



بسم الله الرحمن الرحيم

∞∞∞∞

تم رفع هذه الرسالة بواسطة / مني مغربي أحمد

بقسم التوثيق الإلكتروني بمركز الشبكات وتكنولوجيا المعلومات دون أدنى

مسئولية عن محتوى هذه الرسالة.

ملاحظات: لا يوجد



# **Autophagy and Rapamycin in Preventing Experimental Diabetes Mellitus Complications**

*A Thesis*

*Submitted for the Partial Fulfillment of Philosophy Degree in  
Pharmaceutical Sciences (Biochemistry)*

*By*

**Khaled Mahmoud Ali Gouda**

Assistant Lecturer of Biochemistry, Faculty of Pharmacy,  
Modern University for Technology and Information.  
Master Degree in Pharmaceutical Sciences (Biochemistry), AL-Azhar  
University, 2015.

*Under Supervision of*

**Prof. Dr. Hala Osman El- Mesallamy**

Professor of Biochemistry, Faculty of Pharmacy,  
Ain Shams University, Dean of Faculty of Pharmacy,  
Sinai University (Kantara).

**Prof. Dr. Ahmed Mohamed Mansour**

Professor of Pharmacology, Faculty of Pharmacy (Boys),  
AL-Azhar University.

**Dr. Nesreen Nabil Omar**

Associate Professor of Biochemistry, Acting Head of Biochemistry  
Department, Faculty of Pharmacy, Modern University  
for Technology and Information.

**Dr. Sherihan Galal Abdel Hamid**

Lecturer of Biochemistry, Faculty of Pharmacy,  
Ain-Shams University.

أَعُوذُ بِاللّٰهِ مِنَ الشَّيْطَانِ الرَّجِيمِ

بِسْمِ اللّٰهِ الرَّحْمٰنِ الرَّحِيمِ

﴿فَتَعَالَى اللَّهُ الْمَلِكُ الْحَقُّ وَلَا تَعْجَلْ بِالْقُرْآنِ مِنْ قَبْلِ

أَنْ يَقْضَىٰ إِلَيْكَ وَحْيُهُ وَقُلْ رَبِّ زِدْنِي عِلْمًا﴾

سورة طه ١١٤

# Acknowledgment

First of all I thank "**Allah**" for granting me the power to accomplish this work.

I would like to express my deepest thanks to ***Prof. Dr. Hala Osman El- Mesallamy***, Professor of Biochemistry, Faculty of Pharmacy, Ain Shams University and Dean of Faculty of Pharmacy, Sinai University, for her valuable scientific supervision, constructive advice and continuous guidance throughout the work.

I would also like to thank ***Prof. Dr. Ahmed Mohamed Mansour***, Professor of Pharmacology, Faculty of Pharmacy (Boys), AL-Azhar University, for his active participation and great support during the study.

My deepest gratitude and appreciation are expressed to ***Associate Prof. Nesreen Nabil Omar***, Acting Head of Biochemistry Department, Faculty of Pharmacy, MTI University, for her keen, continuous, enthusiastic support patient guidance, and enlightening advice throughout the whole work.

I owe my deepest sincere gratitude to ***Dr. Sherihan Galal Abdel Hamid***, Lecturer of Biochemistry, Faculty of Pharmacy, Ain Shams University, for her faithful encouragement, invaluable suggestion and advice throughout the work.

Finally, my deepest everlasting thanks and appreciation are for ***my family***, for their continuous support and encouragement during the whole thesis tiring period.

# List of Contents

|  |     |
|--|-----|
| <b>List of Abbreviations</b> .....   | i   |
| <b>List of Tables</b> .....  | iii |
| <b>List of Figures</b> .....   | iv  |
| <b>1. Introduction and Aim of the Work</b> .....   | 1   |
| <b>2. Literature Review</b> .....  | 4   |
| 2.1. Diabetes Mellitus and Its Prevalence .....  | 4   |
| 2.2. Classification .....  | 5   |
| 2.3. Complications .....   | 6   |
| 2.3.1. Microvascular Complications .....   | 8   |
| 2.3.1.1. Diabetic Neuropathy .....   | 8   |
| 2.3.1.2. Diabetic Retinopathy (DR).....  | 11  |
| 2.3.1.3. Diabetic Nephropathy (DN) .....   | 12  |
| 2.3.2. Macrovascular Complications .....   | 16  |
| 2.3.2.1. Atherosclerosis.....  | 16  |
| 2.3.2.2. Coronary Heart Disease and Stroke .....   | 17  |
| 2.4. Management .....  | 18  |
| 2.4.1. Management of T1DM .....  | 13  |
| 2.4.2. Management of T2DM .....  | 13  |
| 2.5 Autophagy .....  | 20  |
| 2.5.1. Diseases Associated with Abnormal Autophagy.....                                  | 21  |
| 2.5.2. Types of Autophagy .....  | 22  |
| 2.5.2.1. Macroautophagy.....   | 22  |
| 2.5.2.2. Microautophagy .....  | 23  |
| 2.5.2.3. Chaperon Mediated Autophagy (CMA).....  | 23  |
| 2.5.3. Molecular Machinery of Autophagy.....   | 24  |
| 2.5.4. Induction of Autophagy.....   | 26  |
| 2.5.5. Autophagy Role in The Maintenance of Normal<br>Islet Structure and Function ..... | 29  |
| 2.5.6. Autophagy in Diabetic $\beta$ Cells.....  | 31  |
| 2.5.7. Autophagy and Oxidative Stress in $\beta$ Cells.....                              | 31  |
| 2.5.8. Crinophagy in $\beta$ Cells .....   | 32  |
| 2.6. Rapamycin.....  | 33  |
| 2.6.1. Mechanism.....  | 33  |
| 2.6.2. Uses .....  | 35  |

|  |           |
|--|-----------|
| 2.6.2.1 Rapamycin and Cancer.....  | 36        |
| 2.6.2.2. Rapamycin and Neurodegenerative Diseases.....   | 37        |
| 2.6.2.3. Rapamycin Effect on Longevity.....  | 38        |
| 2.6.2.4. Rapamycin and Metabolic Diseases.....   | 38        |
| 2.6.2.5. Rapamycin and Immune System .....   | 39        |
| 2.6.2.6. Rapamycin Effect on Lymphangioleiomyomatosis<br>(LAM) and Tuberous Sclerosis Complex (TSC)..... | 39        |
| 2.6.2.7. Rapamycin and COVID-19 Pandemic.....  | 39        |
| 2.6.3. Rapalogs .....  | 40        |
| 2.7. Fasting .....   | 42        |
| 2.8. Microtubule-Associated Protein 1A/1B-Light Chain<br>3 (LC3) .....                                   | 46        |
| 2.9. ATP Binding Cassette Subfamily B Member<br>1(ABCB1).....  | 48        |
| 2.10. p53 .....  | 50        |
| <b>3. Materials and Methods.....</b>   | <b>54</b> |
| 3.1. Experimental Design .....   | 54        |
| 3.2. Materials .....   | 55        |
| 3.2.1. Animals .....   | 55        |
| 3.2.2. Drugs .....   | 56        |
| 3.2.2.1. Rapamycin .....   | 56        |
| 3.2.2.2. Streptozotocin (STZ).....   | 57        |
| 3.3. Methods .....   | 57        |
| 3.3.1. Induction of DM .....   | 57        |
| 3.3.2. Preparation of STZ .....  | 57        |
| 3.3.3. Dose preparation .....  | 57        |
| 3.3.4. Samples Collection.....   | 58        |
| 3.3.5. Tissue Preparation .....  | 58        |
| 3.3.6. Histopathology .....  | 59        |
| 3.3.7. Blood parameters .....  | 60        |
| 3.3.7.1. Determination of Fasting Blood Glucose Levels .....   | 60        |
| 3.3.7.2. Determination of Fasting Serum Insulin Levels .....   | 61        |
| 3.3.7.3. Determination of Serum Total Cholesterol (TC)<br>Levels.....                                    | 65        |
| 3.3.7.4. Determination of Serum HDL-C Levels .....   | 67        |

|   |           |
|---|-----------|
| 3.3.7.5. Determination of Serum LDL-C Levels.....                             | 68        |
| 3.3.7.6. Determination of Serum Triacylglycerol (TAG)<br>Levels.....          | 69        |
| 3.3.7.7. Determination of Serum Urea Levels .....                             | 71        |
| 3.3.7.8. Determination of Serum Creatinine Levels .....                       | 72        |
| 3.3.7.9. Determination of Serum Albumin Levels .....                          | 74        |
| 3.3.7.10. Determination of Urine Albumin Levels .....                         | 75        |
| 3.3.7.11. Determination of Serum Bilirubin (Total And<br>Direct) Levels ..... | 77        |
| 3.3.7.12. Determination of Serum Uric Acid Levels .....                       | 79        |
| 3.3.8. Tissue parameters.....   | 81        |
| 3.3.8.1Determination of Pancreatic Malondialdehyde<br>(MDA) Levels.....       | 81        |
| 3.3.8.2. Determination of Pancreatic Catalase (CAT)<br>Level .....            | 83        |
| 3.3.8.3. Determination of Pancreatic and Renal LC3B-II<br>Protein .....       | 85        |
| 3.3.8.4. Determination of Pancreatic and Renal p53<br>Protein .....           | 88        |
| 3.3.8.5. Determination of Pancreatic and Renal ABCB1<br>Protein .....         | 91        |
| 3.3.9. Transmission Electron Microscope (TEM) .....                           | 94        |
| 3.4. Statistical Analysis.....  | 98        |
| <b>4. Results .....</b>   | <b>99</b> |
| 4.1. Animals Weights .....  | 99        |
| 4.2. Diabetes Mellitus (DM) Markers.....                                      | 100       |
| 4.3. Lipid Profile.....   | 102       |
| 4.4. Kidney Function Tests .....  | 103       |
| 4.5. Nucleic Acid Integrity .....   | 104       |
| 4.6. Liver Function Tests.....  | 105       |
| 4.7. Determination of Pancreatic Oxidative Stress<br>Measures.....            | 106       |
| 4.8. Determination of Cellular Autophagy.....                                 | 107       |
| 4.9. Determination of Cellular Apoptosis.....                                 | 109       |

|  |            |
|--|------------|
| 4.10. Cellular Permeability-glycoprotein; ABCB1    |            |
| Estimation .....                                   | 110        |
| 4.11. Transmission electron microscope (TEM) ..... | 112        |
| 4.12. Hematoxylin and Eosin Stain (H&E) .....      | 117        |
| <b>5. Discussion .....</b>                         | <b>118</b> |
| <b>6. Summary and Conclusion .....</b>             | <b>135</b> |
| <b>7. Recommendations.....</b>                     | <b>140</b> |
| <b>8. References.....</b>                          | <b>141</b> |
| الملخص العربي.....                                 | 1          |



## List of Abbreviations

| <b>Abbreviation</b> | <b>Definition</b>  |
|---------------------|--|
| <b>ABCB1</b>        | Adenosine triphosphate binding cassette subfamily b member 1   |
| <b>AGEs</b>         | Advanced glycation end products  |
| <b>Akt</b>          | protein kinase B   |
| <b>Ambra1</b>       | Autophagy and beclin 1 regulator 1   |
| <b>AMPK</b>         | Adenosine monophosphate-activated protein kinase   |
| <b>Atg</b>          | Autophagy related gene or protein  |
| <b>BHB</b>          | $\beta$ -hydroxybutyrate   |
| <b>CMA</b>          | Chaperone-mediated autophagy   |
| <b>CRP</b>          | C-reactive protein   |
| <b>DFCP1</b>        | Zinc finger FYVE (Fab 1, yotB, vesicle transport protein and early endosome antigen 1) domain-containing protein 1 |
| <b>DM</b>           | Diabetes mellitus  |
| <b>DN</b>           | Diabetic nephropathy   |
| <b>DR</b>           | Diabetic retinopathy   |
| <b>ER</b>           | Endoplasmic reticulum  |
| <b>ESRD</b>         | End-stage renal disease  |
| <b>FBG</b>          | Fasting blood glucose  |
| <b>FDA</b>          | The Food and Drug Administration   |
| <b>FKBP-12</b>      | Immunophilin 12- kda FK506- binding protein  |
| <b>F-STZ</b>        | Fasting-Streptozotocin   |
| <b>GBM</b>          | Glomerular basement membrane   |
| <b>GDM</b>          | Gestational diabetes mellitus  |
| <b>GFR</b>          | Glomerular filtration rate   |
| <b>GSH</b>          | Reduced glutathione  |
| <b>H&amp;E</b>      | Hematoxylin and eosin stain  |
| <b>HDL-C</b>        | High density lipoprotein cholesterol   |
| <b>Hsc70</b>        | Heat-shock cognate 70  |
| <b>IR</b>           | Insulin resistance   |
| <b>LAM</b>          | Lymphangioliomyomatosis  |
| <b>LC3</b>          | Microtubule-associated protein 1A/1B-light chain 3   |
| <b>LDL-C</b>        | Low density lipoprotein cholesterol  |
| <b>LKB1</b>         | Liver kinase B1  |
| <b>LPL</b>          | lipoprotein lipase   |
| <b>MDA</b>          | Malondialdehyde  |
| <b>MDR1</b>         | Multidrug resistance protein 1   |

| <b>Abbreviation</b> | <b>Definition</b>   |
|---------------------|---|
| <b>MS</b>           | Mesangial sclerosis   |
| <b>mTOR</b>         | Mammalian target of Rapamycin   |
| <b>mTORC1</b>       | Mammalian target of Rapamycin complex 1                                 |
| <b>mTORC2</b>       | Mammalian target of Rapamycin complex 2                                 |
| <b>NADPH</b>        | Nicotinamide adenine dinucleotide phosphate                             |
| <b>PAG</b>          | Polyacrylamide gel electrophoresis                                      |
| <b>PI3K</b>         | Phosphatidylinositol 3-kinases  |
| <b>PI3P</b>         | phosphatidylinositol 3-phosphate  |
| <b>PKC</b>          | Protein kinase C  |
| <b>POD</b>          | Peroxidase  |
| <b>PTEN</b>         | Phosphatase and tensin homolog  |
| <b>PVDF</b>         | Polyvinylidene difluoride   |
| <b>Ras</b>          | Rat sarcoma virus   |
| <b>RIPA</b>         | Radioimmunoprecipitation assay  |
| <b>ROS</b>          | Reactive oxygen species   |
| <b>R-STZ</b>        | Rapamycin-treated Streptozotocin  |
| <b>STZ</b>          | Streptozotocin  |
| <b>T1DM</b>         | Type 1 diabetes mellitus  |
| <b>T2DM</b>         | Type 2 diabetes mellitus  |
| <b>TAG</b>          | Triacylglycerol   |
| <b>TC</b>           | Total cholesterol   |
| <b>TEM</b>          | Transmission electron microscope  |
| <b>TSC</b>          | Tuberous sclerosis complex  |
| <b>ULK1/2</b>       | Unc-51 like autophagy activating kinase                                 |
| <b>UNC-51</b>       | Serine/threonine-protein kinase   |
| <b>Vps34</b>        | Phosphatidylinositol 3-kinase   |
| <b>WB</b>           | Western blot  |
| <b>WIPI2</b>        | $\beta$ -propeller repeat domain phosphoinositide-interacting protein 2 |

## List of Tables

| <b>Table no.</b> | <b>Title</b>  | <b>Page</b> |
|------------------|---|-------------|
| <b>1</b>         | Diabetic neuropathies.  | <b>10</b>   |
| <b>2</b>         | Stages of Diabetic Nephropathy (DN).  | <b>15</b>   |
| <b>3</b>         | Mean values of weights at the first day and after 14 days of the experiment.                      | <b>99</b>   |
| <b>4</b>         | Lipids profile of control and diabetic rats of STZ-treated groups; (STZ, R-STZ and F-STZ).        | <b>103</b>  |
| <b>5</b>         | Kidney function tests of control and diabetic rats of STZ-treated groups; (STZ, R-STZ and F-STZ). | <b>104</b>  |
| <b>6</b>         | Liver function tests of control and diabetic rats of STZ-treated groups; (STZ, R-STZ and F-STZ).  | <b>105</b>  |

## List of Figures

| <b>Figure no.</b> | <b>Title</b>  | <b>Page</b> |
|-------------------|---|-------------|
| <b>1</b>          | Polyol pathway.   | <b>8</b>    |
| <b>2</b>          | Sites of action of pharmacological therapies for the treatment of type 2 diabetes.  | <b>20</b>   |
| <b>3</b>          | Types of autophagy in mammalian cells.  | <b>24</b>   |
| <b>4</b>          | Molecular pathways of autophagy.  | <b>26</b>   |
| <b>5</b>          | Signaling pathways regulating autophagy and sites of action of some agents.   | <b>28</b>   |
| <b>6</b>          | Crinophagy pathway in pancreatic $\beta$ -cells.  | <b>33</b>   |
| <b>7</b>          | Rapamycin action with mTORC1 and mTORC2.  | <b>34</b>   |
| <b>8</b>          | Effects of Rapamycin in various diseases.   | <b>36</b>   |
| <b>9</b>          | Structures of Rapalogs.   | <b>41</b>   |
| <b>10</b>         | Beneficial effects of fasting on various human body organs.   | <b>44</b>   |
| <b>11</b>         | Signaling pathways affected by fasting.   | <b>46</b>   |
| <b>12</b>         | Serum insulin standard curve.   | <b>65</b>   |
| <b>13</b>         | Standard calibration curve of MDA.  | <b>83</b>   |
| <b>14</b>         | Blood glucose estimation.   | <b>101</b>  |
| <b>15</b>         | Pancreatic oxidative stress markers in all studied groups.  | <b>106</b>  |
| <b>16</b>         | Cellular autophagy marker LC3B protein level in pancreatic and renal tissue samples of the study groups.                          | <b>108</b>  |
| <b>17</b>         | Cellular apoptosis marker p53 protein level in pancreatic and renal tissue samples of the study groups.                           | <b>109</b>  |
| <b>18</b>         | Cellular permeability-glycoprotein; ABCB1 level in pancreatic and renal tissue samples of the study groups.                       | <b>111</b>  |
| <b>19</b>         | Effect of Rapamycin and fasting on autophagosome formation in kidney tissue of rats after administration of STZ as viewed by TEM. | <b>112</b>  |
| <b>20</b>         | Selected micrographs by TEM of renal cells in the STZ group.  | <b>114</b>  |
| <b>21</b>         | Selected micrographs by TEM of renal cells in the R-STZ group.  | <b>115</b>  |
| <b>22</b>         | Selected micrographs by TEM of renal cells in the F-STZ group.  | <b>116</b>  |
| <b>23</b>         | Renal tissues as stained by H&E (scale bar, 100 $\mu$ m) for STZ rats after administration of Rapamycin or after fasting.         | <b>117</b>  |

## 1. Introduction and Aim of the Work

Diabetes mellitus (DM) is considered a global emergency. Around 537 million people worldwide suffer from DM, and 1.6 million die annually secondary to diabetes (*IDF, 2021*). It is predicted that 643 million people to suffer from DM by 2030 and to 783 by 2045. (*WHO, 2020; Patel et al., 2022*).

Many pathological factors are involved in the prognosis of DM. However, pancreatic  $\beta$  cell dysfunction is considered the core of DM and its complications (*American Diabetes Association, 2014; Antonetti et al., 2021*).

Maintenance of pancreatic  $\beta$  cells could be achieved through two strategies. First, increasing its reproductive capacity. However, the potential risk of cancer emergence may accompany this strategy. Second, enhancing the defense power of the  $\beta$  cells against destructive matters by inducing autophagy. Nevertheless, the subsequent risk of programmed cell death might also arise. Consequently, an optimal induction of autophagy is the key to gain the benefits of this vital biological process (*Stützer et al., 2012; Vetere et al., 2014; Eshraghi et al., 2022*). We aimed in this study to prolong the life span of  $\beta$  cells through induction of

autophagy in an appropriate way. Autophagy has been demonstrated to have a beneficial effect on DM-induced nephropathy (*Khodir et al., 2020*).

Rapamycin, an immunosuppressant mammalian target of Rapamycin (mTOR) inhibitor drug was shown to stimulate  $\beta$ -cell autophagy, but its effects on preventing or ameliorating the diabetic nephropathy (DN) is unclear, an effect worth to be studied.

Research on fasting is gaining attraction based on recent studies that show its role in many adaptive cellular responses such as the reduction of oxidative damage and inflammation (*Visioli et al., 2022*). Fasting forces healthy cells to enter a slow division and highly protected mode that protects them against oxidative stress which is considered a key factor in developing pancreatic insufficiency (*Nencioni et al., 2018*). As fasting is now an attractive protective strategy, its effect will be compared to Rapamycin effects on pancreatic and renal cells.

Induction of autophagy in pancreatic  $\beta$  cells is of an appreciated importance, as it results in indirect activation of CAT biosynthesis that is normally expressed in low level in

the pancreatic  $\beta$  cells (*Lenzen et al., 1996; Pearson et al., 2021*).

Accordingly, the ultimate aim of this study was to:

- 1) Explore the real outcome of autophagy in developing or preventing DM complications, in particular DN.
- 2) Estimate the prophylactic importance of Rapamycin, as a standard inducer for the autophagy, in preventing progression of DN.
- 3) Explore the physiological importance of fasting towards DM and pancreatic  $\beta$  cells.
- 4) Compare the effect of Rapamycin/ fasting in enhancing or worsening DM and its complications.