Sıçanlarda Parsiyel Hepatektomi Sonrası Karaciğer Rejenerasyonunda Gallik Asit Etkisinin PI3K/AKT/mTOR Yolağı Üzerinden Belirlenmesi

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I hereby declare that this thesis titled “Determination of Effect of Gallic Acid on Liver Regeneration of Rat Through PI3K/AKT/mTOR Pathway After Partial Hepatectomy.” has been prepared in accordance with the thesis writing rules of Eskişehir Osmangazi University Graduate School of Science under the academic consultancy of my supervisor Prof. Dr. Mediha CANBEK. I hereby declare that this DOCTOR OF PHILOSOPHY THESIS is an original work prepared by me; that I have respected scientific ethical principles and rules in all stages of my thesis, data collection, analysis, and presentation of my work; that I have cited the sources of all the data and information that could be obtained within the scope of the study and included these sources in the section of references.

I also declare that animal experiments of this study were carried out by Eskişehir Osmangazi University Rectorate. With the decision number 773 and 814 of the Animal Experiments Local Ethics Committee (HADYEK) 9.10.2020 and 27.11.2020 carried out with permission. 23/02/2023.

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Signature

ÖZET

Karaciğer kendini yenileme ve onarma yeteneğine sahip eşsiz bir organdır. Hasar gördüğünde tam çalışma kütlesine ulaşmak için çoğalmaya başlar. Kısmi hepatektomiden sonra, karaciğer ayrıca hücre replikasyonunu ve gelişimini moleküler mekanızmalarını kullanarak aktive eder.

Hepatosit genişlemesi; büyüme hormonları, hücre içi aracılar (EGF, HGF, TGF-, IL-6, vb.) ve moleküler mekanizmalar tarafından indüklenir. PI3K/AKT/mTOR karaciğer rejenerasyonunda rol oynayan çok önemli bir moleküler yolaktır. %70 parsiyel hepatektomiden sonra PI3K/AKT/mTOR yolu aktive olarak hepatosit sayısının normale dönmesini sağlar.

Karaciğerin yenilenme yeteneğini değiştiren yeni farmakolojik ürünler ile karaciğer fonksiyon bozukluğu ile mücadele edilebilir. Oksidatif strese ve serbest radikallere karşı, polifenollerin karaciğeri korumada etkili olduğu kanıtlanmıştır. Birkaç fenolik asit arasında Gallik asit antioksidan özellikleri ile öne çıkmıştır. Kapsamlı araştırmalara rağmen, bu maddenin potansiyel karaciğer yenileyici etkisi ve mekanizması henüz bilinmemektedir.

Bu amaçla çalışmamızda Gallik asitin sıçanlarda (Erkek Wistar albino) rejeneratif etkisine bakıldı. Ayrıca rejeneratif etkisi kanıtlanmış Silmarin maddesi de pozitif kontrol olarak çalışmamızda yer aldı. Deney gruplarımız 4 ana grup ve 13 alt gruptan (n=6) oluştu. Grup I (kontrol), Grup II (PHx+SF), Grup III (PHx+silmarin100 mg.kg-1i.p), Grup IV (PHx+gallik asit 200 mg.kg-1i.p). Daha sonra %70 PHx gerçekleştirildi. Deney hayvanları 6, 12, 24, 48 saatlik sürelere kadar yaşatıldı. Deney sonunda PI3K/AKT/mTOR yolağına ait 6 gen ekspresyonu RT-PCR ile incelendi. Daha sonra PI3K/AKT/mTOR yolağına ait en etkin olan genler seçilerek protein analizleri western blot ile değerlendirildi. PCNA immunohistokimya analizleri yapılarak hücre proliferasyonuna bakıldı. Son olarakta karaciğer hasarının belirteci olan ALT ve AST değerlerinin ölçümleri yapılarak karşılaştırıldı.

Çalışmamız sonucunda PIKCA gen ekspresyonuna baktığımızda SF (II) 6 saat ve SLY (III) 6, 24, 48 saat ve GA (IV) 6, 12, 24, 48 saatleri arasında anlamlı fark (****, P<0.0001, *** P0.0004, *** P0.0002, ** P0.0038, ****P<0.0001, ***P0.0004, ****P<0.0001) bulundu. Protein ifadesinde ise grublar arasında (****<0.0001) olarak fark bulundu.

AKT1 gen ekspresyonunu ise Kontrol ve deney grupları arasında SF (II) 6, 12, 24, 48. saat, SLY(III) 6, 12, 24, 48.saat ve GA (IV) 6, 12, 24, 48 saatlerinde büyük ölçüde farklı p değeri (****, 0,0001) olduğunu gözlemlendi. AKT1 protein ekspresyonu SF (II) 12 ve GA (IV) 12 saat arasında (*P0.019, ***P0.0003) anlamlı bir fark tespit edildi.

mTORC1 gen ekspresyonunun SF (II), SLY(III) ve GA (IV) tüm grupları arasında önemli ölçüde farklı (****P0.0001) olduğu ortaya çıktı. SF (II) 12 saat SLY (III) ve GA (IV) 24, 48 saat sonra protein ekspresyonunu gösteren western blot üzerinde net bir bant ölçüldü.

Siklin D1 gen expresyonunda SF (II) 6, 12, 24, 48 saat ve GA (IV) 24 saat arasında istatistiksel olarak (**P0.0063, ****P0.0001, *P0.0144, ****P0.0001, *P0.0115, ****P0.0001) anlamlı bir fark ölçüldü. Ayrıca gruplar arasında SLY (III) 6, 12, 24, 48 saat ve GA (IV) 6, 24 saat (*P0.0133, ****P 0,0001, *P0.015, ****P0,0001, *P0,0166, ****P0,0001, ***P 0,0003) fark bulundu. GA (IV) 12 ile 24 saat ve GA (IV) 24 ile 48 saat arasında istatistiksel olarak anlamlı fark (**P0.0098, ***P0.0005) belirlendi. Aynı zamanda SF (II) 48 ile SLY (III) 48 ve GA (IV) 48 saatleri arasında CyclinD1 protein ekspresyon seviyeleri karşılaştırmasında gruplar arasında istatistiksel olarak anlamlı fark bulundu (****P0.0001, * P0.0252). Bulgularımıza göre, Siklin-D1 geninin ifade miktarı nispeten yüksek ölçüldü. Bunun nedenini Siklin D1 ve Siklin D ailesinin diğer türleri, hücre büyüme sürecine katkıda bulunmak için birlikte işlev görmesine bağlayabiliriz. Bu nedenle PI3K/AKT/mTOR yolağında yer alan farklı tipte siklin D tipi proteinlerinin görev aldığını düşünmekteyiz.

Çalışmamızda Gallik asitin sıçanlarda %70 parsiyel hepatektomi sonrası 200 mg.kg-1 i.p. dozunun ilk kez keşfedilen yenileyici faydaları çalışmamızda ortaya kondu. Sonuç olarak tüm gruplarda Gallik asit PI3K/AKT/mTOR yolağını kullanarak hücre büyümesini ve proliferasyonu arttırdığını verilerimizle ortaya kondu. Ancak bu yola ait Cyclin D1'i

aktive eden transkripsiyon faktörü S6K701 geni ifade edilmedi. Buda bize Cyclin D1'in aktivasyonunun üçüncü bir bileşenden kaynaklanabileceğini düşündürdü. Ayrıca araştırmamızda ilk defa PIKCA(ser317) protein ekspresyonu kullanılmış ve tüm grupların protein ekspresyonunun yanı sıra gen düzeylerinin de yüksek olduğu saptanmıştır.

ALT ve AST düzeyleri ise Gallik asit gruplarında 48. saatte diğer gruplara göre en düşük olarak kaydedildi. Bu bize karaciğeri koruma yeteneklerini de göstermiş oldu. PCNA değerlendirmesinde ise rejeneratif etkinin tüm gruplarda 48. saatte en yüksek olduğu görüldü. Bazı hücrelerde yoğun boyanma bazılarında ise daha zayıf boyama tespit edildi. Bazı hücrelerin ise mega hücreler oluşturduğunu veya çift çekirdekli hücrelere füzyon yaptığı görüntülendi.

Sonuç olarak çalışmamızda Gallik asit karaciğer rejenerasyonuna etkisi PI3K/AKT/mTOR moleküler yolağı ile tanımlanmış ve hepatosit hücrelerinin proliferasyonunu arttırdığı kanıtlanmıştır. Ayrıca Gallik asid (200 mg.kg-1i.p) dozunun rejeneratif etkisi bilinen Silmarin göre daha etkin proliferatif etki gösterdiğini söyleyebiliriz.

Anahtar Kelimeler: Karaciğer rejenerasyonu, PI3K/AKT/mTOR, gallik asit, silmarin.

SUMMARY

The liver is a unique organ with the ability to regenerate and repair itself. After partial hepatectomy, the liver activates cell replication using its molecular mechanisms. Hepatocyte enlargement; induced by growth hormones and other intracellular mediators (EGF, HGF, TGF-, IL-6, etc.). The PI3K/AKT/mTOR pathway has a very important function in liver regeneration. After 70% partial hepatectomy, the PI3K/AKT/mTOR pathway is activated, allowing the hepatocyte count to return to normal. Polyphenols have been proven to be effective in protecting the liver. Among several phenolic acids, gallic acid have high profile antioxidant properties. Despite extensive research, the potential liver regenerating effect on PI3K/AKT/mTOR pathway of this substance are not yet known.

For this purpose, the regenerative effect of Gallic acid in rats (Male Wistar albino) was examined in our study. In addition, Silymarin substance with proven regenerative effect was included in our study as a positive control. Our experimental groups consisted of 4 main groups and 13 subgroups (n=6). Group I (control), Group II (PHx+SF), Group III (PHx+simarin100 mg.kg-1i.p), Group IV (PHx+gallic acid 200 mg.kg-1i.p). Then PHx was performed. Experimental animals were kept alive for 6, 12, 24, 48 hours. At the end of the experiment, the expressions of 6 genes belonging to the PI3K/AKT/mTOR pathway were examined by RT-PCR. The most active genes belonging to the PI3K/AKT/mTOR pathway were selected and protein analyzes were evaluated by western blot. Cell proliferation was examined by PCNA immunohistochemistry analysis. Finally, ALT and AST values, which are indicators of liver damage, were compared by looking at their enzymes.

As a result, ALT and AST levels were analysed as the lowest in the Gallic acid groups at the 48th hours. In PCNA evaluation, it was observed that the regenerative effect was highest at the 48th hours. GA was first time describes in partial hepactectomy model through PI3K/AKT/mTOR molecular pathway in liver regeneration and its showed proliferation of hepatocytes cells. Moreover, we can say that the dose of gallic acid (200 mg.kg-1i.p) showed a more effective proliferative effect than silymarin, whose positive regenerative effect is known.

Keywords: Liver regeneration, PI3K/AKT/mTOR, gallic acid, silymarin

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LIST OF CONTENTS

Page

ÖZET vi
SUMMARY ix
ACKNOWLEDGMENT x
LIST OF CONTENTS xi
LIST OF FIGURES xvi
LIST OF TABLES xv
LIST OF ABBREVIATIONS AND SYMBOLS xvi
1. INTRODUCTION AND PURPOSE 1
2. LITERATURE REVIEW 4
3. GENERAL INFORMATION 8
3.1. Human Liver Anatomy 8
3.2. Rat Liver Anatomy 9
3.3. Partial Hepactectomy 12
3.4. Liver Regeneration 13
3.4.1. Priming Phase 16
3.4.1.1. Homodinemic changes 16
3.4.1.2. Activation of uPA 17
3.4.1.3. Extracellular Rearrangement 17
3.4.2. Responsive Phase 18
3.4.2.1. Cell cycle 19
3.4.3. Termination of liver regeneration 21
3.5. PI3K/AKT/mTOR signaling pathway: 21
3.6 .Gallic Acid 26
3.7. Silymarin 29
4. MATERIALS AND METHOD 33
4.1. Experimental Animals and Groups 33
4.2. Hepatectomy Procedure: 35
4.3. Anesthesia 36
4.4. Substance Administration to Animals 37
4.5. The wet weights of each of the livers. 37
4.6. Biochemical Analysis 38
4.7. Molecular Biological Analysis 38
4.7.1. RT-PCR Analysis 38

LIST OF CONTENTS (Continued)

Page

4.7.2. RNA Isolation 40
4.7.3. Primers Used 42
4.7.4. Real Time PCR 43
4.7.5. RT-PCR Repeat Number: 43
4.8. Western Blot Analysis 44
4.8.1. Preparation of Samples 45
4.8.1.1. Incubation 45
4.8.1.2. Execution 45
4.8.1.3. Membrane Transfer 46
4.8.1.4. Display 47
4.8.1.5 Antibodies Used 47
4.9. PCNA 49
4.9.1 Immunohistochemistry 50
5. RESULTS AND DISCUSSION 52
5.1. Liver Regeneration Rate 52
5.2. PCNA analysis 53
5.3. Heat Map 58
5.4. RT-PCR and western blot Results 59
5.4.1. PIK3CA Gene Expression: 60
5.4.1.1. PIK3CA protein expression 62
5.4.2. AKT1 gene expression: 64
5.4.2.1. AKT1 protein expression 67
5.4.3. mTORC1 gene Expression 67
5.4.3.1 mTORC1 protein Expression 70
5.4.4. EIF4EBP1 72
5.4.5. P70S6K1 gene expression 74
5.4.6. CYCLIND1 75
5.4.6.1 Cyclin D1 protein expression 77
5.5. AST and ALT analysis 82
6. CONCLUSION AND RECOMMENDATIONS 88
Future Perspective of our Research 89
REFERENCES 91
APPENDIXES 113
APPENDIX- A. Ethics Committee Report 113

LIST OF FIGURES

Figure **Page**

3.1. A lobule of the liver is shown. 9
3.2. A is ventral view of the intraoperative situs..... 10
3.3.Weight distribution of rat liver lobes..... 11
3.4. Intraoperative pictures of the situs before and after resection of defined liver lobes in the 70% liver resection……..……...13
3.5. Displays a timeline of the primary processes that take place during the early stages of liver regeneration after partial hepatectomy. 18
3.6.The cell division cycle..... 19
3.7.Cascade of PI3K/AKT/mTOR signalling pathway 25
3.8. Structure of Gallic acid (3,4,5-trihydroxicbenzoic acid)……27
3.9. Sylimarin and its derivatives effects on Phase I and Phase II……31
4.1. Partial removal of the liver from a rat (partial hepatectomy)..... 36
5.1. Liver regeneration rates ……52
5.2. PCNA rates ……..……..…….…….54
5.3. SF Groups of PCNA. 55
5.4. SLY Groups of PCNA..... 56
5.5. GA Acid Groups of PCNA 56
5.6. Special Character of PCNA cells 57
5.7. Heat Map PI3K/AKT/mTOR patway..... 58
5.8. Heat Map Statistics PI3K/AKT/mTOR patway 59
5.9. PİK3CA gene expression..... 62
5.10. (a) PIK3CA band images from Western Blot analysis 62
5.10. (b) PİK3CA protein expression graph. 63
5.11. AKT1 gene Expression graph……..…… 66
5.12. (a) AKT1 band images from Western Blot analysis……67
5.12. (b)AKT1 protein expression graph…….. 67
5.13. mTORC1 gene expression graph 70
5.14. (a) mTORC1 bands from Western Blot analysis..... 70

LIST OF FIGURES (Continued)

Figure **Page**

5.14. (b) Pictures of mTORC1 protein expression graph 71
5.15. EIF4EBP1 gene expression..... 73
5.16. P70S6K1 gene expression..... 74
5.17. CyclinD1 gene expression 77
5.18. (a) Cyclin D1 band images from Western Blot analysis are shown……77
5.18. (b) Cyclin D1 protein expression graph ……...78
5.19. The involvement of 4E-BP1 in the connection between cyclin D1 and mTORC1. 81
5.20. ALT level in Groups..... 82
5.21. AST level in Groups..... 82
5.22. The mTOR pathway in the liver establishes a connection between cyclins D and E in hypatocytes……..……..86

LIST OF TABLES

Table **Page**

4.1. Insulation Materials 39
4.2. Primer Name…..……...42
4.3. PCNA Materials 49
5.1. Fold change (2-ΔΔCt) Genes…….……..……60
5.2. PiK3CA Gen ifadesi 61
5.3. AKT1 gen ifadesi 64
5.4. mTORC1 gene Expression…..…….……...69
5.5. EIF4EBP1 gene expression…..…….....72
5.6. P70S6K1 gene expression..... 74
5.7. CYCLIN D1 gene expression 76

LIST OF ABBREVIATIONS AND SYMBOLS

Symbols	Descriptions
Kg	Kilogram
µL	Microlitre
µM	Micromolar
µm	Micrometre
Min	Minute
G	Gram
G	Gravity
^{o}C	Centigrade degree
Mg	Miligram

Abbreviations	Descriptions
PBS	Phosphate-buffered saline
PH	Partial Hepatectomy
ROS	Reactive Oxigen species
MDA	Malondialdehite
CAT	Catalase
SOD	Dismutase
ALT	Alanin aminotransferase
AST	Aspartate transaminase
SLY	Silymarin
GA	Gallic Acid
PI3Ks	Phosphatidylinositol 3-kinases
HSCs	Hepatic stellate cells

LIST OF ABBREVIATIONS AND SYMBOLS (Continued)

Abbreviations	Descriptions
AKT	Protein Kinase B,
4E-BP1	Eukaryotic initiation factor 4E-binding protein 1
CCl_4	Carbon tetrachloride
STAT3	Signal transducer and activator of transcription
HGF	Hepatocyte growth factor
EGF	Epidermal growth factor
TGF	Transforming growth factors
ECM	Extracellular matrix
CLDs	Cytoplasmic lipid droplets
FGF	Fibroblast growth factor
LR	Liver Regeneration
ECM	Extracellular matrix
uPA	Urokinase plasminogen activator
MMP9	Matrix metalloproteinase 9
TPA	Plasminogen activator
FoxO1	Forkhead box protein
FoxO3	Forkhead box protein
FoxO4	Forkhead box protein
eNOS	Endothelial nitric oxide synthase
IRS1	Insulin receptor
PMNs	Polymorphonuclear leukocytes
RANKL	Receptor activator of nuclear factor kappa-B ligand
OPG	Osteoprotegerin
MSH	Melanocyte-stimulating hormone
UGDH	UDP-alpha-D-glucose 6-dehydrogenase
COS	Chitooligosaccharide acid
PVA	Polyvinyl alcohol
ADR	Driamycin

LIST OF ABBREVIATIONS AND SYMBOLS (Continued)

Abbreviations	Descriptions
GSH	Glutathione peroxidase
MDA	Mitochondrial malondialdehyde
Phase I	P450
Phase II	Glutathione S-transferase (GST), glutathione reductase (GR), and glutathione peroxidase)
OSI	Oxidative stress index
I/R	Ischaemia-Reperfusion
O.D	Optical density
MTX	Methotrexate
GGT	Gamma Glutamyltransferase
DEN	Diethylnitrosamine
AP1	Activating protein 1
pERKs	Phosphorylated extracellular signal-regulated kinases
C/EBP	CCAAT-enhancer-binding protein
LPS	Lipopolysaccharide
ICAMs	Intercellular adhesion molecules
CDK	Cyclin-dependent kinase
PCNA	Proliferating Cell Nuclear Antigen
TNF	Tumor necrosis factor
IGFBP1	Insulin-like growth factor binding protein 1

1. INTRODUCTION AND PURPOSE

The liver regulates and controls the functions of the brain, spinal cord, immune system, and endocrine glands because it act as the body's metabolic hub. The liver's parenchyma able to regenerated after damage, let the liver to heal itself. The liver is in control of this repairs (de Miguel et al., 2019).

In 1931, Andre and Higgins first time research on liver regeneration in both rodents (rats and mice) by performing a procedure known as a 2/3 partial hepatectomy, in this procedure removing two-thirds of the liver mass that is called partial hepactectomy (PHx) (Kamali et al., 2021). The time range is from five 5-7 days. The coming after, which were started by PHx, took place in a very specific order. Hepatocytes undergo cell division in the early stages of their development in order to make new DNA. 60% of hepatocytes will finish the process of DNA synthesis during the initial round of replication. The first round of replication in rats reach their peak in 24 hours, but in case of mice reach theirs at 36 hours. A minimal greater proportion of cells copy their DNA, which causes the total number of hepatocytes to come back to where it was before its original mass (Michalopoulos, 2007).

Following PHx treatment, there is a time-dependent change in the kinetics of DNA synthesis that takes place in a diffirent type of liver cells. The four basic cell types that responsible the liver develop at distinct times. The liver hepatocytes reach their maximum rate of proliferation after 24 hours, but in other cell types do not reach their maximum until much later. Hepatocytes are responsible for secreting growth factors, which in turn stimulate these cells. Based on these findings, a paracrine mechanism might be responsible for the growth stimulation that secrete by hepatocytes provide to their surrounding cells (Michalopoulos & Defrances, 1997).

The molecular signaling pathways known as PI3K/AKT/mTOR, and p70S6K are responsible for regulating not for the hepatic stellate cells (HSCs) proliferation but also its play a key role in the development of hepatic fibrosis. In addition, the PI3K/AKT/mTOR pathways are involved in the autophagy process. When PI3K is activated its activate to PIP2 and PIP2 is converted into PIP3 by a process called phosphorylation. Moreover to PDK1

and AKT, the cell membrane is regulated by PIP3, which is works as a regulator. By phosphorylating mTOR, the AKT kinase can modulate the activity of the p70S6K protein. mTOR is a protein that can be broken down into two different regulatory subunits known as mTORC1 and mTORC2 (Y. Liu et al., 2022). In human cancer, frequent mutations could found in the PIK3CA gene. This gene is responsible for coding for the catalytic subunit p110a of phosphatidylinositol 3-kinases (PI3Ks) (Bader et al., 2006).

Furthermore, the enzyme AKT, which is called Protein Kinase B, is responsible for regulating metabolic processes as well as cell survival and proliferation. In mostly adipose and skeletal tissues are the first locations where AKT2 is located, in contrast to the widespread distribution of AKT1 in the body. AKT1 is activated by molecular signaling pathway that is mediated by insulin pathways. AKT identified two potential sites for phosphorylation: Thr 308 and Ser 473. mTORC2 is reason for phosphorylating the Ser473 site, while PI3K/AKT/mTOR is responsible for phosphorylating the Thr 308 site(L. Liu et al., 2022; Wan & Helman, 2003).

The PI3K/AKT/mTOR signaling pathways are critically important to the several biological process such as apoptosis, proliferation, autophagy, and liver regeneration. The ability of mTORC1 (ser 2448) to control S6K1 and 4EBP1 was investigated by the researchers as a potential of stimulating mRNA translation and the synthesis of proteins (F. Liu et al., 2022; Ma et al., 2022).

The mTORC1 signaling pathway mostly used in cancer treatment. Rapamycin its inhibitor of mTORC1, inhibits cyclin D 1 regulations in MCF7 breast cancer cells without changing cytoplasmic levels of its mRNA. When compared to leucine deprivation (which more potently inhibits mTORC1 signaling than rapamycin), rapamycin can not affect cyclin D1 expression in various cell types. The behavior of eukaryotic initiation factor 4E-binding protein 1 (4E-BP1, a mTORC1 target that controls translation initiation). The siRNA suppression of 4EBP1 abolishes and the effect of rapamycin on cyclin D1 expression and promotes cyclin D1 mRNA in polysomal association. 4EBP1 is a main trancription factor that regulate cyclin D1 expression, indicates that variations in cyclin D1 mRNA levels in the cytoplasm do not influence this effect (Averous et al., 2008).

In following treatment of liver regeneration, new aspect of pharmacological treatments that can help liver regeneration capability are important. It is important to understand the mechanisms of drug action with pharmacological potential and the cellular events that is involved in liver regeneration in order to develop effective pharmacological methods. A popular phenolic acid with antioxidant characteristics is known as gallic acid. It has been the subject of extensive research, although its potential regenerative impact on molecular pathway is yet unknown. Gallic acid's efficacy was studied by contrasting it to that of silymarin, that is well-known regenerative drug using in this period.

Grapes, green tea, and pomegranates are all good sources of the phenolic compound known as gallic acid (3, 4, 5-trihydroxy benzoic acid). Gallic acid antioxidant which found in several foods. In diffirent studies, gallic acid have a variety of biological effects, such as anti-inflammatory, anti-mutagenic, anti-oxidant, anti-tumor, and anti-neoplastic properties. These are just a few of the many biological actions. In rats with carbon tetrachloride (CCl_4)-mediated hepatotoxicity, gallic acid and catechin (GC) were examined for their effects on biochemical and inflammatory markers; however, the investigations were modest and contradictory (Oriakhi & Orumwensodia, 2021).

In this study, we investigated the potential regenerative effects of gallic acid in liver regeneration following partial hepatectomy. Specifically, we looked at the activation of the PI3K/AKT/mTOR pathway at 6, 12, 24, and 48 hours after the procedure. Additionally, gene expression levels were analyzed using an RT-PCR method that contained 6 genes and 4 proteins. This study fills a gap in the literature by previously unknown research on the PI3K/AKT/mTOR pathway in liver regeneration following partial hepatectomy (PHx).

Our studies aim to add to the growing research of knowledge by illuminating previously unknown data on the PI3K/AKT/mTOR pathway and in addition the molecular mechanisms underlying the process of regeneration. Almost no research has been done on this specific field of gallic acid. What this does to the liver's ability to repair itself is unknown. This is the first study to examine the mechanism of action by tracing molecular signaling pathways. This research fills a gap in the literature by providing previously unknown information on the PI3K/AKT/mTOR pathway's role in liver regeneration following partial hepatectomy.

2. LITERATURE REVIEW

The liver is able to regulating the quantity of glucose and fatty acids that are made available to both the muscles and the brain. It's possible that the fundamental role that the liver plays as the "supplier" of metabolism for all of the body's tissues is responsible the significant correlation between liver weight and total body weight. It is possible that the liver-body weight ratio algorithm will evolve as a result of physiological changes brought on by shifting bodily circumstances. Your "liver to body weight ratio" will alter under the condition of your body, such as condition is cachexia, puberty, pregnancy, or a chronic illness (LBWR) (Liver Regeneration 2020, n.d.).

The liver regeneration process is done by the loss of hepatic tissue. It has been referred to as a mythological occurrence for thousands of years, but modern research has revealed that it is a combind reaction to specific stimuli which involves alterations to gene expression, growth factors synthesis, and morphological structure. In liver regeneration process growth factors and cytokines play an important role, such as hepatocyte growth factor, epidermal growth factor, transforming growth factor- α, interleukin-6, tumor necrosis factor, insulin, and norepinephrine (Michalopoulos & Defrances.,1997).

The majority of the earlier research on the signals that are largely involved in controlling hepatic regeneration in mouse livers that have not been damaged through inflammatory processes which carried out in mice. The livers of rodents are divided into lobes, and each lobe has its independent network of blood arteries (arterial and portal veins) as well as its branch of the bile duct. Higgins and Ande are first to describe the uncomplicated surgical procedure of PHx (Taniguchi et al., 2001).

Additionally, increases in plasma IL-6 levels following PHx occur with increases in circulating tumor necrosis factor (TNF), the first stimulator of IL-6 is release. The genes involved in cell proliferation are upregulated through IL-6 signaling because they are regulated by the signal transducer and activator of transcription factor STAT3. Meanwhile, hepatic stellate cells (HSCs) initiate hepatocyte growth factor (HGF) synthesis, which

triggers cell cycle entry in hepatocytes. Brunner's gland in the duodenum produces epidermal growth factor (EGF), and hepatocytes create transforming growth factors (TGF). Both are autocrine and promote cell proliferation during liver regeneration (Liver Regeneration 2020, n.d.).

Moreover, the inherent roles that mitogenic regulators play in the process of liver regeneration, three major types can be identified. The effects of complete mitogens, such as hepatocyte growth factor (HGF) and its receptor (c-MET) and EGFR ligands, on hepatocyte expansion and DNA synthesis, are detrimental to liver regeneration if they are removed at the same time; (ii) the effects of supplemental mitogens, such as vascular endothelial growth factor, Notch and adrenergic amines like norepinephrine and (iii) Lastly, mitogens such as Wnt/catenin and Hedgehog, as well as signals that direct the Hippo/Yap signaling pathway, the effects of which have still not been known (Liver Regeneration 2020, n.d.).

At an early stage in the process of liver regeneration, the molecular pathways which is activated is called the Wnt/-catenin signaling pathway. During the time of liver regeneration, the -catenin target genes CCDN1 and EGFR are important. It has been shown in several studies that the activation of catenin gene is an essential factor in determining the prognosis for individuals who are suffering from liver failure. Patients with low levels of Wnt catenin gene expression have a poor prognosis for liver regeneration, whereas those whose levels are high have a better chance of effective regeneration (Russell & Monga, 2018).

In the act of hepatocyte regeneration after PHx, signaling pathways like Hedgehog and Yap have been shown to play a role in this molecular process. For example, the signals that are produced by hepatic stellate cells (HSCs) work as a paracrine stimulus for the expression of hedgehog and yap1 in hepatocytes. In the case of acute liver damage, inflammatory responses take place, in addition to a restricted accumulation of extracellular matrix (ECM). However, in cytoplasmic lipid droplets (CLDs), normal parenchymal tissue is abnormally replaced with an excessive amount of extracellular matrix (ECM) produced by HSCs, which leads to a failure in liver regeneration (Swiderska-Syn et al., 2016).

Although cellular metabolism plays key role in the process of cell division, but less information about the metabolic process that underlies the regeneration of the liver. It is possible to shed light on both normal and pathological processes of liver regeneration by gaining an understanding of the metabolic activity controls the division of liver cells. Recent research suggests that metabolic signals might be triggering regenerative mechanisms in the liver in response to liver failure (Solhi et al., 2021).

Moreover, because of its ability to repair itself, the liver has more capabilities than any other organ. It's likely that the liver, in response to several stimuli, might start replicating and multiplying to reach its full functional mass. In response to viral or chemical hepatocyte injury, as well as after partial hepatectomy, there is an increase in the rate of hepatocyte replication (Fausto et al. 2006; Miyajima and Miyaoka 2013).

In the area of liver regeneration research, a significant amount of emphasis has been placed on a wide variety of fundamental cellular and molecular processes. In this processes include cellular hypertrophy, cell division, nuclear division, polyploidy alterations, and organ size control mechanisms. Partial hepatectomy is an excellent model that used in the lab to test ideas about fundamental biological processes such as liver regeneration. The excision of the liver surgically is usually successful in treating liver tumors. The more study that is done on liver regeneration, the better the therapeutic solutions that will come out of it (Miyajima and Miyaoka., 2013).

It is well known that the process of liver regeneration is impacted by number of stimuli, both stimulate and inhibit. A growth response is start out by a conglomeration of hepatocytes and non-parenchymal cells, as well as extracellular matrix, metabolites, and nutrients. All of the following are considered progressive signals such as hepatocyte growth factor (HGF), transforming growth factor (TGF), epidermal growth factor (EGF), and insulin. Insulin and changes to the matrix are considered initial signals. A wide type of different signaling channels are responsible for mediating the regenerative response (Taub., 2004).

Liver-damaging substance, the liver cells are known to respond positively to insulin, which is believed to have a role in encouraging their proliferation. Among insulin's many effects on cells are the promotion of cell proliferation and differentiation as well as the stimulation of protein kinase and phosphatase activity. It's possible that insulin's cellular effect is controlled by signals involving PI3K (phosphoinositide 3-kinase) (Desdouets et al., 2010).

For hypertrophy to take place throughout the process of liver regeneration, the PI3K/AKT/mTOR signaling pathway needs to be activate in molecular process. This protein kinase is responsible for regulating diffirent cellular processes, such as cell growth, proliferation, survival, differentiation, and changes to the cytoskeleton. Activation of the kinases phosphatidylinositol-3 kinase (PI3K), AKT, and mTOR occur following a hepatectomy that removes 70% of the liver (Miyajima and Miyaoka 2013).

Prostate cancer cells rely heavily on PI3K and AKT activity to go forward in the G1 phase of the cell cycle. The mTOR-p70S6K signaling pathway is constitutively activated in most cancer cells for instant small-cell lung cancer cells and pancreatic cancer cell lines (Gao et al., 2015).

According to the diffirent studies, U12a blocked the progression of HepG2 cells through the G0/G1 phase and stopped the activation of the PI3K/AKT/mTOR molecular pathway. This prevented the cells from dividing. In addition, U12a induced apoptosis in HepG2 cells via acting as a mediator along the caspase signaling pathway (Yang et al., 2022).

To effectively treat liver disease, novel pharmacological approaches that influence liver regeneration are required. To protect the liver from free radicals and oxidative stress, polyphenols can be quite benificial in liver regeneration. As a phenolic acid with antioxidant properties, gallic acid has gained famous recognition in the scientific community. Despite extensive research, the potential regenerative impact on the PI3K/AKT/mTOR pathway is unknown. Our findings suggest that activating this pathway has beneficial benefits in liver regeneration.

3. GENERAL INFORMATION

The liver is the most important organ in the body and essential to the metabolic activities that take place within the body. This category of proteins is essential to the functioning of a large variety of biological process. The liver is responsible for a diversity of essential functions, such as the production of cholesterol and bile, the regulation of blood sugar levels, the storage of vitamins, and the biotransformation of both endogenous and exogenous chemicals (Fausto et al., 2006; Kamali et al., 2021).

3.1. Human Liver Anatomy

Liver transplantation between two persons can be difficult since the patient's remaining liver might not be large enough to sustain life. However, there is no known danger when ratios greater than 0.8, thus this should not be a concern (Madrahimov et al., 2006).

Each of the eight sections of the liver that Couinaud identified receives blood from the portal vein and the hepatic artery. The position and direction of the vena cava about the segment that is being removed can affect the level of difficulty associated with excision as well as the necessity of undergoing surgical procedures such as total vascular isolation to stop the loss of blood. If the lesions are located behind and close to the vena cava, then total isolation of the vena cava may be required (G. Chen et al., 2017; Mulaikal & Emond, 2012).

By the reason of the structure of the liver's functional anatomy, it is feasible hepatic segments using portal pedicles and veins. Hepatic veins are responsible for dividing the liver into four equal halves, and each of these halves has a portal pedicle within it part. Periodically, the positions of the portal pedicles and the hepatic veins will switch. These particular areas of parenchyma are referred to as portal sectors because individual portal pedicles are responsible for delivering nutrients to them. Because of this, the blood vessels known as portal scissors are the ones that carry the portal veins, and the blood vessels known as portal pedicles are the ones that carry the portal pedicles (Figure 3.1) (Bismuth, 1982; G. Chen et al., 2017).

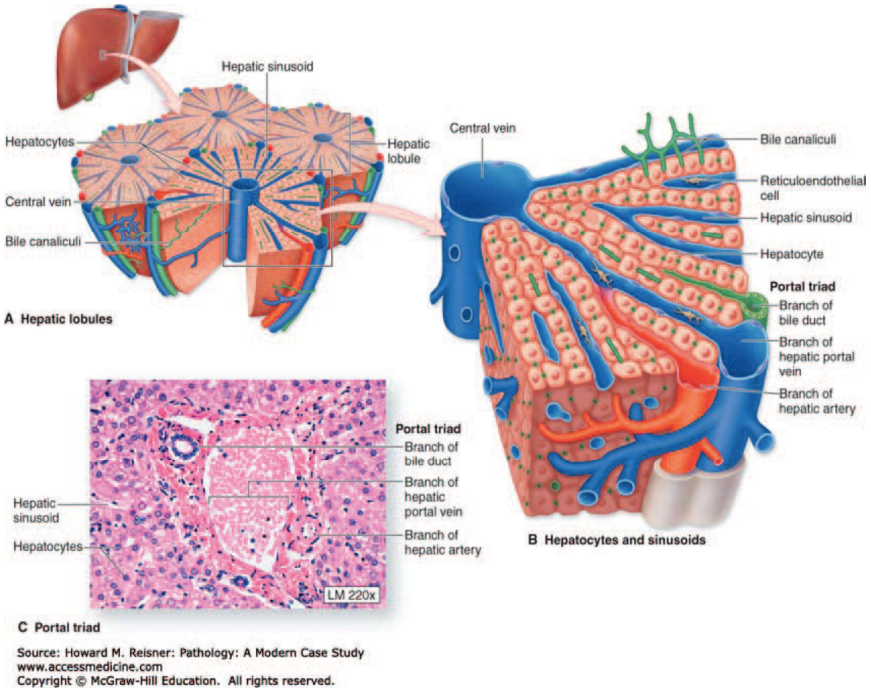


Figure 3.1. A lobule of the liver is shown. (A) Hepatic lobules. (B) Hepatocyte and hepatic sinusoidal arrangement. (C) Portosystemic biliary tree histology (From Figure 26.19 of McKinley & O'Loughlin's Human Anatomy, 2nd Edition) (Bismuth, 1982).

3.2. Rat Liver Anatomy

In the case of a rat liver, four distinct sections can be identified (As shown in Figure 3.2), the portion of the liver which is the left lobe is 30% of the organ's overall mass of rat liver. The intrahepatic vena cava is connected to the left side of the median lobe of the liver, which is where the arteries are found, by a thin pedicle. There is a connection between the left lateral lobe and the caudate gyrus that is made by interlobular ligaments (Madrahimov et al., 2006).

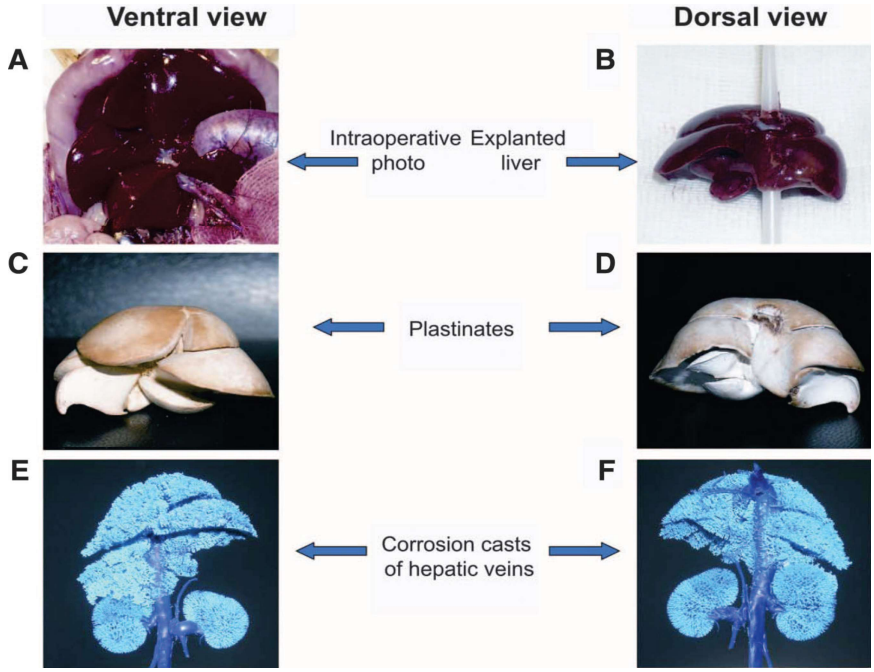


Figure 3.2. (A) is ventral view of the intraoperative situs. (B) is explanted liver-dorsal surface. (C) and (D) is epoxy plastinate ventral and dorsal surfaces. (E) and (F) is corrosion cast of hepatic veins ventral and dorsal views (Madrahimov et al., 2006; Martins & Neuhaus, 2007).

Moreover, weight of approximately 40% of the liver is distributed across the median lobe, which is split into two sections. They are separated from each another by a vast abyss. Through the falciform ligament, the xyphoid and the diaphragm are connected, which results in the formation of the interlobular fissure. The base of the median lobe of the vena cava is large, and it takes up approximately half of the total length of the vena cava. The right lobe of the liver is located on the opposite side of the vena cava. In addition, the cava has two separate lobes, which are mention to as the right superior and right inferior lobes. The right superior lobe accounts for 13 % of the liver and has the shape of an egg. It has a broad base that extends to the paracaval region of the liver, where it sits atop the intrahepatic cava (Figure 3.3) (Madrahimov et al., 2006; Martins & Neuhaus, 2007).

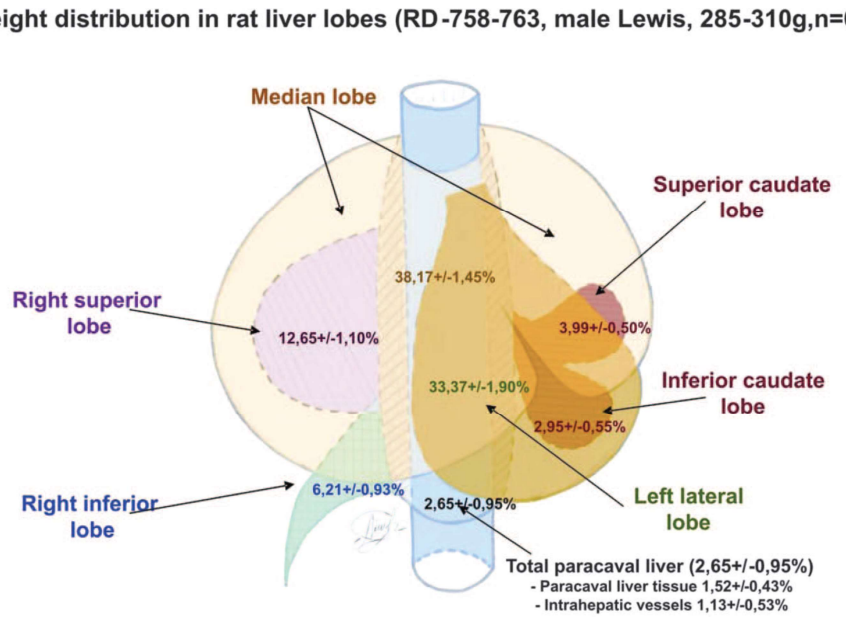


Figure 3.3. Weight distribution of rat liver lobes (Madrahimov et al., 2006; Martins & Neuhaus, 2007).

The dorsal fixation is caused through the thick ligament that connects the liver to the lungs. The right inferior lobe is the smallest of the three lobes in the liver. It accounts for 6% of the liver's total mass and look like shape of a pyramid, with its peak located close to the vena cava. On the other hand dorsal side, it is connected to the diaphragm, and on the ventral side, it is connected to the vena cava. The caudate lobe, also known as the Spiegel lobe, is situated on the left side of the cava, just below the left lateral lobe. It accounts for around 7% of the liver's total mass and is also known as the caudate lobe. The superior lobe, which is the upper lobe, and the inferior lobe, which is the lower lobe, are the two portions that make up the caudate lobe. An interlobular ligament provides the connection between the minor omentum and the lateral left lobe of the brain. The minor omentum completely envelops the superior caudate gyrus (Madrahimov et al., 2006; Martins & Neuhaus, 2007).

3.3. Partial Hepactectomy

Hepactectomy is common practice to use animal models, especially the rat model, to investigate novel features of liver regeneration. In 1931, Higgins and Anderson developed a method for performing standardized partial hepatectomy. Andersen et al., recovery of liver mass Hepatocyte proliferation and expansion are the initial steps toward after partial hepatectomy (Xu et al., 2010). After making a 1-2 cm incision in the midline of the abdomen to expose the liver, the two anterior lobes are exposed, the vascular pedicles are ligated, and the lobes are surgically removed (Taniguchi et al., 2001).

In Rats Hepatocytes start the process of DNA replication approximately 12 hours after a partial hepatectomy has been performed. DNA synthesis initially start around the 24-hour, with a second, less significant peak occurring between the 36^{th} and 66^{th}-hour time diffirence. On the day 7, the regeneration of the liver following PH will have been finished. According to the findings of the study, hepatocytes become activated after 2 hours (that is called immediate early phase); the G0/G1 transition takes place between 6 and 12 hours (the early phase); cell proliferation begins between 24 and 72 (the middle stage); and at 120-168 hours, cell proliferation occurs the study suggests (late-stage) (Taniguchi et al., 2001; Xu et al., 2010).

The lobes of the rat's liver are shaped and sized different compared to any other mammal. Because of their different topographic and circulatory architectures, lobe excisions require individualized care (Figure 3.4) (Madrahimov et al., 2006).

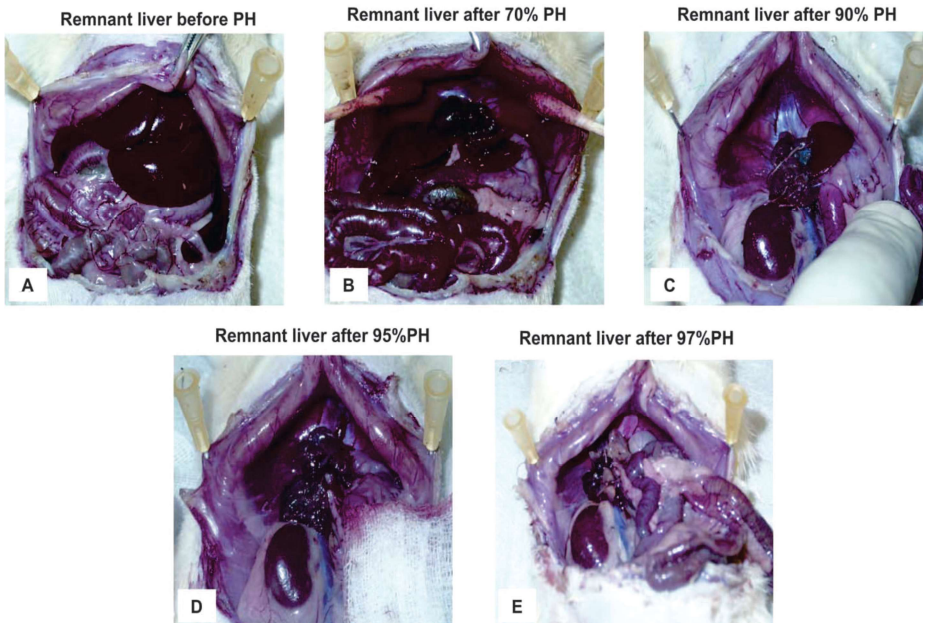


Figure 3.4. Intra-Operative pictures of the situs before and after resection of defined liver lobes in the (B) is 70% liver resection, the (C) is 90% resection model, the (D) is 95% model, and the (E) is 97% PH model (Madrahimov et al., 2006).

3.4 Liver Regeneration

The liver is conduct for directing the transport of glucose to the brain and fatty acids to the muscles. There is a important relationship between the weight of the liver and the total mass of the body. This is most likely because of the role that the liver plays as the metabolic center in the body (Michalopoulos & Defrances, 1997).

The liver is the most weighty organ in terms of its size. The digestive system functions as a sort of gatekeeper, screening and analyzing any chemicals that enter the body from the digestive tract. Surprisingly, regeneration gets continues throughout postnatal life (Michalopoulos & Defrances,1997; Michalopoulos.,2020).

Previous research have shown the slow proliferation of cells from various lobule areas to maintain the number of the liver cells and organ weight consistent under the placid calmness of the hepatic capsule. The specific reason that maintain these processes are not entirely not known. However, for operational reasons, a series of controls involving a "hepatostat" must be accepted which will decide the maintenance of liver weight to where it is necessary (Michalopoulos., 2020).

The EGFR gene has been demonstrated to be expressed in both hepatocytes and cholangiocytes. members of the ErbB receptor family have a role. Proteins called ErbB2 (Her2/Neu) are expressed by cholangiocytes, fetal hepatocytes, ERB1 (EGFR), and ERB3 (adult hepatocytes). Activating EGFR, which was expected to be present in the liver after direct exposure of hepatocytes to EGF, was actually present in the liver. Increased EGF secretion from Brunner's glands is triggered by norepinephrine (Michalopoulos & Defrances, 1997; Michalopoulos., 2007).

According to the another study, fibroblast growth factor (FGF) comes in at least 23 distinct varieties, each of which is produced by a unique collection of cells and they are capable of activating one of the four FGFR receptors. The mature hepatocytes only express FGFR4 and a few other receptors that could be found on non-epithelial hepatic cells. Mature hepatocytes can not express any other receptors. The level of FGF1/2 expression in hepatocytes is at its maximum point during the Liver regeneration (LR) . They have less than 20% of the effect that HGF and EGF have on hepatocyte cultures, although they still have a moderate mitogenic effect. It is unknown whether endothelial cells that express FGFR1/2 have autocrine or paracrine effects during LR. This is particularly true for the situation where autocrine effects are observed (Michalopoulos & Defrances, 1997; michalopoulos., 2020).

Additionally it stimulates a rapid increase in the major "acute phase reaction" ligand and the production of different proteins involved in innate immunity and IL-6 has been the subject of in-depth research in hepatocytes. When circulating IL-6 binds to a soluble receptor, the activation of hepatocyte receptors (such as oncostatin M, LIF, CNTF etc.) always occurs. During period of the Liver regeneration (LR), the main regulator of STAT3 activation is IL-6, that is produced by the lymphocyte response. After IL-6 was removed from the germline because of it STAT3 activation was delayed, which caused the regeneration process to proceed at a more gradual rate. Even additional signals (for example MET and EGFR) have that potential to activate STAT3, LR frequently continues and is ultimately successful in the liver regeneration (Michalopoulos & Defrances, 1997).

In addition, various different regenerating molecular pathways have developed over time to keep the possibility of appropriate liver functions. This is because a stable liver

function is vital for maintaining the body's equilibrium. Both cachexia and weight loss can be prevented or alleviated when an enlarged liver, what is might take place as a result of diffirent physiological conditions (such as pregnancy etc). In spite of the fact that the communication processes of these various systems are not completely yet known, it is evident that there is a 'hepatostat' that employs a method in order to maintain a liver size that is appropriate for the particular frame. This is the case even though the communication processes of these various systems are not fully understood in liver regeneration (Michalopoulos & Bhushan, 2021).

The multilobe arrangement of the rodent such as rat liver is used in the experimental model of liver regeneration after partial hepatectomy (PHx). Because of it enables the removal of individual liver lobes through a basic surgical procedure that is called PHx without inducing necrosis in the remaining lobes, that enables the analysis of solely regenerative activity on the liver regeneration. In addition, the multilobe arrangement of the rat liver makes it easier to study the effects of hepatectomy on liver regeneration. There is also the possibility of timing regenerative events following partial hepatectomy (Michalopoulos & Bhushan, 2021).

Moreover, priming phase occurs when resting hepatocytes undergo a transition from G0 to G1 of the cell cycle in response to multiple stimuli; proliferation phase occurs at hepatocytes are aided by mitogens and move past the restriction point into G1 and then mitosis started; and finally, termination occurs when cells terminate proliferation in response to negative factors like transforming growth factor beta (TGF-) and activin so on. A diffirent type of cytokines and growth factors play important roles in these three stages through the activating cell signaling pathways with a large process of biological effects. Here, provided a brief summary of some of the very well-known and recently discovered signaling molecules involved liver regeneration phases (Tao et al., 2017).

Liver Regeneration divided into three phases

3.4.1. Priming Phase

3.4.2. Responsive Phase (Proliferation Phase)

3.4.3 Termination Phase

3.4.1 Priming Phase

Activation of more than 100 genes takes place at this stage in the process of preparing the liver regeneration and to start molecular pathway. Arround the first 48 hours, the most of the hepatocyte growth will have already taken place. Priming phase is the mechanism by which a quiescent hepatocyte cells enters the cell cycle and continues to the Gl restriction phase (Michalopoulos, 2007). The Priming phase through IL-6 and TNF family cytokines and the cell cycle are two distinct processes that are managed by specific molecular mechanisms. Priming is triggered by cytokines in the IL-6 family (Fausto & Fausto, 2000). In the priming phase, there are three steps of activation during liver regeneration and its mention below.

3.4.1.1 Homodinemic changes

Notwithstanding the absence of total blood extravasation, the hemodynamic changes that follow PHx generate a spectrum of events over the whole liver. These changes happened early on in the priming phase, and they resemble a wound-healing response. In contrast to the portal contribution, that sees an increase in volume that is equivalent to three times the initial amount at 2/3 PHx, the arterial component of blood flow per unit of liver tissue does not change. The portal vein is the pathway that all of the blood takes as it exits the digestive tract, what includes the intestines, the spleen, and the pancreas. It is now necessary for the entire stream to pass through a capillary, the cross-section of that has been mathematically reduced to one-third of its original size of the liver (Liver Regeneration 2020, n.d.; Michalopoulos, 2007).

3.4.1.2. Activation of urokinase plasminogen activator (uPA)

The first alteration noticed was a rise in urokinase plasminogen activator (uPA) activity across the residual lobe tissues in liver (Michalopoulos, 2007). After 1 minute, the activity of the urokinase plasminogen activator (uPA) increases in rats that done a partial hepatectomy. This leads to an increase in the rate at that is plasminogen is converted to plasmin. The hepatectomy causes this activation of metalloproteinases as a consequence of the process od liver regeneration (Michalopoulos & Bhushan, 2021). Urokinase is recognized to be a driver in the process of matrix remodeling, in addition to having the potential to be present in the regenerative processes of the liver and other tissues. There is often a high rate of turnover of the proteins which is found in the extracellular matrix. The first indication that matrix metalloproteinase 9 (MMP9) has been triggered (Michalopoulos, 2007) (Figure 3.5).

3.4.1.3. Extracellular Rearrangement

Hepatocyte growth factor (HGF) activation is not mediated by either tissue plasminogen activator (TPA) or urokinase plasminogen activator (uPA) and also not through the interaction of uPA with its receptor (uPAR). Soluble protein like HGF activators containing a diffirent factor XII can be used to stimulate HGF production called HGFA. Two other enzymes can activate HGF, and those are hepsin and matriptase. In a healthy liver, hepatic stellate cells are responsible for the production of HGF. The HGF is subsequently deposited in the extracellular matrix (ECM), mostally in the periportal region of the lobule. Where it is connected to glycosaminoglycans and specific collagens (Figure .5) (Fausto et al., 2006; Liver Regeneration 2020, n.d.; Michalopoulos, 2007).

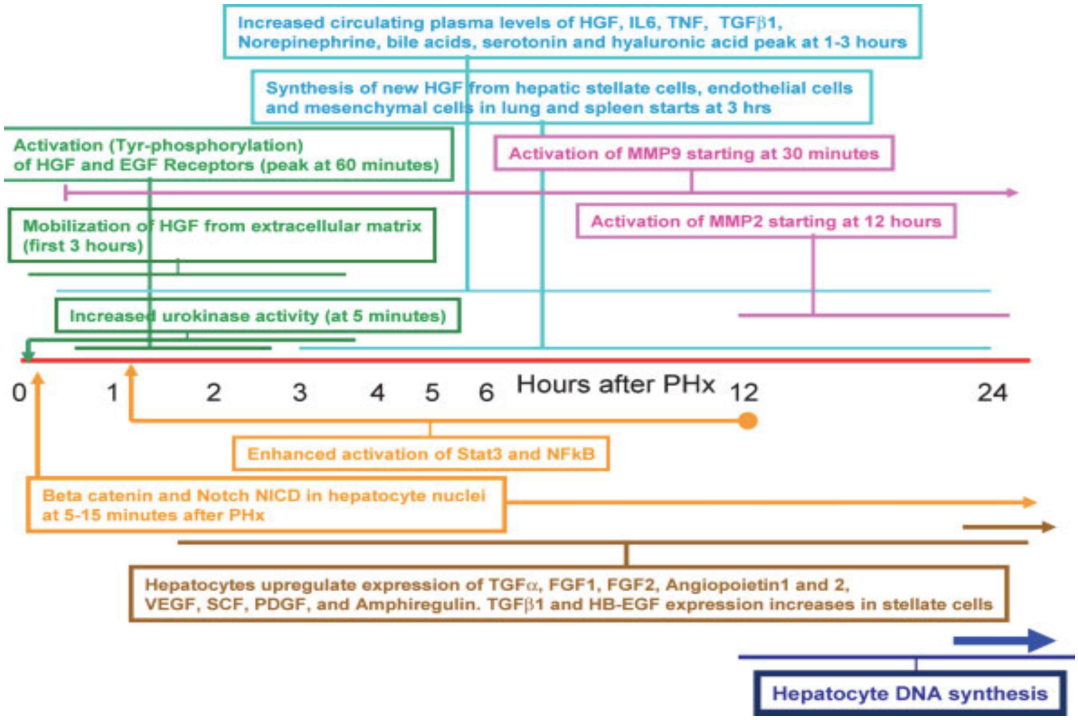


Figure 3.5. Displays a timeline of the primary processes that take place during the early stages of liver regeneration after partial hepatectomy. Cases that have colors that are similar to one another on the map are grouped (e.g., green: growth factor-related events; blue: plasma-related changes, etc.). A horizontal line between the boxes illustrates both the beginning and the conclusion of each signal (Michalopoulos, 2007).

3.4.2. Responsive Phase (Proliferation Phase)

Additionally, with the help of mitogens, hepatocytes are able to exit the G0/G1 phase and going through the progress to the S phase and then the mitotic phase (the proliferation phase) (Tao et al., 2017)

3.4.2.1 Cell cycle

Eukaryotic cells will copy their entire contents during the interphase of their life cycle, and then during the M phase, the cells will divide to form two copies of themselves that are genetically identical and similar to each other. DNA replication and the following separation of replicated DNA are two separate processes that happen at various stages during the cell cycle. DNA replication happens at first. The process of DNA replication takes place during the cell cycle of interphase that is referred to as the "S Phase," and the process of DNA separation takes place during cytokinesis at the end of the "M Phase." At this point, the cell has the option of either exiting the cell cycle or beginning a new round of cell division (Figure 3.6) (Johnson & Walker, 1999; Matthews et al., 2021).

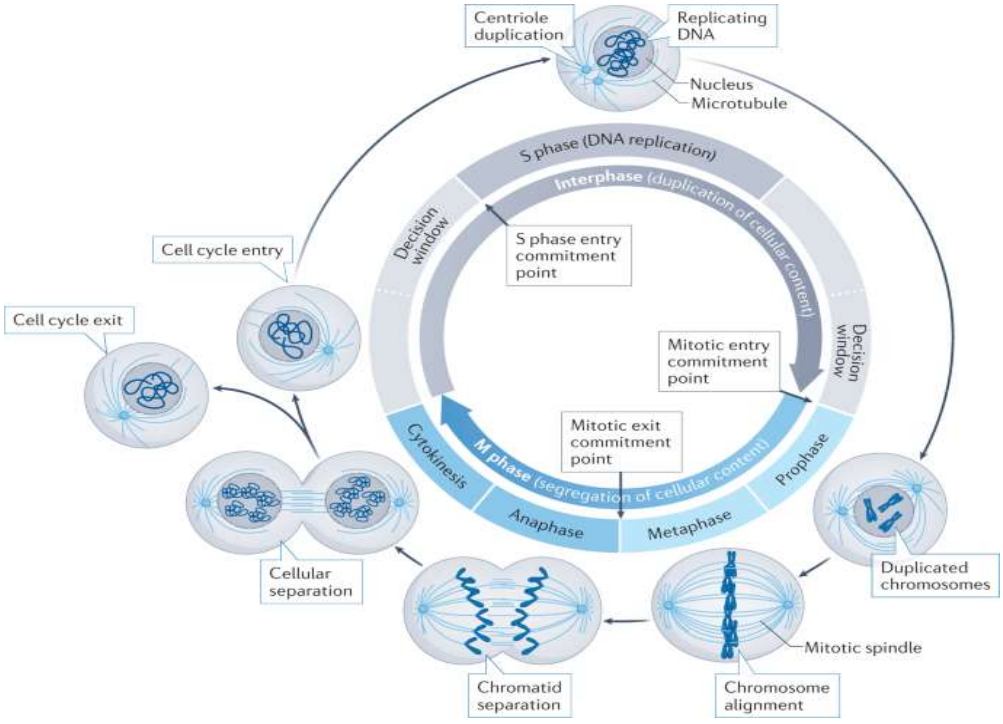


Figure 3.6. The cell division cycle (Matthews et al., 2021).

The cell cycle is regulated both before and after the S phase, that occurs during the interphase. The decision to start a new cell cycle, also known as entering the S phase. Similarly, a cell might decide to enter mitosis during a decision period that happens after the S phase. During the M phase, which occurs during the transition from metaphase to

anaphase, the decision is made to leave the mitotic phase (Johnson & Walker, 1999; Matthews et al., 2021).

CDKs and cyclins are most important factor for cell cycle regulation. The prototype of Cdk inhibitor p21 plays a role in the process of switching kinase complexes between their active and inactive states. A single molecule of p21 is adequate for kinase inhibition, as revealed by several different approaches, including analytical ultracentrifugation of isolated p21/cyclin/Cdk2 complexes. This demonstrates that p21-saturated complexes contain only one securely attached inhibitor molecule. Even after it has been phosphorylated, p21 is still capable of inhibiting the function of CDK (Hengst et al., 1998).

CDKs from a large family of protein kinases that phosphorylate proteins by adding phosphate groups to serine and threonine residues of the target protein. The processing of messenger RNA (mRNA) and the regulation of transcription factor that these proteins play role in eukaryotic cells (Pan et al., 2021).

In mammalian cells, it has been discovered that there are two different families of CKIs that are active at the same time preiod. On the other hand, members of the Cip/Kip family are able to inhibiting a large variety of Cdk/cyclin complexes. Inhibitors belonging to the Ink4 family bind exclusively to the kinases Cdk4 and Cdk6, whereas Cip/Kip family members cannot (Hengst et al., 1998).

During liver regeneration following a 2/3 hepatectomy in rodents such as mice, hepatocytes rapidly express miR-21. Researchers studied that miR-21's function in hepatocyte regeneration in mice by inhibiting it through antisense oligonucleotide. To preserve hepatocyte function, they suppressed the 2/3 PH-induced miR-21 surge while maintaining baseline expression. Hepatocytes were unable to go through S phase because of miR-21 knockdown, which reduced cyclin D1 protein without affecting Ccnd1 mRNA. By upregulating miR-21 expression, they were able to rescue cyclin D1 translation during the early stages of liver regeneration, when Rhob suppression of Akt1/mTOR complex 1 signaling and eIF-4F-mediated translation initiation was at its weakest. MiR-21 promotes hepatocyte proliferation by increasing cyclin D1 translation during liver regeneration (Raymond et al., 2012).

3.4.3. Termination of liver regeneration

In the end of the liver's regeneration process, hepatocytes undergo a transient but important wave of apoptosis. The weight of the liver's expanded lobes close to that of the liver's five lobes (in rats and mice) (Michalopoulos, 2007). In the isolation of the hepatic capsule, recent studies have shown that the various cellular zones are gradually multiplying to maintain the liver's cell quantity and organ weight. Even though the fundamental regulators of these activities are a mystery, must not lose sight of the "hepatostat," that is a collection of processes that guarantees the liver has the appropriate amount of weight (Liver Regeneration 2020, n.d.).

3.5. PI3K/AKT/mTOR signaling pathway

The PI3Ks are a subclass of kinases that consist of heterodimers that are made of regulatory (p50/55/p85) and catalytic (p110 (PIK3CB), p110 (PIK3CD), and p110 (PIK3CG)) subunits. These heterodimers are known as PI3Ks (Jaber et al. 2012; Janku et al. 2011). When human growth hormone or insulin bind to the IRS receptor at the tyrosine sites, type I PI3Ks are activated. Type II PI3Ks are not activated. PIP2 needs to phosphorylate PIP3 for the subunits to be able to carry out their functions (Wymann and Marone, 2005) (Figure. 7). Class II and class III are functionally different from the class I. (Engelman et al. 2006). PKB or AKT (protein kinase B) down signaling has a significant impact on a wide variety of cellular processes, including cell growth, proliferation, differentiation, motility, survival, and intracellular communication. It is common for human malignancies to contain mutations in both classes I PI3K (such as the p110 component) and the class I PI3K inhibitor PTEN (phosphatase and tensin homolog deleted on chromosome 10) (Sung et al. 2012; Samuels et al. 2005; Torices et al., 2022).

The PI3K/mTORC2 and PI3K/PDK1 are involved in the stimulation of cell proliferation that is caused through AKT molecular pathway. PIP3 in the plasma membrane acts as a regulator for the activity of the AKT protein. Signal control is improved further as a direct result of AKT's stimulation of mTORC2 and mTORC1, respectively (Tian et al., 2019).

Src homology 2 domain-containing IRS interacts with PI3K, which phosphorylates tyrosine in order to activate PIP3 (Gao et al. 2005). An increase in the quantity of phosphoinositide 3-phosphate (PIP3) is known to activate the PH (pleckstrin homology) domain-containing kinase as well as the phosphoinositide-dependent kinase 1, also known as PKB/AKT1 (PDK1) (Yang et al. 2002; Jiang et al., 2021). When activated both sites of AKT phosphorylation at Ser 473 and Thr 308 sites, AKT becomes active and its play diffirently in diffirent pathways. In addition to its role as a serine/threonine protein kinase, glycogen synthase kinase 3 (GSK3) is a key component of the AKT signaling pathway (Oshiro et al. 2007).

Cross-talk between the PI3K and MAPK signal transduction pathways is facilitated by AKT as a result of the role it plays and it control Ras pathway (Jaber et al. 2012). AKT is known to target several other proteins in addition to nitric oxide synthase (eNOS) and mTOR. These proteins include Bcl2-antagonist of cell death (BAD), forkhead box protein (FoxO1), forkhead box protein (FoxO3), forkhead box protein (FoxO4), and endothelial nitric oxide synthase (eNOS) (Janku and Polivka 2013). In addition to its role in regulating cell growth and survival, the mTOR pathway is responsible for autophagy. The vast majority of malignancies are caused by mutations in a biological mechanism called the mTOR pathway (Tian et al., 2019).

PKB/AKT activation in the presence of a constitutive activation mechanism suppresses apoptosis. As the process of survival and antiapoptotic activation gets underway, target proteins are turned on and the activity of other proteins in the pathway is both boosted or inhibited. The PKB/AKT pathway regulates cellular growth via mTOR complex 1 (mTORc1) activation (Figure 7). The two mTOR multiprotein complexes are designated as mTORC1 and mTORC2, respectively. It is activated through response to interactions with nutrients, hormones, and growth factors. Class III PI3Ks are involved in the activation of mTORC1 in response to nutrients including amino acids and glucose (hVps34). When either IGF (insulin growth factor) or insulin enters the body, it activates insulin signaling pathway When the insulin receptor (IRS1) is bound, phosphorylation of insulin receptor substrate 1 increases. Lipid metabolism in adipose tissue is activated by insulin receptor (INSR) and insulin receptor substrate (IRS) proteins, Phosphoinositide 3-kinase (PI3K), and protein

kinase B. (H. Chen et al., 2022). Stimulating PDK1 and PKB/AKT increases PIP3 production. The activation of PKB/AKT is linked to the activation of rapamycin-resistant mTORC2 and PDK1. Protein kinase B (PKB) and AKT phosphorylate TSC2, rendering it inactive. The TSC1/2 complex is activated in response to the stimulation of TSC2. The phosphorylation of TCS2 initiates the activation of Rheb-GTP. The (Ras homolog) attaches to mTORC1 and initiates its activity when it is activated in the brain. As a result, the ribosomal protein S6 kinase 1 and S6K1 (phosphorylation of the 4E-binding proteins) are activated, promoting translation initiation and ribosome biogenesis (Janku and Polivka, 2013; Wang et al., 2008; Sancak et al., 2007).

mTORC1 is required for cell growth, progress through the cell cycle, and the ribosome translation of mRNAs encoding proteins that are very important for cellular metabolism. Phosphorylation of 4EBP1 and P70S6 (S6K) kinase by mTORC1 is required to accomplish these tasks. Phosphorylated eukaryotic transcription factor 4E, also known as 4EBP1, is directly related to eIF4E during the G1 cell cycle arrest phase when it serves to prevent the starting point of protein translation. Stimulators like growth hormones can phosphorylate 4EBP1, that is then sends a signal to the mTOR pathway. This pathway is responsible for the cleavage of both 4EBP1 and eIF4E. (Hara et al. 1997; Zaryouh et al., 2022). The translation of proteins including eIF4E, c-myc, cyclin D1, and ornithine decarboxylase is what comes out the process that moves cells from the G1 phase of the cell cycle to the S phase. This task completed by regulating the process by which mRNA is translated into proteins (like cyclin D1 and c-myc,). In addition, phosphorylation of 4EBP1 starts the synthesis of HIF-1a. In addition to the phosphorylation of 4EBP1, mRNA translation could be stimulated by the phosphorylation of S6K1. In addition, S6K1 inhibits IRS-1, which, in turn, exerts a suppressive influence on PI3K, AKT, and mTOR through the mechanism of negative feedback. (Janku and Polivka 2013; Wang et al. 2008; Sancak et al. 2007). The PI3K/AKT/mTOR signaling cascade is responsible for regulating cellular metabolism as well as angiogenesis, proliferation, and progression through the cell cycle. This route may be set in motion by a diffirent of circumstances, including hypoxia, metabolites, and growth factors (Corti et al., 2019).

Liver illnesses have the tragic consequence of taking human lives and medical treatment. Even though the liver can adjust for serious injury, there is still a risk of major decreases in liver function. Deregulation plays an important part in a extensive variety of liver illnesses and conditions related to the liver (Dufour and Clavien., 2010). The PI3K/AKT/mTOR pathway is an most important component in the regulation of cellular metabolism, growth, and survival. The many forms of human cancer, such as renal carcinoma, breast cancer, and neuroendocrine tumors, is a disruption in the PI3K/AKT/mTOR molecular pathway (Corti and et al., 2019).

An overactive PI3K/PTEN/AKT pathway is one of the defining characteristics of a huge variety of illnesses, for example cancer and metabolic disorders etc. The PI3K/PTEN/AKT pathway is activated in cancers in which either PI3K or PTEN is overexpressed more than normal. As a result of the availability of diffirent entry points, PTEN can enter the nucleus from diffirant ways (hence to as nuclear PTEN). Recent research indicates that the protein PTEN, that also has a homolog in the nucleus, performs a unique function in the cytoplasm. PTEN in the nucleus is crucial for inhibiting cell division, alteration of DNA, maintaining the integrity of chromosomes, as well as keeping the integrity of both cells and genomes. (Figure 3.7) provides a diagrammatic representation of the interrelated links that make up the mentioned pathway (Zaryouh et al., 2022).

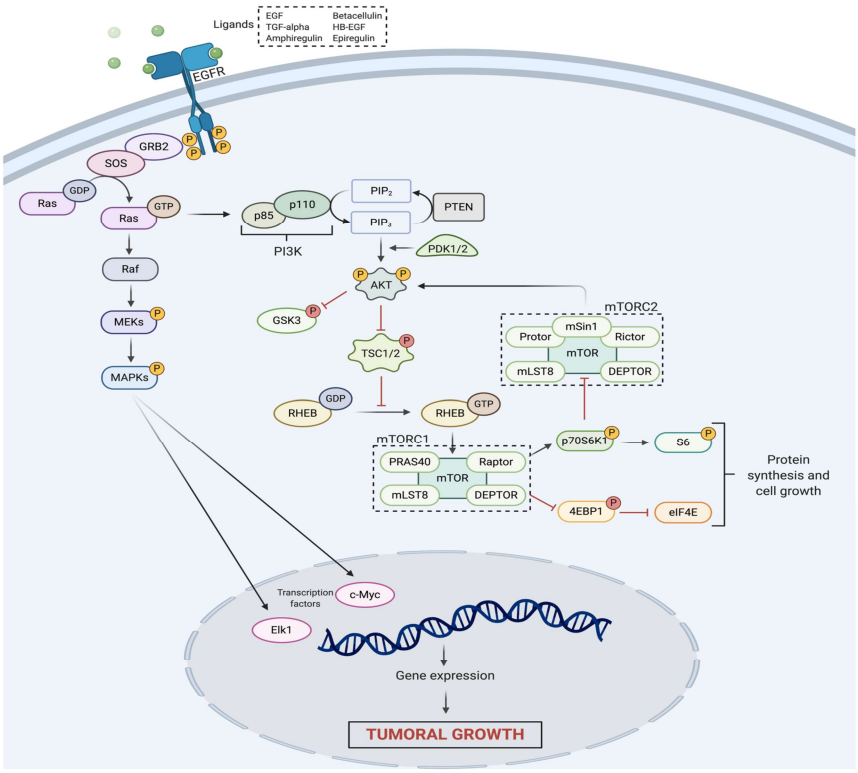


Figure 3.7. Cascade of PI3K/AKT/mTOR signalling pathway (Taken from Zaryouh et al., 2022).

This is done by the activation of the downstream signaling pathway factor such as AKT/mTORC1. Because of the link between hepatic insulin resistance and type 2 diabetes, the PI3K/AKT/mTOR pathway has get attention from researchers (Khamzina et al., 2005). The most common kind of liver disease in western cultures today is called metabolic liver disease, and its is strongly corelate with obesity and type 2 diabetes. An imbalance in the activity level of this enzyme, which is necessary for the digestion of hormones and nutrients, has been associated with metabolic diseases (Gulati and Thomas 2007). An active mTORC1, which is caused by an oversupply of nutrients in the blood and tissues, has a detrimental effect on both the functionality of the liver and the insulin responsiveness of the body. Insulin resistance is linked to (obesity) medical condition. Insulin resistance in the liver of obese mice fed a high-fat diet can be attributed, to increased activation of the mTOR and S6K1 pathways (Um et al., 2006; Marchesini et al., 2008; El-Serag et al., 2006; Khamzina et al., 2005).

Moreover in a liver regeneration model done by PHx of multiple chemicals, activating the mTOR pathway was demonstrated to initiate regeneration. In diffirent study to examine the regenerative effect and potential mechanism of action of Rosmarinic acid in liver regeneration prompted by PHx (0, 2, 4, 6, 8, 10 days). It was reported that rosmarinic acid increased PCNA readings beginning on day 2, and these increases persisted until day 8. In the PHx group, p-mTOR was reported to rise sharply on day 2, then gradually fall over days 4–6. It was shown that p-mTOR was increased in the rosmarinic acid group for a period of 2-6 days. Similarly, p70S6K levels increase on days 2 and 4 following PHx. Rosmarinic acid improves liver regeneration through the mTOR pathway, as shown in this study (Lou et al., 2016). The PI3K/AKT/mTOR pathway was discussed in light of the effect of Panax notoginseng saponins on liver regeneration. The western blot technique was used for the pathway analyses. Phosphorylation of PI3K, AKT, and mTOR is elevated and the PI3K/AKT/mTOR pathway is activated in P. notoginseng-treated mice, suggesting a reparative action of the herb (Zhong et al., 2019).

The MAPK/ERK pathway cross-activation and the PI3K/AKT pathway cross-activation are both required for cell survival, proliferation, and expansion of cells. Multiple cross-activation mechanisms, including the stimulation of PI3K by RAS and the inhibition of TSC activity by ERK and RSK, are cause for the activation of mTORC1. The MAPK/ERK pathway is responsible for activating the PI3K/AKT upstream component. Pathways refers to the phenomenon in which two distinct signaling pathways can have direct effects on the same group of downstream effectors, such as FOXO, GSK3, and BAD (Gomes et al., 2022).

3.6. Gallic Acid

Phytochemically diffirent category of compounds, polyphenols (phenaloids) exist abundantly in nature (Sharad Vermaa, 2013). Polyphenols are a broad and diversified class of chemicals that are found in great supply in plants as secondary metabolites. They are also associated as phenaloids. The most importantly, gallic acid (GA) is a well-known polyphenol that is selectively cytotoxic against a large variety of tumor cells. Its chemical name is 3,4,5-trihydroxy benzoic acid (Figure 3.8). Gallic acid is a type of polyphenol that can be discovered naturally occuring huge combation in foods, such as tea, grapes, berries,

and other fruits, as well as wine. In addition to oak, diffirent other species of plants that produce hardwood also have it this antioxidant. Gallic acid (GA) is like a crystal compound that has a yellowish-white appearance, a molecular mass of 170.12 grams per mole, and a melting point of 250 degrees Celsius. It may be dissolved in water at a temperature of 20 degrees Celsius with a concentration of 1.1%. Gallic acid (GA) possesses too much powerful anticancer, antimutagenic, anti-inflammatory, and antioxidant activities. It also prevents mutations (al Zahrani et al., 2020; Ojeaburu & Oriakhi, 2021; Verma et al., 2013).

In addition gallic acid(GA) role as a precursor of catechins, gallic acid may also undergo metamorphosis in the tea plant, resulting in the production of secondary metabolites. The leaves of the plant contain rich amounts of two different metabolites, namely gallic acid (GA) and methyl gallate. There is an increase in the amount of gallic acid that builds up in the peroxisomes (Zhou et al., 2020).

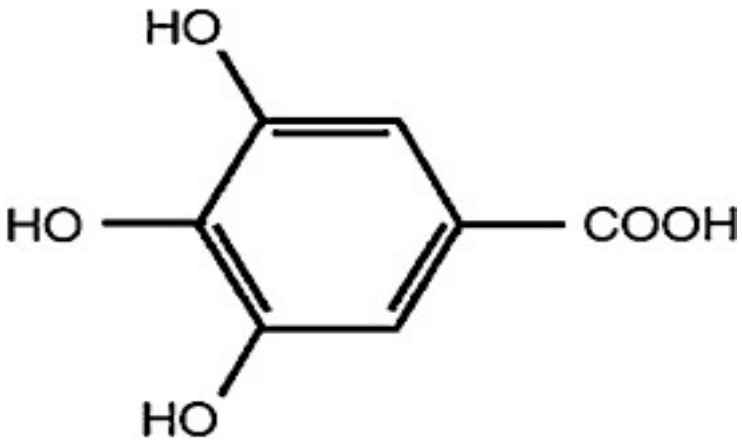


Figure 3.8. Structure of Gallic acid (3,4,5-trihydroxicbenzoic acid).

The vast majority of the phenolic compounds that are present in plants that is oxidation processes which is caused by the action of free radicals and reactive oxygen species (ROS) (Rúa et al., 2017). During the process of cell formation, oxygen can get depleted, which can lead to a condition such as "oxidative stress." This depletion results in the generation of free radicals of oxygen. The free redical kind of radicals that are most powerful and characteristic which help in diffirent type of disease. The phenomenon known as "oxidative stress" refers to an increase in the production of superoxide radicals and

hydrogen peroxide, both of which have the potential to cause direct damage to cells. Dangerous hydroxyl radicals and other oxidizing species can be produced when these oxygen-related products react with one another and certain transition metal catalysts. As a direct consequence of this, calcium levels rise, whilst NADH, GSH, and ATP levels decline, which results in damage to cells as well as disorders such as atherosclerosis, cancer, and ischemia (Yen et al., 2002).

The inhibitory impact of gallic acid on the generation of reactive oxygen species (ROS) may be due to an interference with the action of NADPH-oxidase. Because NADPH-oxidase is necessary for the production of reactive oxygen species by polymorphonuclear leukocytes (PMNs), its assembly is blocked when protocatechuic acid is subjected to 0_2 or H_20_2. This is happens because of NADPH-oxidase is important for the production of reactive oxygen species. It is not possible to disprove the idea that there is a link between the production of oxidized gallic acid and reactive oxygen species (ROS) through PMNs (Kroes et al., 1992).

In additonal research when gallic acid (GA) liposomes are administered to Wistar rats with calvarial abnormalities, there is a considerable increase in the rate rats bones repair. Improvements in wound healing have been decreases in inflammatory response and expression of receptor activator of nuclear factor kappa-B ligand (RANKL), and also increases in expression of osteoprotegerin (OPG) and BMP-2 (Altan et al., 2020). Gallic acid (GA) was present, the level of murine tyrosinase activity in B16 cells that had been treated with a melanocyte-stimulating hormone (MSH) dropped by a rich amount. They discovered that the presence of GA greatly reduced the formation of melanin in developing B16 (human skin cancers) cells and that the degree of suppression connected is also related to the concentration of GA (Kim, 2007).

Moreover gallic acid (GA) could protect the liver and kidneys of rats from the damage caused by lindane that is very well known insecticide. The diagnosis of renal failure was validated by both a histological examination and blood tests, which measured amounts of creatinine and urea, respectively. After treatment with lindane, enzyme activities such as GSH, CAT, SOD, GPx, and GST all went down, while lipid peroxidation and oxidation enzyme activities went up (Vijaya Padma et al., 2011). Mice were provide with gallic acid

(GA) at 100 mg.kg-1, 200 mg.kg-1, and 400 mg.kg-1 for 5 days. All doses of gallic acid (GA) resulted in elevated SOD and GSH levels, although the 100 mg.kg-1 dose was most effective in compare to all other doses (Shruthi & Shenoy, 2020). Hussein et al found that administering gallic acid (GA) (20 mg.kg-1, oral) to animals with thioacetamide-induced liver fibrosis decreased MDA levels and preserved the liver by boosting SOD and CAT levels (Hussein et al., 2020).

Similarly, gallic acid (GA) inhibits carcinogenesis through its antioxidant mechanism that involves the cell cycle, apoptotic cell death, angiogenesis, invasion, and metastasis. This can cure the spread of cancer throughout the body. The anticancer effects of gallic acid are communicated to the body via many different molecular signaling pathways. These pathways include VEGF/VEGFR, NFKB, Bax/Bcl-2, UDP-alpha-D-glucose 6-dehydrogenase (UGDH), ribonucleotide reductase, COX, ADAM17, and ATM Kinase. It is currently common to consume gallic acid (GA) in the form of a dietary supplement (Verma et al., 2013).

According to the findings of another study, chitooligosaccharide acid (COS) demonstrated antibacterial and antioxidant qualities after being conjugated with gallic acid (GA) via a mechanism driven by hydrogen peroxide. This process was carried out by combining COS with GA. After that, the researchers made use of a hydrogel that was constructed from polyvinyl alcohol (PVA) and glucose adducts (GA) (GA-COS). When tested against the P. acnes strain, it was discovered that GA-COS at 5-10 kDa possessed a higher level of antioxidant activity than COS did (Park et al., 2018).

3.7. Silymarin

Milk thistle contains a polyphenolic flavonoid called silymarin, what is one of its active components aslo secondary metabolite. It has been used for a very long time in the treatment of various liver disorders. In addition to having anti-inflammatory, anti-cancer, antioxidant, immunomodulatory, anti-proliferative, hepatoprotective, and anti-diabetic properties, silymarin (SLY) has also been studied for the protective effects that it has on the cardiovascular system, the nervous system, the lungs, and the brain. This research was conducted to better understand how silymarin works to achieve these effects and how its

shows protective effects on liver regeneration (Koçarslan et al., 2016; Malekinejad et al., 2014).

Moreover by increasing glutathione levels and behaving as a powerful antioxidant, silymarin protects the liver from the damaging effects of oxidative stress which is cause by free redicals and enhances its capacity to rid the body of harmful substances. It does this by preventing lipid peroxidation, which is made possible by its capacity to stabilize membranes. The activation of DNA and RNA polymerase facilitates the regeneration of hepatocytes (Aktaş et al., 2019).

A diffirent studies have looked to silymarin (SLY) as a treatment for liver disease. The potential significance of Silymarin in the prevention of metabolic syndrome has been suggested by recent studies due to its anti-atherosclerotic, anti-diabetic, and other favorable cardiovascular activities (Pourová et al., 2019).

In addition, pretreatment with silymarin did not reduce the severity of ADR (adriamycin)-induced hyperlipidemia in individuals who already had hyperlipidemia. The levels of glutathione peroxidase (GSH) and mitochondrial malondialdehyde (MDA) in the kidney were regulated whereas mitochondrial MDA levels were lowered after pretreatment of silymarin (SLY). Heart and kidney tissue samples taken from rats that had been treated with silymarin revealed that ADR caused a slight degree of myocardial damage. In addition to this, it guarded the kidney tubules against the damage that ADR might produce (El-Shitany et al., 2008).

Furthermore, silymarin's effect on phase I (P450) and phase II (glutathione S-transferase (GST), glutathione reductase (GR), and glutathione peroxidase) enzymes converts drugs, procarcinogens, and xenobiotics into active drugs, inactive drugs, toxic metabolites, and carcinogens, all suggesting that silymarin and its derivatives may be effective in the cancer treatments. The detoxification process could be occur with the treatment of silymarin (SLY), what can slow down Phase I enzymes (like P450) but boost Phase II enzymes (Figure 3.9) (Delmas et al., 2020).

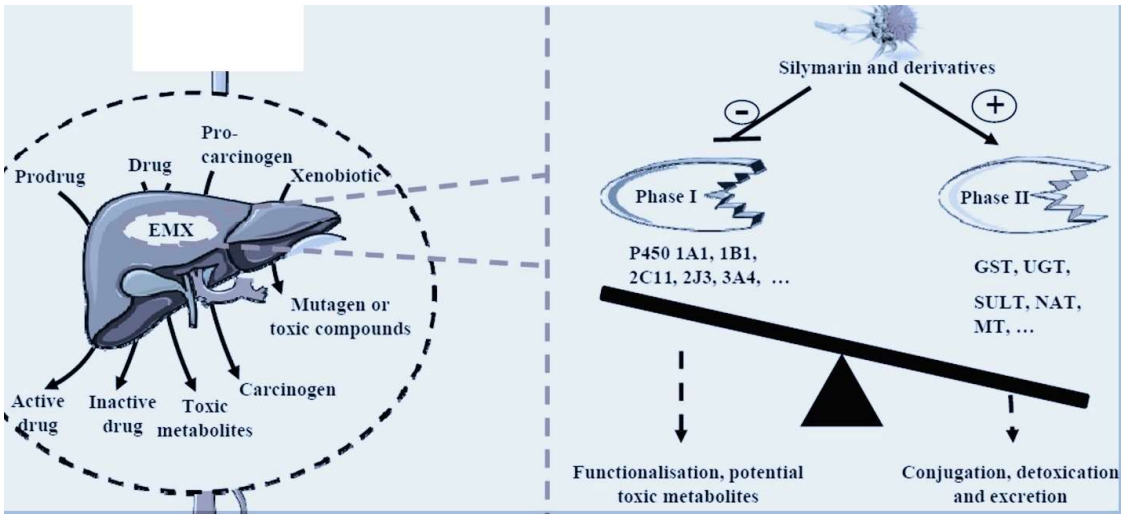


Figure 3.9. Sylimarin and its derivatives effects on Phase I and Phase II (Delmas et al., 2020).

Other research has revealed that silybin, 2,3-dehydrosilybin, and 2,3-dehydrosilychristin's ability to suppress platelet aggregation is only transient state. The degree of this effect depends on how much the inhibitors are being used in particular experiment. Flavonolignan metabolites produced from silymarin (SLY) have been demonstrated to have vasodilatory effects, despite the fact that the constituent components of silymarin have a negligible effect on platelets (Pourová et al., 2019).

In addition to its ability to free radicals, silymarin (SLY) may also limit its production via blocking reactive oxygen species (ROS) producing enzymes. This would be in addition to its ability to scavenge free radicals (removing free rediclas). It may prevent damage to the structure of mitochondria in the envionment of stressful conditions. It has been demonstrated that silymarin can activate the Nrf2 pathway, which then leads to an increase in the synthesis of antioxidant enzymes (Surai, 2015).

In a different study, it was demonstrated that silymarin (SLY) might protect the cardiotoxicity caused by chemical exposure (such as metals, environmental pollutants, oxidative agents, and anticancer drugs) (Razavi & Karimi, n.d.).

Moreover, there is a possibility that it will reduce oxidative stress index (OSI) and tissue damage. Silymarin (SLY, i.p) doses, in this study for the first time, was shown to diminish histological damage in the liver, kidneys, and lungs and oxidative stress in an Ischaemia-Reperfusion (I/R) model of the supra celiac abdominal aorta (Koçarslan et al., 2016).

4. MATERIALS AND METHOD

4.1. Experimental Animals and Groups:

Male Wistar albino rats weighing ±200-250 gm was used in our experiment. In our study, n=6, 4 main groups (Control Group (I), PH+ SF (II) Group, PH+ Silymarin (III), and PH+ Gallic acid (IV) Group) and 4 subgroups were formed by random selection.

Main Group I (Control Groups):

The animals in the Control Group had laparotomies during which the membranes that attached the liver's lobes were surgically removed. Like a 70% partial hepatectomy, the front right, center, and left lobes of the liver were split and threaded. Closure of the laparoscopic incision was performed without doing a partial hepatectomy. Absolutely nothing was provided. At the end of each period, dissections were performed and blood and liver samples were taken for analysis. There was a check on the serum samples for the ALT/AST levels. liver samples were used to investigate the expression patterns of six genes (PIK3CA, AKT, mTORC1, EIF4EB1, P70S6K1 and Cyclin D1) that are part of the PI3K/AKT/mTOR signaling pathway using reverse transcription polymerase chain reaction (RT-PCR). Western blot analysis was used to determine the protein quantities of the genes. In order to calculate the Mitotic Index, the PCNA method has been used.

Main Group II (PH+SF Groups):

In the SF group, and after 70% PH, a single dose of saline (0.5 mL) i.p. given as determined the time (6, 12, 24, 48 hours), blood and liver samples were taken. There was a check on the serum samples for the ALT/AST levels. By using the RT-PCR technique, liver samples were analyzed for gene profiles of 6 PI3K/AKT/mTOR signaling pathway. Western blot analysis was used to determine the protein levels of the genes that passed the Fold2 test. In order to calculate the Mitotic Index, the PCNA method was used.

Subgroup II-1: 6th Hours (SF)
Subgroup II-2: 12th Hours (SF)
Subgroup II-3: 24th Hours (SF)
Subgroup II-4: 48th Hours (SF)

Main Group III (PH+Silymarin Groups):

A dosage of Silymarin (100 mg/kg i.p.) was administered to the positive control group after 70 % PH (Canbek and et al., 2017). As determined by the time (6, 12, 24, 48 hours), blood and liver samples were taken. There was a check on the serum samples for the ALT/AST levels. By using the RT-PCR technique, liver samples were analyzed for gene profiles of 6 PI3K/AKT/mTOR signaling pathway. Western blot analysis was used to determine the protein levels of the genes that passed the Fold2 test. In order to calculate the Mitotic Index, the PCNA method was used.

Subgroup III-1: 6th Hours (SLY)
Subgroup III-2: 12th Hours (SLY)
Subgroup III-3: 24th Hours (SLY)
Subgroup III-4: 48th Hours (SLY)

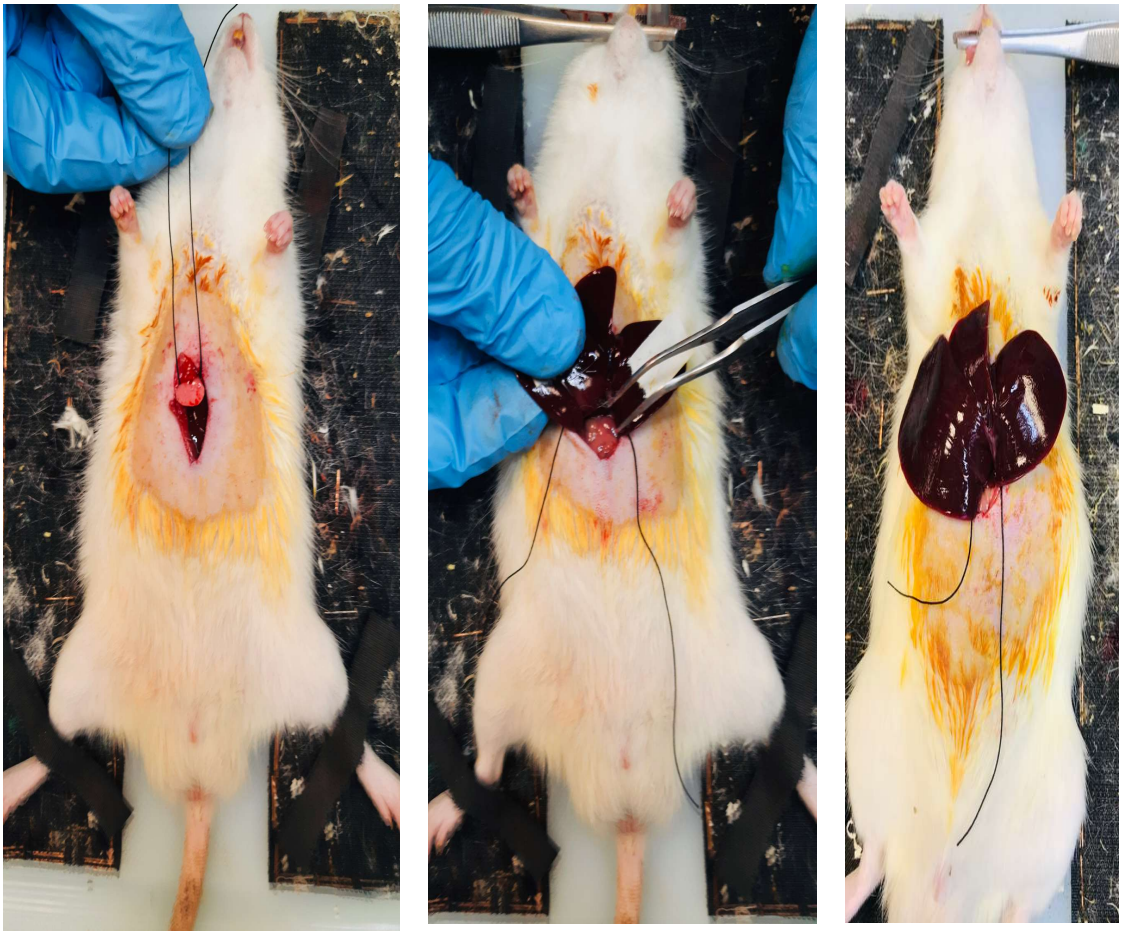
Main Group IV (PH+Gallic Acid Group):

A single dosage of Gallic Acid (200 mg/kg) i.p. was administered to the experimental group after PH of 70% (Canbek et al., 2012). Dissections were accomplished and blood and liver samples were taken at the end of the designated intervals (6, 12, 24, 48 hours). There was a check on the serum samples for the ALT/AST levels. By using the RT-PCR technique, liver samples were analyzed for gene profiles of 6 PI3K/AKT/mTOR signaling pathway. Western blot analysis was used to determine the protein levels of the genes that passed the Fold2 test. In order to calculate the Mitotic Index, the PCNA method was used.

Subgroup IV-1: 6th Hours (GA)
Subgroup IV-2: 12th Hours (GA)
Subgroup IV-3: 24th Hours (GA)
Subgroup IV-4: 48th Hours (GA)

4.2. Hepatectomy Procedure

Each and every one of the experiments was conducted in a sterile condition and using sterile surgical tools. As a result of the possibility of diurnal hormonal changes in rats, all surgical operations were conducted between the hours of 09.00 and 12.00 hrs the experimental animals received intramuscular injections of 10 mg.kg-1 xylazine and 70 mg.kg-1 ketamine anesthesia. In the supine position, the experimental animal to be treated to partial hepatectomy was placed on the dissection table, which was kept at a constant temperature. It was cleansed with 70 percent ethyl alcohol and a Higgins and Anderson partial hepatectomy was done (1931). For this reason, laparotomy will be conducted on the experimental animal with a 2-to 3-cm midline incision perpendicular to its body. The liver lobe membranes were separated, as well as the front right middle and left lateral lobes, which account for 68-70 % of the liver's mass. It was decided to tie the liver's arteries, veins and bile ducts by wrapping them in silk thread and cutting them immediately above the ligament. For each animal, the abdominal cavity was filled with sterile saline in order to prevent hypovolemic consequences from the fluid loss following surgery. Next, the muscle and skin incisions were sutured together with 4/0 silk suture, and the laporoctomy region was closed and cleansed with poviiodex antiseptic solution. Chemically sterilized experimental animals were maintained alive for 6, 12, 24, 48 hours without nutritional change (Figure 4.1).



(A) (B) (C)

Figure 4.1. Partial removal of the liver from a rat (partial hepatectomy). (A) is Incision of the Rat. (B) is taking out the liver of the Rat. (C) is stringing the liver of the Rat as a demonstration.

4.3. Anesthesia

All surgical procedures were performed in a sterile environment and using sterile surgical instruments. The animals belonging to the experimental groups were killed under light anesthesia by intracardiac removal of whole blood from the heart. 10 mg.kg-1 xylazine and 70 mg.kg-1 ketamine anesthesia were administered intramuscularly to the animals belonging to the experimental groups. Then, liver tissue samples were taken by performing a midline laparotomy.

Blood samples were taken with MSE Mistral 2000 brand device for 10 minutes. Serum was obtained by centrifugation at 3000 rpm. Serum samples transferred to Eppendorf tubes were preserved in a deep freezer at -80 ^{0}C for biochemical alanine aminotransferase (ALT) and aspartate aminotransferase (AST) analysis. Some of the samples taken after dissection from the liver tissue remaining after hepatectomy were homogenized to be used in biochemical measurements and molecular biological analyzes in the tissue and stored in a deep freezer at -80 ^{0}C. The other part of the liver tissue was fixed with 10% neutral formaldehyde solution for Proliferating Cell Nuclear Antigen (PCNA) immunohistochemical analysis.

4.4. Substance Administration to Animals

Group I was not given any substance. Rats in Groups II, III and IV were given SF, Silmarin and Gallic acid, respectively.

Gallic acid and silymarin were dissolved in 1 ml of physiological saline. Gallic acid 200 mg.kg-1 as i.p at a time; Silmarin was given to rats as i.p at a dose of 100 mg.kg-1.

4.5. The wet weights of each of the livers and their 68% and 32% amounts were placed in Child's (1953) formula and used to determine the regeneration rate (Tang et al., 1997).

$$\boldsymbol{liver\ regeneration\ rate}\ (\%) = \mathbf{100} \times [(\boldsymbol{A} - \boldsymbol{B})/\boldsymbol{C}]$$

4.6. Biochemical Analysis

ALT and AST measurement was performed with a Crony Airone 200 RA autoanalyzer using commercial kit (Biolabo, Maizy, France).

4.7. Molecular Biological Analysis

4.7.1. RT-PCR Analysis

In this technique, expression profiles of genes belonging to the PI3K/AKT/mTOR (PIK3CA, AKT1, mTORC1, p70S6K1, EIF4EBP1, CYCLIN D 1) signaling pathway were analyzed by RT-PCR analysis. In order for RNA to be amplified by PCR, it must be translated into cDNA. The RNA used as a template in the RT-PCR method was converted into complementary DNA with the reverse transcriptase enzyme. For this, a 3-step procedure was followed, first liver tissue homogenization, then RNA isolation from the homogenate using the RNA isolation kit protocol, and in the last step, the isolated RNA was converted to cDNA.

Table 4.1. Insulation Materials

Used materials	Brand	Cat No
RNA Isolation Kit	A.B.T.™ Blood/Tissue RNA Purification Kit for Leukemia	I04-01-10 100 preps
Beta Mercaptoethanol	M3148 Sigma-Aldrich 2-Mercaptoethanol	CAS Number 60-24-2
Proteinase K	Thermo Scientific™ Proteinase K, recombinant, PCR grade	EO0491
Chloroform Isoamylalcohol	Merck Isoamyl Alcohol	516726
Nuclease Free, Ultra Distilled Water	Biochrom AGUltra Pure Water, Sterile, Suitable for HPLC	L0015-BC
cDNA Kit	High-Capacity cDNA Reverse Transcription Kit	4368814
Nanodrop	Thermo Scientific™ NanoDrop™ 2000/2000c Spectrophotometers	ND-2000
PCR	Applied Biosystems™ MiniAmp™ Thermal Cycler	A37834
Real Time PCR Master Mix	Thermo Scientific™ Maxima SYBR Green/ROX qPCR Master Mix (2X)	K0221
Real Time PCR	Applied Biosystems™ 7500 Real-Time PCR System	4376600
Primer	100 nmol HPLC Grade	
Centrifuge	Neofuge 13R-Refrigerated Centrifuge-Heal Force Bio	
Water	Thermo Scientific™ Water, Nuclease-free, Molecular Biology Grade, Ultrapure	7732-18-5
PBS	Gibco™ PBS, pH 7.2	20012027

4.7.2. RNA Isolation

Lizis Buffer (for 1 sample)
Buffer L 500 µl
14.3 M Beta mercaptoethanol 10 µl

- 200 µl of PBS (Ca Mg free) per 10-20 mg material. 10 µl of Proteinase K (20mg/ml), 500 µl of LIZIS buffer and 60 mg of zirconium bead were added and homogenized in a homogenizer.
- The homogenate was transferred to a new 2 ml centrifuge tube.
- 3 min. was incubated at room temperature.
- Add 500 µl of Buffer P to it and vortex well for 3 min. was incubated at room temperature.
- Add 200 µl of Chloroform isoamylalcohol (49:1) mixture to the mixture. It is mixed by inverting and incubated at room temperature for 2 min. waited longer.
- Incubate the mixture at 12000 x g at 4°C for 15 min. centrifuged. After centrifugation, the supernatant in the uppermost liquid phase (approximately 600 µl) was transferred to a new tube.
- 1.25 times ethanol (96-100%) was added on it (approximately 800 µl) and mixed by pipetting.
- 700 µl of the lysate was transferred to the column and centrifuged for 20 seconds at 12000 x g at room temperature.
- The tube is poured and the column is placed back into the tube. This step was repeated until all lysates were transferred to the column.
- 700 µl of wash buffer1 solution was added to the column, centrifuged at 12000 x g for 20 seconds at room temperature. The tube was poured and the column was placed back on the tube.
- 600 µl of Wash Buffer 2 solution was added on the column, centrifuged at 12000 x g for 20 seconds at room temperature and the column was placed on the tube again.
- 600 µl of Wash Buffer 2 solution is added on the column, centrifuged at 12000 x g for 20 seconds at room temperature. The tube was poured and the column was placed back on the tube.

- The column was centrifuged again for 1 minute at maximum speed and the column was placed on a 1.5 ml eppendorf tube.
- 50-100 µl of nuclease-free, ultra-distilled water or DEPC water kept at 65-70 0C was added to the center of the column and centrifuged at 12000 x g for 1 minute at room temperature.
- RNA was used immediately or stored at -80°C until use.

4.6.3. cDNA Synthesis

The RNAs obtained were measured in the nanodrop device. Since the values measured in the device are in ng/µl, the reaction was established from 20 ng. Necessary calculations have been made.

For each sample;

Calculation

1µl (value measured on the device) ng

x 1000ng

---------- ------

X= 1000/ (value measured on the device) µl

cDNA Standard Reaction (for 1 sample)

10X RT Buffer——————————————— ——————— 2 µl
25X dNTP Mix (100 mM) ——————————————— ———— 0.8µl
10X RT Random Primers ——————————————— ———— 2 µl
MultiScribe™ Reverse Transcriptase——————————————— 1 µl
Nuclease-free H2O——————————————— ———————— 4.2 µl

The above unit value was multiplied by the number of samples the reaction was. It was put into a 1.5 mL Eppendorf tube. The prepared mix was evenly distributed into the wells.

Since the MIX per 1 sample is 10 µl in total, the RNA of each sample was added to subtract the total volume from 20 µl.

According to the data sheet, the temperature and time values were entered into the device.

Settings	Step 1	Step 2	Step 3	Step 4
Temp.	**25°C**	**37°C**	**85°C**	**4°C**
Time	**5 minutes**	**120 minutes**	**10 minutes**	∞

Reconstitution of Primers

Primers were diluted according to the data sheet. The prepared stock primers were diluted to 10 µM.

4.7.3 Primers Used

Table 4.2. Primer Name

Primer Name	Sequence	bp length
BetaActinForward primer	AGCAAGAGAGGCATCCTCACC	21
BetaActin Reverse primer	ACAGGGATAGCACAGCCTGGA	21
AKT1 Forward primer	ATGGACTCAAACGGCAGGAG	20
AKT1 Reverse primer	AGCACCTGAGTTGTCACTGG	20
mTORC1 Forward primer	CAGGCTGCTGGAGTTACCAA	20
mTORC1 Reverse primer	CGTGTGGATCACCACAGACA	20
PIK3CA Forward primer	TCATGGATGCTTTGCAGGGT	20
PIK3CA Reverse primer	TGCCGTAAGTCATCGCCATT	20
EIF4EBP1Forwardprimer	ACTAGCCCTACCAGCGATGA	20
EIF4EBP1Reverse primer	AGCATCACTGCGTCCTATGG	20
P70S6K1 Forward primer	TATGCCTTTCAGACCGGTGG	20
P7OS6K1 Reverse primer	CCCCTTTTGATGTAAATGCCCC	22
CyclınD1 Forward primer	AGTAGTGGCATCGCGAAGAG	20
Cyclın D1 Reverse primer	AACAACACATCCCAAGGGGAC	21

4.7.4. Real Time PCR

Real Time PCR Standard Reaction (for 1 sample)

10 µl Master Mix

2 µl cDNA

2 µl Primers

Total 14 µl

20-14= 6 µl DNA free water

Samples were prepared and distributed into the wells. RT-QPCR Protocol Used:

5min at 95°C; 15s at 95°C, 30s at 60°C, 30s at 72°C; 40 cycles.

4.7.5. RT-PCR Repeat Number

PCR-array analysis was performed as 4 biological replicates for each subgroup with different time and conditions. In the literature researches, it was stated that biological repetition is more valid than technical repetition (Rapaport, et al., 2013). Besides the biological repetition, technical repetition was not preferred because it exceeded the cost of the project. Analysis of the results was performed with ABI Step One Plus brand RT-PCR and the software program (Biogazalle q+) recommended by the manufacturer. By calculating the data analysis house keeping genes and performing "Ratio Statistics Normalization", gene expression data were gradually combined using clustering method and agglomerative technique, starting from single-member clusters.

Methods for RT-PCR Data Analysis

ΔCt in calculating gene expression differences in RT-PCR Array analyzes and ΔΔCt method (Livak method) was used. Reference genes whose expression is presumed constant are never used in this analysis approach. The goal is to use RNA to create cDNA with as little variations as possible in terms of processing and synthesis. Furthermore, current research has uncovered a reference genes, which, when used, are said to produce more reliable results (Silver et al., 2006). Reference genes were chosen using HEAT map analysis. The calculation was performed using Excel and Graphpad 8's Tukey test Analysis.

4.8. Western Blot Analysis

Western blot of 4 genes with high gene expression and statistically significant ($p \leq 0.05$)selected for their analysis: Phospho-PIK3CA (Ser317) (100-150 kDa), Cyclin D1 (36 kDa), Phospho-mTOR (Ser2448) (289 kDa), Phospho-Akt (Thr308) (60 kDa).

The following steps were followed in Western blot analysis:

- Preparation of samples
- Incubation
- Execution
- Membrane transfer
- Antibody applicatıon
- Display

4.8.1. Preparation of Samples

First, the concentrations of the samples were adjusted.
$M_1.V_1=M_2.V_2$
Required solutions after calculation:

Amount of Solution Required for a Sample	
LDS Sample Buffer	5 µl
Sample Reducing Agent	2 µl
Sample	Calculated amount- µl
Distilled Water	6,5 µl- (sample) µl
Total	20 µl

4.8.1.1. Incubation

- Prepared tubes at 70 °C for 10 min. incubated.
- At the end of the incubation, it was kept on ice or at +4 °C for a while.

4.8.1.2. Execution

- Bolt ™ 4-12% Bis-Tris Plus Gel is prepared for use.
- Execution was performed with Running Buffer 1X solution.
- A marker was placed in the first well.
- Approximately 30-35 minutes at 200 volts. Execution has been done.

4.8.1.3. Membrane Transfer

- Thermo Fisher Scientific IBlot 2 Dry Blotting System was used for the transfer process.
- In order to remove the gel from its reservoir, it is rinsed in a tub filled with pure water. Remove the top cover slowly by lifting it from the corners with a spatula. It is carefully separated.
- iBlot™ 2 Transfer Stacks.
- Using the IBlot 2 device's own sandwich shaped membrane transfer system (IBlot™ 2 Transfer Stacks) at 20 volts for 7 minutes. Transfer from gel to membrane was performed.
- After the transfer process, Bloking Solution was prepared.
- To prepare Bloking solution from iBind™ Flex Solution Kit;
- 40 ml distilled water + 10 ml 5X Buffer + 500 µl 100X Additive Solution
- After the transfer process was completed, the gel was discarded. The gel image on the transfer paper was cut by cutting. It was put into the prepared blocking solution and left for 5 minutes kept on hold.

Antibody Application

- Antibody administration was performed on the Invitrogen™ iBind™ Flex Western instrument. For this purpose, antibody, washing and blocking were prepared from solutions suitable for the device.
- The binding process of primary and secondary antibodies on the card specific to iBind™ Flex was performed in a short time like 2.5 hours, and the binding process was terminated.
- Antibody Preparation.
- How many antibodies, 2 ml each, from the blocking solution were to be prepared, were put into 2.5 ml tubes or falcon tubes and vortexed.
- Wet the iBind™ Flex card with blocking solution (10ml). Another 1 ml of the same solution was added to the place where we would put the membrane. It was fixed by going over it with the roller.

- It was left in the iBind™ Flex device for 3 hours. ECL solution was prepared.
- ECL Solution Preparation.
- It was prepared by adding 1 ml of Luminol/Enhancer solution + 1 ml of Peroxidase solution.
- The membrane removed from iBind™ Flex was placed in solution.

4.8.1.4. Display

- A chemiluminescence imaging system was used for imaging. Since the basis of this imaging system is the chemical luminol, a secondary antibody-specific substrate (ECL) was used. The membrane was incubated for 5 minutes in the dark in the ECL solution prepared 1:1, and the imaging process was started.
- The picture of the membrane placed on the table in the Genbox device was taken with the program on the PC.

Sample No.	Sample Group	Qubit Protein Measurement (µg/µl)
1	SF (II) 12	9,5
2	SF (II) 24	33
3	SF (II) 48	32
4	SLY (III) 12	68,5
5	SLY(III) 24	48
6	SLY (III) 48	42,5
7	GA (IV) 12	32
8	GA (IV) 24	100
9	GA (IV) 48	51

The samples were run in the gel by calculating 70 µg into the gel well.

4.8.1.5. Antibodies Used

- β-Actin Antibody (N-21), Rabbit, sc-130656, Santa Cruz Biotechnology (43 kDa).
- Phospho-PIK3CA (Ser317) pAb, Ref. no. PA5-105113, Invitrogen (100-150 kDa).

- Cyclin D1 (92G2) Rabbit mAb (#2978), Cell Signaling Technology (36 kDa).
- Phospho-mTOR (Ser2448) (D9C2) Rabbit mAb (#5536), Cell Signaling Technology (289 kDa).
- Phospho-Akt (Thr308) (D25E6) Rabbit mAb (#13038), Cell Signaling Technology (60 kDa).
- HRP Goat Anti-Rabbit IgG (H+L), Catalog No.: AS014, ABclonal

Ladder Used

- Invitrogen SeeBlue Plus 2 Prestained Standard

4.9. PCNA

Table 4.3. PCNA Materials

Microtome	Thermo Shandon
Slide	Thermo Menzel- Glasser Polysine Slides Lot :0889
oven	Mecan Technic
Xylene	A.D.R Group Cat: 129007
Alcohol	% 96 Ethanol
Hydrogen peroxide	Riedel-de Haen %35 Lot: 1130A
Citrate Buffer	SCYTEK Lot:44833
PBS	BBI LIFE SCIENCES Lot : EA17KA9844
Primary Antibody	PCNA Antibody (F-2): sc-25280
Secondary Antibody	HRP Goat Anti-Mouse IgG (H+L) AB-clonal Cat: AS003
Albumin	BioShop Lot: 8E54929
ABC Kit	Vectastain PK-6100
AEC Chromogen / Substrate Kit	SCYTEK Lot: 50373
Hematoxylin Mayer	Biognost Lot: HEMM-21/16
Consul-Mount Adhesive	Thermo Scientific Lot: 214586

- Primary Antibody: 1/100 dilution was used.
- Secondary Antibody: 1/250 dilution was used.

4.9.2. Immunohistochemistry

The liver tissue samples were obtained at the end of the 6, 12, 24, and 48-hour regeneration periods and fixed in 10% formaldehyde for 24 hours for PCNA immunohistochemical staining. Following fixation, the tissues were washed for 24 hours in running water and followed as per the standard procedure.

- Samples were first passed through xylene and alcohol, respectively. (5 min xylene-5 min xylene- 1 min alcohol 96%- 1 min alcohol 80%- 1 minute alcohol 70%- 1 minute alcohol 50%) was taken into distilled water.
- **NOTE:** Prior to this procedure, slides were incubated at 60°C for 2 hours.
- 20 ml of H_2O_2+180 ml of distilled water was prepared (10 min).
- 2-3 minutes washed in distilled water.
- Citrate buffer is prepared (75ml citrate buffer + 675ml distilled water) for 15 minutes. Boiled in the microwave.
- **NOTE:** If it will be made in a pressure cooker, it will take 5 minutes when 120°C is reached. Kept and cooled.
- 15 minutes for how long it was left in the microwave. It's so cold outside.
- It was kept in distilled water for 3 minutes, then drawn with a marker.
- 2x3 minutes. washed with 1X PBS.
- 15 min with 8-2% BSA. Was treated.
- The first antibody prepared with 9-1X PBS was applied.
- 2x3 minutes with 1X PBS. Washed away.
- Secondary antibodies were placed. It was kept at room temperature for 3 hours.
- ABC kit was prepared. In 1X PBS and 15 min. kept at room temperature.
- 2x3 minutes. washed away. (With 1X PBS)
- The prepared ABC kit was placed on the slides. (Room temperature 1 hour)
- 2x3 min. washed with 1X PBS.

- Chromogen was dropped on it. (8 min)
- It was stained with Mayers Hematoxylin as 17-Counterstain. (2 minutes.)

Statistical Evaluation

"GraphPad Prism 8" software was used to evaluate the data obtained as a result of our studies. One-way analysis of variance (ANOVA) was used for statistical analyzes of regeneration rate according to liver weights and PCNA positive cell count. Tukey Test was used to reveal the differences between the groups. RT-PCR gene expression analyzes were evaluated according to the Livak Method and the coefficient of gene expression differences between groups was expressed by $2^{-\Delta\Delta CT}$. In all analysis results, $p \leq 0.05$ was considered significant and ± SEM (standard error of the mean) was used.

5. RESULTS AND DISCUSSION

5.1. Liver Regeneration Rate

SLY (100 mg) and GA (200 mg) were given after PHx is done, and the regeneration rates were determined at the time period of 6, 12, 24, and 48 hours using liver weight method. Group comparisons are shown in (Figure 5.1). The liver regeneration process start at the 12th hour, despite the fact there was no statistically significant difference between the 0th and 6th hours in the experimental groups PH (II), SLY (III) and GA (IV). At the 24th hours of time the liver regeneration was much higher across all over groups. According to previous study, DNA replication peaks around 24th hours after PHx and its reaches maximum level within 48th hours to its original mass. Arround 48th hours, the GA group showed the second-highest peak of DNA replication in recent study.

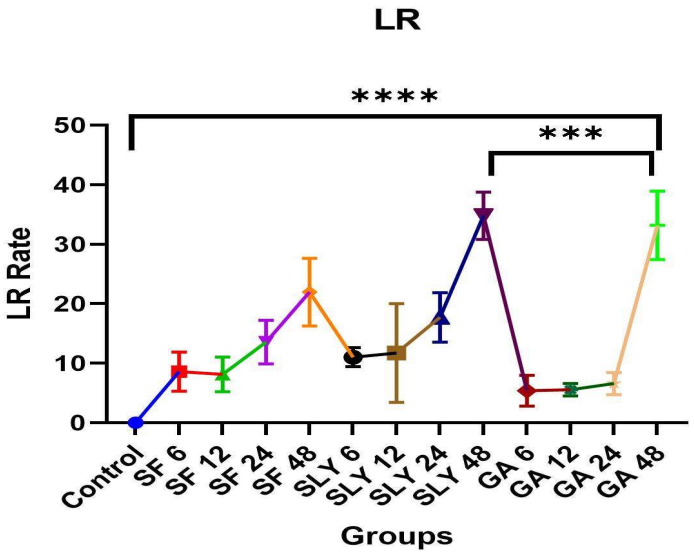


Figure 5.1. Liver regeneration rates (Mean±SEM) of Control (I), PH+SF (II), PH+SLY (III) and PH+GA(IV) Groups. The time difference between Control (I) and SLY (III) 48 hours (****, <0.0001) shows a significant difference.

The liver's ability to heal itself after damage sets it compare to other organs. Despite severe damage, the liver can continue to perform its complex tasks and to reach its original

mass (Shennen et al., 2014). After PHx, changes are made in the initial trigger such as urokinase elevation, before Notch and nuclear migration of beta-catenin, etc. In the case od Fully differentiated hepatocytes keep the body in a state of equilibrium and help regenerate liver mass at 36th hours after surgery done (Michalopoulos, 2007). According to the study, genes related to the circadian-dependent cell cycle may influence the transition between G2 and mitosis. The remaining liver returned to its normal size within 5-7 days after PHx is done (Shennen et al., 2014).

In our study, each group showed regeneration after 12th hours, and when we comparison of all groups after 24th hours revealed that hepatocyte proliferation was greater than after 12th hours. After 48th hours of partial hepectectomy, the peak of GA groups is highest compare to all other groups. Despite the large amount of research done on liver regeneration molecular pathways, there are still many aspects of this phenomenon that require additional research in this particular area.

5.2. PCNA analysis

PCNA was examined positive cell counts in the all the groups like Control (I), PH+SF (II), PH+SLY(III), and PH+GA(IV) groups. The number of PCNA-positive cells in all groups between 12, 24, and 48th hours have significant diffirence. GA (IV) had the most positive cells compare to all other groups. At 6th hours, SF (II), SLY (III) and GA (IV) did not vary significantly. The control (I) group and GA (IV) 48 hours differed significantly (**, P 0.001). Moreover, SLY (III) 12th hour and GA (IV) 48th hour differed significantly (**, P 0.001). When SF (II) 12, 24, 48, SLY (III) 12, 24, 48, and GA (IV) 12, 24, 48 hours were evaluated for PCNA large amount of positive cell numbers, GA (IV) 48 hours had the most positive cells. The P value is (**, P 0.001) difference shown in (Figure 5.2).

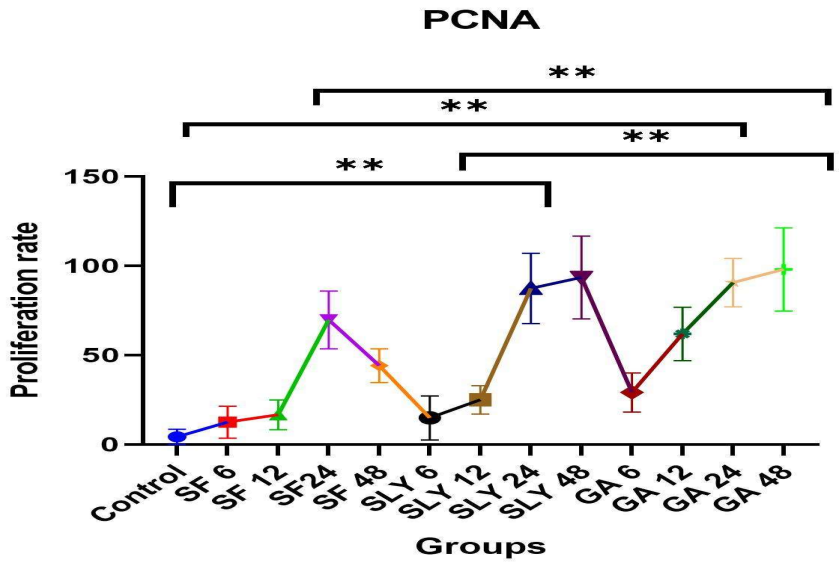


Figure 5.2. PCNA rates (Mean±SEM) of Groups Control (I), PH+SF (II), PH+SLY (III) and PH+GA (IV) Groups. The time difference between the control (I) and GA (IV) 48 hour is (**, P <0.001) and between SLY (III) 12 and GA (IV) 48 hour is (**, 0.001) differed significantly.

Following cell injury, PCNA provides us with important data on cell tissue proliferation, transformation, and repair. Although some cells stained more intensely than others cells in our study, all visible staining was considered as a positive cells in our analysis. In our study the counts of cells, we observed that 34 positive cells in the control group. While there were much lower PCNA positive cells in the group SF (II) group at the 6th hour. On the other hand, SF(II) 12, 24 and 48 hours were reported to have a large number of positive cells. A higher % of PCNA-positive cells was observed in the group SLY (III) compared to group SF (II) groups shown in (Figure 5.2). Group GA (IV) had the highest number of diffuse positive cells which shows Brown color. When the group GA (IV) was evaluated within itself, it was seen that the most positive cell group was at the time preiod 48th hour.

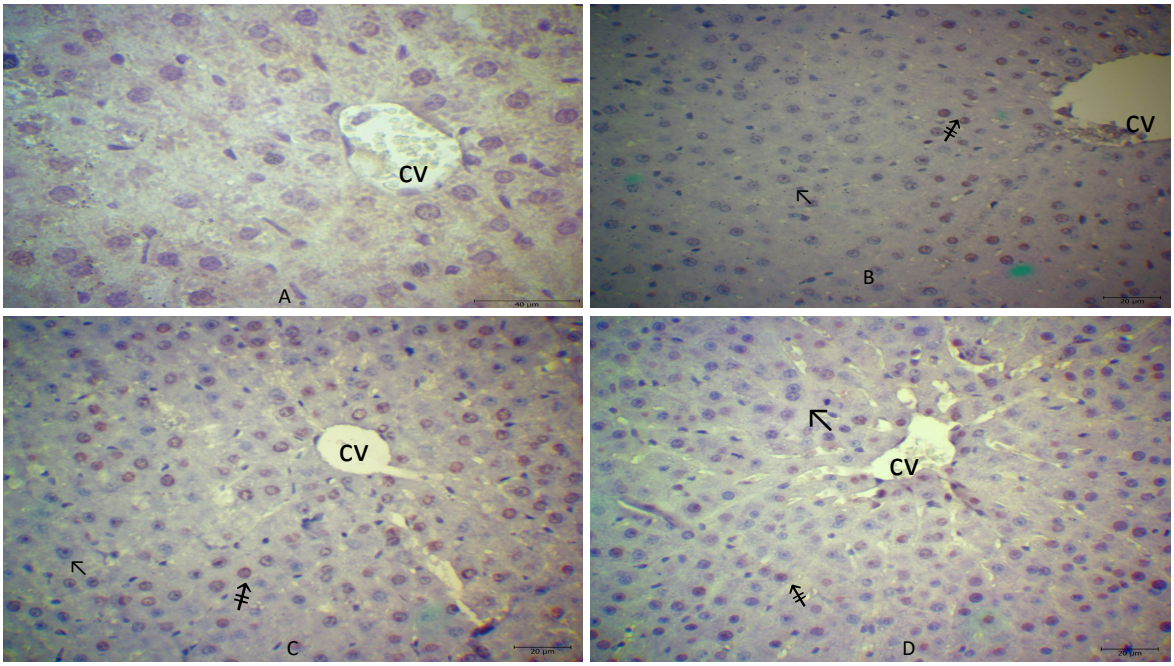


Figure 5.3. A: Group (I) PCNA from liver tissue of rats in the control group. B: Following partial hepatectomy, the SF (II) group exhibited (↑) PCNA-negative cells and (↟) PCNA-positive cells 12 hours later. C: Following partial hepatectomy, the SF (II) group at 24 hours showed PCNA negative cells (↑) or (↟) positive cells, respectively. D: Following partial hepatectomy, SF (II) 48 hours group exhibited (↑) PCNA negative cells and (↟) PCNA positive cells.

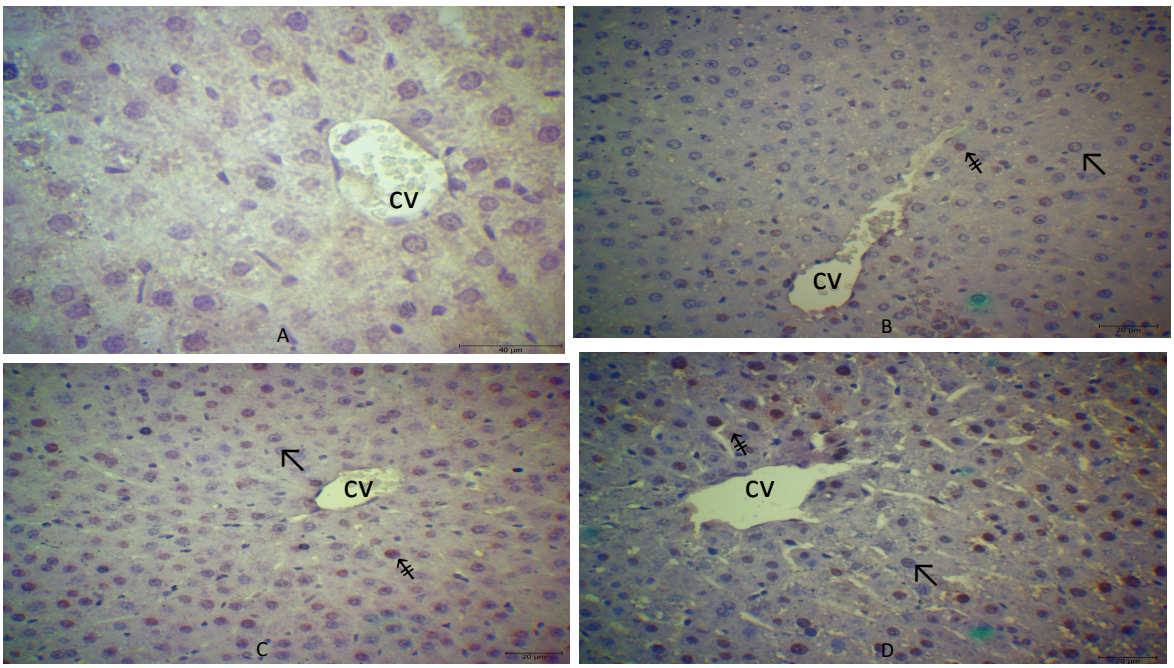


Figure 5.4. A: control (I) group PCNA from liver tissue of rats. B: Following partial hepatectomy, Group SLY (III) group exhibited (↑) PCNA-negative cells and (ǂ) PCNA-positive cells 12 hours later. C: Following partial hepatectomy, the SLY (III) group at 24 hours showed PCNA negative cells (↑) and (ǂ) positive cells, respectively. D: Following partial hepatectomy, 48 hours post SLY (III) group exhibited (↑) PCNA negative cells and (ǂ) PCNA positive cells.

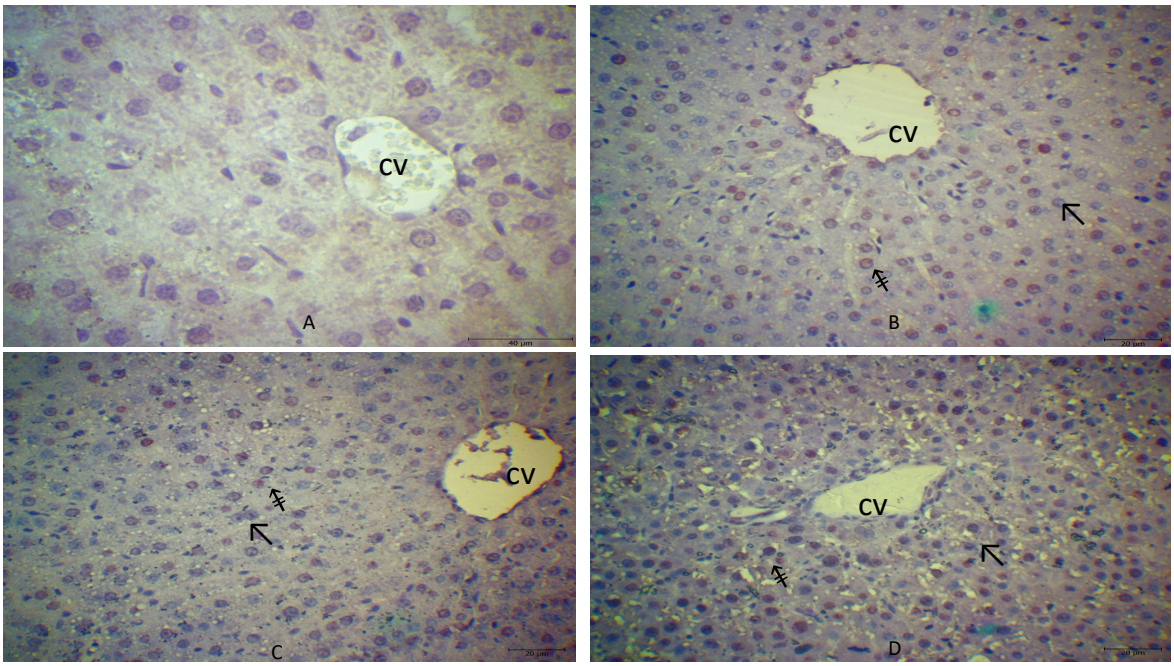


Figure 5.5. A: PCNA from liver tissue of rats in the control (I) group. B: Following partial hepatectomy, the GA (IV) group exhibited (↑) PCNA-negative cells and (ǂ) PCNA-positive cells at 12 hours. C: Following partial hepatectomy, the GA (IV) group at 24 hours showed PCNA negative cells (↑) and (ǂ) positive cells, respectively. D: Following partial hepatectomy, 48 hours post GA (IV) group exhibited (↑) PCNA negative cells and (ǂ) PCNA positive cells.

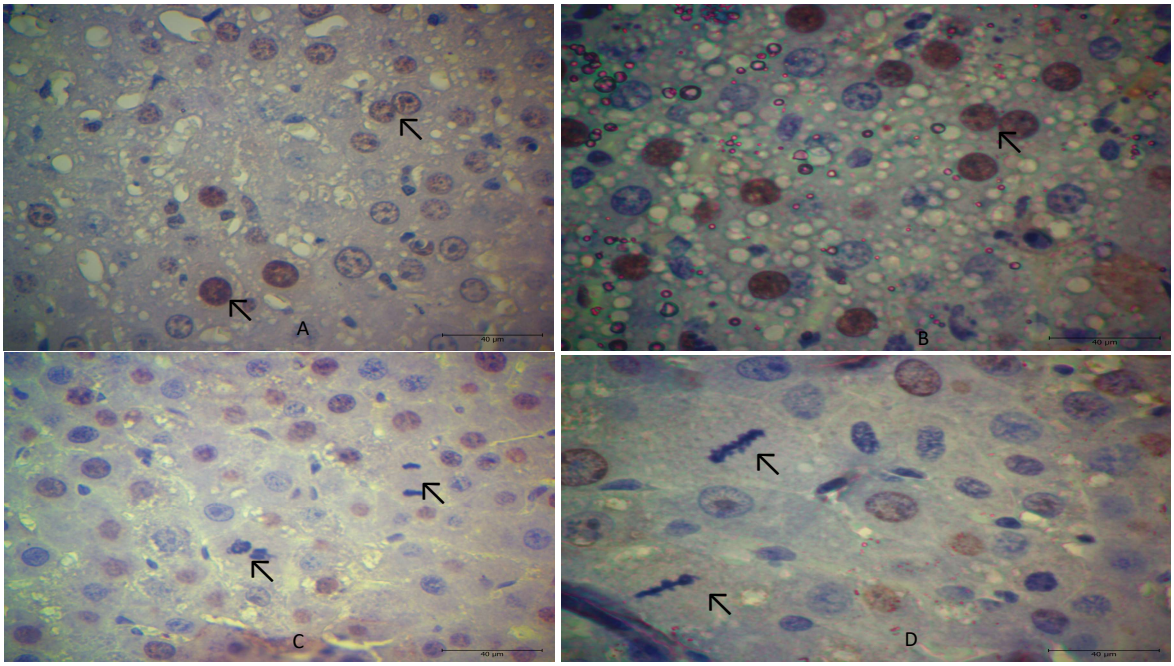


Figure 5.6. A: PCNA-positive and binuclear cell from liver tissue of rats in the 12 hours SLY (III) group (←) B: After partial hepatectomy, GA (IV) group after 12 hours (←) PCNA-positive cells and bipolar cell fusion about to do. C: GA (IV) group at 48 hours, Anaphase (←) following partial hepatectomy. D: Following partial hepatectomy, group 48 (←) PCNA positive cells and metaphase were seen 48 hours later.

In diffirent study shows that is increased PCNA transcription and mRNA stabilization in proliferating cells, but not in quiescent cells. The PCNA results indicate that protein accumulates at all stages of the cycle (Stamatios et al., 1994).

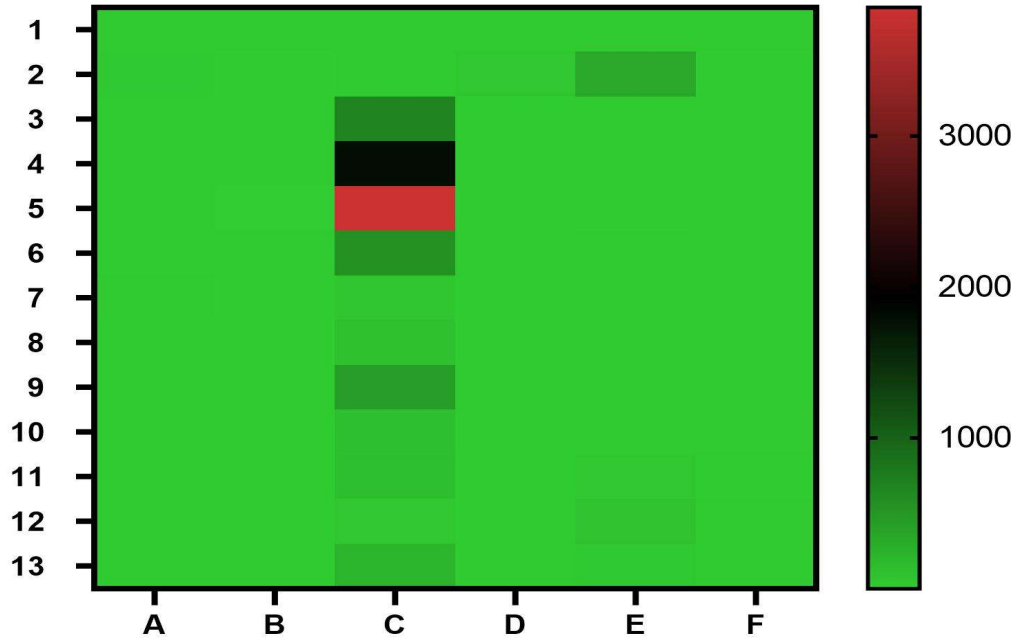
At days 3 and 7 following hepatectomy, the regeneration rate in cirrhotic rats was significantly lower than in the control groups. The PCNA index was higher in cirrhotic livers before hepatectomy. The PNCA labeling index of the cirrhotic rat liver remained stable, but the case of control liver grew significantly. The higher values were discovered on days 1, 2, and 3, with the greatest value on day 2 after hepatectomy. In cirrhotic rats, plasma fibronectin reduction and Kuffer cells before and after hepatectomy have been reported between 40 and 72 hours (Chijiiwa et al., 1994).

In our study, hepatocytes at different rates showed PCNA expression in randomly in zoned area of cells. In our recent study was observed that the larger hepatocytes had quite large nuclei compared to the other hepatocytes and some of them had binuclei. Kuffer cells were found before the 12^{th} and 24^{th} hours of the regeneration period and that is something

so unique. In addition, statistical analyzes found that the highest number of PCNA-positive cells at the PH 48th hour compared to the other hours of groups such as SF, SLY. While the highest number of positive cells was observed at the GA (IV) 48th hour. As shown in (Figure 5.2, 5.3, 5.4, 5.5).

5.3. Heat Map

The use of a heat map to present information regarding gene expression is generally favorably appreciated. The levels of gene expression are shown in this heat map, which includes all of the genes expression listed below.



(A)PIK3CA;(B)AKT1;(C)mTORC1;(D)EIF4EBP1;(E)P70S6K1;(F)CYCLIND1

Figure 5.7. Heat Map PI3K/AKT/mTOR pathway.

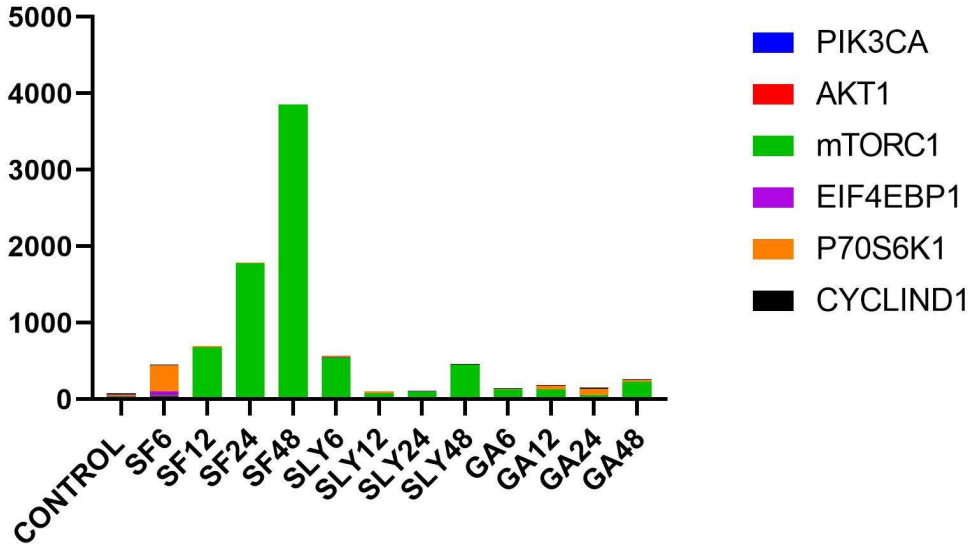


Figure 5.8. Heat Map Statistics PI3K/AKT/mTOR pathway

5.4. RT-PCR and western blot Results

Absolute and relative quantification are two ways in what quantitative gene expression could be presented. Absolute quantification uses a PCR signal method to compare with a standard curve to get an accurate count of how many copies of a gene are present in particular research. The relative expression of a gene is measured by comparing with its activity to an internal standard value or reference gene. The 2ΔΔCT approach is commonly known as the comparative CT method that is one of the most widely used methods to compare gene expression levels. The quantitative gene expression comparative CT approach is described in this protocol. (Schmittgen & Livak, 2001). In our study, 2ΔΔCT (2Fold values) Livak method was used to obtain CT values from RT-PCR method. Microsoft Excel was used to perform the calculations. Those with fold values ≤ 0.5 and ≥2 were considered significant. In Table 5.1, fold change values of the gene are given.

Table.5.1. these three groups, PH+SF (II) (6, 12, 24, 48 hours), PH+SLY (III) (6,12, 24, 48 hours) and PH+GA (IV) (6, 12, 24, 48 hours), each representing 2-ΔΔCT (fold values). The red fold value indicates ≥2, while the green fold value ≤0.5.

Table 5.1. Fold change (2-ΔΔCt) Genes with increased (↑) and decreased (↓) expression

Fold change ($2^{-\Delta\Delta Ct}$) Expresyonu artan (↑) ve azalan (↓) genler												
Gen	SF 6	SF 12	SF 24	SF 48	SLY6	SLY 12	SLY 24	SLY 48	GA6	GA 12	GA 24	GA 48
PİK3CA	17.1	2.3	0.3	0.1	2.3	14.3	5.2	1.8	12.7	0.2	2.6	0.7
AKT1	8.22	0.14	0.3	0.01	0.15	0.21	0.42	0.1	0.10	0.11	0.1	0.12
MTORC1	11.2		16	44	24	38	87	78	89.7	40.1	67	33
P70S6K1	6.8	9.6	6.2	15.6	9.1	19	8.7	9.4	30	42.2	73.1	44.4
EIF4EB1	12.6	2.9	2.8	0.2	5.7	2.4	2.3	3.2	1.1	3.1	2.3	1.1
CYCLIND1	9.43	4.64	0.15	0.00	0.0	0.19	0.1	6.3	17.6	8.1	20	7.5

In RT-PCR method the CT values were compared with the CT values obtained the Comparative CT method, also known as the Livak method. ANOVA and Tukey's test for multiple comparisons were performed using the GraphPad Prism 8 program to compare the CT values of each biological sample (Control (I), SF(II), SLY(III), GA(IV)).

5.4.1. PIK3CA Gene Expression

In our study PIK3CA gene expression calculation, SF (II) 6th hour; SF (II)12th hour, SF (II) 24th hour and SF (II) 48th hour groups significant diffirent is (****, P <0.0001). Comparing SF (II) 6th hour and group to SLY (III) 6, 12, 24, 48 hours and GA (IV) 6, 12, 24, 48 hours, statistically significant differences were identified is (****, P<0.0001, *** P0.0004, *** P0.0002, ** P0.0038, ****P<0.0001, ***P0.0004, ****P<0.0001). Shown in Table 5.2.

Table 5.2. PIK3CA Gene expression

PİK3CA					
Tukey's multiple comparisons test	Mean Diff,	95,00% CI of diff,	Significant?	Summary	Adjusted P Value
SF 6 vs. SF 12	35.87	13,38 to 58,36	Yes	***	0.0001
SF 6 vs. SF 24	36.69	14,20 to 59,19	Yes	****	<0,0001
SF 6 vs. SF 48	36.97	14,48 to 59,46	Yes	****	<0,0001
SF 6 vs. SLY 6	36.04	13,55 to 58,54	Yes	***	0.0001
SF 6 vs. SLY 24	33.19	10,70 to 55,69	Yes	***	0.0004
SF 6 vs. SLY 48	35.36	12,87 to 57,85	Yes	***	0.0002
SF 6 vs. GA 6	28.57	6,077 to 51,06	Yes	**	0.0038
SF 6 vs. GA 12	36.9	14,40 to 59,39	Yes	****	<0,0001
SF 6 vs. GA 24	35.98	11,68 to 60,27	Yes	***	0.0004
SF 6 vs. GA 48	36.73	14,24 to 59,22	Yes	****	<0,0001

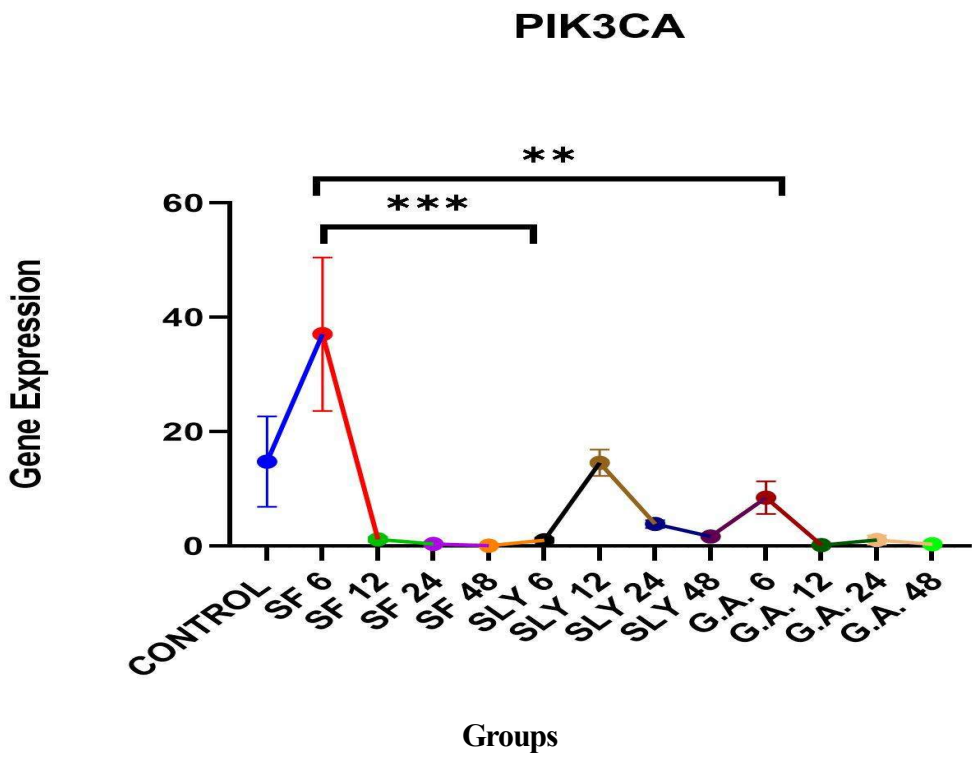
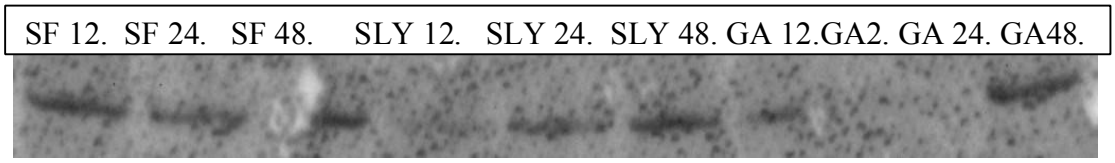


Figure 5.9. PIK3CA gene expression: SF (II) 6, 12, 24 with 48 significant diffirence is (***P 0.0001). SF (II) 6, SLY (III) 6, SLY(III) 12, SLY (III) 24, SLY (III) 48; and GA (IV) 6, GA (IV) 12, GA (IV) 24, GA (IV) 48 (****, P<0.0001,*** P0.0004, *** P0.0002, ** P0.0038, ****P<0.0001,***P0.0004, ****P< 0.0001) significant diffirence (Mean±SEM).

5.4.1.1. PIK3CA protein expression



100-150 kDa

Figure 5.10. (a) PIK3CA band images from Western Blot analysis are shown.

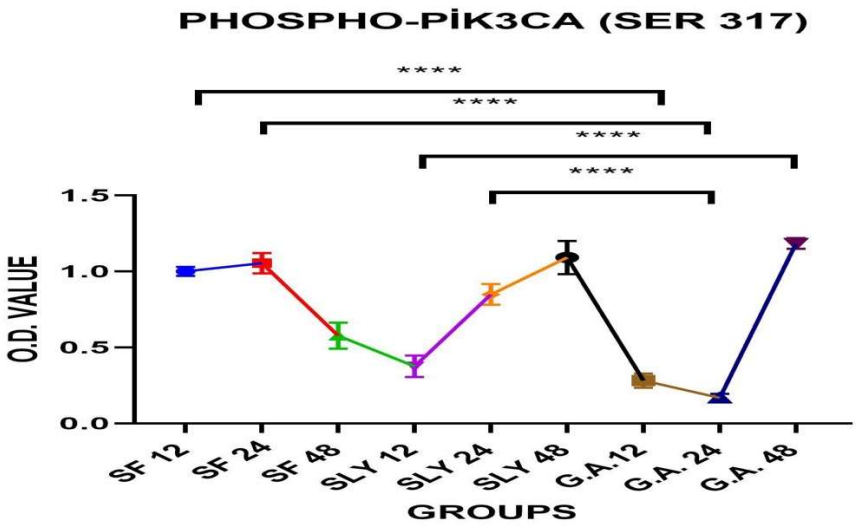


Figure 5.10. (b). PIK3CA protein expression: SF (II) 12 with GA (IV) 12; SF (II) 24 with GA (IV) 24; SLY (III) 12 with GA (IV) 48; SLY (III) 24 with GA (IV) 24 hours ****P<0.0001 significant difference (Mean±SEM).

In diffirent study, the PI3K/AKT/mTOR/p70S6K signaling pathways are responsible for regulating both the proliferation of HSCs (hepatic stellate cells) and the development of hepatic fibrosis. When PI3K is activated its phosphorylate to PIP2 and then PIP2 is phosphorylated, which results in the production of PIP3. PIP3, PDK1, and AKT are all examples of downstream effectors which present in the cell membrane. AKT is responsible for regulating the phosphorylation of mTOR as well as the activity of p70S6K. mTOR is formed by two units known as mTORC1 and mTORC2 (Peng et al., 2022). In contrast to mTORC1, which has had its molecular signaling well researched but mTORC2 is still largely a mystery. However, a number of mTORC2 effectors and substrates have been found. That suggests that mTORC2 may exist in a number of distinct functions according to its functional role (Fu et al., 2020).

Additionally in the tropane alkaloid which is the first step in the production of the hydroxylated nontropane alkaloids, which are also known as calystegine. When human adipose-derived stromal stem cells (also known as ASCs) are exposed to hyperglycemia, calystegines play a role in protecting the cells through preventing apoptosis, inflammation, and oxidative damage. Diabetes is obesity and metabolic syndrome are all conditions that could potentially benefit from calystegines as a result to their ability to maintain the AKT/PI3K/mTOR molecular pathway. A decrease in Endoplasmic reticulum (ER) stress, an

increase in the percentage of adult stem cells that are able to survive, and it shows metabolic function are among these benefits (Anna at el., 2022).

In this work, we observed that the PI3K/AKT/mTOR gene pathway (PIKCA, AKT mTORC1, EIF4EBP1, P70S6K1, Cyclin D1) is the most effective method to explore hepatocytes proliferation after liver partial hepatectomy. Despite of that the study showed PIKCAC1, AKT1, mTORC1 and CyclinD1 gene expression ocur in good amount but P70S6K1 did not expressed. It suggest that its might be P70S6K1 expression is activated by another factor. On the other hand, the findings of our research indicate that the pharmacological interaction between silymarin (SLY) and gallic acid (GA) does not shows in the same pattern as we might expect. When we compared with the expression of gallic acid (GA) gropus, the expression of the silymarin (SLY) group reveals a number of diffirent characteristics that are recognizable.

Mutations in PIK3CA, which encodes the PI3K catalytic subunit p110a, are frequently seen in human cancer. (Bader et al., 2006). According to the findings of our study, statistically significant gene expression of PIK3CA1 is 0.0001, <0.0001, <0.0001, 0.0001, 0.0004, 0.0002, between the groups belonging to the groups SF(II) 6th hours and SLY (III) 6, 24 ,48 hours and GA (IV) groups 6, 12, 24, 48 0.0038, <0.0001, 0.0004, <0.0001. Group GA (IV) 24th hours statistical analysis for protein expression shows that the ****P<0.0001 value. In addition, PIKCA (Ser317) protein expression was investigated for the first time in the scope of our research. We observed a thick band in western blot and discovered that it was highly expressed in 48th hours group GA (IV). Our hypothesis predicts that PIKCA will be expressed early in the stages of our study, however we find that its expression is early with the respect of gene and late of protein expression. Furthermore, as this is the first investigation into this protein PIKCA (ser317) we have no references for the conclusion.

5.4.2. AKT1 gene expression

AKT1 gene expression control (I) group and group SF (II) 6, 12, 24, 48 experimental groups, SLY (III) 6, 12, 24, 48 and GA (IV) 6, 12, 24, 48 the mean p value difference between in all groups is (****, <0.0001) as shown in (Figure 5.11) and were found significant as shown in Table. 5.3.

Table. 5.3. AKT1 gene expression

AKT1					
Tukey's multiple comparisons test	Mean Diff,	95,00% CI of diff,	Significant?	Summary	Adjusted P Value
Control vs. SF 6	6.234	1,819 to 10,65	Yes	***	0.0009
Control vs. SF 12	11.16	6,748 to 15,58	Yes	****	<0,0001
Control vs. SF 24	11.23	6,814 to 15,64	Yes	****	<0,0001
Control vs. SF 48	11.25	6,838 to 15,67	Yes	****	<0,0001
Control vs. SLY 6	11.17	6,759 to 15,59	Yes	****	<0,0001
Control vs. SLY 12	10.99	6,572 to 15,40	Yes	****	<0,0001
Control vs. SLY 24	10.99	6,580 to 15,41	Yes	****	<0,0001
Control vs. SLY 48	11.17	6,752 to 15,58	Yes	****	<0,0001
Control vs. GA 6	11	6,588 to 15,42	Yes	****	<0,0001
Control vs. GA 12	11.07	6,660 to 15,49	Yes	****	<0,0001
Control vs. GA 24	10.61	5,840 to 15,38	Yes	****	<0,0001
Control vs. GA 48	11.1	6,687 to 15,52	Yes	****	<0,0001
SF 6 vs. SF 12	4.928	0,5140 to 9,343	Yes	*	0.0174
SF 6 vs. SF 24	4.995	0,5804 to 9,409	Yes	*	0.0151
SF 6 vs. SF 48	5.019	0,6043 to 9,433	Yes	*	0.0143
SF 6 vs. SLY 6	4.94	0,5257 to 9,355	Yes	*	0.017
SF 6 vs. SLY 12	4.752	0,3379 to 9,167	Yes	*	0.0252
SF 6 vs. SLY 24	4.761	0,3467 to 9,176	Yes	*	0.0248
SF 6 vs. SLY 48	4.932	0,5178 to 9,347	Yes	*	0.0173
SF 6 vs. GA 6	4.769	0,3542 to 9,183	Yes	*	0.0244

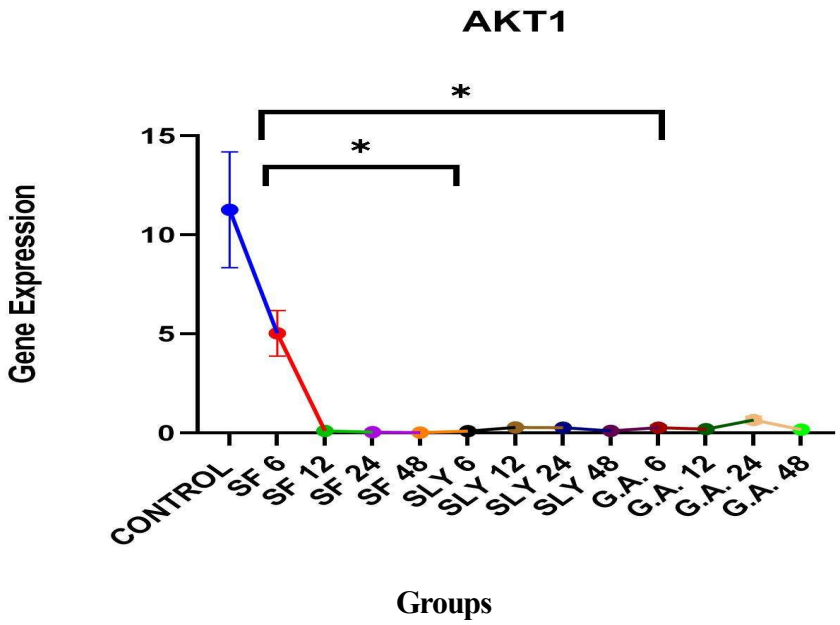


Figure 5.11. AKT1 gene Expression Control (I) group and group SF (II) 6, 12, 24, 48 hours experimental groups SLY (III) 6, 12, 24, 48 hours and GA (IV) 6, 12, 24, 48 Significant diffirence is (****, P<0.0001) (Mean ± SEM).

The activity of an enzyme called AKT. That is also known as Protein Kinase B its affects metabolism as well as cell survival and cell proliferation. AKT2 is found in adipose and skeletal tissue, while AKT1 is found over all the body. Molecular Signaling that is mediated by insulin is what turns it on. AKT was cause for the two phosphorylation sites such as Thr 308 and Ser 473 (Herman et al., 2022).

According to the findings of the study the phosphorylation of AKT1 on Thr308 decreased in 1 to 4th hours after reperfusion, while phosphorylation of AKT1 on Ser473 increased during the same period. The total AKT1 levels were stable when estimated 1 and 4th hours after reperfusion. However, when the levels were measured 24th hours after reperfusion a considerable decline was seen. It was discovered that the quantity of p70S6K protein was lower at 1, 4 and 24th hours after reperfusion. But, it was also discovered that p70S6K phosphorylation was lower through AKT1 Thr308 residues (Janelidze et al., 2001; Zheng et al., 2016).

In our study, AKT1 gene expression was found to be significantly different between the control (I) and experimental groups SF (II) 6, 12, 24, 48 hours and SLY (III) 6,12, 24, 48 hours and GA (IV) 6,12, 24, 48 hours was the significant mean (P) difference is (**** P<0.0001). Additionally, in the case of the AKT1 gene, the expression level at group GA (IV) 24 hours is higher compared to other groups.

Futhermore group SF (II) 12th hours in AKT1 protein expression with GA (IV) 12th hours significant P-value is (*P<0.019). Group SF (II) 48th hours with the group GA (IV) 48th hours significant diffirence is (***P0.0003) as shown in (Figure 5.12(b)). However, in the case of protein expressions, all experiment groups at 12, 24, and 48 hours were found to have thick bands on the western blot shown in (Figure 5.12 (a)

5.4.2.1. AKT1 protein expression

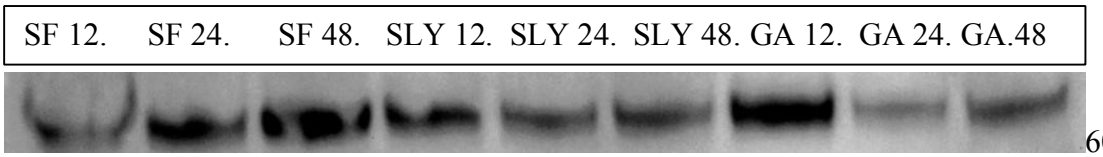


Figure 5.12 (a). AKT1 band images from Western Blot analysis are shown.

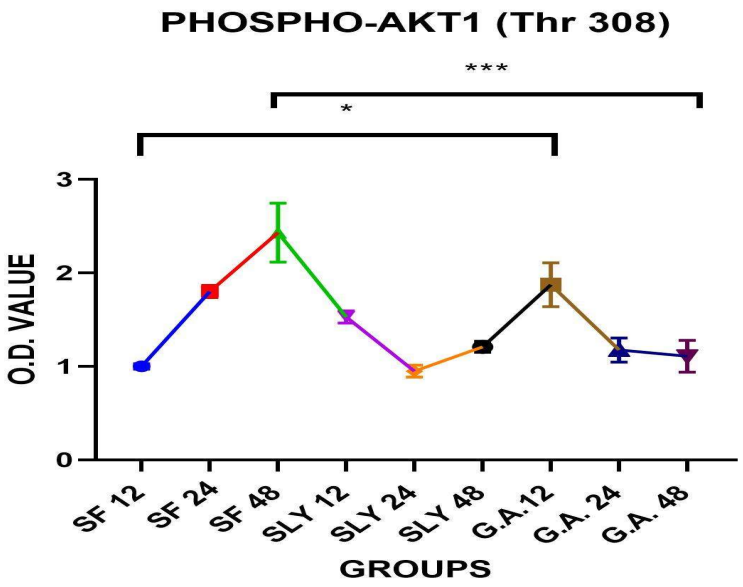


Figure 5.12. (b). AKT1 protein expression: SF (II) 12 with GA (IV) 12; GA (IV) 48 hour with SF (II) 48; hours significant difference is (*P<0.019, ***P0.0003) (Mean±SEM).

5.4.3. mTORC1 gene Expression

The mTORC1 gene expression compared to control (I) group with group SF (II) 24th and 48th hours significant diffirence is (****, P <0.0001). In addition SF (II) 6th with SF (II) 24, 48 hours were compared p value is (****, <0.0001). In the time difference between SF (II) 12th hour with SF 24th hours (**, P <0.0002) was found. The difference between SF (II) 24th hours and SF (II) 48th hours (****, P<0.0001) was found to be more significant. Group SF (II) 24 hours with group SLY (III) 6, 12, 24, 48 hours and group GA (IV) 6, 12, 24, 48 hours (****, P <0.0001) significant difference was found. The significant difference between SF (II) 48 hours and SLY (III) 6, 12, 24, 48 hours and GA (IV) 6, 12, 24, 48 hours is (****, P<0.0001) as shown in the table 5.4 and (Figure 5.13).

Table 5.4. mTORC1 gene Expression

MTORC1					
Tukey's multiple comparisons test	Mean Diff,	95,00% CI of diff,	Significant?	Summary	Adjusted P Value
Control vs. SF 24	-1764	-2561 to -966,4	Yes	****	<0,0001
Control vs. SF 48	-3838	-4635 to -3040	Yes	****	<0,0001
SF 6 vs. SF 24	-1762	-2560 to -964,9	Yes	****	<0,0001
SF 6 vs. SF 48	-3836	-4634 to -3039	Yes	****	<0,0001
SF 12 vs. SF 24	-1102	-1899 to -304,5	Yes	**	0.0012
SF 12 vs. SF 48	-3176	-3973 to -2378	Yes	****	<0,0001
SF 24 vs. SF 48	-2074	-2871 to -1276	Yes	****	<0,0001
SF 24 vs. SLY 6	1229	431,3 to 2026	Yes	***	0.0002
SF 24 vs. SLY 12	1714	916,2 to 2511	Yes	****	<0,0001
SF 24 vs. SLY 24	1674	877,1 to 2472	Yes	****	<0,0001
SF 24 vs. SLY 48	1328	530,8 to 2126	Yes	****	<0,0001
SF 24 vs. GA 6	1660	862,1 to 2457	Yes	****	<0,0001
SF 24 vs. GA 12	1647	849,4 to 2444	Yes	****	<0,0001
SF 24 vs. GA 24	1724	747,6 to 2701	Yes	****	<0,0001
SF 24 vs. GA 48	1552	754,8 to 2350	Yes	****	<0,0001
SF 48 vs. SLY 6	3303	2505 to 4100	Yes	****	<0,0001
SF 48 vs. SLY 12	3788	2990 to 4585	Yes	****	<0,0001
SF 48 vs. SLY 24	3748	2951 to 4546	Yes	****	<0,0001
SF 48 vs. SLY 48	3402	2605 to 4199	Yes	****	<0,0001
SF 48 vs. GA 6	3733	2936 to 4531	Yes	****	<0,0001
SF 48 vs. GA 12	3721	2923 to 4518	Yes	****	<0,0001
SF 48 vs. GA 24	3798	2821 to 4775	Yes	****	<0,0001
SF 48 vs. GA 48	3626	2829 to 4424	Yes	****	<0,0001

Futhermore in diffirent study apoptosis, proliferation, autophagy, and liver regeneration are all affected by the AKT/mTOR signaling pathways. To understand how mTORC1 (ser 2448) controls S6K1 and 4EBP1, which in turn trigger mRNA translation and protein synthesis, the researchers looked at mTORC1 (Chen et al., 2022; Ma et al., 2022).

In our research, we observed that there was a statistically significant difference between the mTORC1 gene expression SF (II) group in all hours and the SLY (III) and GA (IV) in all hours is significant difference was the (****P<0.0001).

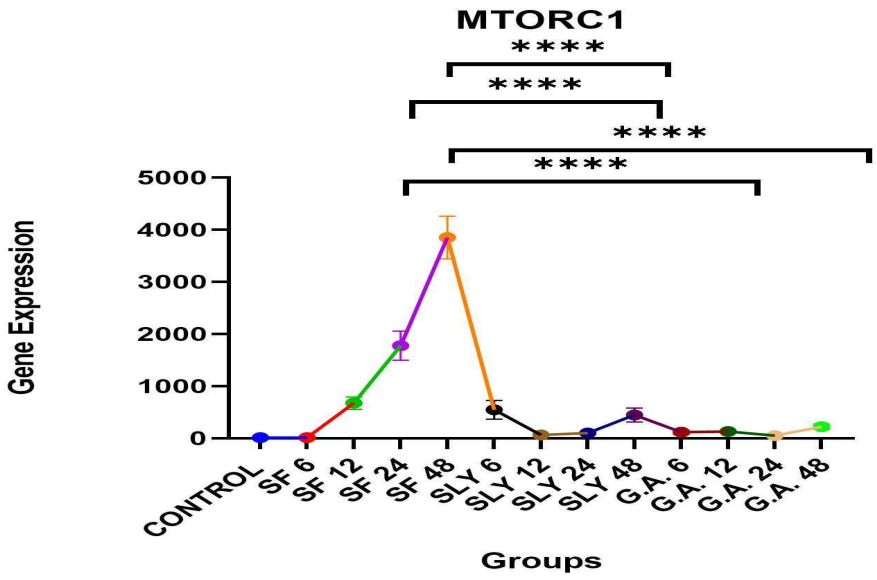


Figure 5.13. mTORC1 gene expression: significant difference between group SF (II) 24 hours and group GA (IV) 6, 12, 24, 48 hours (****, P <0.0001). Significant difference between SF (II) 48 hours and SLY(III) 6, 12, 24, 48 hours (****, P<0.0001) (Mean ± SEM).

5.4.3.1. mTORC1 protein Expression



289kDa

Figure 5.14. (a). mTORC1 bands from Western Blot analysis.

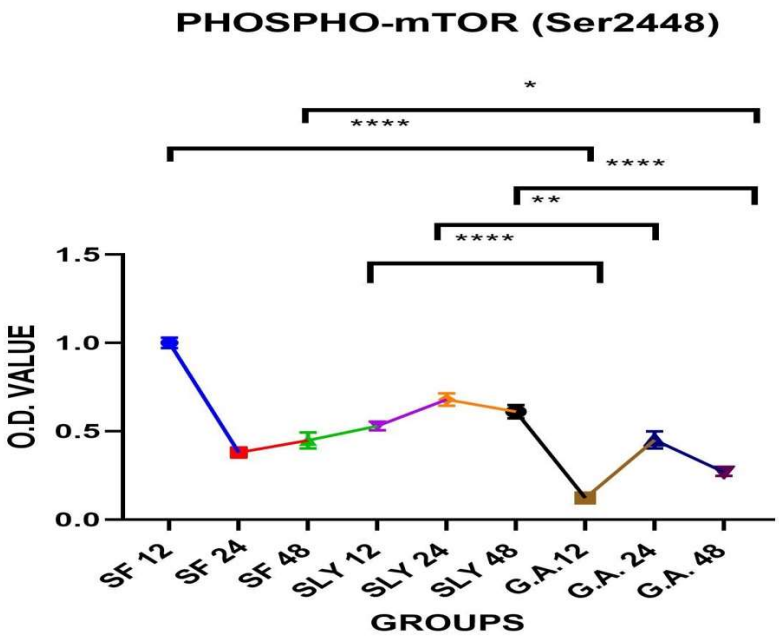


Figure 5.14. (b). mTORC1 protein expression: SLY (III) 12 with GA (IV) 12; SLY (III) 24 with GA (IV) 24; SLY (III) 48 with GA (IV) 48 hours; SF (II) 12 with GA 12 hours; SF (II) 48 hours and GA 48 hours significant diffirence is (****P<0.0001, **P0.0028, ****P<0.0001, ****P<0.0001, *P0.0252) (Mean± SEM) .

When we looking at the protein expressions in our study the group SF (II) 12th hours and group SLY (III) at 12, 24, and 48 hours were compared to the group GA (IV) 24th showed a thick band on the western blot performed after 48th hours of partial hepactectomy.

Futhermore in diffirent studies have found that modifications in mTORC1 cell culture can cause changes in CDK8-CycC (Cyclin C) levels in patients who suffering from non-alcoholic fatty liver disease (NAFLD). Mice in whom the raptor which belong to mTORC unit gene was deleted only in the liver had increased levels of CDK8 expression. At the last, rapamycin can repair CDK8-CDC in the mice. mTORC1 is the reason for the physiological and molecular regulation of hepatic CDK8-CDC (Feng et al., 2015).

In another study, L-ornithine (Orn) was investigated in study and found to affect mTORC1 downstream targets in rat liver. Orn stimulates an increase the phosphorylation of mTORC1 downstream targets in rat initial hepatoma cells and liver which contain low amino acids. Based on the findings of studies that used mTOR inhibitors such as rapamycine, that is this pathway is to showed these results. The production of proteins in tissues could be stimulated using this method (Kokubo et al., 2015).

Futher research shows that, the mTOR protein play as the key positive regulator of ovarian follicle growth and development in vivo. The mTOR protien is cause of this function. There appear to be irregularities in the mitotic activity of granulosa cells and an enhanced cell cycle arrest in the G1 phase during mTOR protein inhibition is prevented, both of help to limit follicle growth (Yu et al., 2011).

5.4.4. EIF4EBP1 gene expression

As shown in (Figure 5.15) and (Table 5.5) significant difference was found between EIF4EBP1 gene expression is none compared to other groups.

Table 5.5. EIF4EBP1 gene expression

EIF4EBP1					
Tukey's multiple comparisons test	Mean Diff,	95,00% CI of diff,	Significant?	Summary	Adjusted P Value
GA 6 vs. GA 12	-0.5381	-14,47 to 13,39	No	ns	>0,9999
GA 6 vs. GA 24	-0.4788	-15,37 to 14,41	No	ns	>0,9999
GA 6 vs. GA 48	0.1004	-14,79 to 14,99	No	ns	>0,9999
GA 12 vs. GA 24	0.05929	-13,87 to 13,99	No	ns	>0,9999
GA 12 vs. GA 48	0.6385	-13,29 to 14,57	No	ns	>0,9999
GA 24 vs. GA 48	0.5792	-14,31 to 15,47	No	ns	>0,9999

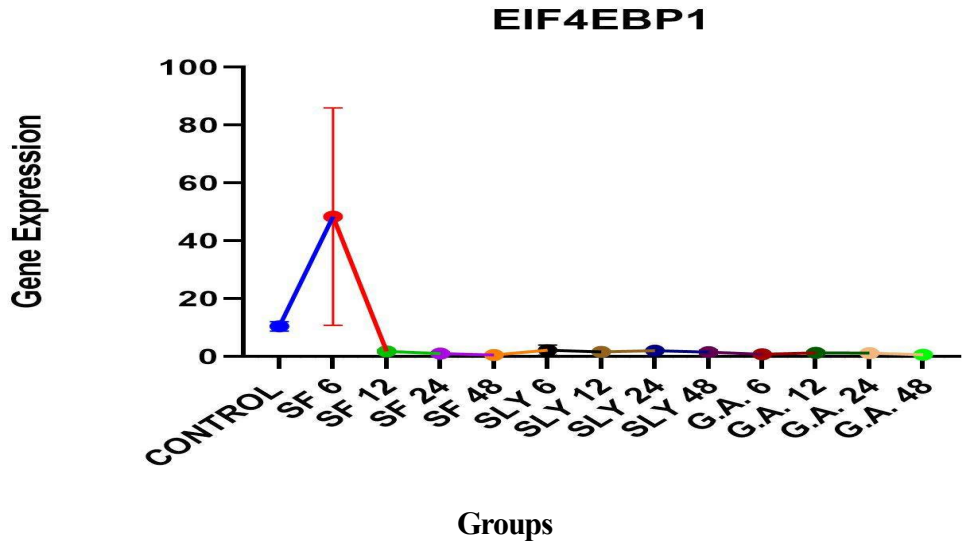


Figure 5.15. EIF4EBP1 gene expression: There was no significant difference (Mean±SEM) between groups expression.

In diffirent study, the actions of rapamycin-responsive mTOR complex 1 are under the control of the mammalian target of rapamycin (mTOR inhibitor). That is controlling the cell function (mTORC1). Because rapamycin induces G1 arrest and its suppressed tumor cell growth, it is more effective that blocking mTORC1 would be useful in the cancer therapy. In MCF7 breast cancer cells, rapamycin reduces cyclin D1 without altering mRNA levels. Leucine deprivation is more affecting mTORC1 signaling that has a greater effect on cyclin D1 levels than does rapamycin in compared to cell types. The inhibiting 4E-BP1, the effect of rapamycin on cyclin D1 synthesis is distroyed and mRNA polysomal binding is improved. It identifies 4E-BP1 as a most important regulator of cyclin D1 expression during protein synthesis. It suggesting that this effect is not mediated by changes in cytoplasmic cyclin D1 mRNA levels. That is in some cell types it interferes with amino acid entry into mTORC1 complex. It can reduce proliferation compared to rapamycin inhibitor (Averous et al., 2008). In our research, we looked at the expression of the mTORC1 gene and protein and we found that both are expressed very well in all groups.

5.4.5. P70S6K1 gene expression

P70S6K1 gene expression showed a significant difference between the Group SF (II) 6 versus SF 24, 48 hours groups. (Respectively*, P<0.0395,0.0256). Group SF (II) 6 hour and SLY(III) 24 hour ,48 hours as shown in (Table 5.6) and (Figure 5.16).

Table 5.6. P70S6K1 gene Expression

P70S6K1					
Tukey's multiple comparisons test	Mean Diff,	95,00% CI of diff,	Significant?	Summary	Adjusted P Value
SF 6 vs. SF 24	92.84	2,469 to 183,2	Yes	*	0.0395
SF 6 vs. SF 48	97.23	6,860 to 187,6	Yes	*	0.0256
SF 6 vs. SLY 24	91.93	1,552 to 182,3	Yes	*	0.0431
SF 6 vs. SLY 48	95.58	5,203 to 186,0	Yes	*	0.0302

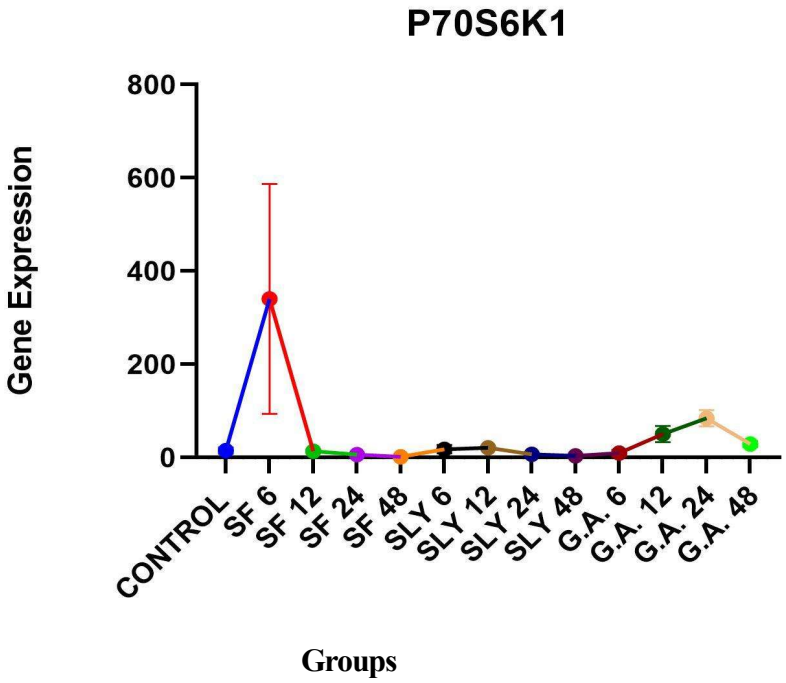


figure 5.16. P70S6K1 gene expression, significant (Mean± SEM) difference was observed between SF (II) 24 and 48 hours compared to SF (II) 6 is (Respectively*, P<0.0395,0.0256).

Findings from recent studies indicate that mTORC1 protein expression is high in most of the liver regeneration research. This is shown that all study groups had a high molecular weight band size in western blot analysis. As part of our study, we expected the P70S6K protein to become active, but this did not ocur in all group accept in some groups. Moreover, cyclin D1 gene activation occurs along this molecular pathway. However might not via P70S6K protein/gene expression, it may suggesting the presence of a third component is there which play a role this molecular phenomena.

5.4.6. CYCLIND1 gene Expression

Cyclin D1 gene expression was significantly different group SF (II) 6, 12, 24, 48 hours between groups GA(IV) 24,6 hours is (**,P0.0063,****P<0.0001,*P0.0144, ****P<0.0001,*P<0.0115,****P<0.0001). Moreover group SLY (III) 6, 12, 24, 48 hours and group GA (IV) 6, 24, 24 hours significant is found that (*, ****,*,****,*,****,*** P0.0133, P <0,0001, P 0.015, P<0,0001, P 0.0166, P <0,0001, P 0.0003). The significant difference between GA 12, 24 and GA 24, 48 hours was calculated as (**, ***, P 0.0098, 0.0005) (Table 5.7) and (Figure 5.17).

Table 5.7. CYCLIND1 gene Expression

CYCLIND1					
Tukey's multiple comparisons test	Mean Diff,	95,00% CI of diff,	Significant?	Summary	Adjusted P Value
Control vs. SF 12	9.815	1,317 to 18,31	Yes	*	0.012
Control vs. SF 24	11.06	2,562 to 19,56	Yes	**	0.0028
Control vs. SF 48	11.26	2,763 to 19,76	Yes	**	0.0022
Control vs. SLY 6	11.13	2,633 to 19,63	Yes	**	0.0026
Control vs. SLY 12	11.03	2,530 to 19,53	Yes	**	0.0029
Control vs. SLY 24	10.93	2,437 to 19,43	Yes	**	0.0032
SF 6 vs. GA 24	-11.2	-20,38 to -2,022	Yes	**	0.0063
SF 12 vs. GA 24	-16.11	-25,29 to -6,934	Yes	****	<0,0001
SF 24 vs. GA 6	-9.654	-18,15 to -1,156	Yes	*	0.0144
SF 24 vs. GA 24	-17.36	-26,54 to -8,178	Yes	****	<0,0001
SF 48 vs. GA 6	-9.855	-18,35 to -1,357	Yes	*	0.0115
SF 48 vs. GA 24	-17.56	-26,74 to -8,379	Yes	****	<0,0001
SLY 6 vs. GA 6	-9.725	-18,22 to -1,227	Yes	*	0.0133
SLY 6 vs. GA 24	-17.43	-26,61 to -8,249	Yes	****	<0,0001
SLY 12 vs. GA 6	-9.622	-18,12 to -1,124	Yes	*	0.015
SLY 12 vs. GA 24	-17.33	-26,50 to -8,147	Yes	****	<0,0001
SLY 24 vs. GA 6	-9.529	-18,03 to -1,031	Yes	*	0.0166
SLY 24 vs. GA 24	-17.23	-26,41 to -8,054	Yes	****	<0,0001
SLY 48 vs. GA 24	-13.99	-23,16 to -4,807	Yes	***	0.0003
GA 12 vs. GA 24	-10.79	-19,97 to -1,616	Yes	**	0.0098
GA 24 vs. GA 48	13.42	4,242 to 22,60	Yes	***	0.0005

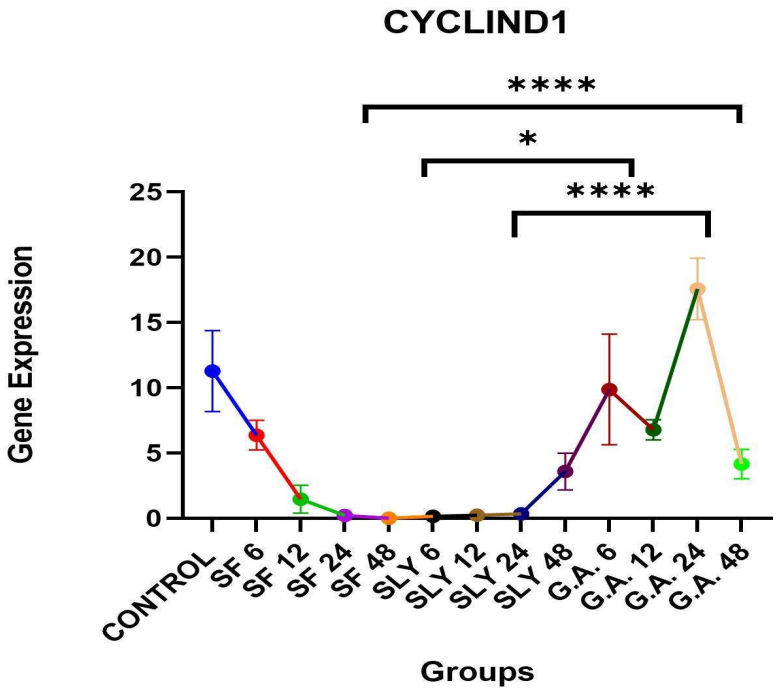
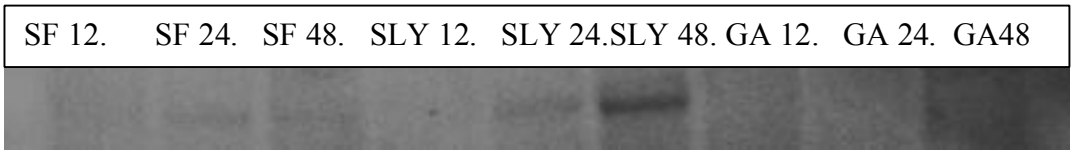


Figure 5.17. CyclinD1 gene expression group with SF (II) 12 GA (IV) 24: ****, P <0.0001; SF (II) 24 with GA (IV) 24: ****,*, P<0.0001, P< 0.0115; SF (II) 24, 48 with GA (IV) 24, 6 : (*,****,*,****,*,****,*** P0.0133,P <0,0001,P 0.015, P<0,0001,P 0.0166,P <0,0001, P 0.0003) SLY(III) group 6, 12, 24, 48 hours with group GA (IV) 6, 24, 6,24,6,24,24; and **,***,P 0.0098, 0.0005. GA 12, 24 to GA 24, 48 hours are significant as shown in Table 5.7 (Mean±SEM).

Our recent study results support these conclusions the highest levels of cyclin D1 gene expression were found in GA (IV) 6th and 24th. Based on our findings, we noticed that the Cyclin D1 gene is expressed high level in the GA (IV) groups (Figure 5.17).

5.4.6.1. Cyclin D1 protein expression



36kDa

Figure 5.18. (a). Cyclin D1 band images from Western Blot analysis are shown.

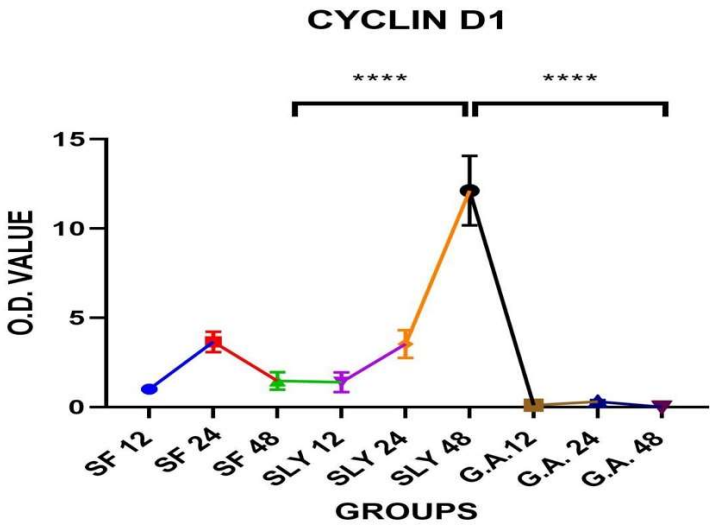


Figure 5.18. (b). CYCLIN D1 protein expression: Group SF (II) 48 with SLY (III) 48 hours; GA (IV) 48 with SLY (III) 48 hours significant difference is ****P<0.0001, *P0.0252 (Mean±SEM).

Interestingly, protein expression was only detected in diffirent type of the study groups. Nevertheless, when a western blot was performed on the sample collected at the 48-hour of time after PHx. It was found that group SLY(III) had a distinguished band. There is a possibility that group of the GA (IV) will have exceptionally high cyclin D1 gene expression. However, there is might be chance that the protein will modify in some aspect if it is expressed. In the all members of the cyclin D family protein, share a similar function. They all participate in cell division and proliferation.

In diffirent study shows that in growth factor-stimulated hepatocytes, it has been considered that the PI3K inhibitor LY294002 and the FRAP/mTOR inhibitor rapamycin can able to completely destroy the process of DNA replication and cyclin induction. Despite of this they are unable to postpone entrance into the S phase of the expression of cyclin D1. Both LY294002 and rapamycin were able to reduce the level of cyclin D1 mRNA and also protein found in hepatocytes. The fact that the expression of cyclin D1 is dependent on the PI3K, FRAP/mTOR, and MEK/ERK cascade15 is the importance of this pathways (Coutant et el., 2002; Huang et el., 2020).

Additionally maintaining the liver's size and function requires regenerative signaling that is done by the Wnt/catenin pathway. The production of Wnt protein by sinusoidal endothelial cells and macrophages is the earliest response to liver injury and desease. These

proteins then activate hepatocyte Frizzled and LDL-related protein-5/6 coreceptors. Which stabilize-catenin and trigger its translocation into the nucleus. This protien interactions with T-cell transcription factors and nuclear catenin is able to promote target genes such as cyclin D1 for proliferation and other genes that are essential for hepatocyte function during the liver regeneration (Hu et al., 2021). As a results of the significant rise in cyclin D1 gene expression that did not cause correspond to a rise in P70S6K1 expression. Our working hypothesis that suggests cyclin D1 could be activated by the Wnt pathway because according to recent research Wnt pathway play a important role in liver regeneration.

It was previously research showed that an increase in the level of cyclin D1 mRNA and protein that was degraded was the cause of the rapamycin-induced suppression of cyclin D1 expression (Hahemolhosseini et al., 1998). D-type cyclins (D1-D3) bind to and activate cyclin-dependent kinases (CDK4 and CDK6). These kinases are most important for the phosphorylating retinoblastoma protein and initiating DNA synthesis. The progression of the cell cycle through the G1-S phase. During cell cycle, the de novo formation of cyclin proteins takes place and then these cyclin proteins are finally removed (Thomas et al., 2007). In our research, we noticed that the cyclin D1 gene is strongly expressed in GA (IV) groups. However, we did not find any thick or high molecular weight band on the western blot. Which leads us to believe that there are multiple types of cyclin D, such as D1, D3 may be participate in our experiment by gallic acid and it may activate cyclin D3 rather than D1.

According to the diffirent study, endogenous 4E-BP1 may be important for modulating cyclin D1 levels in response to decreased mTORC1 signaling. However, still this is not fully known. The rapamycin has on the level of cyclin D1 produced is mitigated when 4E-BP1 is inhibited. For instance, treatment of HEK293 cells with rapamycin results in a reduction in S6K activity but does not affect the levels of cyclin D1. 4E-BP1 deletion is sufficient to prevent this effect from happening (Averous et al., 2008).

Moreover in the progression of mammalian cell development from the G1 phase to the S phase is stimulated through the activation of CDK4 and CDK6 by D-type cyclins such as D1, D2, and D3. Researchers still tryed to understand that the functional overlap that happens among D-type cyclins protein. However, they focused their research mostly on cyclin D3. Since cyclin D3 is involved in both cell proliferation and terminal differentiation

biological phenomena. This provides support for the idea that D-type cyclins do not perform repetitive tasks (Bartkova et al., 1998). The recent work provides strong evidence that D3 is what stimulates cell growth beacause D3 is participate into the molecular phenomena according to research. Our investigation into this it is possibility that may provide similar results.

Another study showed that epidermal growth factor such as insulin on their own did not have much effect on cultured hepatocytes. However, the combination of these two factors increased cyclin D1 synthesis and may increases cell turnover. When Mek1/PI3K was blocked, both cyclin D1 expression and replications decreased. The mitogens had little effect on cyclin D3 expression. These results support the concept that factors affecting proliferation regulate the liver protein cyclin D1, but not cyclin D3 and this liver protein responsible for a different pathway (Rickheim et al., 2002). According to this research, most of the time cyclin D1 is controlled by external influences, whereas cyclin D3 is entirely determined by the molecular pathways. As a result, the molecular pathway is the foundation of also our research, and this might be indicate that D3 is active in PI3K/AKT/mTOR pathway.

In another research, leucine amino acid demonstrated to influence protein synthesis in the rats. Leucine effect protein synthesis via eIF4G phosphorylation and its interaction with eIF4E, whether eIF4G phosphorylation mediates these effects is still unknown. However, it is unclear if leucine affects eIF4G phosphorylation, whether eIF4G phosphorylation is important for leucine's influence on protein synthesis. mTOR (the mammalian target of rapamycin) or some other component of mTOR is involved in leucine-specific signaling pathway (Figure 5.19) (Martha et al., 2007).

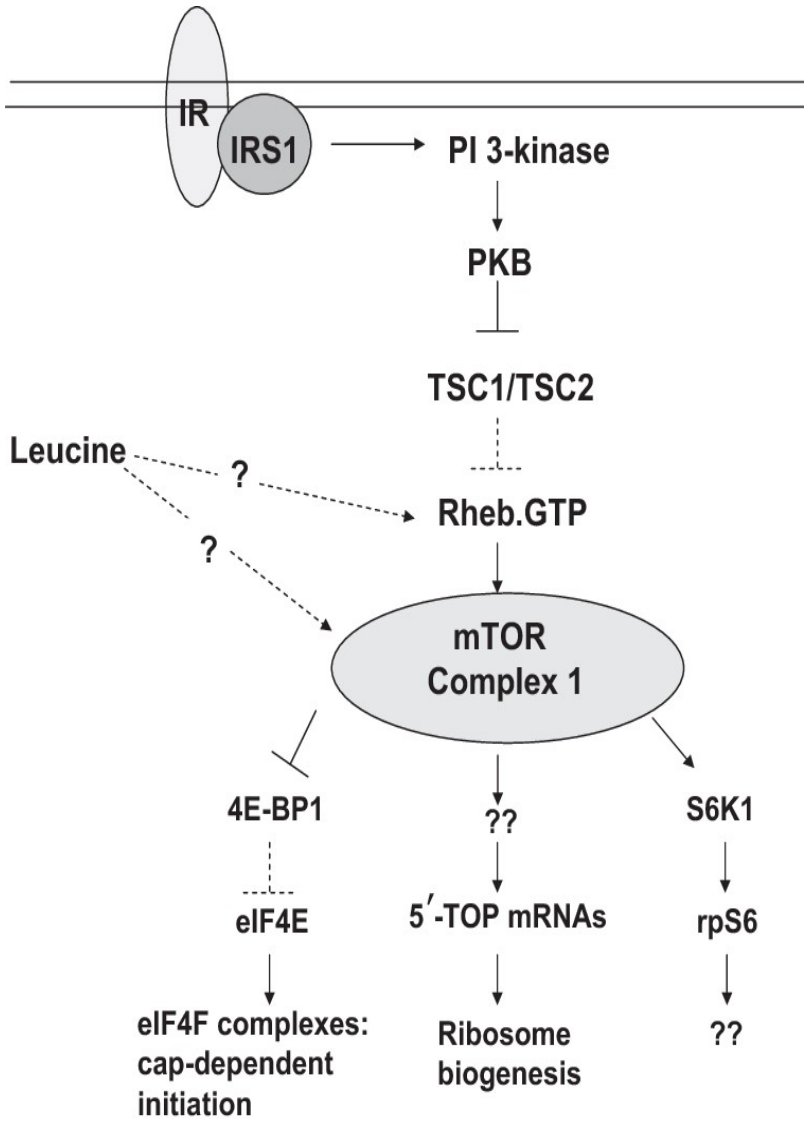


Figure 5.19. The involvement of 4E-BP1 in the connection between cyclin D1 and mTORC1(Martha et al., 2007).

According to diffirent research, 4E-BP1 is most important component of the interaction that exists between cyclin D1 and mTORC1 molecular pathway. Overexpression of 'constitutively active' 4E-BP1 in MCF7 cells led to cell cycle arrest and decreased expression of cyclin D1. This is findings showing that 4E-BP1 activation (dephosphorylation) is required for protein kinase C to inhibit cyclin D1 expression. Overexpression of 'constitutively active' 4E-BP1 in MCF7 cells led to cell cycle arrest. The decreased expression of cyclin D1 of the most important targets is to find other mRNAs that, like cyclin D1 mRNA can be controlled similarl pattern (Jiang et al., 2003; Hizli et al., 2006; Averous., 2008).

5.5. ALT analysis

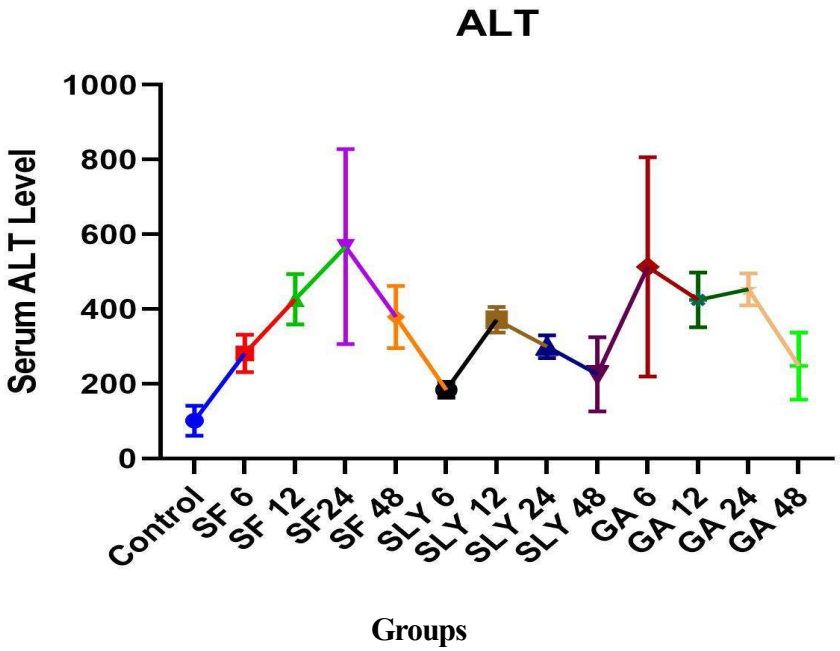


Figure 5.20. ALT level in Group Control (I) and SF (II), SLY (III), GA (IV) (6, 12, 24, 48 hours groups).

5.5.1. AST analysis

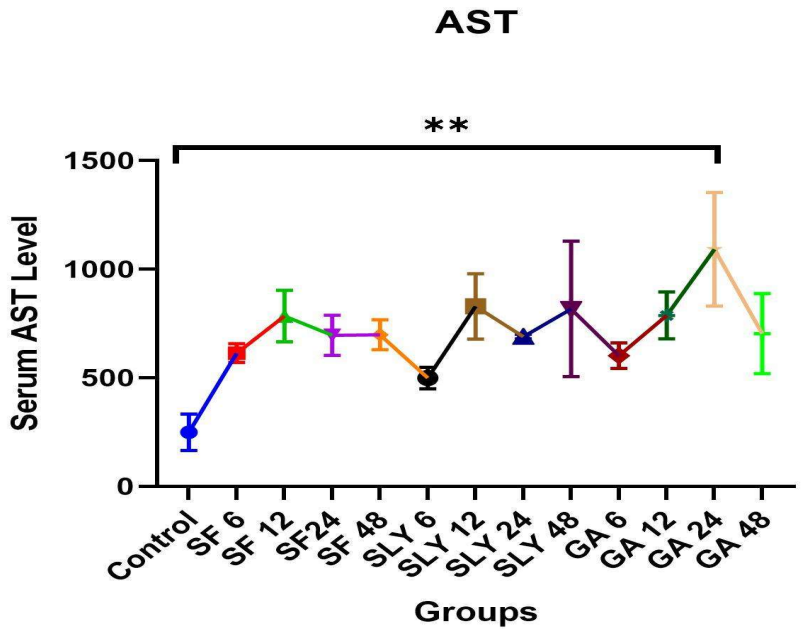


Figure 5.21. AST level in Group Control (I) and SF (II), SLY (III), GA (IV) (6, 12, 24, 48 hours groups).

In diffirent study Show that, Methotrexate (MTX) has been shown to cause hepatotoxicity in rat. However, pretreatment with GA effetively reduced AST and ALT levels at 24 hours of time preiod in the MTX group. The results suggesting that the hepatotoxicity was at least partially reduced by GA. (Safaei., 2018). We found that the levels of AST and ALT in the GA-treated group were significantly lower than those in the comparison group like (Control, SF (II), SLY (III)) (Figure 5.20, Figure 5.21).

According to the diffirent Ischaemia-Reperfusion (I/R) study, which was treated with GA at a dose of 100 mg/kg, was found to have significantly lower levels of AST, ALT, and LDH in the blood than compare to the other two groups (Bayramoglu et al., 2015). According to the results of our research, an effective dose of gallic acid is 200 mg / kg.

According to the findings of our experiment, After 6, 12, and 24 hours had passed the ALT and AST levels increased in all groups. However, it decreased at 48 hours in all groups Control (I), SF (II), SLY(III), and GA (IV) and also we noticed that with the highest reduction in ALT and AST enzyme expression occurring in the GA (IV) 48 hour group. Suggestions indicate it could help in liver regeneration and hepatocyte damage.

Additionally, Patients with renal ischemia-reperfusion who were given GA doses showed decreased levels of malondialdehyde (MDA) enzyme in the kidney and liver. In the case of kidneys, both low and high dosages of GA treatment reduced antioxidant superoxide dismutase (SOD) activity, but in the case of liver its shows diffirently and only low doses of GA treatment increased SOD activity. A high dose of GA decreased liver SOD levels. The levels of the enzyme catalase (CAT) in the kidney were not altered by GA treatment. These studies suggest that different doses of GA shows diffirent results in protect against oxidative damage (Canbek et al., 2011).

According to the another studies, GA and dodecyl gallate (DGA) are beneficial to rats that have been exposed to carbon tetrachloride (CCl_4) which create toxicity in liver. These effects include lowering plasma levels in blood AST, ALT, and gamma Glutamyltransferase (GGT) and reducing liver weight gain while increasing body weight loss. Lowering levels of liver lipid peroxidation, necrosis, and fibrosis however enhancing nonenzymatic antioxidant status, and increasing expression of the p53 gene. That is might

play a key role in liver cell regeneration (Raimunda et al., 2017). The high antioxidant potentiality of gallic acid (GA) recover mice who have been damaged with paracetamol. This as a results of the hepatoprotective effect that gallic acid has done on the liver (Rasool et al., 2010).

Addionally, the fibrosis of the liver that is formed by carbon tetrachloride (CCl_4) could be prevented with the help of Gallic acid. GA lowers serum levels of enzymes produced by the liver such as AST, ALT and also lowers the expression of pro-inflammatory cytokines such as IL-1B, IL-6, COX-2, and TNF and increases expression levels of antioxidant genes superoxide dismutase and catalase (SOD). Gallic acid is a naturally founding antioxidant that might help in treament of liver disorders (Ojeaburu et al., 2021).

Additional research was conducted to investigate the effects of gallic acid (GA) on led (Pb) toxicity in the kidney, liver and blood. In wistar rats were first given Ethylenediaminetetraacetic acid (EDTA) (55 mg/Kg i.p.) and GA (13,5 mg/Kg i.p.), and then they were given led (Pb) nitrate (50 mg/Kg/day, i.p.). Following the rats were given either GA or EDTA. The GA have chelating property that is not responsible for its ability to protect against the oxidative damage which is caused by Pb. However rather the antioxidant activity of GA and its also to boost the body's antioxidant status (Reckziegel et al., 2016).

Further study found that gallic acid (GA) (50, 100, and 200 mg/kg, i.p.) was more effective doses at recovering biochemical parameters compare to TB (Terminalia belerica Roxb) extract (200, 400, and 800 mg/kg, i.p.) doses. The effective protection against carbon tetrachloride-induced liver and kidney damage was found to be given doses is gallic acid 200 mg/kg (Jadon et al., 2007). In our recent study also effective dose is 200 mg/kg.

Further investigation showed that some hepatic stellate cells (HSCs) could revert to a quiescent stellate after being cultured in vitro with proliferation and activity suppressed by GA. Hepatic fibrosis was slowed down considerably because GA has capacity to reduce HSC activation, proliferation, and apoptosis in vivo. When the effects of GA on HSC activation (TGF-1 and -SMA) and hepatocyte proliferation (PCNA) were compared to those of silymarin, the results showed that GA was stronger than silymarin (Lakkany et al., 2019).

According to diffirent research, lindane is insecticide which is long-lasting effetive biochemical. Gallic acid (GA) prevents liver and kidney damage caused by lindane in rats. Protecting the kidneys and liver against lindane's harmful effects required lowering their lipid peroxidation product (LPO) levels, increasing their enzyme activity, and giving them antioxidants. Organ damage caused by oxidation is reduced in lindane-treated rats when gallic acid is given to the animals because GA act as antioxidant. This provides protecting organs from oxidative damage like the kidneys and liver (Padma et al., 2011).

Gallic acid given dose of 10 mg/kg of body weight, was able to inhibit the development of hepatocellular carcinoma (HCC) in Male Wistar albino rats that had been exposed by diethylnitrosamine (DEN). The lowering the levels of carcinoembryonic antigen, alpha-fetoprotein, bilirubin, aspartate transaminase, alanine transaminase, alkaline phosphatase, acid phosphatase, lactate dehydrogenase, gamma-glutamyltransferase, and 5′-nucleotidase, gallic acid inhibits the proliferation of DEN (Ramakrishnan et al., 2008).

Moreover, how the growth factor process functions and attachment of VEGF to endothelial cells stimulates near to stellate cells to secrete pro-HGF. The cleavage of pro-HGF into active HGF are catalyzed through plasminogen and urokinase plasminogen activator (uPA) which is play role during the liver regeneration. By binding to Met receptors on the surface of hepatocytes at the same time HGF triggers the activation of PI3K, AKT, and S6 kinase signaling. The molecular signals generated by HGF signaling such as transforming growth factor (TGF), activating protein 1 (AP1), phosphorylated extracellular signal-regulated kinases (pERKs), CCAAT-enhancer-binding protein (C/EBP), and insulin-like growth factor binding protein 1 (IGFBP1). Yet there is no proof exists that these chemicals actually trigger TOR induction (target of rapamycin) in this particular research. After that the results showed cyclins D and E become more abundant while p27 becomes less abundant and that is causing changes in the cell cycle (Figure 5.22) (Heinrich et al., 2003; Taub et al., 2004). Cell cycle proteins which control cell cycle phases such as cyclin-dependent kinase (CDK), cyclin D, and cyclin E are important for regulating the rate of hepatocyte proliferation during the process of liver regeneration (Wei et al., 2019).

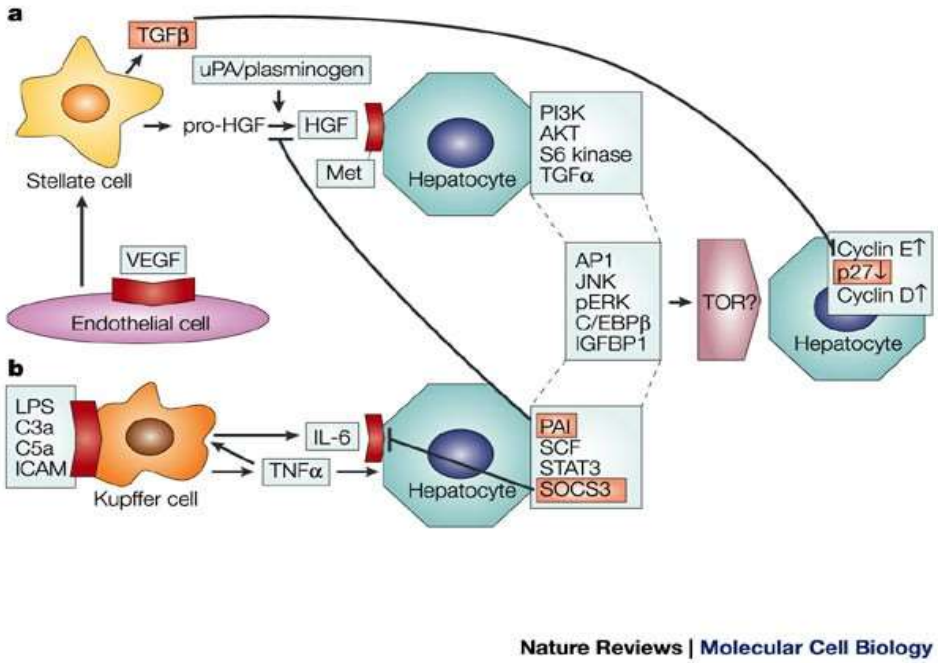


Figure 5.22. The mTOR pathway in the liver establishes a connection between cyclins D and E in hypatocytes (Taub et al., 2004).

Moreover another study showed that in kupffer cell which is liver macrophage apoptosis mediated by cytokines Kupffer cells could be activated to produce the cytokine tumor necrosis factor (TNF) in response to many different stimuli, togather with lipopolysaccharide (LPS), complement components C3a and C5, and intercellular adhesion molecules (ICAMs) and TNF. Correspondingly, Kupffer cells start producing more interleukin-6. Additionally, liver stellate cells produce transforming growth factor (TGF) and p27 and this is two cyclin-dependent kinase (CDK) inhibitors. Which is present on cellular surface receptors on erythrocytes (Leu et al., 2001; Taub et al., 2004; Maeda et al., 2003). According to the findings of this research, they found that the TNF, ICAMs, TGF in particularly has able to regenerating capabilities of the liver. This research might be supporting the recent study.

Our investigation concluded that gallic acid 200 mg.kg-1 i.p. after 70% partial hepatectomy in rats showed that the GA this dose activated the PI3K/AKT/mTOR pathway. As a result of that promotes cell growth and liver regeneration. A key component of this

pathway, the transcription factor S6K701 gene, was not expressed in all groups as we expected. S6K701 gene is responsible for Cyclin D1 from being activated but it did not happened. It might possible Cyclin D1 activation through a third factor. Moreover, previous studies have shown that proteins related to the cyclin D family all have diffirent role and its responsible for the activation of the molecular pathway such as MAPK, AKT etc. The time period of 48th hours after partial hepactectomy, Gallic acid (GA) groups had the lowest levels of ALT and AST compare to the other groups such as SF, SLY. According to PCNA analysis the mitotic index peaked at the 48th hour of time after partial hepactectomy. We are able conclude that gallic acid (200 mg.kg-1'i.p) has a proliferative impact on our partial hepactectomy model with the compared to silymarin, which is well-known for its beneficial regeneration effect.

6. CONCLUSION AND RECOMMENDATIONS

Based on our result, we propose that gallic acid (GA) stimulates cell growth and hepatocyte proliferation through the PI3K/AKT/mTOR pathway. In our trials, the gallic acid (GA) group had the highest rates of hepatocytes proliferation according to the results. Furthermore, after 48th hours of partial hepactectomy, the gallic acid (GA) groups showed the lowest level of ALT and AST liver enzymes. We conclude that all of the our experiment groups had high levels of gene expression as well as protein expression. In our recent study we used in our experiment PIKCA(ser317) protein expression that is for the first time use till now. As a conclusion we need more investigation into this topic to explain how cyclin D1 activates and what mechanisms it takes to do so.

Moreover, we administered treatments including silymarin (SLY) and gallic acid (GA) to rats that had following partial hepatectomy to explain the extent to which the PI3K/AKT/mTOR pathway stimulated hepatocyte proliferation. Based on our research, we conclude that gallic acid (GA) showed is competitive with the positive control silymarin (SLY) and its showed effective results. That both have a multiplicative effect on different groups of animals such as GA 6,12,24,48 and GA 6,12,24,48 hours after partial hepactectomy. Surprisingly, the results of this investigation showed that the two drug had very distinct interactions and might be pharmacologically they intract with each other diffirently. According to our PCNA and liver regeneration rate gallic acid significantly increased the rate of cell proliferation compared to that of silymarin as a positive control group and SF as a negative control groups.

Furthermore, we conclude that the PI3K/AKT/mTOR pathway was activated in response to gallic acid administration at a dose 200 mg/kg, however that the S6K701 gene was not expressed in all groups as we expected and S6K701 gene is responsible for the activation of Cyclin D1 activation therefore, might require the presence of a third component in our recent research. According to the previous research we try to understand that that there are other forms of the cyclin protein and that most experiments demonstrate that Cyclin D1 is not acting alone and mostly is working with cyclin D3 protein as we mentioned above.

According to our above research discussion, there might be several pathways by which gallic acid influences hepatocyte proliferation after partial hepatectomy. In addition according to another reserch activating the AP1 complex, its also possible GA may also stimulate protein expression via the c-myc pathway. Another possibility is that it triggers protein translation through activating the NFkB pathway because in our pathway AKT1 gene and protein expression is too high and this protien is key to activate several pathway such as NFkB, MAPK etc.

Future Perspective of our Research

Our findings indicate that the gallic acid via PI3K/AKT/mTOR pathway needs some additional research.

- To begin with, why does Gallic Acid behave pharmacologically diffirent from SLY we will utilize the Docking tool to figure out how its interactions with each other work. Futhermore when drug enter to the body absorbtion done by Cytochrom C which is present in mitochondria so might be its related to each other.

- Since P70S6K1 gene activation is observed in only the GA^{th} 24 hours group, and because the Kegg pathway database lists only two factors capable of activating Cyclin D in all groups such as P70SK1 and 4EBP1 so third factor must be present in this research. So in future investigation into the PI3K/AKT/mTOR pathway may provide all three parameters.

- As gallic acid (GA) was utilized for the first time in the partial hepatectomy model, it activated a large number of pathways in the hepatocytes and may we do not know. The third factor is that in this research the expression of AKT1 is too high so previous research has shown that AKT is the hub of several other pathways such as c-myc, NFkB etc. However futher reserch needed to explain this theory.

- In our future research, we need to take special emphasis on the dosage, as previous studies have shown that the effects of gallic acid are proportional to the given doses

and dose quantity is most important. Therefore, additional research is required on the topic.

- We notice that during our investigation the majority of genes show early expression in the experimental groups that were administered drugs such as GA and SLY but in the case of the SF groups it had normal expressions. However, this antioxidant can help in liver regeneration after PHx model.

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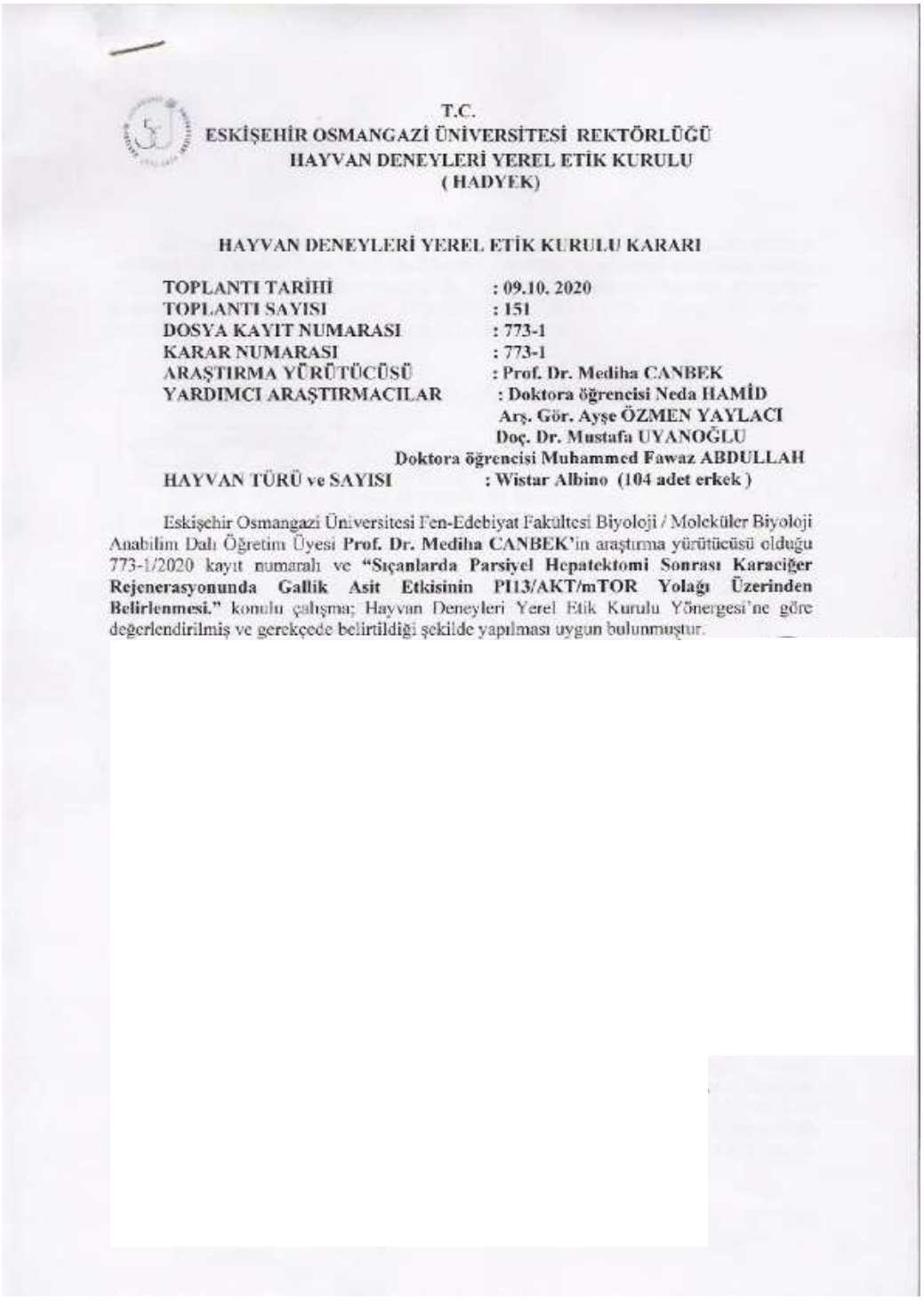
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APPENDIXES

APPENDIX- A. Ethics Committee Report



T.C.
ESKİŞEHİR OSMANGAZİ ÜNİVERSİTESİ REKTÖRLÜĞÜ
HAYVAN DENEYLERİ YEREL ETİK KURULU
(HADYEK)

HAYVAN DENEYLERİ YEREL ETİK KURULU KARARI

TOPLANTI TARİHİ	: 09.10. 2020
TOPLANTI SAYISI	: 151
DOSYA KAYIT NUMARASI	: 773-1
KARAR NUMARASI	: 773-1
ARAŞTIRMA YÜRÜTÜCÜSÜ	: Prof. Dr. Mediha CANBEK
YARDIMCI ARAŞTIRMACILAR	: Doktora öğrencisi Neda HAMİD Arş. Gör. Ayşe ÖZMEN YAYLACI Doç. Dr. Mustafa UYANOĞLU Doktora öğrencisi Muhammed Fawaz ABDULLAH
HAYVAN TÜRÜ ve SAYISI	: Wistar Albino (104 adet erkek)

Eskişehir Osmangazi Üniversitesi Fen-Edebiyat Fakültesi Biyoloji / Moleküler Biyoloji Anabilim Dalı Öğretim Üyesi **Prof. Dr. Mediha CANBEK**'in araştırma yürütücüsü olduğu 773-1/2020 kayıt numaralı ve **"Sıçanlarda Parsiyel Hepatektomi Sonrası Karaciğer Rejenerasyonunda Gallik Asit Etkisinin PI13/AKT/mTOR Yolağı Üzerinden Belirlenmesi."** konulu çalışma; Hayvan Deneyleri Yerel Etik Kurulu Yönergesi'ne göre değerlendirilmiş ve gerekçede belirtildiği şekilde yapılması uygun bulunmuştur.

T.C.
ESKİŞEHİR OSMANGAZİ ÜNİVERSİTESİ REKTÖRLÜĞÜ
HAYVAN DENEYLERİ YEREL ETİK KURULU
(HADYEK)

GEREKÇE

Eskişehir Osmangazi Üniversitesi Fen-Edebiyat Fakültesi Biyoloji / Moleküler Biyoloji Anabilim Dalı Öğretim Üyesi **Prof. Dr. Mediha CANBEK'**in araştırma yürütücüsü olduğu 773-1/2020 kayıt numaralı ve **"Sıçanlarda Parsiyel Hepatektomi Sonrası Karaciğer Rejenerasyonunda Gallik Asit Etkisinin PI13/AKT/mTOR Yolağı Üzerinden Belirlenmesi."** konulu çalışma; tarafımızca değerlendirilmiştir.

Ana Gruplar	Alt Gruplar	Yedek sayısı
Ana Grup I (Kontrol Grubu)	Grup I (n=6)	2
Ana Grup II (PH+SF Grupları)	Sham Alt Grup I: 6. Saat (n=6) Sham Alt Grup II: 12. Saat (n=6) Sham Alt Grup III: 24. Saat (n=6) Sham Alt Grup IV: 48. Saat (n=6)	2'şer hayvan x 4 Grup
Ana Grup III (PH+ Silmarin Grupları)	PH+Madde Alt Grup I: 6 Saat(n=6) PH+ Madde Alt Grup II: 12. Saat(n=6) PH+ Madde Alt Grup III: 24. Saat(n=6) PH+ Madde Alt Grup IV: 48. Saat(n=6)	2'şer hayvan x 4 Grup
Ana Grup IV (PH+Gallik Asid Grubu)	PH+SF Alt Grup I: 6. Saat (n=6) PH+SF Alt Grup II: 12. Saat (n=6) PH+SF Alt Grup III: 24. Saat (n=6) PH+SF Alt Grup IV: 48. Saat (n=6)	2'şer hayvan x 4 Grup

1. **Deney Hayvanları ve Gruplar:** Deneyimizde 200-250 gr ağırlığında erkek Wistar albino ırkı sıçanlar kullanılacaktır. Çalışmamızda n=8 olmak üzere rastgele seçimle 4 ana grup (Kontrol Grubu, PH+ SF Grubu, PH+Silmarin ve PH+ Gllik asid Grubu) ve 4 alt grup oluşturulacaktır.

Ana Grup I (Kontrol Grupları): Kontrol Grubundaki hayvanlara laparatomi yapılıp karaciğer loblarını birbirine bağlayan zarlar ayrılacaktır. %70 partiyal hepatektomi yapılacakmış gibi karaciğerin öndeki sağ orta ve sol yan lopları ayrılarak iplik geçirilecektir. Parsiyal hepatektomi yapılmadan laparotomi bölgesi kapatılacaktır. Hiçbir madde verilmeyecektir. Belirlenen sürelerin (6, 12, 24, 48 saat) sonunda diseksiyonları gerçekleştirilerek kan ve karaciğer örnekleri alınacaktır. Serum örneklerinde ALT ve AST değerlerine bakılacaktır. Karaciğer örneklerinden Mini Array yöntemi ile PI3K/Akt/mTOR sinyal yolağına ait 13 genin (Igf1, Irs1, Pik3ca, Pdk1, PTEN, Akt, TSC1, TSC2, Rheb,

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mTORc1, p70S6K1, EIF4e, EIF4ebp1) profilleri incelenecektir. Fold 2 değerini geçen genlerin western blot analizi ile protein miktarlarına bakılacaktır. Mitotik İndeks için PCNA analizi yapılacaktır.

Ana Grup II (PH+SF Grupları): SF kontrol grubu olup, % 70'lik PH sonrası tek doz SF (0,5 mL) i.p. olarak verilecektir. Belirlenen sürelerin (6, 12, 24, 48 saat) sonunda diseksiyonları gerçekleştirilerek kan ve karaciğer örnekleri alınacaktır. Serum örneklerinde ALT ve AST değerlerine bakılacaktır. Karaciğer örneklerinden Mini Array yöntemi ile PI3K/Akt/mTOR sinyal yolağına ait 13 genin profilleri incelenecektir. Fold2 değerini geçen genlerin western blot analizi ile protein miktarlarına bakılacaktır. Mitotik İndeks için PCNA analizi yapılacaktır.

PH+SF Alt Grup I: 6. Saat
PH+SF Alt Grup II: 12. Saat
PH+SF Alt Grup III: 24. Saat
PH+SF Alt Grup IV: 48. Saat

Ana Grup III (PH+Silmarin Grupları): Silmarin positive kontrol grubu olup, % 70'lik PH sonrası tek doz Silmarin (100 mg/kg) i.p.(Canbek et al., 2017) olarak verilecektir. Belirlenen sürelerin (6, 12, 24, 48 saat) sonunda diseksiyonları gerçekleştirilerek kan ve karaciğer örnekleri alınacaktır. Serum örneklerinde ALT ve AST değerlerine bakılacaktır. Karaciğer örneklerinden Mini Array yöntemi ile PI3K/Akt/mTOR sinyal yolağına ait 13 genin profilleri incelenecektir. Fold2 değerini geçen genlerin western blot analizi ile protein miktarlarına bakılacaktır. Mitotik İndeks için PCNA analizi yapılacaktır.

PH+Silmarin Alt Grup I: 6. Saat
PH+Silmarin Alt Grup II: 12. Saat
PH+Silmarin Alt Grup III: 24. Saat
PH+Silmarin Alt Grup IV: 48. Saat

Ana Grup IV (PH+Gallik Asid Grubu): Deney grubu olup, % 70'lik PH sonrası tek doz Gallik Asid (200mg/kg) i.p.(Canbek et al., 2012) olarak verilecektir. Belirlenen sürelerin (6, 12, 24, 48 saat) sonunda diseksiyonları gerçekleştirilerek kan ve karaciğer örnekleri alınacaktır. Serum örneklerinde ALT ve AST değerlerine bakılacaktır. Karaciğer örneklerinden Mini Array yöntemi ile PI3K/Akt/mTOR sinyal yolağına ait 13 genin profilleri incelenecektir. Fold2 değerini geçen genlerin western blot analizi ile protein miktarlarına bakılacaktır. Mitotik İndeks için PCNA analizi yapılacaktır.

PH+Madde Alt Grup I: 6 Saat
PH+ Madde Alt Grup II: 12. Saat
PH+ Madde Alt Grup III: 24. Saat
PH+ Madde Alt Grup IV: 48. Saat

2.Hepatektomi İşlemi: Tüm deneysel çalışmalar steril ortamda ve steril cerrahi aletler kullanılarak gerçekleştirilecektir. Diurnal hormonal değişimlerin sıçanlar üzerine olası etkileri dikkate alınarak tüm cerrahi işlemler 09.00 ile 12.00 saatleri arasında yapılmıştır. Deney gruplarına ait hayvanlara intramuskular olarak 10 mg.kg-1 ksilazin ve 70 mg.kg-1 ketamin anestezisi uygulanacaktır. Parsiyal hepatektomi yapılacak deney hayvanı, sırt üstü pozisyonda, sıcaklığı ılık ve sabit olan diseksiyon tablasına tespit edilip işlem başlatılacaktır. Cerrahi uygulama bölgesi %70'lik etil alkol ile temizliği yapılıp Higgins ve Anderson'un (1931)

T.C.
ESKİŞEHİR OSMANGAZİ ÜNİVERSİTESİ REKTÖRLÜĞÜ
HAYVAN DENEYLERİ YEREL ETİK KURULU
(HADYEK)

tekniğiyle parsiyal hepatektomi gerçekleştirilecektir. Buna göre, deney hayvanının karın bölgesinde vücuda paralel 2-3 cm'lik bir orta hat kesisi ile laporoktomi yapılacak ve karaciğer loplarını birbirine bağlayan zarlar ayrılarak karaciğerin yaklaşık %68-70 oranında kütlesini teşkil eden öndeki sağ orta ve sol yan lopları ayrılacaktır. Bu loplara ait hepatik arter, ven ve safra kanallarının etrafından ipek iplik geçirilerek bağlanacak ve bağın lop tarafından ve hemen üstünden kesim gerçekleştirilecektir. Her bir hayvana yapılan cerrahiden sonra kaybolan sıvının hipovolemik etkilerine engel olunması için karın boşluğuna steril serum fizyolojik verilecektir. Sonra, kas ve deri kesileri ayrı ayrı fakat devamlı olarak 4/0 ipek sütürle dikilerek laporoktomi bölgesi kapatılacaktır ve poviiodeks antiseptik solüsyon ile laporoktomi bölgesi temizlenecektir. Cerrahi işlem görmüş her bir deney hayvanı kimyasal sterilizasyonu yapılmış, tek bireylik, polikarbonat bileşimli ve şeffaf nitelikteki kafeslere ayrı ayrı koyularak diyet değişikliği yapılmaksızın 6, 12, 24, 48 saat boyunca yaşatılacaktır.

3. Anestezi: Tüm cerrahi işlemler steril ortamda ve steril cerrahi aletler kullanılarak gerçekleştirilecektir. Deney gruplarına ait hayvanlar hafif eter anestezisi altında, intrakardiyak olarak kalpten bütün kanın alınması yoluyla öldürülecektir. Deney gruplarına ait hayvanlara intramuskular olarak 10 mg.kg-1 ksilazin ve 70 mg.kg-1 ketamin anestezisi uygulanacaktır ve midline laporotomi gerçekleştirilerek karaciğer doku örnekleri alınacaktır.

Kullanılan yöntemler uluslararası standardı olan yöntemlerdir. Gerekli literatürler verilmiştir. Çalışmanın yapılmasında, Komisyonumuzun olumsuz görüşü bulunmamaktadır.

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(HADYEK)

HAYVAN DENEYLERİ YEREL ETİK KURULU KARARI

TOPLANTI TARİHİ	**: 27.11. 2020**
TOPLANTI SAYISI	**: 152**
DOSYA KAYIT NUMARASI	**: 814**
KARAR NUMARASI	**: 814**
ARAŞTIRMA YÜRÜTÜCÜSÜ	**: Doç. Dr. Mustafa UYANOĞLU**
YARDIMCI ARAŞTIRMACILAR	**: Yüksek Lisans Öğrencisi Pınar YÜCEL**
	Arş. Gör. Dr. Ayşe ÖZMEN YAYLACI
	Prof. Dr. Mediha CANBEK
	Doktora öğrencisi Neda HAMİD
	Doktora öğrencisi Muhammed Fawaz Abdullah

HAYVAN TÜRÜ ve SAYISI : 14.11.2019 kayıt tarihli 773-2019 no'lu Etik kurulu onayına sahip çalışmadaki Wistar albino (104 adet erkek) **hayvanın dokuları kullanılacaktır.**

Eskişehir Osmangazi Üniversitesi Fen-Edebiyat Fakültesi Biyoloji / Moleküler Biyoloji Bölümü Öğretim Üyesi **Doç. Dr. Mustafa UYANOĞLU**'nun araştırma yürütücüsü olduğu 814/2020 kayıt numaralı ve **" Gallik asitin parsiyel hepatektomi yapılmış sıçanlarda oluşan oksidatif stres hasarına karşı Nrf-2 yolağı üzerinden etkilerinin araştırılması"** konulu çalışma; Hayvan Deneyleri Yerel Etik Kurulu Yönergesi'ne göre değerlendirilmiş ve gerekçede belirtildiği şekilde yapılması uygun bulunmuştur.

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HAYVAN DENEYLERİ YEREL ETİK KURULU
(HADYEK)

GEREKÇE

Eskişehir Osmangazi Üniversitesi Fen-Edebiyat Fakültesi Biyoloji / Moleküler Biyoloji Bölümü Öğretim Üyesi **Doç. Dr. Mustafa UYANOĞLU**'nun araştırma yürütücüsü olduğu 814/2020 kayıt numaralı ve **" Gallik asitin parsiyel hepatektomi yapılmış sıçanlarda oluşan oksidatif stres hasarına karşı Nrf-2 yolağı üzerinden etkilerinin araştırılması"** konulu çalışma; tarafımızca değerlendirilmiştir.

Hayvan Deneyleri Yerel Etik Kurulumuzun 14.11.2019 tarihli 143 sayılı toplantısı ve 773-2019 no'lu karar numarası ile önceki tarihlerde onaylanan "Sıçanlarda Parsiyal Hepatektomi Sonrası Karaciğer Rejenerasyonunda Gallik Asit Etkisinin PI3K/Akt/mTOR yolağı üzerinden Belirlenmesi" başlıklı çalışmaya **Wistar albino (104 adet erkek) hayvanın dokuları kullanılacaktır.** Bu çalışmada ek sıçan kullanılmayacaktır.

1. Deney Grupları ve Uygulamalar
Deneyimizde 200-250 gr ağırlığında erkek Wistar albino ırkı sıcanlar kullanılacaktır. Çalışmada n=8 olmak uzere rastgele secimle 4 ana grup (Kontrol Grubu, PH+ SF Grubu, PH+Silmarin ve PH+ Gllik asid Grubu) ve 4 alt grup oluşturulacaktır.

Ana Gruplar	Alt Gruplar
Ana Grup I (Kontrol Grubu)	Grup I (n=6)
Ana Grup II (PH+SF Grupları)	Sham Alt Grup I: 6. Saat (n=6) Sham Alt Grup II: 12. Saat (n=6) Sham Alt Grup III: 24. Saat (n=6) Sham Alt Grup IV: 48. Saat (n=6)
Ana Grup III (PH+ Silmarin Grupları)	PH+Madde Alt Grup I: 6 Saat(n=6) PH+ Madde Alt Grup II: 12. Saat(n=6) PH+ Madde Alt Grup III: 24. Saat(n=6) PH+ Madde Alt Grup IV: 48. Saat(n=6)
Ana Grup IV (PH+Gallik Asid Grubu)	PH+SF Alt Grup I: 6. Saat (n=6) PH+SF Alt Grup II: 12. Saat (n=6) PH+SF Alt Grup III: 24. Saat (n=6) PH+SF Alt Grup IV: 48. Saat (n=6)

Ana Grup I (Kontrol Grupları): Kontrol Grubundaki hayvanlara laparatomi yapılıp karaciğer loblarını birbirine bağlayan zarlar ayrılacaktır. %70 partiyal hepatektomi yapılacakmış gibi karaciğerin öndeki sağ orta ve sol yan lopları ayrılarak iplik geçirilecektir. Parsiyal hepatektomi yapılmadan laparotomi bölgesi kapatılacaktır. Hicbir madde verilmeyecektir. Belirlenen surelerin (6, 12, 24, 48 saat) sonunda diseksiyonları gerçekleştirilerek kan ve karaciğer örnekleri alınacaktır.

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HAYVAN DENEYLERİ YEREL ETİK KURULU
(HADYEK)

Ana Grup II (PH+SF Grupları): SF kontrol grubu olup, % 70'lik PH sonrası tek doz SF (0,5 mL) i.p. olarak verilecektir. Belirlenen surelerin (6, 12, 24, 48 saat) sonunda diseksiyonları gerçekleştirilerek kan ve karaciğer örnekleri alınacaktır.
PH+SF Alt Grup I: 6. Saat
PH+SF Alt Grup II: 12. Saat
PH+SF Alt Grup III: 24. Saat
PH+SF Alt Grup IV: 48. Saat
Ana Grup III (PH+Silmarin Grupları): Silmarin positive kontrol grubu olup, % 70'lik PH sonrası tek doz Silmarin (100 mg/kg) i.p.(Canbek et al., 2017) olarak verilecektir. Belirlenen surelerin (6, 12, 24,48 saat) sonunda diseksiyonları gerçekleştirilerek kan ve karaciğer örnekleri alınacaktır.
PH+Silmarin Alt Grup I: 6. Saat
PH+Silmarin Alt Grup II: 12. Saat
PH+Silmarin Alt Grup III: 24. Saat
PH+Silmarin Alt Grup IV: 48. Saat
Ana Grup IV (PH+Gallik Asit Grubu): Deney grubu olup, % 70'lik PH sonrası tek doz Gallik Asid (200mg/kg) i.p.(Canbek et al., 2012) olarak verilecektir. Belirlenen surelerin (6, 12, 24, 48 saat) sonunda diseksiyonları gerçekleştirilerek kan ve karaciğer örnekleri alınacaktır.
PH+ Gallik asit Alt Grup I: 6 Saat
PH+ Gallik asit Alt Grup II: 12. Saat
PH+ Gallik asit Alt Grup III: 24. Saat
PH+ Gallik asit Alt Grup IV: 48. Saat

1.1. Hepatektomi İşlemi: Tüm deneysel çalışmalar steril ortamda ve steril cerrahi aletler kullanılarak gerçekleştirilecektir. Diurnal hormonal değişimlerin sıçanlar üzerine olası etkileri dikkate alınarak tüm cerrahi işlemler 09.00 ile 12.00 saatleri arasında yapılmıştır. Deney gruplarına ait hayvanlara intramuskular olarak 10 mg.kg-1 ksilazin ve 70 mg.kg-1 ketamin anestezisi uygulanacaktır. Parsiyal hepatektomi yapılacak deney hayvanı, sırt üstü pozisyonda, sıcaklığı ılık ve sabit olan diseksiyon tablasına tespit edilip işlem başlatılacaktır. Cerrahi uygulama bölgesi %70'lik etil alkol ile temizliği yapılıp Higgins ve Anderson'un (1931) tekniğiyle parsiyal hepatektomi gerçekleştirilecektir. Buna göre, deney hayvanının karın bölgesinde vücuda paralel 2-3 cm'lik bir orta hat kesisi ile laporoktomi yapılacak ve karaciğer loplarını birbirine bağlayan zarlar ayrılarak karaciğerin yaklaşık %68-70 oranında kütlesini teşkil eden öndeki sağ orta ve sol yan lopları ayrılacaktır. Bu loplara ait hepatik

arter, ven ve safra kanallarının etrafından ipek iplik geçirilerek bağlanacak ve bağın lop tarafından ve hemen üstünden kesim gerçekleştirilecektir. Her bir hayvana yapılan cerrahiden sonra kaybolan sıvının hipovolemik etkilerine engel olunması için karın boşluğuna steril serum fizyolojik verilecektir. Sonra, kas ve deri kesileri ayrı ayrı fakat devamlı olarak 4/0 ipek sütürle dikilerek laporoktomi bölgesi kapatılacaktır ve poviiodeks antiseptik solüsyon ile laporoktomi bölgesi temizlenecektir. Cerrahi işlem görmüş her bir deney hayvanı kimyasal sterilizasyonu yapılmış, tek bireylik, polikarbonat bileşimli ve şeffaf nitelikteki kafeslere ayrı ayrı koyularak diyet değişikliği yapılmaksızın 6, 12, 24, 48 saat boyunca hayatta kalmaları sağlanacaktır.

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ESKİŞEHİR OSMANGAZİ ÜNİVERSİTESİ REKTÖRLÜĞÜ
HAYVAN DENEYLERİ YEREL ETİK KURULU
(HADYEK)

1.2. Anestezi ve Ötenazi: Tum cerrahi işlemler steril ortamda ve steril cerrahi aletler kullanılarak gerçekleştirilecektir. Deney gruplarına ait hayvanlar hafif eter anestezisi altında, intrakardiyak olarak kalpten bütün kanın alınması yoluyla öldürülecektir. Deney gruplarına ait hayvanlara intramuskular olarak 10 mg.kg-1 ksilazin ve 70 mg.kg-ı ketamin anestezisi uygulanacaktır ve midline laporotomi gerçekleştirilerek karaciğer doku örnekleri alınacaktır. Kan ornekleri MSE Mistral 2000 marka cihaz ile 10 dk 3000 rpm devirde santrifujlenerek serumları elde edilecektir. Ependorf tuplere aktarılan serum ornekleri biyokimyasal alanine aminotransferase (ALT) ve aspartate aminotransferase (AST) miktar analizleri icin -80 oC derin dondurucuda korunacaktır. Hepatektomi sonrası kalan karaciğer dokusundan diseksiyon sonrası alınacak örneklerden bir kısmı dokuda superoxide dismutase (SOD) ve catalase (CAT) endojen antioksidan enzimlerinin biyokimyasal ölçümlerinde ve moleküler biyolojik analizlerde kullanılmak üzere homojenize edilerek -80 oC derin dondurucuda korunacaktır. Karaciğer dokusunun diğer kısmı ise Proliferating Cell Nuclear Antigen (PCNA) immunhistokimyasal analizleri icin %10 notral formaldehit çözeltisi ile tespit edilerek korunacaktır.

Kullanılan yöntemler uluslararası standardı olan yöntemlerdir. Gerekli literatürler verilmiştir. Çalışmanın yapılmasında, Komisyonumuzun olumsuz görüşü bulunmamaktadır.

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