 2000; Anson *et al.*, 2003; Mattson *et al.*, 2004). Reduced lipid peroxidation i.e. TBARS levels and increased SOD, CAT, GPx activities were found in night-time food restricted rats (Jayakumar, *et al.*, 2009). Timed food restriction also increases the ratio of unsaturated to saturated fatty acids in hepatocytes thus decreasing the lipid peroxidation (Eiam-ong *et al.*, 1996). The study revealed improvement in hepatic CAT levels in both groups under the temporal feeding regimens but no difference in POD and SOD activities of regenerating livers had been observed.

A decreasing trend in liver TBARS levels was noticed under the second feeding regimen in both groups. Interestingly vehicle rats under tRF1 had high MDA levels than fibrotic rats. These rats had more hepatic lipid content too. Due to high hepatic lipid, its oxidation rate may also be high resulting in high TBARS levels in this group. GSH and GPx were also high in these rats, that rises are may be to counteract the effect of increased malondialdehyde as GSH and GPx are the important components of cellular antioxidant defense system (Guerin *et al.*, 2001; Wang, 2005). Comparatively more rise in GSH was observed in fibrotic rats under the first regimen. These rats had very low GPx content that may result in relatively less utilization of GSH in them as it reduces peroxidaes using GSH as a substrate (Wang, 2005). Further the increased GSH levels in these rats are might be due to increasing demand of the hepatectomized fibrotic liver to challenge the increased oxidative stress caused by the surgery. Interestingly for unknown reasons low GSH levels in control livers under tRF2 had been monitored despite the fact they had significantly high GSR levels.

All these results revealed that the exposure of liver to timed feed restriction before partial hepatectomy produces specific alterations in the antioxidant defense mechanism of regenerating liver especially in the catalase content and lipid peroxidation system.

Temporal feed restriction produced important alterations in different minerals during the hepatic regenerative process

Minerals play important role during hepatic regeneration after partial hepatectomy (Milin *et al.*, 2005); hepatic fibrosis results in impaired mineral metabolism (George 2006; García-Fernández *et al.*, 2005) which might affect the regeneration process. Essential elements alterations in both serum and liver have been observed during hepatic fibrosis. Serum levels of essential metals often decrease (Podolsky *et al.*, 1973; Sullivan *et al.*, 1979; Koller and Rosenkranz, 2005; George 2006), while Fe and Cu levels are raised during hepatic fibrosis and cirrhosis (Rodríguez-Moreno *et al.*, 1997; García-Fernández *et al.*, 2005). Hepatic fibrosis has affected almost all the estimated metals in the study as was observed in pre PHx mineral analysis. It led to reduced hepatic Na, K, Ca, Mg and Zn content while Fe and Cu levels were raised. However after sham surgery Na, K, and Zn liver levels remained unchanged; Ca and Mg were reduced while Fe and Cu contents were increased indicating that sham surgery may also result in mineral alterations. Decreased albumin synthesis, gastrointestinal malabsorption and disturbances, and interactions between elements may involve in the defective mineral metabolism during hepatic fibrosis (Papadakis and Arieff, 1988; George 2006).

Significant and interconnected mineral changes take place during hepatic regeneration not only in the regenerating liver but also in the lymphatic tissues and submandibular gland, indicating their importance in the regenerative process (Milin *et al.*, 2005). Partial hepatectomy tend to significantly or non-signicantly increase the hepatic Na, K, Ca and Mg levels while Fe and Zn contents were decreased in both vehicle and fibrotic rats however Cu levels were decreased in fibrotic rats only with little or no increase in vehicle rats. An accumulation of different minerals including Ca, Mg, Fe and Zn in regenerating liver following partial hepatectomy has been observed (Milin *et al.*, 2005). Srivastava *et al.*, (1988) detected a decrease in hepatic iron and copper content following PHx while Al-Othman *et al.*, (1986) observed decreased plasma Zn in regenerating liver after PHx. The difference in hepatic mineral alterations post PHx in different studies including the present study may be due to the extent of hepatectomy and time point of the tissue collection post-surgery.

Circadian rhythms of the different essential, trace and toxic metals has been found in blood, plasma and urine (Bhattacharya, 1986; Lifschitz and Henkin, 1971; Yokoyama *et al.*, 2000). Data on the effect of feed restrictions on minerals is scarce however it has been found that the hepatic subcellular distribution of Ca, Fe, Cu and Zn is subjected to feed restriction (Miranda *et al.*, 1984).

Na and K are the major cations of intracellular fluid that are involved in cell cycle regulation and proliferation (Koch and Leffert, 1979; Urrego *et al.*, 2014). No significant change in hepatic Na and K contents were observed in the study except a considerable decrease under the second feeding regimen in vehicle rats that tend to raise their levels during the regenerative process, but that levels were not high compared to the control hepatectomized rats. As their levels remained comparatively unchanged therefore no effect of these minerals on hepatic regeneration had been considered.

Hepatic Ca was decreased in vehicle rats by tRF regimens, while in fibrotic rats treatments had no effect and Ca levels remained unchanged. Calcium plays important role in collagen metabolism (Evans and Drouven, 1983). Decreased serum, while increased hepatic Ca was observed during hepatic fibrosis in rats (George, 2006). Increased intracellular Ca ions influx in hepatic stellate cells mediated by TGF-β1, may be involve in increased collagen synthesis (McCarthy *et al.*, 2001). Decreased Ca ions alongwith decreased Fe and Cu in vehicle rats under the temporal feed restriction may be one of the reasons for less collagen content in these animals compared to fibrotic rats which showed no response in the above mentioned minerals content under the tRF regimens. It must be noted that calcium is necessary for optimum activity of matrix metalloproteinases, the collagen degrading enzymes (McCarthy *et al.*, 2001; Reuben *et al.*, 2002) so a precise balance of Ca is very necessary for optimal collagen content in regenerating liver.

Interestingly the first feeding regimen elevated while the second schedule has depressed the hepatic magnesium levels during regeneration. Magnesium is an important micronutrient that plays vital role in regulating metabolism, growth and proliferation of mammalian cells (Rubin, 1975; Rubin *et al.*, 1979). Mg promotes cell proliferation as it stimulates DNA and protein synthesis. Its levels remain low in non-proliferating cells

(Wolf and Cittadini, 1999). The increased inhibitory effect of tRF2 on hepatocyte proliferation may also involve the markedly decreased Mg levels in these rats compared to untreated hepatectomized rats. The first regimen has significantly high Mg content than the respective controls but still they have less proliferation index due to some other unknown reasons. The increased lipid may be one of the reasons as was discussed above.

Fe and Cu were reduced under the tRF treatments in vehicle rats and may be responsible for decreased ECM in these rats as they promote oxidant forces and are involve in collagen biosynthesis (Bacon and Britton, 1989; Sokol, 1996). Their levels rise during cirrhosis (García-Fernández*et al.*, 2005). As fibrotic rats were unresponsive to these metals under tRF regimens so no considerable decrease in collagen content in these rats could be detected.

Timed feed restriction tends to raise the hepatic Zn levels in fibrotic rats during the liver regenerative process. Metallothionein, metalloproteinases and tissue inhibitors of metalloproteinases all are involved in the ECM regulation during liver regeneration (Mohammed and Khokha, 2005; Oliver *et al.*, 2006) and zinc play important role in this system (Cherianand Kang, 2006). Important growth factors, including hepatocyte growth factor, are bind to hepatic ECM that are released by this system (Mohammed *et al.*, 2005). The increased zinc levels in fibrotic rats may help the fibrotic liver to remodel its ECM in this way as to release more growth factors necessary for regeneration.

From the above discussion it could be concluded that temporal feed restriction tend to produce important hepatic mineral alterations during the regenerative process especially in normal liver.

Conclusion

Hepatic regenerative assessment following PHx under the physiological effect of time restricted feeding revealed that the feeding regimens inhibited hyperplasia and hypertrophy in regenerating liver but at the same time they improved the liver regeneration rate with no reduction in hepatic relative weight, DNA and protein content alongwith other tissue constituents. Rather a shift from binuclearity to mononuclearity and decreased ploidy had been observed indicating that tRF potentiated the pluripotency

and replicative capacity of regenerating liver. The tRF regimens improved the hepatic functional and antioxidant capacity and results in specific mineral alterations that lead to hepatic extracellular matrix remodeling during regeneration. Temporal feed restriction had produced different alterations in adrenal and thyroid hormones with respect to vehicle and fibrotic rats, which further influence liver regeneration being bound in a complex close relation with liver. In the study sampling was done on one time point only where increased hepatic lipid levels were observed that might mask the proliferative potential of regenerating liver as the phenomenon includes alternative waves of lipid and DNA synthesis. Further studies with sampling at different circadian time points can better reveal the effect of temporal feed restriction on regenerative process especially in normal liver.

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